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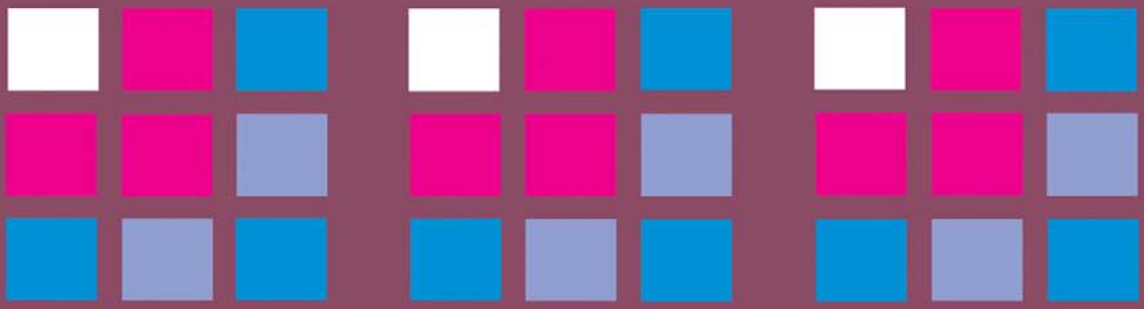

Plant Genetic Engineering

Towards the Third Millennium

Edited by

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Plant Genetic Engineering

Towards the Third Millennium

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Preface

The International Symposium on Plant Genetic Engineering: Towards the Third Millennium continues to show the excellent gathering power of the Center for Genetic Engineering and Biotechnology of Havana, that attracts first level speakers from all over the world, and delegates from many countries, all interested in the latest advances of this vigorous field where new knowledge mixes with technology and products, and where Cuba continues to occupy an outstanding place among developing countries.

Plant biotechnology offers important opportunities for agriculture, horticulture, pharmaceutical, diversification of plant products, and the food industry by generating transgenic varieties with altered properties. This is likely to change farming practice and reduce the potential negative impact of plant production on the environment.

This volume shows the potential benefits of Plant Biotechnology focused on the Third Millennium. The book contains 39 edited papers commissioned for the International Symposium on Plant Genetic Engineering held in Havana from December 6 to 10, 1999. Authors discuss the production of transgenic plants resistant to biotic and abiotic stress, the improvement of plant qualities, the use of transgenic plants as bioreactors, and the use of plant genomics for genetic improvement and gene cloning. All the contributors are actively engaged in research in plant genetic engineering and several are concerned directly with its commercial applications. Important issues are the consideration of regulatory processes to govern the safe release of genetically modified plants into the environment, the management of intellectual property as well as the public acceptance of the transgenic plants.

The Organizing Committee of the International Symposium on Plant Genetic Engineering: Towards the Third Millennium, would like to dedicate this volume to Professor Marc Van Montagu. Professor Marc Van Montagu is well-known in the international scientific community. He is among those who discovered the molecular mechanisms that rule the transfer of DNA from *Agrobacterium* to the plant nucleus, and assembled the first practical systems for plant genetic engineering. His enormous effort to encourage plant research and to stimulate scientists worldwide has been outstanding. We are sure that history will recognize the great value of this generous person who has contributed so much to the development of Plant Biotechnology.

Carlos Borroto
Deputy Director

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Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Global Status of Transgenic Crops: Challenges and Opportunities

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Global Distribution of Transgenic Crops

Between 1996 and 1998, eight countries, 5 industrial and 3 developing, have contributed to more than a fifteen fold increase in the global area of transgenic crops. Adoption rates for transgenic crops are some of the highest for new technologies by agricultural industry standards. High adoption rates reflect grower satisfaction with the products that offer significant benefits ranging from more flexible crop management, higher productivity and a safer environment through decreased use of conventional pesticides, which collectively contribute to a more sustainable agriculture. In 1998, the global area of transgenic crops increased by 16.8 million hectares to 27.8 million hectares, from 11.0 million hectares in 1997 (Table 1). Five principal transgenic crops were grown in eight countries in 1998, three of which, Spain, France and South Africa, grew transgenic crops for the first time in 1998. Data for China has not been included in the global database because only tentative estimates were available which suggest that <100,000 hectares of transgenic crops were grown in 1998, representing <1 % of global transgenic area, with *Bt* cotton being the principal crop.

Table 1. Global area* of transgenic crops in 1996, 1997 and 1998.

	Hectares (million)	Acres (million)
1996	1.7	4.3
1997	11.0	27.5
1998	27.8	69.5

* Excluding China. Increase in area from 1996 to 1997 was 9.3 million hectares (23.2 million acres). Increase in area from 1997 to 1998 was 16.8 million hectares (42.0 million acres)
Source: Clive James, 1998.

Distribution by Country

The countries listed in descending order of transgenic crop area on a global basis in 1998 (Table 2) are: USA 20.5 million hectares representing 74 % of the global area, Argentina with 4.3 million hectares equivalent to 15 % of global area; Canada 2.8 million hectares representing 10 %; Australia with approximately 0.1 million hectares equivalent to 1 % and finally Mexico, Spain, France and South Africa each with <0.1 million hectares, equivalent to less than 1 % of the global area of transgenic crops in 1998. (Table 2). The proportion of transgenic crops grown in industrial countries was 84 %, about the same as 1997 (86 %) with 16 % grown in the developing countries, with most of that area in Argentina, and the balance in Mexico and South Africa. As in 1997, the largest increase in transgenic crops in 1998 occurred in the USA (12.4 million hectares) where there was a 2.5 fold increase, followed by

Argentina (2.9 million hectares) with a 3.0 fold increase, and Canada (1.5 million hectares) with a 2.1 fold increase. USA continued to be the principal grower of transgenic crops in 1998 and its share of global area was the same (74 %) in 1997 and 1998. Argentina's transgenic crop area increase was the largest relative change, increasing 3.0 fold from 1.4 million hectares in 1997 to 4.3 million hectares in 1998; thus Argentina's global share of transgenic crop area increased from 13 % of global area in 1997 to 15 % in 1998. Canada's share of global transgenic crop area decreased marginally from 12 % in 1997 to 10 % of global area in 1998.

Table 2. Global area of transgenic crops in 1997 and 1998: By country (millions of hectares).

Country	1997	%	1998	%	Increase 1997 to 1998 (Ratio)
USA	8.1	74	20.5	74	12.4 (2.5)
Argentina	1.4	13	4.3	15	2.9 (3.0)
Canada	1.3	12	2.8	10	1.5 (2.1)
Australia	0.1	1	0.1	1	<0.1 (1.0)
Mexico	<0.1	<1	<0.1	<1	<0.1 (- -)
Spain	0.0	0	<0.1	<1	<0.1 (- -)
France	0.0	0	<0.1	<1	<0.1 (- -)
South Africa	0.0	0	<0.1	<!	<0.1 (- -)
Total	11.0	100	27.8	100	16.8 (2.3)

Source: Clive James, 1998.

Distribution by Crop and Trait

The five principal transgenic crops grown in 1998 (Table 3) were, in descending order of area, soybean, corn/maize, cotton, canola/rapeseed, and potato. Transgenic soybean and corn continued to be ranked first and second in 1998, accounting for 52 % and 30 % of global transgenic area, respectively. Cotton and canola shared third ranking position in 1998 each occupying 9 % of global area.

Table 3. Global area of transgenic crops in 1997 & 1998: By crop (millions of hectares).

Crop	1997	%	1998	%	Increase (Ratio)
Soybean	5.1	46	14.5	52	9.4(2.9)
Corn	3.2	30	8.3	30	5.1(2.6)
Cotton	1.4	13	2.5	9	1.1(1.8)
Canola	1.2	11	2.4	9	1.2(2.0)
Potato	<0.1	<1	<0.1	<1	<0.1(-.-)
Total	11.0	100	27.8	100	16.8 (2.5)

Source: Clive James, 1998.

The relative ranking of the principal transgenic traits were the same in 1997 and 1998 (Table 4), with herbicide tolerance being by far the highest, increasing from 63 % in 1997 to 71 % in 1998. Insect resistant crops decreased from 36 % in 1997 to 28 % in 1998. Stacked genes for insect resistance and herbicide tolerance increased from <0.1 % in 1997 (<0.1 million hectares) to 1 % or 0.3 million hectares in 1998 with quality traits occupying less than 1 % and <0.1 million hectares in both 1997 and 1998.

Table 4. Global area of transgenic crops in 1997 & 1998: By trait (millions of hectares).

Trait	1997	%	1998	%	Increase (Ratio)
Herbicide tolerance	6.9	63	19.8	71	12.9 (2.9)
Insect resistance	4.0	36	7.7	28	3.7 (1.9)
Insect res. & Herbicide tolerance	<0.1	<1	0.3	1	0.2 (-.-)
Quality Traits	<0.1	<1	<0.1	<1	< 0.1 (-.-)
Global Totals	11.0	100	27.8	100	16.8 (2.5)

Source: Clive James, 1998.

Major Changes in 1998

In reviewing the shift in global share of transgenic crops for the respective countries, crops and traits, the major changes between 1997 and 1998 were related to the following trends: growth in area of transgenic crops between 1997 and 1998 in the industrial countries continued to be significant and almost 5 times greater than in developing countries (13.9 million hectares versus 2.9 million hectares); in terms of crops, soybean contributed the most (56 %) to global growth of transgenic crops, equivalent to 9.4 million hectares between 1997 and 1998, followed by corn at 30 % (5.1 million hectares), canola at 7 % (1.2 million hectares) and cotton at 6 % (1.1 million hectares). There were three noteworthy developments in terms of traits, herbicide tolerance contributed the most (77 % or 12.9 million hectares) to global growth, and insect resistance contributed 22 % equivalent to 3.7 million hectares; the multiple or stacked traits of insect resistance and herbicide tolerance increased by 0.2 million hectares in 1998 representing 1 % of global area with significant prospects for further growth in future. Of the 5 major transgenic crops grown in 8 countries in 1998, (Table 5) the two principal crops of soybean and corn, represented 82 % of the global transgenic area.

Table 5. Dominant transgenic crops 1998

Crop	Million Hectares Areas	% Transgenic
Herbicide tolerant Soybean	14.5	52
Bt Corn	6.7	24
Insect resistant/herbicide tolerant Cotton	2.5	9
Herbicide tolerant Canola	2.4	9
Herbicide tolerant Corn	1.7	6
Total	27.8	100

Source: Clive James, 1998.

In 1998 herbicide tolerant soybean was the most dominant transgenic crop (52 % of global transgenic area) followed by insect resistant corn (24 %), herbicide tolerant canola (9 %), and insect resistant/herbicide tolerant cotton at 9 % and herbicide tolerant corn at 6 %.

It is noteworthy that 1998 was the first year for a commercialized transgenic crop to be grown in the countries of the European Union. Estimates suggest that introductory quantities of insect resistant maize were grown primarily in Spain (20 000 hectares) and France (2000 hectares); this is judged to be potentially a very significant development because it could have important implications for the further adoption of transgenics in countries of the European Union.

Estimated Benefits from Transgenic Crops

More information on the benefits associated with transgenic crops is becoming available following the substantial area of transgenic crops planted in the USA and Canada in 1997. Multiple benefits have been reported by growers for selected transgenic crops; these include more flexibility in terms of crop management (particularly important for herbicide tolerant crops), decreased dependency on conventional insecticides and herbicides, higher yields and cleaner and higher grade of grain/end product.

As expected, net economic returns to the grower vary by year, by crop product and by location, depending on factors such as level of infestation of the targeted pest, the epidemic level of a disease or the weed density. For the USA in 1996, economic benefits to growers from the following transgenic crops were estimated conservatively at $128 million for *Bt* cotton, $19 million for *Bt* corn, and $12 million for herbicide tolerant soybean, for a collective national benefit of $159 million. Similarly, in 1997, economic benefits were estimated at $119 million for *Bt* corn, $109 million for herbicide tolerant soybean, $133 million for *Bt* cotton, and $5 million for herbicide tolerant cotton and <$1 million for *Bt* potato, for a collective national benefit in the USA of $366 million. In Canada, benefits at a national level, due to the use of herbicide tolerant canola, were estimated at $5 million in 1996, and $48 million in 1997, plus $5 million for *Bt* corn for a total of $53 million. Thus, in 1996 and 1997, selected transgenic crops in the USA and Canada resulted in economic benefits to growers, conservatively estimated at $583 million.

Current and Future Global Markets

Global sales of transgenic crop products have grown rapidly during the period 1995 to 1998 (Table 6).

Table 6. Estimated value of global transgenic crop market, 1995 to 1998 (US $ millions)

Year	Market value $	Increase $	Increase %
1995	75[1]		
1996	235[1]	160	+ 213
1997	670[1]	435	+ 185
1998	1,200 to 1,500[2]	530 - 830	+ 79 to 124

[1] Wood Mackenzie 1998 (Personal Communications). [2] Projection by Clive James
Source: Compiled by Clive James, 1998.

Global sales from transgenic crops were estimated at $75 million in 1995; sales tripled in 1996 and again in 1997 to reach $235 million and $670 million respectively, and doubled in 1998 to reach an estimated value of between $1.2 to $1.5 billion. Thus, revenues for transgenic crops have increased by approximately twenty fold in the four year period 1995 to 1998. The global market for transgenic crops is projected to increase to $3 billion or more in 2000, to $8 billion in 2005, and to $25 billion in 2010.

The number of countries growing transgenic crops has increased from 1 in 1992, to 6 in 1996, to 9 in 1998, and is expected to continue to grow to the year 2000 and beyond. In 1999, countries in North and Latin America already growing transgenic crops are expected to significantly expand the area of current products and also to introduce new single and multiple trait products and Brazil will probably grow transgenic crops for the first time in 1999. Similarly, China is expected to expand its transgenic crop area aggressively, with growth and diversification continuing in Australia and South Africa. Whereas public acceptance, including labeling of foods derived from genetically modified plants, will continue to be dominant issues that will impact on adoption of transgenic crops in countries of the European Union, the initial approval of several products in 1998 was encouraging. However, more recent developments have delayed plans for early expansion. India and several countries in Eastern Europe have transgenic crops that are ready for commercialization. As expansion of transgenic crops continues, a shift will occur from the current generation of "input" agronomic traits to the next generation of "output" quality traits, which will result in improved and specialized nutritional food and feed products that will satisfy a high-value-added market; this will significantly affect the value of the global transgenic crop market and also broaden the beneficiary profile from growers and consumers to food, feed and fiber processors.

Consolidation in the Agri-biotech Industry

Biotechnology-driven consolidations in the form of acquisitions, mergers and alliances continue to be a dominant feature of the biotechnology industry. In the last three years alone, corporations commercializing transgenic crops and involved with seeds, agricultural chemicals, and the life sciences have been engaged in more than 25 major acquisitions and alliances valued at >$15 billion. This consolidation is expected to continue. Genomics is pivotal to the growth of the industry and is catalyzing a new generation of alliances, acquisitions and mergers.

Global Food Security

Transgenic crops are proprietary, developed almost exclusively by the private sector in the industrial countries, with the majority of the global transgenic crop area to-date grown in countries of the North. However, it is important to note that developing countries such as China played a pioneering role by being the first country to introduce a commercialized transgenic crop in the early 1990s.Argentina is a global leader in the accelerated adoption of transgenic crops with significant expansion imminent in Mexico and South Africa.

Given that the food gap of many developing countries, including China, is expected to more than double in the next 25 years and that some developing countries like Argentina can meet some of those needs through exports, the long term potential and importance of transgenic crops for developing countries is evident. There are three considerations that underpin the strategic importance of transgenic crops for developing countries.

Firstly, developing countries have potentially more to gain from transgenic crops than industrial countries because the area of almost all crops is far greater in developing countries than in the USA and Canada where adoption has been highest to date. Secondly, yields of almost all crops are significantly lower in developing than industrial countries. Yields are low in developing countries for many reasons but one of the principal causes is that crops in developing countries suffer much more from biotic stresses, due to pests, weeds and diseases, for which current transgenic crops already offer improved protection. Thirdly, and most importantly, it is in the developing countries, not the industrial countries, where 800 million people suffer from malnutrition today and where transgenic crops could increase crop productivity and contribute to the alleviation of hunger and poverty which are inextricably linked. Transgenic crops have much to offer developing countries and should be an essential component of a global food security strategy that integrates conventional and biotechnology crop improvement applications to produce more food where the need is greatest, and where the welfare value of food is the highest.

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Can the Biotechnology Revolution Feed the World?

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Introduction

For many years we have been told that biotechnology has an important role to play in 'feeding the world'. In many industry internet homepages and brochures, the life science industry shows its conviction that biotechnology is indispensable in view of some worrying demographic tendencies. At present around 800 million people, equivalent to 15 per cent of the world population, are chronically undernourished, and their number is increasing rapidly. Since a significant expansion of cultivated area is not feasible in most parts of the world, increased food supply must be achieved by a more efficient use of land already under cultivation. This requires new crop plants that can be designed by genetic engineering (GE) techniques.

However, now that the first products are entering farmers' fields and the international market, public opinion in various parts of the world seems all but favourable to the new scientific achievements. In Europe, one of the principle markets for biotechnology products, the entire population seems to have lost its appetite for 'Frankenstein Food'. Some supermarket chains are removing GE food from their shelves, while Unilever and Nestlé are removing GE ingredients from their product lines, at least in several EU states. In June 1999, the EU Commission decided not to authorize any new GE organisms in Europe until the introduction of strict environmental standards (Business Week, April 12 1999; The Economist, June 19 1999). The discontent with biotechnology in Europe does not seem an isolated case. Also in Japan and the USA, consumers have begun to express their concerns about GE food and to question the production of GE crops.

In a recent wave of articles and presentations, scientists, aid officials, and government members criticize the social opposition to biotechnology (see e.g. Glickman 1999; Mann 1999; Sachs 1999; Nairobi Daily Nation, Kenya, July 15, 1999; Deccan Herald, Bangalore, India, July 6, 1999). They point out the critical need for new pest and climate resistant plants, for which genetic engineering is required. It is argued that the affluence in industrialized countries enables its consumers to be choosy. The protests, however, would slow down technological development and consequently deprive the world's poorest population from adequate nutrition.

Whatever is true of this argument, it seems to disregard the controversy over biotechnology in many developing countries itself. For years, the life science transnational corporations (TNCs) have been subjected to criticism, as they would monopolize food supply, steal plants from the Third World, and privatize what used to be commonly owned by claiming patent protection for plants and animals. This controversy is less related to the safety of the technology itself, but rather to the social changes in crop development and agriculture that

have been accompanying the emergence of biotechnology. Its impact on the development and acceptance of new biotechniques may be considerable. We will discuss the social implications of the rise of biotechnology in view of (a) the relations within the crop development industry, and (b) subsistence farmers in developing countries.

Changing relations in the crop development industry

Ever since the mid nineteenth century, when governmental agencies in all major capitalist countries began to intervene in seed supply, the public sector has played a pivotal role in the breeding and releasing of new plant varieties. This picture began to change in the 1980s, when, for a number of reasons, advances in genetic engineering attracted private sector interest in crop development. Firstly, new techniques made it possible to pass on characteristics to the progeny under controlled, industrial circumstances. These techniques are considered to be time saving and more accurate than conventional breeding, and they also enable crossing between different species. Secondly, biotechnology offered the prospect of finding solutions to some serious problems in Western agriculture: the heavy reliance on oil-derived and contaminating agro-chemicals, and the need to find new outlets for agricultural surpluses in the major OECD countries. Finally, the expanding world population promised an emerging market for seed of food crops, especially in developing countries (Pistorius and Van Wijk, 1999).

Agro-industry stepped up its research investment in the 1980s by an annual growth rate of over 5 per cent. Private agricultural R&D expenditure in OECD countries increased from US$ 3.9 billion in 1981 to more than US$ 7 billion in 1993, almost equaling total public R&D expenditure on agriculture (Alston et al., 1998). The majority of biotechnology investments are made by a small number of life science TNCs. Monsanto, for example, spent US$ 1.2 billion on R&D in 1998 (www.monsanto.com), while Novartis, in the same year, announced the establishment of the Novartis Agricultural Discovery Institute, which is dedicated to genomics studies, at a cost of US$ 600 million (AgBiotech reporter, August 1998). The private investment contrasts sharply with the public spending on biotechnology, especially in view of developing country agriculture. The largest organization in this respect, the Consultative Group on International Agricultural Research (CGIAR), has a total annual budget of around US$ 300 million, of which only 15 per cent is allocated to biotechnology.

The increased private investment in biotechnology is changing the global organization of crop development considerably. Pharmaceutical, chemical and food processing TNCs are becoming key developers of the technology that is crucial for plant breeding. Through acquisitions, mergers and various forms of alliances, they become the steering forces in what can be called 'crop development conglomerates': industrial networks in which life science TNCs, plant breeding firms, new biotechnology firms, genomics firms, and software companies are integrated.

The emerging new organization of crop development has contributed considerably to the controversy over biotechnology. Worldwide, small and medium-sized seed companies, as well as farm-oriented NGOs, have questioned the increasingly oligopolistic structure of the crop development industry. They dislike the prospect of six to eight Western industrial conglomerates that make key decisions about the research directions in crop development. They oppose even more the policy of the leading TNCs to claim patent protection for whole

plant varieties. Patenting plants eliminates the fundament of the breeding industry's traditional innovation strategy that allowed the free use of commercial varieties for breeding purposes.

What is at stake in this controversy is not plant patents or biotechnology as such, but the question of the optimal organization of crop development worldwide. Due to industrial concentration, the world's decentralized crop development system, consisting of manifold local breeding companies and institutes, is slowly being replaced by a more centrally organized crop development system. The relevant question is now whether the hundreds of millions of undernourished people in the world will benefit from a small number of industrial conglomerates that 'feed the world', or that food production is better off by decentralized breeding efforts.

As data about welfare effects of the organization of crop development are hardly available, our answer can only be explorative. Advantages can be attributed both to centralized and decentralized systems. The situation in which large corporations make most investment in biotechnology may be beneficial in that economies of scale in R&D can be achieved. Because of their strong financial capacity and the partial integration of pharmaceutical, bio-chemical, and agricultural research through genetics, the crop development conglomerates have the capacity to design research programs that are beyond the reach of any small or medium-sized seed company or institute. On the other hand, a decentralized crop development industry has the advantage that it can better take into account the countless local differences in ecosystem, culture, farm capacity, and farmer and consumer preferences throughout the world. In fact, by sustaining decentralized breeding capacity with a large number of local small and medium-sized companies, agriculture would continue to benefit from the combined knowledge and innovative spirit of thousands of plant breeders. Their varieties may also be biologically more diverse, at the varietal as well as the genetic level, than those bred in a centralized system.

Obviously, the organization of the crop development industry will never be either completely central or decentralized, but rather will be subject to tendencies in both directions. The question as to which direction is to be preferred is, nevertheless, relevant, because the answer will hold the clue for several controversial regulatory issues that affect the acceptance of biotechnology. If dominance of life science TNCs were considered necessary to optimize crop development, the negative association of these corporations as monopolists in agro-food production would diminish. The TNCs efforts to recoup their massive investment by broad patent claims of plants and their progeny would also be more justifiable. As a consequence, the small and medium-size seed companies, as well as the public breeding institutes, would become less important for breeding. Their main job would be to test and adapt the (foreign) transgenic varieties, distribute them, and assist farmers to grow them.

If, in contrast, a decentralized industry is to be preferred, the regulatory measures should differ accordingly. In this case, local seed companies and institutes (in both developing and industrialized countries), NGOs, and farmer-breeders should be supported as core actors in breeding. Science, including the scientific departments of the life science TNCs, should be responsive to the manifold local and specific breeding activities in the various parts of the world. Commercial plant varieties should be considered as a common pool, freely available for breeding purposes. Patent protection for genetic information should not exceed the level of the specific techniques or modified genetic information.

In short, the rejection of biotechnology can partly be explained as a projection of discontent with changing relations between the old and new actors in the crop development industry. The

issue of the optimal organization of crop development should therefore be addressed, rather than issues concerning (the regulation of) the technology itself.

Rejection of a further industrialization of agriculture

Another explanation for the opposition to biotechnology can be found in the rejection of the process of agro-industrialization by subsistence farmers in developing countries. The industrialization of agriculture involves a long-term process that started in the nineteenth century. Due to the inability to eliminate the risks and discontinuities intrinsic to a natural or biological reproduction process, an integral transformation of craftwork into an industrial production process failed to occur. Family farms remained the central engines of agro-food production. Private industry involvement in agriculture took place when companies began to invest in discrete elements of farming. They transformed these elements into industrial activities and re-introduced them into agriculture as inputs (Goodman et al., 1987). Horses were replaced by tractors, manure by synthetic fertilizer, landraces by high yielding varieties, and on-farm saved seed by commercial seed. Agro-industrialization has expanded agro-food production worldwide, but also reorganized the organization of production. Ever fewer farmers produced ever more food. In most industrialized countries the rural population decreased dramatically after the Second World War, when superfluous farmers found their way to the expanding industry in urban areas.

A similar route has been followed by most developing countries, but often with a different outcome. The rationalization of farming inevitably results in many small farms being did not consider as non-viable production units, in which neither the government nor established farm unions wish to invest any longer. Since alternative (urban industrial) employment opportunities are not available, and since social security schemes are insufficient - if they exist at all - superfluous farmers are left to fend for themselves. It is this process that is at stake when biotechnology is introduced in developing countries. Biotechnology heralds a new phase in agro-industrialization, which implies the marginalization of a considerable part of the rural population.

Not surprisingly, developing countries can be strongly divided over the issue of biotechnology. Interviews we conducted some time ago in Chile and Colombia indicated that advocates of a further industrialization of the agro-food sector, such as the fruit and cut flower exporters, and the producers of commercial national crops, welcomed biotechnology and accepted intellectual property rights (IPR) for plant material (Pistorius and Van Wijk, 1999). In order to remain competitive on foreign, and ever more open national markets, it is imperative for these commercial producers to grow highly productive varieties (including transgenic varieties) that meet industrial processing requirements, international quality standards, and the preferences of both domestic and foreign consumers.

Opposition to biotechnology was voiced particularly by nature and farm-oriented NGOs and indigenous people's organizations. These organizations act as a last resort for all those farming and indigenous communities that have little to gain from biotechnology and concentration in the crop development industry. They simply lack the land, appropriate soil, capital, markets, and infrastructure. Subsistence farmers focus not on commercial agriculture, but *necessarily* on the maintenance and improvement of their small-scale farming systems. Their strategy aims at more farmer autonomy in agricultural production by avoiding dependence on the use of commercial, industrial inputs. They use on-farm saved seeds and other locally available natural

resources, and a large diversity of native varieties, adapted to specific local needs and tastes. The reason that the communities and NGOs have turned against biotechnology and the life science TNCs is because these are considered as exponents of industrialized, export-oriented agriculture from which they have little to expect. For this reason they oppose the introduction of transgene varieties, the efforts to prevent on-farm propagation of commercial plant varieties by means of IPR, hybrid varieties, or 'terminator' technology, as well as the free collection of plants and seeds from their territory by foreign institutions. In other words, the reason that biotechnology is being challenged in developing countries seems related to the marginalization of farmers, rather than a dislike for advanced technology itself.

Discussion: Challenges for the life science industry

In a recent address the US Secretary of Agriculture Glickman was exploring ways that scientists, farmers, and consumers would "learn to love biotechnology" (Glickman, 1999). In a world that is increasingly hostile to genetic engineering his effort seems a "mission impossible". What is clear, however, is that the life science industry has lost contact with many stakeholders. The agro-food sector is not a mere suppliers market. The enterprises must show corporate citizenship and earn a "licence to operate" in society. In view of the industry's goal to feed the world, a number of policy measures could be considered:

Examine the changing organization of crop development

It questionable whether the present concentration tendency in the crop development industry automatically leads to an optimal structure required for increasing the world's food production. The pros and cons of this concentration need to be closely examined, especially in view of the world's poorest population. Such research should guide governments and private enterprises in their policies regarding public agricultural research, and protection of plant-related IPR.

Support research for non-industrial agriculture

The overemphasis on genomics and genetic engineering carries the risk of underfunding other approaches to crop development. Scientific support, particularly for the non-industrial production strategies of subsistence farmers, seems to have a low priority. The low-cost efforts to improve the efficiency of traditional varieties, on-farm saved seed, and organic fertilizer contrast sharply with the fascinating new technological achievements, and they are consequently perceived as backward forms of production that should already have been replaced. Nevertheless, improvement of the non-industrial approach deserves to be considered as a goal in itself by both governments and industry, because it is the *inevitable* survival strategy of the poorest category of farmers.

Lift restrictions on the diffusion of new varieties for non-industrial farming

It is not obvious what benefits can be attributed to restrictions in using plant varieties for propagation or plant breeding in respect of non-industrial agriculture. Does the creation of new hybrids and 'terminator' varieties, and patenting of plant-related inventions help to expand agro-food production of under-nourished people? It seems more obvious in this respect to exempt public research institutions from legal restrictions in the use of protected technology and genetic information, while subsistence farmers should be enabled and allowed to propagate new varieties on-farm.

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Plant Genetic Engineering: Towards the Third Millennium
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Biotechnology Can Help Crop Production to Feed an Increasing World Population-Positive and Negative Aspects Need to be Balanced: A Perspective from FAO

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Fifteen years ago, plant biotechnology comprised only a few applications of tissue culture, recombinant DNA technology and monoclonal antibodies. Today, transformation, and marker-aided selection and breeding are just a few of the examples of the applications of biotechnology in crop improvement with profound implications for a region as Latin America and The Caribbean (LAC) with increasing demands in terms of food security, socio-economic development as well as for the conservation and sustainable use of plant genetic resources basic inputs to sustain the future agriculture of the Region. The end of a century, or, as now, of a millennium, offers a unique and "magic" time to reflect on any particular human activity. In the case of plant biotechnology, to examine past occurrences and learn lessons that could help in the further development of an appropriate biotechnology ensuing from it, is needed considering the social actors, market forces, user demands, and public views to address basic and strategic research issues.

Being the author of this article, a classical plant physiologist and breeder, that have had the opportunity of serving as the technical secretary of an extensive plant biotechnology network (REDBIO/FAO) from his on- going technical duties at the LAC FAO'Regional Office for the past 15 years, the motivation behind black dots is to discuss, learn, share and accept innovative views and concepts that would surely enhance the appreciation for this technology to improve sustainable crop production.

The Food Security Context

Agriculture is expected to feed an increasing human population, forecast to reach 8 000 million by 2020, of whom 6 700 million will be in the developing countries. Although the rate of population growth is steadily decreasing, the increase in absolute numbers of people to be fed may be such that the carrying capacity of agricultural lands could soon be reached given current technology (FAO, 1999). New technologies, such as biotechnologies, if properly focused, offer a responsible way to enhance agricultural productivity for now and the future. Hence as suggested by the Nobel Peace Laureate, Norman Bourlag, (1997), new biotechniques in addition to conventional plant breeding are needed to boost yields of the crops that feed the world.

Classic methods of crop improvement and other selection methods and advances in plant breeding led, during the mid sixties and seventies, to the "Green Revolution" in cereal production, which accounts today for more than 50 % of the total energy intake of the world's

poor. In the last decade, 370 kg of cereals per person were harvested as compared to only 275 kg in the 1950s; i.e., in excess of 33 % per capita gain (FAO, 1995). There are 150 million fewer hungry people in the world today than 40 years ago, though there are twice as many human beings. Despite this splendid progress in crop productivity, even greater progress must be made in order to feed an additional two billion people by the early part of the 21st century (Anderson 1996), to cope with food insecurity, and to promote income and employment generation.

In the case of the Latin American Region, in spite of abundance of natural resources and continued investments on development from the LAC countries, rural poverty has persistently affected more than 55 percent of the rural population with one third of the latter being below the extreme poverty line which means an income below a basic food basket. Furthermore in six out of eleven countries for which there was information for several years, extreme poverty showed relative increases between the beginning of the eighties and the mid nineties. This establishes the magnitude of the poverty and food insecurity problems to which research in agricultural production, by the application and complement of plant biotechnology, has to find a way to contribute to its alleviation (Izquierdo and de la Riva, 1998).

However, whether any benefits of current agriculture biotechnology research will actually reach poorer farmers and consumers without major public sector intervention is an open question. Resource poor farmers, by definition, are unlikely to have easy financial access to agricultural inputs such as improved seeds, pesticides, fertilizers or irrigation. Considering that over 70 % of the people in developing countries living below the poverty line are women, the majority of whom live in rural areas (UNDP, 1998), science alone is unlikely to provide a "technical fix" for alleviating such poverty. There are many processes, factors and socio-economic structures underlying rural peoples poverty such as; lack of access to land and other productive resources, low purchasing power, political powerlessness, fragile environments, peripherally from markets, etc. Biotechnology must be integrated to serve as complement to agricultural technology leading to better seeds and improved sustainable production.

Biotechnology is taken by FAO to cover the application of tissue culture, immunological techniques, molecular genetics and recombinant DNA techniques in all facets of agricultural production and agro-industry.

Struggling with this complexity, plant biotechnology is but one factor, which could have differential impacts on rural poverty. Indeed, the potential contribution of biotechnology to developing country agriculture or to poverty alleviation is considered to have been overstated, in the short term at least. Yet over the longer term there is little doubt that some biotechnological approaches to crop improvement could generate social, economic and environmental benefits if specifically targeted at specific needs, especially those of poorer groups. Such needs might for instance include the reduction in pesticide use via insect/disease resistant crops; improved nutritional/medicinal composition of crops; elimination of toxic substances or allergens; developing early maturing varieties; hybrid vigour fixation through apomixis; reducing post-harvest storage losses; salinity, aluminium, drought, heat and cold abiotic stress tolerant crops, and/or reducing labour demands at appropriate times during the cropping cycle. But, considering the many resource poor farmers in developing countries as those in Latin America, who depend on an income of less than a USD 1 per day, they are not likely to be a near term target market for most of the bio-agricultural companies. If agriculture research, including biotechnology, is to be better targeted to addressing the needs of poorer

farmers, it will be necessary for relevant public sector institutions to more transparently identify which farmers needs and products are of concern to their research and effectively face the challenge (FAO, 1999b).

Opportunities and Scenarios for Plant Biotechnology Applications

Plant biotechnology represent one of a number of competing technological approaches to addressing a particular agronomic problem but however, as an example, a particular pest problem might equally be addressed through conventional plant breeding, through a transgenic approach, or through an integrated crop management (ICM) approach or any combination of these.

Andean scenarios in countries of Bolivia, Chile, Peru, Ecuador and Venezuela, for instance, are depositories of important genetic resources that could constitute inputs to food security and sustainable development. Andean "orphan" food crops as arracacha *(Arracacia xanthorrhiza),* achira (*Canna edulis*), yacon (*Polymnia sonchifolia*), mashua (*Tropaeolum tuberosum*), oca (*Oxalis tuberosa*), ulluco (*Ullucus tuberosus*), quinoa (*Chenopodium quinoa*), amaranto o kiwicha (*Amaranthus caudatus*), popping beans -"nuñas" (*Phaseolus vulgaris*), tarwi (*Lupinus mutabilis*), goldenberry-"capuli" (*Physalis peruviana*), cherimoya (*Annona cherimola*) and passion -fruit (*Passiflora sp.*) are highly under-utilised and face the need to strengthen the use of modern plant biotechnology for the conservation and sustainable agricultural use of those essential genetic resources. For these purposes, biotechnologies as cell and tissue culture, molecular genome analysis, plant genetic transformation, molecular plant disease diagnosis and germplasm cryo-conservation coupled with plant breeding and physiological integrated crop management, can be successfully used to cope genetic erosion, to reinforce *ex-situ* collections and in *in-situ* conservation, to upgrade the supply of improved and healthy seed and planting materials to farmers and to integrate a new approach into the development programmes for food production and food security in mountain lands (Izquierdo and Roca, 1998). In the meantime, populations of Andes suffer severely the effects of poverty, food insecurity, malnutrition and the health problems derived from it.

The Amazon Basin, is one of the richest areas in the world. The high vegetal and animal biodiversity, gives this area a real potential to be sustainable in terms of food security, nutrition, and economy. However, Amazon area in all the countries where this very especial humid forest exist, has security problems, under nutrition and bad-nutrition problem, as well as poverty is increasing, specially in areas near the biggest cities of the Amazon. The biggest problem in the Amazon has been the colonisation path. Countries in the Amazon Area have a very intense migration dynamics and have been penetrated with culture systems, especially animal production, that have caused severe impact in this very fragile bio-system. The management of Amazon soils, their poor structure, low fertility and acidity, is one of the most important subject to be studied and this is the principal dedication of several R&D institutions in Amazon Countries. However, this is not the only subject to focalise the research in the Amazon. There are several crucial other items that have to be included in the production system to sustainable convert the production from just harvesting ("extractivism") to a technical production, where all the agronomic productivity factors are considered. Plant biotechnology applications for sustainable fruit, vegetable, medicinal, and forestry production considering micropropagation and biotechnology-enhanced breeding are some of the subject that today have to be reinforced.

Biotechnology is a powerful tool in agricultural development with great potential - both positive and negative. Coupled with other technologies, biotechnology could surely provide new solutions for some of the old problems hindering sustainable rural development and achievement of food security. Biotechnology also offers unique opportunities to solve environmental problems, some of which derive from unsustainable agricultural and industrial practices. Plant biotechnology requires both human resources ad infrastructure. The Technical Co-operation Network on Plant Biotechnology (REDBIO/FAO) for the Latin American Countries, sponsored by FAO since 1991, have detected for this region technical constrains in terms of scarcity of human resources, limitation in technology development and use, infrastructure and lack of information/data banks on plant biotechnology.

FAO recognises the need to take a balanced and comprehensive approach to biotechnological development by considering its integration into various areas of the Organization's work programme. Since the scope for potential action is huge, while FAO resources are limited, it is important that close partnerships with other bodies be forged, including the CGIAR.

Tissue culture was developed in the 1950s and became popular in the 1960s. Today, micropropagation and *in vitro* conservation are standard techniques in most important crops, especially those with vegetative propagation. Tissue culture is seen as a main technology for developing countries for the production of disease-free, high-quality planting material. In commercial applications, such as fruit-export production and floriculture, it also generates much-needed employment, particularly for women. Tissue culture includes micropropagation; embryo rescue; plant regeneration from callus and cell suspension; and protoplast, anther and microspore culture. These techniques are being used particularly for large-scale plant multiplication. Micropropagation has proved especially useful in producing high quality, disease-free planting material of a wide range of crops.

DNA-based techniques include isolation, amplification, modification and recombination of DNA; genetic engineering to obtain Genetically Modified Organisms (GMOs); use of markers and probes in gene mapping and in functional and structural genomics; and unambiguous identification of genotypes through DNA fingerprinting. Recombinant DNA techniques are used for the production of transgenic individuals, which involves isolation, cloning, recombination and reinsertion of genetic material by various techniques. Several transgenic cultivars of major food crops have been released incorporating genes for resistance to herbicides and insects. The area planted with transgenic crops went from 2.8 million ha in 1996 to 28 million ha in 1999 (FAO, 1999). In 1996, there were about 3 million ha of transgenic crops grown in the world (mainly in North America) whereas in excess of 34 million ha (a 12-fold addition) of transgenic crops will be harvested this year in North America, Argentina, China, and South Africa among other countries. Argentina is the leading developing country with an excess of 4 million ha of transgenic herbicide-resistant soybean. There are 4.4 million ha of transgenic corn (14 % of total acreage), 5 million ha of transgenic soybean (20 %), and 1.6 million ha of transgenic canola (42 %) grown only in North America (Moore, 1998). It has been calculated that in 1998 US farmers are growing over 50 % of their cotton fields with transgenic seeds, the largest percentage for any crop ever. Trees are the next target in the agenda of genetic engineering (Ortiz, 1998). Selection of biotechniques as well as a realistic assessment of their potential in crop improvement are needed to avoid not only the criticism of the anti-science lobbyists but also the permanent distrust of pragmatic traditional

breeders (Ortiz, 1998). Plant biotechnology has been regarded as a priority area for technology transfer, because genetically modified food, feed, and fibre are of vital concern to the developing world (Ives and Bedford, 1998; Alman, 1999).

The Plant Biotechnology - Plant Breeding Bridge

Achievements today in plant biotechnology have already surpassed all previous expectations, and the future is even more promising. The full realisation of the agricultural biotechnology revolution depends on both continued successful and innovative research and development activities and on a favourable regulatory climate and public acceptance. Biotechnology should be fully integrated with classical physiology and breeding: (1) as an aid to classical breeding, (2) for generation of engineered organisms, (3) for integration of micro-organisms into agricultural production systems. Biotechnology will, by the production of genetic modified plants, impact the agricultural and plant scene in three major areas: (1) growth and development control (vegetative, generative and reproduction/propagation), (2) protecting plants against the ever-increasing threats of abiotic and biotic stress, (3) expanding the horizons by producing specialty foods, biochemicals and pharmaceuticals (Alman, 1998). DNA-based molecular markers in various forms can be used to construct linkage maps of different species so as to locate particular genes. The mapped markers are used for speeding up selection in conventional breeding procedures. Allozymes were available as the first biochemical genetic markers in the 1960s. Population geneticists took advantage of such marker system for their early research. Since then, marker-aided analysis based on PCR has become routine in plant genetic research and marker systems have shown their potential in plant breeding. Furthermore, new single nucleotide polymorphic markers based on high density DNA arrays, a technique known as 'gene chips', have recently been developed. With 'gene chips', DNA belonging to thousand of genes can be arranged in small matrices (or chips) and probed with labelled cDNA from a tissue of choice.

Plant breeders will change their modus operandi with the development of objective marker-assisted introgression and selection methods. Backcross breeding will be shortened by eliminating undesired chromosome segments (also known as linkage drags) of the donor parent or selecting for more chromosome regions of the recurrent parent. Parents of elite crosses may be chosen based on a combination of DNA markers and phenotypic assessment in a selection index, such as best linear unbiased predictors. To achieve success in these endeavours, cheap, easy, decentralised, and rapid diagnostic marker procedures are required (Ortiz, 1998). In this way, plant breeders can develop new cultivars with the appropriate genes that improve fitness of the promising selections. Fine-tuning plant responses to distinct environments may enhance crop productivity. Development of cultivars with a wide range of adaptation will allow farming in marginal lands. Likewise, research advances in gene regulation, especially those processes concerning plant development patterns, will help breeders to fit genotypes in specific environments. Photoperiod insensitivity, flowering initiation, vernalization, cold acclimation, heat tolerance, host response to parasites and predators, are some of the characteristics in which advanced knowledge may be acquired by combining molecular biology, plant physiology and anatomy, crop protection, and genomics. Multidisciplinary co-operation among researchers will provide the required holistic approach to facilitate research progress in these subjects.

Plant Genomics

This new term, defined by the development of biotechnology, refers to the investigations of whole genomes by integrating genetics with informatics and automated systems. Genomic research aims to elucidate the structure, function and evolution of past and present genomes. Some of the most dynamic fields concerning agriculture are the sequencing of plant genomes, comparative mapping across species with genetic markers, and objective assisted breeding after identifying candidate genes or chromosome regions for further manipulations. As a result of genomics, the concept of gene pools has been enlarged to include transgenes and native exotic gene pools that are becoming available through comparative analysis of plant biological repertoires (Lee, 1998). Understanding the biological traits of one species may enhance the ability to achieve high productivity or better product quality in another organism.

Farming in Environmentally Friendly Systems

The aims of applied plant science research for agriculture are to enhance crop yields, improve food quality, and preserve the environment where human beings and other organisms live. The best way for conservation of plant biodiversity and its environment would be to achieve high crop productivity per unit area. There has been a significant yield improvement owing to enhanced crop husbandry, but in the next years progress will be achieved by changing plants that could be more suitable to sustainable and environmentally friendly farming systems. Agro-chemical corporations are developing pest and disease resistant transgenic crops to avoid pollution with pesticides in the farming system. Furthermore, food quality will become more important than crop productivity in a wealthy society. Consumers will prefer transgenic crops if they have the desired characteristics (Ortiz, 1998). In the next decades meiotic-based breeding will still generate cultivars for farmers. Genetic improvement through biotechnology needs conventional breeding because (1) the elite cultivars will be the parents of the next generation of improved genotypes, (2) field testing across locations or cropping systems and over years will be needed to determine the best selections due to the genotype-by-environment interaction. Indeed, genetic engineering may provide a means to add value by introducing synthetic or natural genes that enhance crop quality and yield, as well as protect the plant against pest and diseases. Farmers will pay more for transgenic crop propagules if they obtain extra-income after adopting biotech-derived products. For example, seeds of insect resistant transgenic crops will be more expensive than those of available cultivars but the farmer will not need to apply pesticides in their transgenic fields. Of course, patents make transgenic seeds more expensive but also farmer's benefits may be higher.

Farmer-ceuticals and Nutra-ceuticals

Growth of cities in the developed world has already replaced farmland with shopping malls, parking lots, and housing developments. Peri-urban agriculture and home gardening are also becoming very important for national food security in the developing world as a result of rapid urban expansion. Hence, new cultivars will be needed to fit into intensive production systems, which may provide the food required satisfying urban world demands of the next century. Specific plant architecture, tolerance to urban pollution, efficient nutrient uptake, and crop acclimatisation to new substrates for growing are, among others, the plant characteristics required for this kind of agriculture. Genes controlling these characteristics may be available in gene banks for further cross breeding, which can be assisted by genomics. Peri-urban and

home garden "farmers" will have to adapt to new demands from emerging urban populations with higher income. These consumers may request a more varied diet. For example, food crops with low fats, and high in specific amino acids may be needed to satisfy people who wish to change their eating habits. If genes controlling these characteristics do not exist in a specific crop pool they may be incorporated into the breeding pool using transgenics.

Often plants provide the raw materials for agro-industry, and not only for food or fibre processing. Active ingredients of plants have been transformed into commercial products such as medicines, solvents, dyes, and non-cooking oils for many years. Hence, it would not be surprising to see, in few years from now, entire farms without food crops but growing transgenic plants to produce new products, e.g. edible plastic from peas or plant oils to manufacture hydraulic fluids and nylon (Grace, 1997). This new rural activity may result in important changes in the national economic sector.

For example, oral vaccines appear to be a convenient delivery system for vaccination throughout the world and it has been recently demonstrated the ability of transgenic food crops to induce protective immunity in mice against a bacterial enterotoxin such as cholera toxin B subunit pentamer with affinity for GMI-ganglioside (Arakawa et al., 1998).

Public Perception and Risk Assessment of Transgenic Crops

The advances in plant transgenics and genomics described above have not been isolated from society. Some of these achievements have been acclaimed by end-users whereas other accomplishments, e.g. release of genetically modified organisms (GMO), are being attacked, not only in words but also in deeds, by political activists. Some of these educated middle-class campaigners are expressing in this way their rampant "eco-paranoia", while others hide their real agenda to manipulate the fashionable ecological movement. This controversy has attracted the attention of non-scientific partisans to each side. There have been negative comments about transgenic plants by a crown prince and contrasting positive comments by a former president, both of whom may not have the required technical knowledge to assess the potential of biotechnology for crop improvement. Irrespective of this ideological dispute and ensuing democratic disagreements, biotechnology products will be accepted by people who support scientific-based progress, in a similar way that new cultivars or innovative crop husbandry techniques have previously become integral parts of farming systems elsewhere. However, without end-user's consent, the impact of a new technology in the society will be small or nil.

Lack of scientific data, non-scientific partisan views, uncertainty of potential risks, and ignorance confounds rational discussion concerning the release of GMO. The issue of releasing genetically modified plants (GMP) into the farming system has become particularly agitated by lobbyist groups in Europe despite widespread cultivation of such crops in North America and elsewhere. Scientists must realise that the general public is concerned that an incautious approach to the manipulation and cultivation of transgenic crops may affect biodiversity and its sustainable utilisation in the farming system, e.g. loss of variability and viability. People also want that their views about applications of biotechnology for improving agriculture are listened irrespective of their knowledge in the subject. Moreover, farmers are afraid that negative propaganda jeopardises the public image of their products. Scientists and policy markers should not forget that people's acceptability is the most important component of the general public assessment of risk, which includes both uncertainty and negative

consequences. This acceptability depends on cultural factors because people's views change according to time and location.

The process of risk assessment in agro-chemical consists of (i) hazard identification, (ii) exposure assessment, (iii) effect's management, (iv) risk characterisation, and (v) risk management. However, transgenic crops may be able to invade (or colonise) and multiply in many habitats. Hence, this risk assessment of a genetically modified living organism (also known as GMLO) must consider other characteristics not included when assessing the release of non-living compounds to the environment, e.g. horizontal gene transfer between transgenic crops and wild related species. Scientific risk assessment of transgenic crops must be strictly performed and precautionary principles should be considered in the decision making process. In the industrialised world, this precautionary principle is a key component of the response to the unforeseen (and sometimes irreversible) human and environmental impact, which may occur by introducing into the system new advances ensuing from research and technology development.

In Norway, a unique legislation advocates that "the production and use of GMO should be ethically and socially justifiable in accordance with the principle of sustainable development" as well as "safe to humans and to the environment". By applying this framework, marketing applications of GMO could be rejected if insufficient documentation regarding ecological and heath aspects was submitted by the producer. What are the potential ecological risks associated with the release of GMP into the farming system?. These are of course a very large number of potential risks, However, perhaps the two most important risks are: (i) GMP establishes in semi- or natural habitats, and (ii) inserted transgenes incorporate into other species, thereby affecting non-target organisms in farms or natural habitats.

Hierarchical test protocols have been proposed to assess the risks of releasing GMP. Such protocols require knowledge about evolutionary history, morphology, life-history characteristics, pollination or breeding system, gene-transfer likelihood, natural hybridisation, recruitment and vegetative propagation of a chosen species. Likewise, producers should provide, to facilitate this risk assessment, additional information regarding biochemical, physiological, and morphological changes owing to inserted gene(s), along with a list and description of marker and reporter genes included in the transgenic plant. It would also be important to add details concerning when and in which plant tissues or organs will be expressed the modified function or phenotype. Nonetheless, people must also know that scientists assessing risks of transgenic crops may extrapolate the outcome or results from simple short-time experiments into complex long-term natural- or farming systems. Investigations about gene flow and competing ability of transgenic crops may be easily addressed through short-term experiments. However, the assessment of the environmental impact of GMP requires a long-term, expensive, holistic research. Computer modelling, which integrates knowledge about gene flow, competing ability, spread of transgenes to weedy species, and cultural practices in the farming system, may provide an alternative means for long-term risk assessment of releasing GMP into the environment.

Consumer concern about transgenic crops also focuses on their safety as food, especially if modifications could influence their metabolism or health. In this regard, transgenic plants without selectable markers, such as antibiotic resistance genes, are needed to convince GMP-sceptics of the advantages of genetic engineering for crop improvement. In this way, their criticism concerning the potential risks of transgenic crops could be overcome. For example,

molecular or metabolic markers may provide a means to identify transgenic plants with desired trait(s). Of course, these alternative markers should be safe from an environmental and health perspective.

Biotechnology Issues and Concerns of Developing Countries

FAO has been involved in biotechnology in order to help developing countries adopt useful methodologies and monitor developments in the area. It is recognised that there are major differences among developing countries in relation to biotechnology and its application. Sustainable food production is uncertain in many developing areas of the world because of their particular climatic, land and water limitations; thus, developing low-cost applications will be an important element in increasing food production and stability for rural poor. Biotechnology offers a potential solution for many problems affecting crops and livestock production in developing countries. National programmes should ensure that biotechnology benefits all sectors, including resource-poor rural populations, particularly in marginal areas where productivity increases would be more difficult to achieve. Biotechnology-derived solutions for biotic and abiotic stresses built into the genotype of plants could reduce use of agrochemicals and water, thus promoting sustainable yields. Durability of resistance to pests, as always, depends on natural evolution processes. A number of issues are of special concern to members aiming at increasing their involvement in biotechnology and thus developing the agricultural sector, which is an integral element of rural development and overall national food security (FAO, 1999; 1999b).

Priority setting

Countries need to develop knowledge appropriate to their own situations and to decide if they need to use biotechnological approaches. Biotechnology expertise should complement existing technologies and be output-driven. Much biotechnology is more expensive than conventional research, so it should be used only to solve specific problems where it has comparative advantage. In many developing countries funding for research in agriculture is being reduced, and often research is being privatised with the consequence risk that it could be aimed mainly at resource rich farmers. Biotechnology research and policy should also address the needs of the poor who depend on agriculture for their livelihoods, particularly in marginal areas where productivity increases will be difficult to achieve. In addition to technical considerations, priority setting should take into account national development policies, private sector interests and market possibilities. The diverse stakeholders should be involved in the formulation of national biotechnology strategies, policies and plans.

Agricultural problems are multidisciplinary in their nature and biotechnology in isolation is unlikely to solve them. Each country should decide how much of the technology should be developed nationally and how much imported and adapted. A good mix of the two can be synergistic, and reduce both the time and cost of developing products for the market.

Infrastructure and capacity

Biotechnology research requires skilled staff, backed up by well-equipped laboratories with proper working conditions, a constant supply of good quality water, a reliable electricity supply, and organised institutional support including timely delivery of reagents and access to Internet and other international networks. A minimal technology base is required even to adapt technology tried and tested elsewhere to local ecological and production conditions and

to meet national obligations for biosafety, release of GMOs and sale of products derived from them. In deciding to adopt biotechnology a country must be prepared therefore to commit itself to guaranteeing substantial ongoing financial support. Biotechnology research does not finish with a laboratory product. To have useful results it must reach the end user. Biotechnology research needs strong and organised outreach services and suitable institutions and infrastructures to facilitate its application. A variety of institutions may be required, depending on the technology.

Biotechnology generates a large amount of data needing analysis and interpretation. Analysis software is available but requires adequate computer facilities. Access to informatics technology via Internet and existing databases is also needed to minimise duplication of effort, such as comparing DNA sequencing data. For any research to be truly productive there must be a critical mass of expertise, knowledge and facilities. Biotechnology is no exception. Individuals working in isolation are unlikely to produce either a process or a product.

Intellectual property rights (IPRs)

With the establishment of the World Trade Organisation (WTO) in 1995 all members are bound by the *Agreement on Trade Related Aspects of Intellectual Property Rights* (TRIPS). TRIPS Article 27, on patentable subject matters, requires countries to grant patents for "inventions whether products or processes, in all fields of technology, provided that they are new, involve an inventive step and are capable of industrial applications." The article allows for the exclusion of diagnostic, therapeutic and surgical methods for animals and humans; and of plants and animals other than micro-organisms, but it obliges members to provide protection to plant varieties either by patents or by "an effective *sui generis* system, or a combination thereof". The provisions of the subparagraph are to be reviewed in 1999. Most processes and many products of biotechnology research are therefore patentable: "Patents shall be available and patent rights enjoyable without discrimination as to the place of invention, the field of technology, and whether products are imported or produced locally." Following entry into force of the agreement in 1995, developed countries had a one-year transition period to allow the necessary legislative changes; developing countries have five years; and the least developed countries have eleven years, with the possibility of an extension.

IPRs are critical for growth of the biotechnology industry, and lack of patent protection in a country can limit access to the results of biotechnology originating elsewhere, blocking inward investment. The issues are complex, with implications for trade, technical investment and access to biotechnology outputs. Countries need to evaluate carefully their positions and, as appropriate, to introduce legislation, as foreseen in the WTO Agreement. In particular, they will need to evaluate the most appropriate form of protection to be given to plant varieties in the light of these implications.

Biosafety, food safety and the environment

Biosafety means the safe and environmentally sustainable use of all-biological products and applications for human health, biodiversity and environmental sustainability in support of improved global food security. Biotechnology provides new and powerful tools for research and for accelerating the development of new and better foods. FAO's position that modern biotechnologies should be used as adjuncts to, and not as substitutes for, conventional technologies in solving problems related to food production or processing. Within the past few

years, a variety of foods produced using technology have been approved in many countries. Examples are crops such as maize, potatoes, soybeans, tomatoes and oilseeds. The benefits of biotechnology are many and include providing resistance to crop pests to improve production and reduce chemical pesticide usage, thereby making major improvements in both food quality and nutrition. However, just as with any new means of food production, there are potential human health risks that must be considered when foods are developed using biotechnology. It is vitally important to encourage worldwide efforts to develop and apply appropriate strategies and safety assessment criteria for food biotechnology research and to ensure the wholesomeness and safety of the food supply. Adequate biosafety regulations, risk assessment of biotechnology products, mechanisms and instruments for monitoring use and compliance are needed to ensure that there will be no harmful effects on the environment or for people. Potential environmental hazards from new products of biotechnology, mainly involving GMOs, have raised concerns that, in the absence of adequate legislation, companies may use developing countries as test sites for their products.

Some of the potential environmental risks concern plant pests. Gene escape from GMOs may result in increased weediness in sexually compatible wild species. The inclusion of novel genes for herbicide resistance in plants may increase the occurrence of weeds with resistance to certain agrochemical. The inclusion of pest resistance in plants should be carefully evaluated for potential development of resistance in pests and possible side effects on beneficial organisms. Were a GMO to be classified as a plant pest, it would fall within the ambit of the International Plant Protection Convention (IPPC).

The use of biotechnological process, particularly genetic modification, is extremely important in devising new ways to increase food production, improve nutrient content, and provide better processing or storage characteristics. It follows that when new foods or food components are developed using biotechnology, there are both national legal requirement and consumer expectations for effective systems and procedures to assess the safety of the food or food component for consumption. Traditional food-safety assessment techniques, based on toxicological testing as used for food additives, for example, may not always apply to foods or food components produced by biotechnology. Another worry about GMOs is the possible inadvertent production of toxins and allergens. The Codex Alimentarius Commission (CAC) was formed in 1962 to implement the Joint FAO/WHO Food Standards Programme, the purpose of which is "to protect the health of consumers and ensure fair practices in the food trade."Codex standards, guidelines and other recommendations are explicitly recognised under the WTO *Agreement on the Application of Sanitary and Phytosanitary Measures.* As concluded by FAO/WHO, 1996, food safety consideration regarding organisms produced by techniques that change the heritable traits of an organism, such as DNA technology, are basically of the same nature as those that might arise from other ways of altering the genome of an organism, such as conventional breeding. On this, the application of the concept of substantial equivalence is the basic tool in the assessment used to establish the safety of food products derived from genetically modified organisms. It is not a safety assessment in itself, but is a dynamic, analytical exercise in the assessment of the safety of a new food or food component relative to an existing food/component guidance for the conditions required for foods prepared through biotechnology. Foremost among these are considerations of potential allergenicity, possible gene transfer from GMOs, pathogenicity deriving from the organism used, nutritional considerations and labelling.

Biodiversity issues

Biodiversity is the primary source of useful variation for use in breeding and biotechnology and an important component of sustainable agriculture; thus, without biodiversity biotechnology becomes academic. New gene transfer techniques have made it possible to transfer genes between species and even between kingdoms. Biotechnology can contribute to the conservation, characterisation and utilisation of biodiversity, thus increasing its usefulness. Some techniques, like *in vitro* culture, are very helpful for maintenance of *ex situ* germplasm collections of plant species that have asexual propagation (bananas, onions, garlic) or polyploid species where fertility is often very low, and of species that are hard to keep as seeds or in field gene banks. However, they all presuppose the existence of an effective infrastructure.

Biotechnology may reduce genetic diversity indirectly by displacing landraces and their inherent diversity as farmers adopt genetically uniform varieties of plants and other organisms. At the same time it increases the potential to preserve and sustainable use diversity. In the case of endangered animal breeds, for example, cryopreservation and somatic cloning can strengthen traditional conservation strategies.

Perspectives

In many LAC countries governments and institutions are establishing biotechnology research facilities. However, the type of research and its application vary considerably among countries, generally being far below the level of developed countries. Small-scale private sector investment in biotechnology has also begun in some developing countries; for example, a number of private laboratories are working on artificial insemination, embryo transfer in animals, and micro-propagation of plants to produce disease-free planting material.

Successful application of biotechnology is possible only when a broad research and knowledge base exists in biology, breeding, agronomy, physiology, pathology, biochemistry and genetics. Benefits offered by the new technologies cannot be realised without a continued commitment to conventional agricultural research. Biotechnology programmes, if they are to succeed, must be fully integrated into the existing research system without depriving other research of funding.

Within the next 10 or 20 years, the following research areas may become very important for crop improvement: (i) apomixis to fix hybrid vigour, (ii) male sterility systems with transgenics for hybrid seed in self-pollinating crops, (iii) parthenocarpy for seedless vegetables and fruit trees, (iv) short-cycling for rapid improvement of forest and fruit trees, (v) converting annual into perennial crops for sustainable agricultural systems, and (vi) abiotic stress tolerant crops. The development of perennial crops will be especially important to protect the soil from erosion.

Banning transgenic crops in the farming system will be foolish because the potential benefits are so great. Environmentalists should re-read 'Silent Spring' and think that any effort scientists do to develop crops that eliminate or reduce the utilisation of polluting agro-chemicals in the farming systems must be welcome by farmers and consumers. For example, one interesting approach for developing resistant transgenic crops may be through the improvement of the plant's own defence system. Inducible and tissue specific promoters could assist in this endeavour.

Collective approval may lead to new partnerships, co-operation or joint ventures in research and development between scientists in the public and private sectors that will benefit farmers and consumers with profits and high quality products, respectively. Any potential risk in human development associated with biotechnology applications in agriculture will be easily resolved in a democratic society. The public needs to choose between being safely self-regulated or to follow safety regulations as agreed by lawmakers after listening to the views of scientists, producers, and consumers.

FAO seeks to realise fully the positive impact of biotechnology and to minimise possible negative effects. It is proposed that FAO concentrate on priority areas mentioned above, acting as facilitator in concert with appropriate entities. Currently, FAO's involvement does not reflect the growing importance of biotechnology. If FAO is to strengthen its involvement and respond to members' requests, budgetary adjustments and building its critical mass will be essential. As recently endorsed by the last FAO Council, 1999 it is the Organisation intention to establish active research and development partnerships and linkages among biotechnology institutions and between countries, and emphasise closer co-operation between private and public sectors.

FAO should concentrate on helping strengthen national capabilities in biotechnology research and application as an integral element of overall agricultural research, focusing on increasing and sustaining agricultural production, including marginal conditions. Training in biotechnology should have as its objective the resolution of actual constraints and the exploitation of real opportunities as well as to promote seminars, meetings, visits, workshops, courses on the use of emerging technologies for technology transfer and access to biotechnology outputs.

Considering that the spectrum of activities associated with biotechnology is so broad that no one organisation can be competent in them all; effective partnerships are being established. FAO's comparative advantage in such partnerships lies in its intergovernmental status, its direct links with public and private entities in member countries, and its comprehensive experience in the agriculture sector. Although not yet effectively exploited in respect of biotechnology, this comparative advantage provides a basis for FAO to foster international information exchange via networks involving members' institutions, international bodies, academic centres, NGOs and the private sector.

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Plant Genetic Engineering: Towards the Third Millennium
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Molecular Markers in Variety and Seed Testing

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Variety and Seed Testing - Introduction

Before they can be sold within the member states of the European Union (EU), newly bred varieties (cultivars) of crop plants of agricultural and horticultural importance have to undergo statutory testing. There are two components to this testing in most crops (Turner, 1998). Firstly, aspects of the agronomic performance of a new variety are tested and compared to those of existing varieties. Secondly varieties must be shown to be Distinct (D) from others known to exist, and to demonstrate Uniformity (U) and Stability (S) in the characteristics used to distinguish and describe them. Plant breeders may protect their intellectual property in new varieties of agricultural, vegetable, fruit and ornamental crops through the statutory Plant Breeders' Rights (PBR) schemes, based on the same DUS principles (Bailey, 1983). Many countries outside of the EU also operate PBR systems and PBR can be viewed as an important instrument that encourages innovation and private investment in plant breeding by rewarding breeders for their efforts and providing them with a measure of security for their germplasm. In most countries with appropriate schemes, PBR is established through national Seeds Acts, which enact the provisions of the International Union for the Protection of New Varieties of Plants (UPOV) Convention. UPOV is the driving force behind the development of techniques for DUS testing, the introduction of new test methods, and the legal implications of such changes for plant variety protection.

Seed of successfully tested varieties can now be marketed. However, in order for there to be a sufficient volume of seed available, there has to be a process of seed multiplication or production, which again is subject to controls through statutory or voluntary Seed Certification schemes. Certification can be envisaged as a process of quality control and consumer protection, designed to ensure that the seed sold is of a sufficiently high standard with regard to germination, purity and freedom from disease and is also of the correct variety. Many aspects of seed testing are co-ordinated by organisations such as ISTA (the International Seed Testing Association), which publishes International Rules governing how the tests should be conducted and the results reported.

Much of the technology used in variety and seed testing has been established for many years and is based on well proven and tested approaches. For instance, variety identification is commonly carried out by the observation and recording of a range of morphological (botanical) descriptors (Cooke, 1995a). Whilst in practice this is largely successful and forms the basis, for instance, of most current testing procedures for DUS and the granting of PBR, it can be an expensive and time-consuming process. Many of the morphological descriptors used are continuous characters, the expression of which is affected by environmental factors. Again, in some species the number of useful descriptors is limited. There are thus good

grounds for finding alternatives to this morphologically based approach. The advent of genetically modified (GM) - transgenic - varieties adds a further complication, in that the phenotype may not be altered substantially from that of the 'parent' variety, introducing the concept of the 'dependent' or 'essentially derived' variety.

One potentially attractive alternative means of variety identification would be to use biochemical and/or molecular markers (Cooke, 1995b). These methods could also be useful in seed testing, especially with regard to the determination of seed quality and seed health (Blakemore et al., 1993; Reeves, 1995; McDonald, 1998; Smith and Register, 1998).

DNA Profiling in Variety and Seed Testing

Hybridisation-based methods (restriction fragment length polymorphisms, RFLP) were the first widely reported means of revealing DNA sequence variations in a diverse range of organisms, including varieties (Ainsworth and Sharp., 1989; Weising et al., 1995; Karp et al., 1996) and the potential of RFLP for variety identification purposes has been confirmed (e.g. Lee et al., 1996). A literature survey from 1993-97 found reports of RFLP analysis for variety discrimination in over 60 agricultural and horticultural species, including all the major cereals, vegetables, fruits, and trees (Cooke and Reeves, 1998). The large number of restriction enzyme/probe combinations available makes RFLP a powerful tool, but the technology has been in decline since the advent of PCR based marker systems, which overcame most of the limitations of this technique (reviewed in: Morell et al., 1995; Karp et al., 1996; Caetano-Anolles and Trigiano, 1997). The earlier PCR-based techniques such as RAPD (random amplified polymorphic DNA) and AP-PCR (arbitrarily primed-PCR) (Welsh and McClelland, 1990; Williams et al., 1990) have been extensively used to fingerprint well over 75 different plant species (unpublished data), but soon showed limitations in reproducibility across laboratories and a lack of polymorphisms in some important crops such as wheat (Devos and Gale, 1992). At present, two techniques dominate the scene of variety profiling and hence identification: AFLP (amplified fragment length polymorphism) (Vos et al., 1995) and STMS (sequence-tagged microsatellite sites, reviewed in: Morgante and Olivieri, 1993), which offer several advantages (Powell et al., 1996) over a multitude of other markers (Caetano-Anolles and Trigiano, 1997). AFLPs have also proved valuable in cereals research, where they overcome the limitations of RAPD while offering a wide range of applications in breeding (Donini and Ridout, 1999). Techniques of DNA analysis have also been shown to be valuable for the detection of pathogens and in particular seed-borne pathogens in the context of seed health testing (Blakemore et al., 1993; Reeves, 1995). The remainder of this paper considers some of the research on the application of molecular markers to variety and seed testing work at NIAB.

Variety Discrimination and Identification

NIAB has research programs investigating the use of various types of molecular markers for variety discrimination and identification in a range of crops, including wheat, barley, oilseed rape, tomatoes, potatoes and chrysanthemums. Much of this work is funded by the UK Ministry of Agriculture, Fisheries and Food (MAFF) or by the EU. For instance, we are part of an EU project which aims to demonstrate the technical viability of molecular markers for varietal identification and discrimination in two important European crop species, tomato (*Lycopersicon esculentum*) and wheat (*Triticum aestivum*). The approach is to use a set of

STMS primer pairs that have been shown to reveal polymorphisms within a small collection of reference varieties (Figure 1) to construct and test databases containing the molecular description of the most common varieties of tomato and wheat grown in Europe during the last ten years.

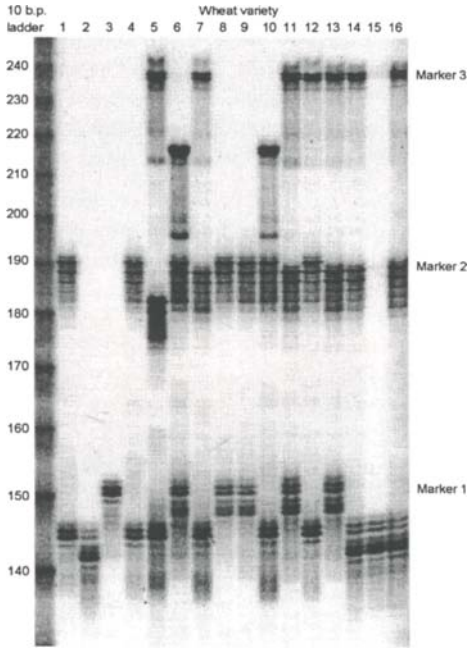

Figure 1. Polymorphisms between wheat varieties as revealed by three STMS markers multiplexed and run on the LI-COR.

The project will demonstrate that both markers and databases can be used for a range of applications, including all aspects of varietal identification, quality control, genetic diversity measurements and DUS testing. Important advances in semi-automated high throughput profiling of multiplexed STMS have been achieved through the use of fluorescence-based sequencing gel systems such as the LI-COR (Gene ReadIR 4200).

DNA profiling techniques are also being examined for their utility in DUS testing of high value crops such as ornamentals. In addition to the use of STMS, work is being carried out to investigate the use of 5'-anchored inter-simple sequence repeats (ISSR; Zietkiewicz, 1994) and retrotransposon sequences (inverse sequence-tagged repeats, ISTR; Rohde, 1996) to fingerprint and identify closely related varieties of Chrysanthemum (Jackson et al., 1999). The development of these techniques and their ability to distinguish between closely related varieties and sports is essential if they are to be integrated into DUS testing of ornamentals. Identifying individual varieties is particularly challenging due to the fact that many new varieties are spontaneous mutations (sports) of existing ones and have little sequence divergence.

An important element of all of this research is to develop a large element of automation. This is to aid characterization of existing varieties (>5000 in some species) and to ease the

pressure of the high annual throughput of samples submitted for DUS testing. The existence of DNA databases of varieties would also facilitate the testing of new varieties against all the existing ones, reducing the need for different countries to maintain large reference collections.

Diversity and Distances

An important question in DUS testing is the determination of the 'distance' between two varieties so that they can be said to be truly distinct. The use of molecular markers and suitable statistical tools allows new approaches to this question to be taken and objective data generated (Law et al., 1998). Of particular importance is the concept of 'minimum distance', which effectively forms a boundary of protection around listed varieties.

Similar approaches can be used to address issues of genetic diversity. Intensive plant breeding is commonly thought to lead to a reduction in crop genetic diversity, which, if true, would have serious consequences, both on the vulnerability of crops to changes in the spectrum of pests and diseases, and on their plasticity to respond to future changes in climate or in agricultural practices. The accurate quantification of the genetic diversity of major agricultural crops is therefore important, both scientifically and socio-economically. In collaboration with the John Innes Centre, Norwich, we have assessed temporal trends in the genetic diversity of UK wheat and barley crops over the past 60-70 years, by AFLP fingerprinting and STMS profiling (Donini et al., 1998; Law et al., 1999). Analysis using a range of statistical methods including AMOVA and PCO indicated that there has been no significant quantitative shift in the genetic diversity of the UK wheat and barley crops over the past 70 years. This approach could be extended to other crops and other countries to answer important questions related to genetic diversity and conservation.

Genetic Purity Testing

Hybrid varieties of crops offer numerous benefits to agriculture, but purity of the hybrids is essential to ensure that these benefits are made available to the industry. Hybrids pose additional seed testing challenges compared to traditional varieties, because the hybrid is re-created each year. This gives the opportunity for outcross events and non-hybrid seed production through self-pollination of the female line to occur. The determination of hybrid purity is thus an important element in seed quality control testing. At the moment, hybrid testing methods largely rely on the examination of morphological characteristics, although some crops (e.g. maize) are examined using isozymes. Morphological assessment requires the plants to be grown to maturity, which means that results are not available to the farmer before the seed is sown. NIAB is co-ordinating an EU project, which is evaluating the potential benefits those molecular markers, may offer to the speed and accuracy of testing hybrid seed lots. STMS and AFLP markers are being developed for hybrid purity testing of oilseed rape, maize, sunflower and white cabbage (Figure 2). The objective is to produce 'variety test sets' of primer pairs that can be effectively used for hybrid testing in these crops and to compare the results from their use with those obtained from conventional field trials.

For example, in sunflowers, microsatellite markers obtained from the Oregon State University website (http://www.css.orst.edu/research/oilseed/sunf%2Db2.htm) have been evaluated for their informativeness in a set of 30 sunflower varieties, leading to a choice of five markers (the variety test set) for the analysis of hybrids. For this phase, we have analyzed approximately fifty individuals of the hybrid variety and twenty individual plants of each of

the parental lines used to generate the hybrid. Microsatellites that show polymorphisms between the parental lines can be used to confirm the hybridity of individuals in a variety, and the uniformity of the parent lines and the resulting hybrid. Offtypes can be detected which are caused by self-pollination and outcross events (Figure 2).

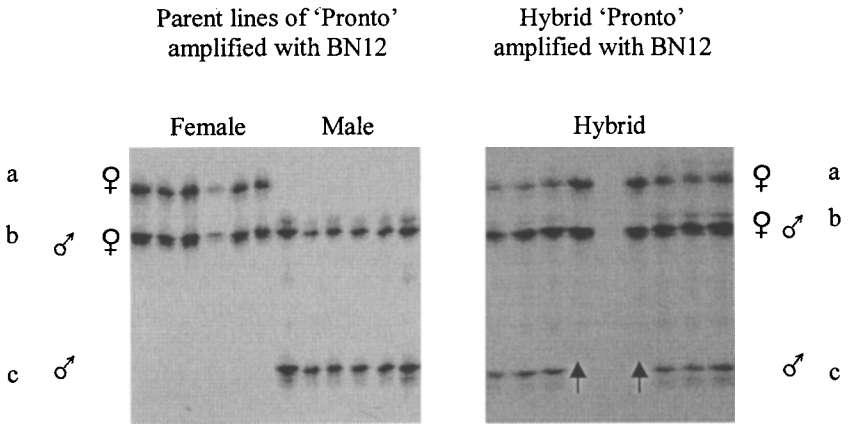

Figure 2. Oilseed rape hybrid variety "Pronto" tested with a pair of microsatellites primers. The female parent has alleles a and b, while the male has alleles b and c. Hybrids show bands a, b and c, but some plants showing only bands a and b are a clear product of self-pollination (arrows).

Seed Health Testing

The most effective method for controlling seed-borne diseases is via seed health testing and certification (Thomas et al., 1998), which thus forms an important element of seed quality control. Traditional testing methods involve lengthy pathogen isolation and infection processes and do not allow effective management of seed treatments. At NIAB, interest has focused on the use of PCR-based methods for the detection of important organisms such as *Erwinia stewartii*, a bacterial pathogen of maize (Blakemore et al., 1999). Current research has concerned the *Pyrenophora* complex, which cause an array of diseases in barley. *P. graminea* is strictly seedborne but *P. teres* may be also transmitted by crop stubble. There is, therefore, a requirement for a seed health test that can distinguish between these two species thereby enabling the appropriate disease management to be carried out. The currently used agar plate or blotter tests are slow and labour intensive (Blakemore et al., 1998). *P. graminea-specific* PCR primers were designed from a cloned RAPD fragment. An optimized extraction and PCR protocol enabled *P. graminea* to be detected and distinguished from other closely related *Pyrenophora* species and saprophytes (Stevens et al., 1998).

Detection in this PCR test has been carried out on a Lightcycler™ (Idaho Technology) PCR fluorescent detection system (Ririe et al., 1997). The Lightcycler can perform a typical PCR program in less than 15 minutes, making the assay extremely rapid. The thermal cycler feeds data to a dedicated computer allowing real time detection of amplicons during amplification and is suitable for large-scale automation in commercial applications. This approach is currently being extended to other important pathogens.

The user-friendly format of the assay as well as its speed are of relevance for potential seed testing and commercial applications of molecular markers, increasing the capacity for processing more seed samples. This is important, as there are seasonal peaks of activity in seed testing according to the sowing and harvest times of particular crops.

Future Prospects

The potential of molecular markers in variety and seed testing is thus clear. Further progress towards automation and high throughput analysis can be anticipated. Other approaches to DNA profiling are being developed and variations on the theme of AFLP, such as S-SAP (sequence-specific amplification polymorphisms) (Waugh et al., 1997) and other retrotransposon-based markers such as IRAP (inter-retrotransposon amplified polymorphism), REMAP (retrotransposon-microsatellite amplified polymorphism) (Kalendar et al., 1999), and RBIP (retrotransposon-based insertion polymorphisms) (Flavell et al., 1998), may come to be as useful as AFLP and STMS in the short and medium-term. The longer term future in plant genotyping will probably exploit ASH (allele-specific hybridisation) markers for the detection of SNP (single nucleotide polymorphism, Gu et al., 1995; Nikiforov et al., 1994), and commercial systems such as TAQMAN™, based on the real time detection of fluorescently labelled probes hybridised to PCR amplicons (Heid et al., 1996; Kalinina et al., 1997), are now being routinely used in other areas. This kind of approach, together with other real-time detection systems such as the use of molecular beacons (Piatek et al., 1998) is at present a valid option for practical uses in diagnostic services, such as seed testing, although the appropriate protocols need to be developed and tested. Progress in nanotechnology and robotics offers new perspectives to hybridization-based methods that are now highly automatable, making the future of high density DNA microarrays technology (Chee et al., 1996) and DNA chips (Gerhold et al., 1999) a closer reality. Hence it is apparent that molecular methods of various sorts will continue to be developed and used in variety and seed testing. The continuing emphasis on quality at all levels and on such concepts as traceability and identity preservation, particularly in relation to GM crops, ensure that these techniques will be more widely used in the future.

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Plant Genetic Engineering: Towards the Third Millennium
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The Genetic Basis of Drought Tolerance in Maize and Options for Improvement Via Marker-Assisted Selection

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Introduction

Drought is a major cause of maize yield loss in the tropics, and for this reason breeding for drought tolerance has become a major focus of the International Maize and Wheat Improvement Center's (CIMMYT) Maize and Applied Biotechnology Programs (for review, see Heisey and Edmeades, 1999). Loss to drought alone in the tropics is thought to exceed 20 million tons of grain per year, or around 17 % of well-watered production (Edmeades et al., 1992), but has been as high as 60 % in severely affected regions such as southern Africa in 1991-92. With soil infertility, drought represent the major cause of grain yield reduction in developing countries (Beck et al., 1997), and considering ongoing climatic changes caused principally by global warming (Curry et al., 1995), the pressure on food production in water-limited environments should increase in the near future.

The global impact of water-limited conditions is not easy to evaluate, especially considering the spillover effect of drought on grain production when combined with insect or pathogen attack and/or non-optimal soil conditions. Conventional breeding for yield improvement under drought conditions is time consuming and laborious, because carefully managed field conditions are required. In addition, there is a decrease in the genetic variance and heritability of yield components that parallels an increase in environmental stress (Ribaut et al., 1997a). Considering these limitations to efficient selection, and the fact that only one relatively rainfree crop season per year is available for selection in most tropical countries, the use of molecular markers could provide a useful tool to complement phenotypic selection.

After the presentation of quantitative trait loci (QTL) identification for target traits involved in maize drought tolerance and a description of two successful marker-assisted selection (MAS) experiments for both line and population improvement, prospects for plant improvement through new MAS will be discussed. The emergence of molecular genetics and associated technologies represents a major new breeding tool; the current challenge is to integrate this tool and the information it generates into breeding schemes to further the development of efficient MAS strategies.

Genetic Dissection of Target Traits Observed Under Drought

By crossing a drought tolerant (P1, Ac7643S5) with a susceptible line (P2, Ac7729/TZSRWS5), with the respective lines showing considerable contrasts, especially for anthesis-silking interval (ASI) under drought, a segregating population was developed. Using

this segregation population, the genetic dissection of several traits related to maize performance under drought —essentially the identification and characterization of the principal genomic regions involved in the expression of those traits— has been performed at two different inbreeding levels.

At the F2:3 (s2) level, flowering parameters, plant and ear height, leaf size and number, and yield components have been studied under three different water regimes: well-watered (WW), intermediate (IS), and severe water stress condition (SS). A genetic map was constructed with DNA extracted from F2 plants and the field evaluation was conducted on the corresponding F3 families. The most relevant results of this experiment have been presented in two papers (Ribaut et al., 1996; 1997a). From this QTL analysis, it was concluded that: (1) a MAS experiment based only on the QTL involved in the expression of yield components would not be the most efficient because only a few of the QTLs are stable across environments; and (2) a MAS experiment should consider the QTLs involved in the expression of secondary traits of interest correlated with yield under drought, such as ASI and plant height. The selected QTLs should be stable across environments and account for a large percentage of the phenotypic variance. Therefore, an efficient MAS strategy should take into account the most suitable QTLs from different traits as an index.

At the F6 (s5) level, and in addition to the morphological traits measured on the F2:3 material, physiological parameters like relative water content, osmotic adjustment, root conductivity, and chlorophyll content have also been measured. In 1996, an initial set of field evaluations was conducted under three water regimes (SS, IS, and WW), and in November 1998–May 1999, a second set was evaluated under the same field conditions. In addition, two ongoing collaborative projects are using the same RIL population to quantify the ABA content in the ear at the flowering stage (Tim Setter, Cornell University, USA), and to evaluate root growth under hydroponics (Roberto Tuberosa, Bologna University, Italy). Although most of these physiological traits are not useful for routine screening purposes because they are too time consuming, the osmotic adjustment measurement being a typical example. Once DNA markers closely linked to the QTL involved in the expression of a physiological trait are identified, they can be used efficiently in a MAS experiment. Identification at the same genomic location of QTL related to physiological and morphological traits should be expected, given that changes in physiological pathways have an impact on the plant phenotype. As an example from our first field evaluation, a QTL for chlorophyll content was identified on chromosome 2 close to a QTL for ASI (under IS and SS) and grain yield (under IS only). This QTL for chlorophyll content was consistent when measurements were conducted on the ear leaf and on a young leaf close to the tassel. On chromosome 6, a QTL for relative water content corresponds exactly to a QTL for ASI (under IS and SS) and grain yield (under IS and SS). At the same chromosomic region, the identification of a dehydrin gene (dhn1) has also been reported (Campbell and Close, 1997). Since several physiological pathways involved in the drought tolerance mechanism are well known (e.g. ABA biosynthesis), the characterization of the gene(s) corresponding to identified QTL can be achieved and the candidate gene approach appears to be an attractive option.

MAS for Maize Line Improvement Under Drought

Based on the QTL and mapping information described above, a backcross marker-assisted selection (BC-MAS) project began in 1994. The line P1 was used as the drought-tolerant

donor and CML247 was used as the recurrent parent. CML247 is an elite tropical inbred line developed by CIMMYT, with outstanding combining ability and good yield per se under well-watered conditions. It is susceptible to drought, in part because its ASI is large under drought. Genetic data from a segregating F2 population derived from the P1 x CML247 cross were combined with F3 evaluations in the field under different water regimes to identify QTLs for traits of interest. The QTLs for ASI identified in this cross were quite consistent with those in the original P1 x P2 cross. Of the five QTLs originally identified from P1 that conferred a short ASI, only the QTL on chromosome 6 was not detected in the second cross. The QTL on the short arm of chromosome 1 was shifted by 40 cM in the new cross, and the three other QTLs on chromosomes 2, 8, and 10 were in similar positions in both. A new QTL for ASI was detected on the short arm of chromosome 3. These results demonstrate the need to make a new genetic map when the recurrent line is changed in BC-MAS schemes. A single good-quality trial under drought conditions, however, might be enough to identify QTLs of interest, providing QTL identification has been previously carried out in another cross involving the donor line.

Five genomic regions involved in the expression of a short ASI were selected to be transferred from P1 into CML247. The screening of large populations (about 2,000 plants) at each selection cycle during backcrossing has been possible because of the development of reliable PCR-based markers, used here as preselection tools (Ribaut et al., 1997b). After two BCs and two self-pollinations, the best genotype was fixed from the donor line for the five target regions (12 % of the genome), as well as for 7 % of the genome lying outside the QTL regions (Figure 1). The 70 best BC2F3 (i.e., S2 lines) were identified and crossed with two CIMMYT tester inbreds, CML254 and CML274. These hybrids, as well as the BC2F4 families (S3 lines) derived from the selected BC2F3 plants, were evaluated in 1997-98 under several water regimes.

Preliminary results show that the mean of the 70 selected genotypes performed better than the control crossed with CML254 and CML274, and the best genotype among the 70 selected (BC2F3 x testers) performed two (x CML274) to four times (x CML254) better than the control (Table 1). No yield reduction was observed under well-watered conditions. A second field evaluation was conducted during the most recent dry cycle (December 1998–May 1999) and results are being analyzed.

Table 1. Grain yields per plot (kg ha^{-1} ± SD) under different water regimes of MAS-improved versions of CML247 versus the original CML247 when crossed with two CIMMYT tester inbred lines (CML254 and CML274). The mean of the control was calculated based on 10 identical entries, while the mean of the improved genotypes represents 70 different entries selected after MAS. "Best" is the yield of the highest yielding individual cross out of the 70.

Genotype	Drought conditions		Well-watered conditions	
	CML254	CML274	CML254	CML274
CML247 (control)				
Mean	798 ± 478	1995 ± 898	6185 ±804	7648 ± 688
CML247 (MAS)				
Mean	1995 ± 957	2195 ± 878	7182 ± 862	7648 ± 918
Best	4522	4057	8715	9909

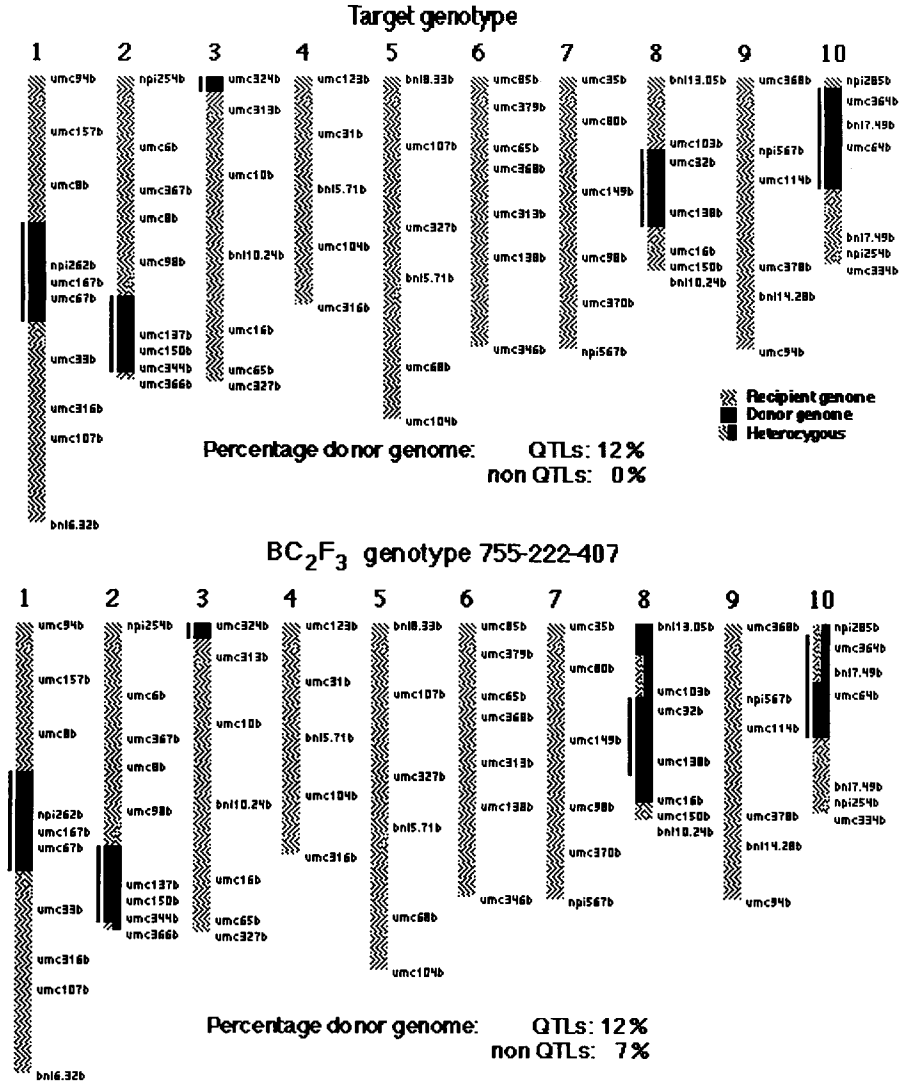

Figure 1. With five selected regions, the target genotype of the BC-MAS for drought improvement conducted at CYMMYT is presented in the top of the figure. Those five regions, at which fixed donor genome contribution is expected, represent all together 12 % of the mapped genome (70 loci/RFLP probes) and presented the best genotype identified after 4 cycles of MAS (2 BCs and 2 self-pollinations), which the remnant donor genome contributions out of the target regions represents about 7 % of the mapped genome.

MAS in Open-Pollinated Populations

The second MAS project involves the improvement of drought tolerance in the open-pollinated population Tuxpeño Sequía (Bolaños and Edmeades, 1993). Varieties derived from such populations remain one of CIMMYT's major products from its maize-breeding program.

Changes in frequencies of drought-adaptive alleles over cycles of recurrent selection at loci of known map position were quantified using DNA markers and 116 individuals randomly chosen from each of C0, C4, and C8 of Tuxpeño Sequía (Table 2). Loci scattered over the maize genome were assayed using random fragment length polymorphisms. Special attention was given to genomic regions responsible for the expression of ASI and grain yield identified in the P1 x P2 study because the two inbreds are derived partially from Tuxpeño germplasm. There is strong evidence that the alleles associated with short ASI in P1 and P2 are also present in this population. Allelic frequencies that either increased, decreased or remained stable were recorded. Genomic regions were classified into those presenting marked, moderate or no changes in allelic frequency due to recurrent selection. Some major frequency changes were detected at loci previously identified as regions responsible for expression of ASI. These alleles could provide a rapid diagnostic tool for screening lines or individuals with potential drought tolerance in Tuxpeño germplasm, and selection based on the presence of these alleles should provide a significant improvement in the drought tolerance of this population.

Table 2. Allele frequency changes at two loci segregating in C_0, C_4, and C_8 of Tuxpeño Sequía, a lowland tropical maize population subjected to full-sib recurrent selection for improved drought tolerance.

Chromosome 2	*umc*371	C_0	C_4	C_8
	Allele 1	0.18 ± 0.03	0.18 ± 0.03	0.06 ± 0.02
	Allele 2	0.11 ± 0.02	0.09 ± 0.02	0.01 ± 0.01
	Allele 3	0.04 ± 0.01	0.08 ± 0.02	0.10 ± 0.02
	Allele 4	0.32 ± 0.03	0.31 ± 0.03	0.56 ± 0.03
	Allele 5	0.36 ± 0.03	0.32 ± 0.03	0.25 ± 0.03
Chromosome 10	bnl7.49			
	Allele 1	0.01 ± 0.01	0.05 ± 0.01	0.00
	Allele 2	0.37 ± 0.03	0.35 ± 0.03	0.22 ± 0.03
	Allele 3	0.22 ± 0.03	0.41 ± 0.03	0.44 ± 0.03
	Allele 4	0.17 ± 0.03	0.05 ± 0.01	0.14 ± 0.03
	Allele 5	0.01 ± 0.01	0.01 ± 0.01	0.06 ± 0.02
	Allele 6	0.22 ± 0.03	0.12 ± 0.02	0.10 ± 0.02

To test this hypothesis, 21 DNA markers were used to screen 400 plants from C0 and C4. Based on their allelic composition, the 40 "best" and "worst" genotypes from each of the two cycles were selected and were evaluated in 1997-98 under several water regimes. Preliminary analysis of those results in this extremely dry season showed a significant difference in yield performance under drought conditions between the two groups of genotypes selected using markers. If those results are confirmed, they will show that MAS is of real value for improving the performance of open-pollinated varieties under drought.

MAS Strategies

For approximately 10 years, genetic dissection of polygenic traits has been hailed as a promising application of DNA markers, resulting in extensive mapping experiments aimed toward the development of MAS (Lee, 1995). However, because of the limited number of

QTL identified per trait, the reduced amount of phenotypic variance that they generally express individually, their interaction with the environment, plus the difficulty of epistasis evaluation, few concrete MAS results have been published that would justify the initial enthusiasm (Mohan et al., 1997, Ribaut and Hoisington, 1998). Until recently, a clear technical limitation has been the restricted population sizes that can be handled, limiting the flexibility and the power of selection. With the development of reliable PCR-based markers, a substantial increase in segregating population size that can be screened is now feasible (Ribaut et al., 1997b). Another limitation was certainly the reduced flexibility of the "good by bad" concept, the transfer of genomic segments from a donor to a recipient elite genome through BC, which presents some constraints in time and cost as we came to realize in our BC-MAS for drought tolerance. However, when this project was initiated five years ago, it then appeared to be an attractive option, given the reduced amount of information available in relation to elite line performance under drought. Today, the situation has evolved at both the germplasm and technology levels. Considering this progress, new MAS strategies should be considered for the improvement of drought tolerance; some of these strategies are already being employed at CIMMYT. If the suitable germplasm is available, the pyramiding of favorable alleles through DNA markers in new germplasm by crossing two elite lines that perform well under the target environment conditions, "good by good," should open new doors for MAS (Ribaut and Betran, 1999).

Given the plethora of ongoing experiments and the explosion of new molecular technologies and applications, new or improved selection schemes should be developed and applied very soon. Notable among these developments will be the multiplication of QTL studies and the expanded identification of gene functions combining EST databases and reverses genetic analyses. More data make gene pyramiding easier and more efficient, while also enabling the development of new genome concepts like gene clustering of developmental functions (Khakin and Coe, 1997). Recent efforts in comparative genetic analysis allow the identification across different plant species of gene sequences involved in the expression of target traits. The superior alleles identified among genomes at those target genes can be used as DNA markers to develop efficient screening techniques. Finally, technological developments including automation, allele specific diagnostics, and DNA chips will make MAS approaches based on large-scale screening much more powerful and effective.

Conclusion

Today, the optimism of a decade ago has been tempered somewhat by constraints and limitations encountered by some current MAS approaches. However, MAS is a dynamic area of investigation, and optimal strategies have likely to evolve together with an increase in available information and new marker technologies. Considering the information available at the QTL and gene levels in relation to drought tolerance in maize, as well as the application of new MAS strategies, germplasm improvement for drought tolerance can count on new molecular tools to complement efficiently conventional breeding. This complementarily should become more important in the near future, given the quantity and quality of the basic genomic research now being conducted at the global level.

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Plant Genetic Engineering: Towards the Third Millennium
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Analysis of Quantitative Trait Locis (QTL) Based on Linkage Maps in Coconut (*Cocos nucifera* L.)

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Introduction

The Coconut palm (*Cocos nucifera* L.) is one of the mayor perennial oil crops of the tropics. Due to the many aspects of its utilization, cocnut represents an important plant for the rural community in developing countries, as it provides the basis for food production, byproduct utilization as well as industrial processing (Persley, 1992). Among the oil crops, the coconut palm plays a special role due to the high lauric acid content of its oil (Jones, 1991). However, coconut oil as the economically most important product of coconut processing is facing heavy competition in the world market. A further area of concern for coconut production is the sensitivity of the coconut palm to biotic and abiotic stress conditions. Thus, breeding in coconut has to aim for high-yielding hybrids with tolerance or resistance to biotic and abiotic stress factors.

In view of the time-consuming process in coconut breeding, modern molecular technologies like DNA markers have a great potential to assist in and accelerate breeding programmes. Various DNA marker technologies have been applied to coconut biodiversity analysis through DNA fingerprinting (ISTR: Rohde et al., 1995; Duran et al., 1997; RFLP: Lebrun et al., 1998; SSR: Perera et al., 1999). With the availability of F1 mapping populations from controlled crosses involving (partially) heterozygous parents from "Tall" and "Dwarf" coconut types, linkage mapping of polymorphic markers has become possible, and linkage map construction based on different molecular marker types has been described recently (Rohde et al., 1999, Herran et al., 1999). In this paper we present QTL analyses for leaf production and girth height, which represent important breeding characters.

Materials and Methods

Plant material

An F1 population of 52 genotypes descending from a cross between a Laguna Tall (LAGT07) and a Malayan Dwarf genotype (MYD20) was used for linkage mapping and QTL analyses. Establishment of the mapping population has been described in detail previously (Rohde et al., 1999). Genomic DNA was extracted from young spear leaf material as described by Rohde et al. (1995).

Molecular methods

For QTL analysis using interval-mapping methods a linkage map was necessary, which had been produced earlier as described in detail by Herran et al. (1999). This linkage map was based on different molecular marker types.

RAPD markers were produced as described by Duran et al. (1997) with some modifications using random 10-mer primers (Operon Technologies, Alameda, California). A total of 28 primers listed in table 1 were used for linkage mapping. The amplification products were separated by agarose gel electrophoresis in TBE buffer and visualized by ethidium bromide staining applying standard methodology (Sambrook et al., 1989).

ISTR analyses were performed as described by Duran et al. (1997) using a total of 24 primer pairs. Amplified ISTR fragments generated in the presence of ^{33}P-labelled ISTR primers were separated on sequencing gels and detected by autoradiography (Rohde, 1996) or by silver staining following the protocol of Bassam et al. (1991).

AFLP fragments were produced with 44 primer combinations according to the protocol of Vos et al. (1995). AFLP fragments were visualized by autoradiography or silver staining. Some primers were also labelled with the fluorescent infrared dye IRD800 (LI-COR, Lincoln, Nebraska, USA) and labeled amplification products were detected by a laser system on a LI-COR 4200-S1 DNA sequencer.

ISSR fragments were generated as described by Fang et al. (1997). They were detected by silver staining (Bassam et al., 1991).

Data analysis and Linkage mapping

Polymorphic DNA fragments were scored for presence or absence in parents and F1 progenies. Linkage analysis between marker fragments, estimation of recombination frequencies, and determination of linear orders between linked loci including multipoint linkage analysis and the EM algorithm for handling missing data were performed as described by Ritter et al. (1990) and Ritter and Salamini, (1996). The MAPRF program was applied for the computational methods. Firstly, linkage groups were constructed based on fragments specific to either parent. In the following, fragments common to both parents were integrated into linkage groups as anchor points as described by Ritter et al. (1990).

The mapping population was examined for the characters leaf production (no of leaves) and girth height. These characters were recorded following the coconut breeders standardised research techniques (Santos et al., 1995).

Initially, QTL analyses were performed at single marker loci according to Soller et al. (1976). Progeny genotypes were divided into two subgroups based on the presence or absence of a fragment. The trait means of these two marker classes were tested for significant differences with the two-sample t-test using SAS software.

In addition QTLs were mapped using least square interval mapping methods developed for backcross progeny according to Knapp et al. (1990) and Knapp and Bridges (1990) and applied to intervals composed of individual markers for both parents. SAS Software (SAS Institute Inc., 1989) and particularly the procedure PROC NLIN were used for the computational analysis.

Results

For QTL analyses based on t-tests and for interval-mapping methods, linkage maps based on different molecular marker types were used which had been obtained earlier (Herran et al., 1999). The 24 ISTR, 5 ISSR, 44 AFLP and 28 RAPD primer combinations generated a total of 382 segregating DNA markers. Linkage maps of 16 linkage groups each were obtained for the two parents of the mapping population based on 343 markers in total. Linkage groups of the LAGT map contained 6 to 21 markers each and were between 99.3 and 173.2 cM in length. The total LAGT map length was 2226 cM. The MYD map was 1266 cM in length and was made up of linkage groups with 4 to 32 markers each. The sizes of the linkage groups varied between 35.4 and 148.3 cM (Herran et al., 1999). The results of QTL analysis are shown in figure 1 were the locations of the obtained QTLs are displayed on the linkage groups from the available linkage map. The codification of marker names is shown in table 1 and table 2 summarizes the results of the QTL analyses.

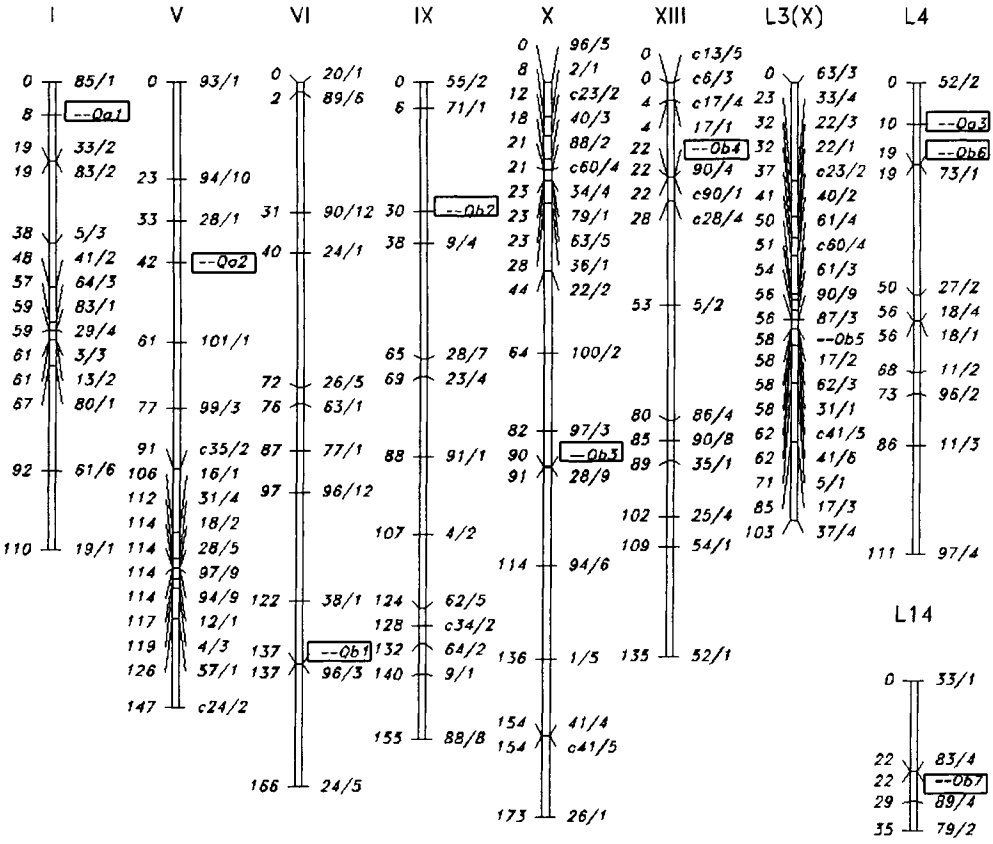

Figure 1. Location of QTL for leaf production (Qai) and girth height (Qbi) on linkage groups of coconut genotypes LAGT (Xi) and MYD (Li). For complete linkage maps see Herran et al. (1999). Common markers are indicated with the prefix "c". For marker names refer to table 1. Distances are given in cM (Kosambi units). See also table 2 for details on QTL.

Table 1. Molecular marker types (M-Type) and primer names of linkage map in figure 1.

No:	M-Type	Primers	No:	M-Type	Primers	No:	M-Type	Primers
1	ISTR	B5/F7c	35	AFLP	E34/M49	69	RAPD	W-5
2	ISTR	B5/F1a	36	AFLP	E79/M81	70	RAPD	W-11
3	ISTR	B5a/F7	37	AFLP	E63/M66	71	RAPD	W-14
4	ISTR	B5a/F3	38	AFLP	E65/M66	72	RAPD	W-15
5	ISTR	B5a/F1	39	AFLP	E63/M64	73	RAPD	X-20
6	ISTR	B3/F7a	40	AFLP	E65/M64	74	RAPD	Ai-18
7	ISTR	B2b/F4	41	AFLP	E63/M65	75	ISTR	B2a/F6
8	ISTR	B2a/F4	42	RAPD	A - 12	76	ISTR	B2b/F6
9	ISTR	B2a/F1	43	RAPD	A - 18	77	ISSR	(TCC)5RY
10	ISTR	B1a/F4	44	RAPD	A - 19	78	ISSR	BDB(CA)7
11	ISTR	B1a/F3	45	RAPD	AQ - 9	79	AFLP	E86/M83
12	ISTR	B1a/F1	46	RAPD	AS - 11	80	AFLP	E85/M86
13	ISTR	B9/F6a	47	RAPD	B - 1	81	AFLP	E85/M85
14	ISTR	B7/F7a	48	RAPD	B - 19	82	AFLP	E83/M86
15	ISTR	B8/F1	49	RAPD	B - 20	83	AFLP	E83/M85
16	ISTR	B9/F7	50	RAPD	C - 11	84	AFLP	E83/M84
17	ISTR	B2b/F5	51	RAPD	C - 12	85	AFLP	E83/M83
18	ISTR	B2b/F3	52	RAPD	C - 13	86	AFLP	E94/M95
19	ISTR	B2a/F5	53	RAPD	C - 14	87	AFLP	E94/M92
20	ISTR	B1a/F9a	54	RAPD	D - 20	88	AFLP	E94/M91
21	ISTR	B5a/F7c	55	RAPD	D - 6	89	AFLP	E94/M90
22	ISTR	B5/F9a	56	RAPD	D-14	90	AFLP	E94/M89
23	AFLP	E35/M33	57	ISSR	(GA)8YG	91	AFLP	E94/M88
24	AFLP	E35/M36	58	ISSR	HVH(CA)7	92	AFLP	E94/M87
25	AFLP	E35/M41	59	ISSR	HVH(TCC)	93	AFLP	E94/M84
26	AFLP	E37/M34	60	AFLP	E79/M80	94	AFLP	E94/M83
27	AFLP	E37/M36	61	AFLP	E81/M81	95	AFLP	E94/M82
28	AFLP	E43/M33	62	AFLP	E81/M82	96	AFLP	E94/M81
29	AFLP	E43/M36	63	AFLP	E81/M80	97	AFLP	E94/M79
30	AFLP	E39/M33	64	AFLP	E82/M81	98	RAPD	X10
31	AFLP	E39/M36	65	AFLP	E82/M82	99	RAPD	AX11
32	AFLP	E39/M41	66	RAPD	P-10	100	RAPD	AX13
33	AFLP	E33/M49	67	RAPD	W-3	101	RAPD	X5
34	AFLP	E33/M54	68	RAPD	W-4			

With respect to the character expressions, progeny genotypes produced on average 3.1 leaves after 14 months and showed a girth height of 34.1 cm (Table 2). However, there was considerably variation of the trait expressions among the different progeny genotypes as reflected in the corresponding minimum and maximum values and the coefficients of variation given in table 2, which were 28 and 73 %, respectively.

Three QTL could be determined for leaf production. Two of them descended from the LAGT parent. Their absolute effects increased leaf production by 0.65 to 0.81 leaves. The individual QTL explained between 11.3 and 15.3 % of the total variation. For Girth height seven QTLs, each one on a different chromosome and three of them present in the Malayan Dwarf parent were detected. Their estimated effects on girth size varied between 17.7 and 22.3 cm. Variance components explained by the QTLs ranged from 10.3 to 20.1 % (Table 2).

Table 2. Results of QTL analyses for the traits leaf production and girth height.

QTL	DES	Effect	Interval	Chrom	I-length [cM]	Pos	Prob. [%]	R2 [%]
A LEAF PRODUCTION								
Qa1	LAGT	0.78	85/1-33/2	I	18.5	7.8	2.2	14..2
Qa2	LAGT	0.81	28/1-101/1	V	28.8	9.9	1.4	15.3
Qa3	MYD	0.65	52/2-73/1	L4	19.2	9.8	4.8	11.3
Trait Mean:		3.1	Min:	2	Max:	7	CV:	28
B GIRTH HEIGHT								
Qb1	LAGT	17.7	38/1-96.3	VI	14.7	14.7	4.5	10.9
Qb2	LAGT	22.6	71/1-9/4	IX	31.8	24.3	3.4	13.4
Qb3	LAGT	23.3	97/3-28.9	X	8.5	8.2	2.9	20.1
Qb4	LAGT	18.7	17/1-90/4	XIII	18.1	18.1	2.9	10.1
Qb5	MYD	19.4	87/3-17/2	L3(X)	2.2	2.2	1.4	15.7
Qb6	MYD	20.2	52/2-73/1	L4	19.2	19.2	1.3	12.9
Qb7	MYD	19.1	83/4-89/4	L14	7	0	1.9	10.3
Trait Mean:		34.1	Min:	16	Max:	114	CV:	73.1

Legend: DES = descendance of the QTL, Chrom = name of linkage group containing QTL, I-length = intervall length, Pos = position of QTL with respect to the upper flanking marker of the interval (Figure 1), Prob = probability for the null hypothesis of no QTL . R2 = portion of total variance explained by the QTL.

Discussion

Although a large number of fragments was obtained in total, only around 16% of them were segregating (Herran et al., 1999) and therefore useful for QTL analyses. Nevertheless they could be arranged into 16 linkage groups corresponding to the 16 chromosomes of the haploid coconut genome. Therefore, with respect to QTL analyses, the whole genome could be covered.

The presence of markers common to both parents and the availability of codominant marker types like the recently developed SSR markers (Rivera et al., 1999; Perera et al., 1999) allows to identify homologous chromosomes and to align them in each parent. In this way information of markers from different individuals can be combined as described by Ritter and Salamini, (1996) and the number of markers available per chromosome for QTL analysis can be increased.

Molecular methods have only been recently introduced into coconut breeding and the plant material required for these applications differs from that used in classical breeding. Therefore, unselected progenies of large size and elevated age are currently not available for this perennial crop. The early germination trait has been evaluated previously (Herran et al., 1999). Data of other characters like girth size and leaf production corresponds to that which was at our disposal from young genotypes. Repeated measurements over longer time periods would be necessary to draw more precise conclusions (Santos et al., 1995).

Despite of the relatively small size of the mapping population, a number of QTLs were detected for the trait under study, since a considerable amount of variation is present in the data as reflected in the corresponding coefficient of variation. QTL analysis was performed by single marker t-tests and interval regression that involves four marker classes. QTLs indicated in table 2 refer only to those detected by interval mapping and were also highly significant with the t-tests. On the other hand, several additional significant trait mean differences were

detected using t-tests. This approach, however, is less sensitive, particularly with small sample sizes and does not allow estimation of the recombination frequency between the marker and the QTL (Lander and Botstein, 1989). The small progeny size decreases the sensitivity of detecting QTLs and only main QTLs can be detected, while smaller ones might remain unrevealed. On the other hand, smaller QTLs might be less stable across different environments and different germplasm and therefore their use is also limited.

Six QTL had been detected previously in coconut for the trait early germination (Herran et al., 1999). In this study several other QTL have been detected for leaf production, which is related to growth vigour and nut production, as well as for girth size, related to the economically more interesting "Dwarf" coconut type. In this way a set of QTL markers is meanwhile available closely linked to several important characters for coconut breeding. Such markers provide an opportunity to select promising parental genotypes or progeny genotypes at early seedling stages through marker assisted selection (MAS). Furthermore, such markers are the basis for map based cloning of QTL genes via BAC libraries.

Acknowledgements

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Molecular Characterization of the Sugarcane Variability for Genetic Improvement

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Introduction

Sugarcane cultivars are complex interspecific hybrids, descendants of a reduced number of foundation clones, mostly from *Saccharum officinarum* and *S. spontaneum* species (Arceneaux, 1967).

S. officinarum main contribution to cultivars is associated to sugar yield components and as it is regularly employed as female recurrent parent in introgression programs, it is almost the exclusive source of organellar diversity. As it is called the *noble* parent, the introgression process is called *nobilisation*. *S. spontaneum* is the main source of genetic variability for breeding purposes, especially for vigor and disease resistance (Glaszmann and D'Hont, 1999).

Modern sugarcane breeding is aimed to obtain new cultivars expressing mechanisms for specific and general adaptation, to overcome the biotic stresses due to the occurrence of new diseases and the abiotic stresses to increase the productivity in marginal affected areas.

The nobilisation program is a long lasting process. In each step, the resulting hybrids are complex autopolyploids and chromosome irregularities in number and transmission are frequent. Thus, the real amount of genetic variation present in these progenies is masked by those phenomena and the phenotypic selection is hazardly conducted. Increase efficiency of nobilisation can be expected by the use of molecular markers as auxiliary tools of phenotypic characteristics in the genetic diversity assessment and selection. These markers are highly polymorphic, with wide genome coverage and environmental and epistatic effects do not influence their molecular phenotype. They are being employed in sugarcane to study phylogenetic relationships and genome structure of members of the so-called *Saccharum* complex (Glaszmann et al., 1990; Burnquist, 1991; D'Hont et al., 1993; Al-Janabi et al., 1994; Sobral et al., 1994; Lu et al., 1994a; Harvey and Botha, 1996; Cornide et al., 1996; Besse et al., 1997; Alix et al., 1998).

Present contribution summarizes the main results in the use of molecular markers for the characterization, identification and assisted management of the basic and the new germplasm of the Cuban sugarcane nobilisation program.

Monitoring Genetic Diversity of the Germplasm for the Nobilisation

The reduced genetic base as a limiting factor for sugarcane genetic improvement has been discussed by several authors (Arceneaux, 1967; Price, 1967; Allison, 1984; Berding and Roach, 1987; Heinz, 1987; Roach and Daniels, 1987; Deren, 1995; Pérez et al., 1997; de Prada et al., 1998). The Cuban program is not an exception; among its basic germplasm; there

are 10 foundation clones widely represented in sugarcane varietal pedigrees from different countries (de Prada, 1998).

The molecular characterization by RFLP of 27 clones among the most used of the Cuban basic germplasm was performed using a group of maize single copy dispersed probes. The results evidenced the higher diversity and the presence of 2 distinct pools in *S. spontaneum* clones (groups A and B) in relation to *S. officinarum* clones (group C) studied (Figure 1, Coto et al., 1999).

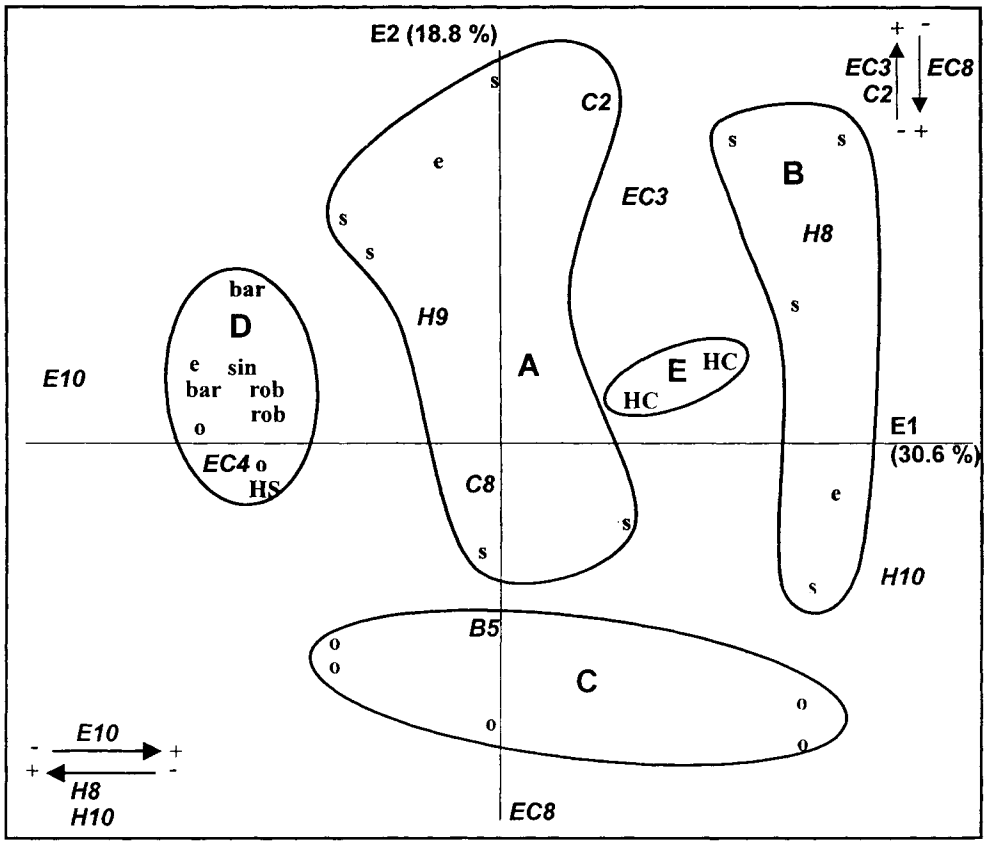

Figure 1. Distribution of foundation clones studied in plane (1,2) of the factor analysis of correspondence (FAC).s,o: *Saccharum spontaneum* and *S. officinarum* foundation clones. rob, sin, bar, e: representatives of *S. robustum, S. sinense, S. barberi* and *Erianthus* clones, respectively. HC: Cuban commercial hybrids. HS: Simple hybrid. EC3, EC8, C2, E10, H8, H10: RFLP bands with a high contribution to total variability.

These results also confirmed previous reports concerning the genetic diversity of *S. spontaneum* (Burnquist, 1991; Lu et al., 1994a; Besse et al., 1997). The clonal groups obtained were confirmed by a factorial analysis (STATICF 4.0, 1988) incorporating to

molecular data the stalk weight components and the disease resistance to smut (*Ustilago scitaminea* Sydow), rust (*Puccinia melanocephala* H. & P. Sydow), and SCMV (sugarcane mosaic virus) as attributes (Coto et al., 1999).

On the other hand, a work collection composed by 172 *S. officinarum*, 50 *S. spontaneum* and 98 representatives of other members of the *Saccharum* complex (*S. sinense*, *S. barberi*, *S. robustum, Miscanthus* and *Erianthus* clones) was evaluated for brix, plant height, the stalk weight components and, disease-resistance to smut, rust and SCMV in 4 localities (Prada, 1998). A discriminant analysis was performed (SPSS/PC, 5.0.1, 1992) on the divided matrix according to each *Saccharum* species and a group for the related genera. An 81.5% of good classification was obtained; the best-typified groups being *S. officinarum, S. robustum* and *S. barberi*. These results confirmed the utility of the selected traits as phenotypic descriptors for germplasm bank management as well as the convenience to complement with other criteria the within group genetic diversity assessment for nobilisation purposes.

A group of 18 *S. officinarum* clones was selected from this work collection by breeders, to study their nuclear and cytoplasmic diversity using RFLP, in order to assist their phenotypic election as female recurrent parents in nobilisation. RFLP combinations were previously chosen by their high polymorphism and scorability. Nuclear RFLP polymorphism was revealed by 16 maize and sugarcane low copy probes from different linkage groups (Grivet *et al.*, 1996). Organellar polymorphism was obtained by 5 mitochondrial and 1 chloroplast heterologous probes. To determine clonal groupings, two independent cluster analyses were performed (STATITICA 4.5, StatSoft Inc., 1994) on the pairwise distance estimates (Nei and Li, 1979).

S. officinarum, *S. spontaneum* and *Erianthus* spp. clones were distributed among 3 well differentiated groups in both analyses. The bands with a major contribution to the general molecular diversity were determined by various Factor Analyses (FAC) iteratively performed (STATITCF 4.0, 1988). Synthetic representations of the nuclear and cytoplasmic diversity exhibited by the clones studied are presented in figure. 2. A group of 36 nuclear RFLPs was totally associated to one of those groups, indicating that the molecular diversity between these groups is partially new and is not merely the product of genetic recombination. Similarly, 4 cytoplasmic RFLPs were associated, 1 to *S. spontaneum* and 3 to *Erianthus* spp. clones; *S. spontaneum* clones can be distinguished by the presence of a band common to these clones and to *S. officinarum* and the absence of the RFLPs associated to the latter species.

Within *S. officinarum* group, clones were tightly clustered in nuclear and in cytoplasmic analyses in relation to other species; their internal distribution was sustained when two cluster analyses were independently performed on nuclear and cytoplasmic RFLP data considering only *S. officinarum* clones.

Three nuclear diversity subgroups (A, B and C) can be considered among *S. officinarum* clones studied (Figure 3). These clones distributed by their cytoplasmic diversity in other 3 subgroups (A_1, A_2 and A_3) with a different clonal composition to the nuclear ones (data not shown). One of the clones, Ashy Mauritius, denoted a relatively high distance to the other ones as it was the only member of A_3 and one of the two members in C.

These results illustrated the low diversity among *S. officinarum* clones studied and the necessity to incorporate new materials. For example, *Erianthus* spp. clones (group D) and wild clones (ECL) (groups C and E) for improving cytoplasmic diversity.

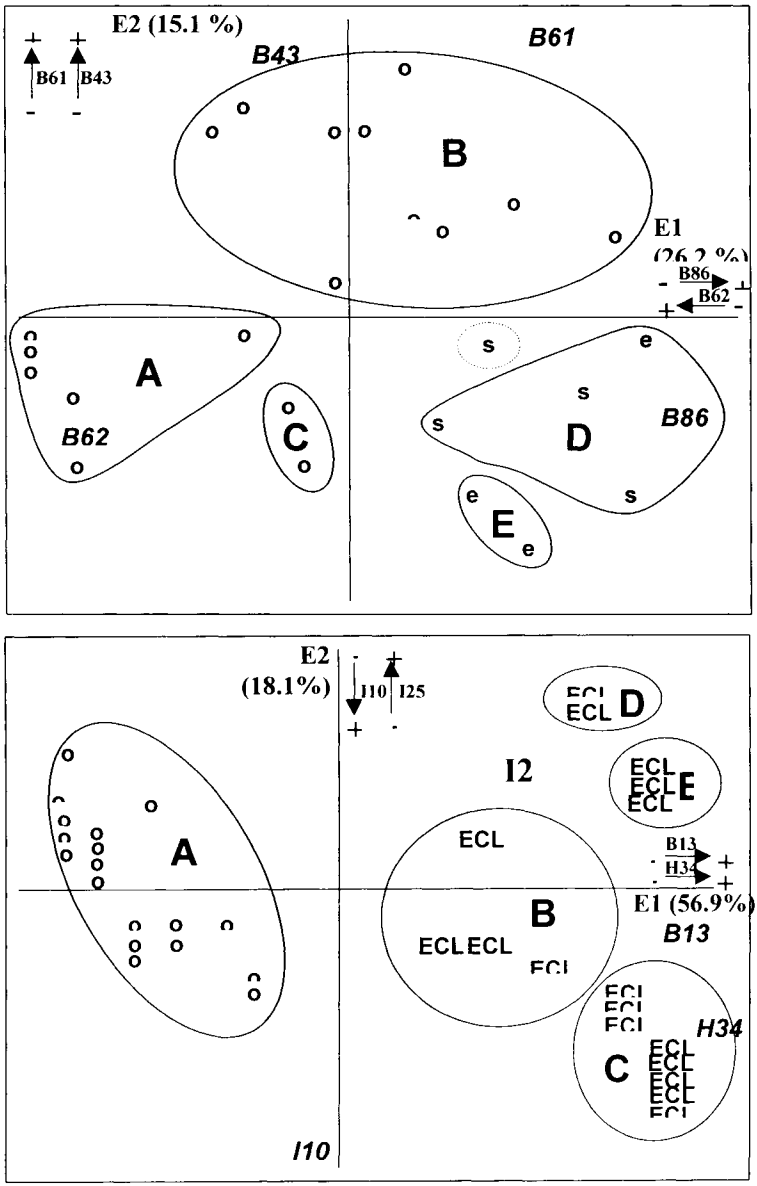

Figure 2. Distribution of 18 *Saccharum officinarum* (o) and representatives of *S. spontaneum* (s), *Erianthus* (e) and ECL collection clones in plane (1,2) of two factor analyses of correspondences (FAC) on nuclear (Upper) and cytoplasmic (Bottom) RFLP data. B61, B43, B86, B62: RFLP bands with a high contribution to total nuclear variability. I10, I25, B13, H34: RFLP bands with a high contribution to total cytoplasmic variability.

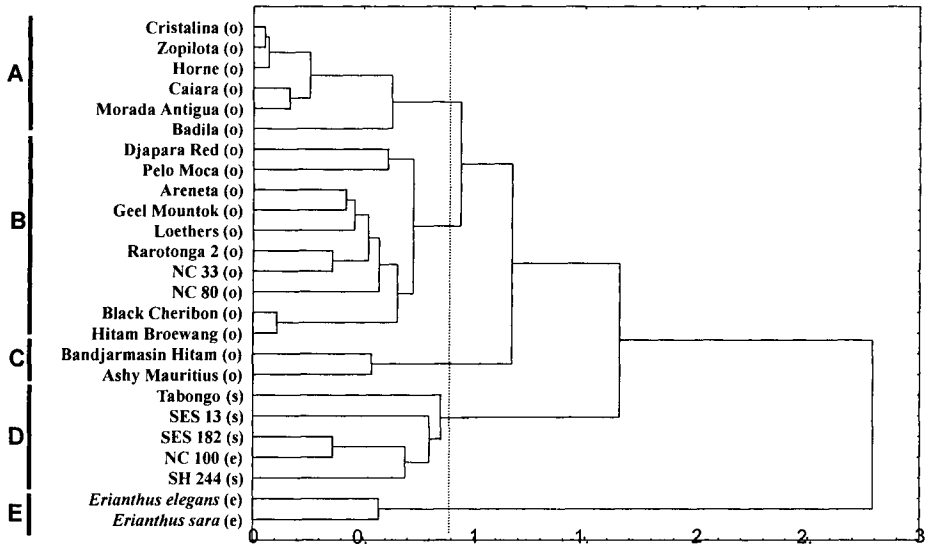

Figure 3. Cluster analysis of 18 *Saccharum officinarum* (o) and representatives of *S. spontaneum* (s) and *Erianthus* (e) clones based on the matrix of nuclear RFLP pairwise distance estimates (Nei and Li, 1979).

In the meanwhile, it can be taken a best profit of the genetic variation available, considering the 6 diversity subgroups determined in this study when selecting *S. officinarum* parents by traditional methods.

A group of 35 wild *Saccharum* complex clones was studied for its nuclear diversity using RFLP, to determine their genetic relationship, their possible origin and to assist their election as new genetic sources (Coto et al., manuscript in preparation).

RFLP and Alu-like PCR amplifications allow to determine the genetic relationship of 7 ECL clones to *Erianthus* spp. and their higher similarity of 8 ECL clones to *S. spontaneum* representatives includes in the study. These results evidenced the occurrence of introgression and outcrossing in wild conditions among members of the *Saccharum* complex and the presence of highly favorable conditions to their diversification in the localities where they were collected.

Monitoring Genetic Diversity of Commercial Hybrids

Genetic diversity among parents

Breeders need accurate methods to obtain progenies with high genetic variance and mean performance. The former is partially dependent on the genetic diversity between parents; that is commonly estimated by phenotypic, genealogical or molecular marker data.

A study was performed to compare these three types of parent distance estimates among a group of 10 sugarcane varieties regularly employed as parents in the commercial program.

Cluster analysis is presented in figure 4. Clonal groupings were different (Cornide et al., 1999a). Although distance estimates based on RFLP data and genealogical information have been reported as highly associated and/or leading to equivalent genotype groupings in several crops (Smith et al., 1990; Boppenmaier et al., 1992; Moser and Lee, 1994; Martin et al., 1995), the lack of association between these estimates in our study is in agreement with previous results by Cornide et al. (1996) for a different set of sugarcane genotypes; by Barbosa-Neto et al. (1996) for wheat; by Graner et al. (1994) for barley and by O'Donoughue et al. (1994) for oat.

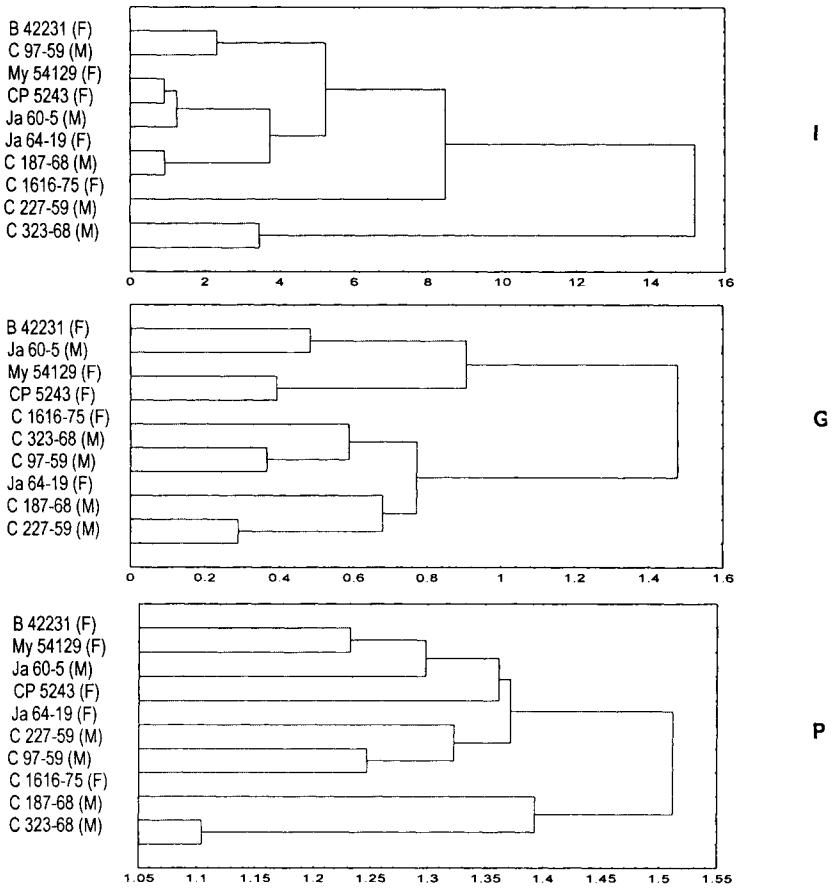

Figure 4. Ward's minimum variance cluster analyses of parental genotypes based on multivariate (I), genealogical (G) and nuclear RFLP distance (P) estimates. (M, F): Male and female parent, respectively.

Violation of genetic assumptions for the coefficient of coancestry estimation (Cox et al., 1985), distance underestimation due to the presence of common variants in non-related genotypes (Bernardo, 1993) and to the presence of different bands with identical electropho-

retic mobility, sampling errors caused by reduced marker and, genotype numbers are the most frequent sources of this bias.

Shared highly conserved variants are probably not the main cause in present study as their proportions were relatively low (0.04-0.08) and modified DP estimates (Bernardo, 1993) to remove this bias were negative in 30 % of the parent combinations. Also, in sugarcane other factors may contribute to these discrepancies, such as the major contribution of *S. officinarum* in some crosses and the polysomic segregation (Deren, 1995).

The presence of environmental and genotype-environment effects was expressed by three different clonal distribution patterns on plane (1,2) of the 5 Factor Analyses of Correspondence Multiple, based on the RFLPs with a major contribution to the total molecular variability and the phenotypic trait means: seedling (PP, PR) and first clonal stages (CP, C1R and C2R) and evidenced that the traits studied do not accurately reflect the genetic diversity among parents.

This factor and the presence of multiple dose fragments in polyploids may explain the lack of correspondence observed between parent groupings according to RFLP distances and to phenotypic classes. (Sorrells, 1992; Wu et al., 1992). A limited number of probes or the lack of linkage between them and the QTLs of interest could also influence these results. Similar results and their possible causes have been reported (Souza and Sorrells, 1991; Moser and Lee, 1994; Lee, 1995).

Present results suggested the usefulness of RFLP patterns to evaluate the true genomic variability present in sugarcane genotypes with independence of its origin, identical by descendance or similar by state, in relation to the coefficient of coancestry and the phenotypic distances.

Associations between parent genetic distance and family performance

Empirical and theoretical results supported the efficiency of family selection at early stage of the sugarcane variety program. Chang and Milligan, (1992) among others, demonstrated that the potential of a cross to produce elite progeny for a trait could be accurately predicted by its family mean at seedling stages. Thus, methods for predicting family means for brix and stalk weight components based on parent evaluation would facilitate the implementation of family selection. To determine the relationship between parent diversity estimators and family mean and heterosis predictors based on progeny tests, 10 sugarcane varieties and their hybrids were evaluated for brix, plant height and the stalk weight components on seedling (PP, PR) and first clonal stages (CP, C1R and C2R). Correlations among RFLP (P), genealogical (G) and multivariate (I) parent distances, the observed family means and 5 predictors of family performance were calculated (Cornide et al., 1999b).

Moderate significant associations between observed family means and the family estimates of the heterosis predictors based on progeny test were observed, the highest values corresponding to brix means. Parent pairwise distance estimates were not associated to observed progeny means. The BLUP model proposed by authors to estimate family mean considering RFLP distances consistently associated to midparent heterosis for brix in all stages (r = .59-.63*) and for other traits in clonal plant cane stage (r = .45-.46*); they were also associated (r= .40-.52*) to brix high-parent heterosis. So, RFLP parent distance had a limited practical value for predicting family means or heterosis among parents studied. These results confirmed previous reports on the predictive value of RFLP diversity estimates.

Although considerable research is currently conducted, correlation's of molecular marker diversity with hybrid performance and heterosis have been too low (Melchinger et al., 1990, Bernardo, 1992; Boppenmaier et al., 1992; Lee, 1995; Zhang et al., 1996). Further research is needed to confirm these associations across a wide range of genetic materials. Better results can be expected increasing the number of markers and selecting them for their linkage relations to QTLs of interest.

Molecular Identification of Accessions

Traditionally, cultivars are identified using morphological and agronomic markers; sometimes, physiological, biochemical or cytogenetic markers complement them. These are basically used for their registration, but are often highly influenced by the environment, and extensive field trials or greenhouse facilities are needed for these evaluations.

New cultivars must be distinct from the already registered ones by the expression of at least one characteristic and they must achieve fixed standards of uniformity and stability. The cultivar trials for registration and identification can be slow and expensive.

In the last years molecular markers have added a powerful new dimension to genetic studies and to the breeding management of plant genetic resources. It can be expected that molecular descriptors will be regularly included in germplasm databases if they guarantee the distinctiveness of each accession in a reproducible and reliable way. They may assist the establishment of core collections and germplasm management for breeding purposes if they are also informative. So, distinctiveness, reproducibility, representatively and informatively are the main requirements to be fulfilled by a descriptor (IPGRI, 1995).

Distinctiveness

The ability of the marker system to distinguish between genotypes is related to it polymorphism and gene diversity. The RFLP system proposed for the identification of sugarcane hybrid and related material was selected for their polymorphism expressed by the number of variants per genotypes and the number of unique variants, and for their high gene diversity estimate according to Nei (1973).

Reproducibility

Reproducibility can be obtained by selecting the marker system for its stability within different individuals of the same genotype and between different experimental repetitions. RFLP, RAPD, AFLP and RAMP have been employed to identify sugarcane commercial hybrids (Lu et al., 1994b; Harvey and Botha, 1996; Sánchez and Cassalett, 1997; Fernández et al., 1998; Canales et al., 1999); somaclones (Canales et al., 1992; Dinkova et al., 1992; Ramos et al., 1996; Cornide and Gálvez, 1999), and elite transgenic plants (Arencibia et al., 1999).

Representativity

Considering the genomic structure of *Saccharum* complex members, the molecular descriptors can be very useful for those purposes. Ancestors and relative forms of sugarcane, as already discussed, are difficult to recognize by a few morphological and agronomic descriptors evaluated in a stool basis in germplasm collections.

Low copy probes may provide a mean to their identification when they are selected by a previous phylogenetic study or diversity analysis (Lu et al., 1994a; Besse et al., 1996, 1997;

Cornide and Gálvez, 1999). Molecular variants associated to groups, such as taxonomic units, diversity groups or geographic origin, and individual variants, presented in one genotype, can be combined to identify an accession. This may reduce the sampling error due to the exclusive use of rare variants in the population that can be present in individuals of different genotypes (Cornide et al., manuscript in preparation).

Our experience employing RFLP confirmed this application. The cytoplasmic RFLP polymorphism was especially useful to individual identification of wild hybrids of unknown origin, within group polymorphism increased when considering each different pattern of bands per RFLP combination as a molecular phenotypic variant. This polymorphism, as it can be expected, revealed a high number of unique patterns. For instance, the mitochondrial ATP subunit 9 (atp-9) probe, allowed the individual identification of 29 clones from a group of 35, including bands associated to the 2 diversity groups represented in the ECL clones studied (Coto et al., manuscript in preparation).

Alix et al. (1998) demonstrated that tandemly repeat satellite DNA sequences are species or genome-specific and that may produce interesting information on chromosome structural organization and may provide useful PCR markers for identification and monitoring sugarcane introgression programs. Recently, Alix *et al.* (1999) reported inter-Alu like species-specific sequences in the *Saccharum* complex. Two pairs of these *Miscanthus*-specific and *Erianthus*-specific Alu-like sequences were employed in the identification of wild ECL clones discarding their relationship with the first genus and confirming their hybrid or derived condition from *S. spontaneum* and *Erianthus* spp. Their *Erianthus*-specificity was also confirmed in clones of at least 3 other species (Coto et al., manuscript in preparation).

Informativity

Long-term breeding is aimed at achieving a balance between continuous genetic gains for economical characters and the maintenance of adequate levels of genetic variation. Thus, recombinant progenies for main selection criteria may exhibit transgressive expression if a highly variable genetic background is maintained.

This balance between specific and general diversity is also important in recurrent selection programs where each cycle is repeated to increase the frequency of favorable alleles at loci controlling the economic traits. So, the third requirement to be accomplished by a descriptor, the informatively, must be achieved using markers associated directly to a specific trait or indirectly to a diversity group, associated at its turn to the trait.

In the *Saccharum* complex the association between a molecular variant and a specific trait is rarely determined from diversity or a phylogenetic study. Sugarcane mapping studies and their comparative genome mapping with other plants, such as sorghum and maize, will provide more informative markers linked to important traits, than can be used to characterize the specific variation among the germplasm bank (Xu et al., 1999). Such is the case of the rust resistance mapped from cultivar R570 (Daugrois et al., 1996; Tomkins et al.,1999).

Concluding remarks

DNA polymorphism revealed by molecular marker techniques such as RFLP, AFLP, RAMP and Alu-like amplification, have demonstrated to be useful complement to traditional methods

for the identification and the diversity assessment of the germoplasm that constitutes the genetic foundation of our sugarcane breeding program.

On the other hand, the demonstration of a high genome synteny in the grasses will provide a powerful mean for understanding and manipulating these species like a single genetic system. Comparative mapping of maize, sorghum and sugarcane has already demonstrated the potentiality of this strategy for sugarcane research and breeding.

Economic characters in sugarcane are quantitatively inherited. Although the ability to map and characterize sugarcane genes has advanced considerably in the past years, it remains a hard work to obtain practical results in selection efficiency and in the gene cloning.

At present, there is a highly positive balance in the molecular marker applications to sugarcane genetic research. Sugarcane breeders and biotechnologists must be aimed in the future to achieve a high resolution in mapping experiments and to the development of practical means to introduce molecular markers of a major and stable contribution to phenotypic expression as regular selection criteria in breeding.

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Somaclonal Variation in Transgenic Sugarcane Plants: Practical Implications

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Introduction

Plant cell culture in the dedifferentiated state is recognized to induce and/or evidence significant somaclonal variants (Larkin and Scowcroft, 1981; Karp, 1991). Some studies regarding with the somaclonal variation within the transgenic plants, showed the presence of polymorphism DNA in control plants regenerated following the tissue culture steps for transformation without DNA. These evidences strongly point that the foreign gene insertion is not the major cause of genome disturbance in transgenic plants (Arencibia et al., 1998b). This phenomenon has already been described in potato (Dale and McPartland, 1992), rice (Schuh et al., 1993; Bao et al. 1996; Arencibia et al., 1998b), poplar (Wang et al., 1996) and sugarcane (Arencibia et al., 1999).

In case of sugarcane, modern hybrids are the product of a breeding program in which, progressively, progenies are repeatedly crossed-back with *Saccharum officinarum*. This process of recurrent introgressive hybridization result in highly heterozygous polyploids with a complex chromosome number (Heinz et al., 1969; Heinz and Mee, 1970; Moore and Fitch, 1990). This mean that the somatic cells of modern hybrids of sugarcane display a significant genetic variability, termed preexisting variability or somaclonal variation (Evans and Sharp, 1986), which is an additional source of variance into the transgenic plants.

Implications of this phenomenon regarding with novel strategies for production of transgenic plants will be discussed below. It is expected that an understanding of a role of somaclonal variation, independent of its origin, within an improvement program through genetic engineering, may help to increase the selection efficiency of the transgenes expressing for agronomic traits. Additionally, the optimizing of the transformation procedures avoiding the calli induction steps, must give transgenic sugarcane plants carrying lowest genomic changes. On the other hand, somaclonal variation could be useful for map-based cloning of agricultural genes by using the chromosome landing approach.

Studying the Somaclonal Variation within Transgenic Sugarcane Populations and Cultured Cells

A population of transgenic sugarcane (*Saccharum hybrid* cv. Ja60-5) genotypes expressing a *tcryIA(b)* gene introduced by cell electroporation were evaluated under severe borer (*Diatraea saccharalis* Fab.) infection. As control populations were used plants regenerated from dedifferentiated culture without transformation (C1-control) and, plants that were clonally propagated in the field (C2-control). Results of field trial provided evidences both for the expression of the insect-resistant trait and for the occurrence of limited, but consistent,

morphological, physiological and phytopathological variation. Molecular tools verified DNA changes in the selected five elite clones. A total of 51 polymorphism bands (out of the 1237 analyzed bands) were produced by AFLP and RAMP analysis, some of these, between control plants. Polymorphism bands found both in C1-control and in the transgenic plants can be interpreted as the sum of genomic variation pre-existing in the original cultivar (C2-control) with that induced during the dedifferentiated cell culture (Arencibia et al., 1999)

To study this phenomenon in plants gathered with different transformation systems, transgenic sugarcane populations produced by *Agrobacterium tumefaciens* were analyzed by AFLP. Transformation was made according with previously reported methodologies (Arencibia et al., 1998a; Enriquez et al., 1998). Summary of AFLP experiments using fourth copy primer is show in table 1.

Table 1. Summary of the results of the AFLP analysis performed on transgenic sugarcane plants

Transgenic population (*)	No. of analyzed bands (**)	No. of polymorphism bands	P.I	No. of polymorphism bands between C1 and C2 controls.
I	74	6	8.1	1
I	80	8	10.0	2
I	68	9	13.2	2
I	76	4	5.2	1
II	72	6	8.3	2
II	80	9	11.2	0
II	82	7	8.5	2
II	63	8	12.6	3

* - AFLP analyses were made with fourth different copy primers. ** - Analyzed bands considering those in the range of best resolution in the gel. P.I: Polymorphism index = (No. of polymorphism bands/ No. of analyzed bands) x 100. I- Transgenic population contains the 35S CaMV-*bar* and ubi1-*omta* cassette. II- Transgenic population contains a combination of AP24, chitinase and glucanase genes.

A total of 595 bands were amplified from which 57 were polymorphism bands. From the lasts, only 13 were identified as polymorphism between C1 and C2-controls and, could be associated with the tissue culture procedure.

Bands on the gels were scored at present or absent and, a dendograms showing genomic similarity were constructed. Data from each transgenic population were analyzed together with C1 and C2-control. Example for 10 transgenic genotypes is shown in figure 1. The resultant dendogram grouped the transgenic genotypes, C1 and C2-control plants in diverse clusters. Non-transgenic plant from the tissue culture procedure (C1) was also classified most at center of the transgenic ones. This fact could explain the presence of somaclonal variants due to the tissue culture within the transgenic clones. Results intended focused the attention that, at least some of these genomic changes could also be present in the transgenic plants, but without a phenotypic expression at least in the primary steps. This assumption aimed to the establishing of appropriate selection schemes for transgenic plants taking into consideration the somaclonal variations independently of it origin.

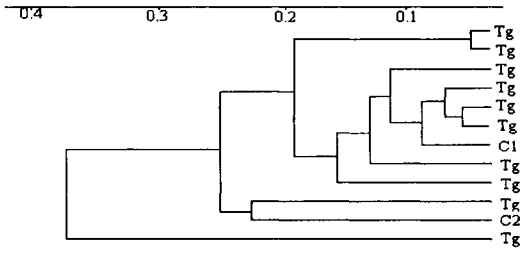

Figure 1. UPGMA dendogram based on the Dice similarity index (SD) illustrating the similarity among the 10 sugarcane transgenic genotypes (Tg) containing both *bar* and *omta* gene. C1 and C2.: Controls plants.

Management of the Somaclonal Variation within an Improvement Program through Genetic Engineering

Molecular analysis of the different sources of somaclonal variation was made for sugarcane. This variability could be considered undesirable in any improvement program through genetic engineering. We suggest that must also be take into consideration when selecting transgenic plants in the field, in this way, a selection scheme for sugarcane transgenic plants is propose in figure 2. This approach has the advantages that notice in primary transgenic plants any eventual somaclonal variation combined with the phenotypic expression of the foreign gene.

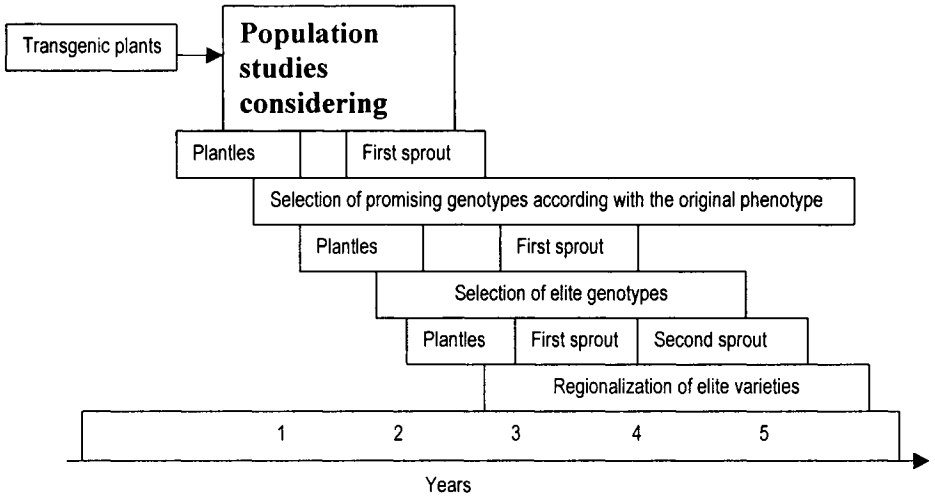

Figure 2. Selection scheme for sugarcane transgenic plants taking into consideration the somaclonal variations.

Avoiding the Somaclonal Variation in the Production of Transgenic Sugarcane Plants

Considering the sources of somaclonal variation, it is unpractical the management of the pre-existing variability into the sugarcane modern genotypes. Both the choice of the starting

material for transformation and the cell targeted for transformations are random events. However, somaclonal variation induced by tissue culture could be management.

Genomic variability induced or evidenced by tissue culture has been demonstrated. Results of polymorphism indexes by studying non-regenerable calli and albino plants could be considered high (manuscript in preparation) and, point that these polymorphisms could be present, at least partially, within the transgenic plants. It is well know that callogenesis is a source of additional variability in plant tissue cultures. All available transformation protocol for sugarcane used the callogenesis as way for propagates the transformed cells and it further plant regeneration (Arencibia et al., 1992; 1995; 1998; Bower and Birch, 1992; Enriquez et al., 1998). For that reason, our recent work has been based on the hypothesis that direct organogenesis must give shoots carrying and evidencing only the *pre-existing* variability, and not inducing a new variability.

As result, we developed a novel approach for sugarcane transformation based on *the A. tumefaciens* infection of the basal zone of *in vitro* plants. Repetitive selective-micropropagation steps eliminated probability for the recovery of mosaic plants. GUS staining was uniform in all parts of the hygromicin-resistant plants. Optimized transformation conditions were plant size between 1.1-3 cm, co-cultivation time ranging 72-96 hours in the dark and bacterial concentration of DO_{620nm}= 1. Frequency for hygromicin-resistant (30-mg/L)-plant recovery was 1,87 % and 2,5 % using EHA105 (pCambia1301) and LBA4404 (pTOK233), respectively.

Advantages of this methodology regarding with the presence of somaclonal variants into the transgenic plants will be studies based on high-volume molecular tools for genome exploration as AFLP.

Map-Based Cloning of Agronomic Somaclonal Variants

There are some reports on the identification and cloning of genes correlated with specific mutant phenotypes using a map-based strategy (Breyne et al., 1997). Since chromosome walking can be very tedious, chromosome landing is the preferred method for gene identification. This approach requires the identification of markers that are very tightly linked to the mutant locus.

Applications of AFLP are very extends for plant breeding, molecular biology and genetics. Because of the high multiple radio of AFLP, linked markers can be screened for. Independent positional cloning experiments have confirmed the efficiency of the AFLP technique for chromosome landing (Ballvora et al., 1995; Meksem et al., 1995; Thomas et al., 1995). Accuracy and powerful of it technique have also been demonstrated by the chromosome landing at the *Arabidopsis* TORNADO1 locus (Cnops et al., 1996) and, the amplification of differential pattern between template DNA from different plant organs of wheat (Donini et al., 1998).

Somaclonal variation has been widely employed for plant genetic improvement (Karp, 1991). For sugarcane, this source of variability has been used in the selection of clones resistant to biotic (Ramos et al., 1996) and abiotic stress. Somaclonal variants of sugarcane (*Saccharum* spp.) have been obtained resistant to either eyespot (*Helmintosporium sacchari*), rust (*Puccinia melanocephala*) and high-salt concentration. Some of these genotypes have

been demonstrated resistant by biochemical and genetic mechanisms in field trials for at least three years.

Considering the somaclonal variation as mutation, we are involved in the isolation and characterization of some of the sugarcane genes correlated with the stress resistant. AFLP technique supports the chromosome landing strategy considering the agronomic-somaclonal variants and its donor genotypes. Others molecular markers as RFLP and microsatellite are used as complementary chromosome walking approach. Until now, taking into consideration the presence of the pre-existing variability of sugarcane, some polymorphism bands has been identified between the selected genotypes (somaclonal variants) and the original clone. These bands could be presumptively correlated with DNA region associated with the resistant to fungi and salts stress, independently. Backcrossing between the somaclonal variants and the donor genotype are in progress.

Remarks

Somaclonal variation is an intrinsic phenomenon that must be taken into consideration in any program for sugarcane improvement through genetic engineering. We suggest the study of this source of variation for other species with similar characteristics, which means, plants of high genome complexity where the regeneration event is achieve by callogenesis. On the other hand, primary results are very promising and point to the use of somaclonal variation as an exciting source for gene cloning.

Acknowledgments

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Plant Genetic Engineering: Towards the Third Millennium
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On the Mechanism of Horizontal Gene Transfer by *Agrobacterium tumefaciens*

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Introduction

Our current interest is on elucidating the promiscuous DNA transfer system of *Agrobacterium tumefaciens*. This organism uses DNA transfer in pathogenesis, which culminates in the formation of crown gall tumors on the plant host. The processes involved in preparing the DNA for transfer and for the biosynthesis of the transmembrane DNA-protein transport system are encoded by *vir* genes on a large resident Ti plasmid. The *vir* genes are arranged into six-major operons clustered within the 38.6 kb *vir* regulon on the Ti plasmid (Rogowsky et al., 1990). The *vir* genes become fully expressed upon perception by *A. tumefaciens* of chemical signals from the host plant. These signals are generated by the plant especially at wounded sites to initiate wound healing. The compounds elaborated are precursors of cork or lignin, of which the condensation precursors are dimethoxyl phenols such as sinapinic acid, ferulate and coumarate, including sinapyl alcohol, coumaryl alcohol and coniferyl alcohol (Kado, 1991). These signal molecules are detected by a transmembrane histidine kinase: VirA. The signal is transduced from VirA to the transcriptional activator VirG by transphosphorylation.The resulting effect is the expression of the remaining *vir* genes via this two-component signal transducing system (Winans, 1992; Das, 1998).

The expression of the *vir* genes results in the initiation of the processing of the 25 kb T-DNA and in the biosynthesis of a transmembrane channel through which this DNA is specifically transferred out of the bacterial cell and into the host plant cell. The T-DNA processing reaction appears to be analogous to the processes involved in the transfer of DNA of broad-host-range plasmids such as RP4, whereby a nicking reaction takes place at the right and left borders of the T-DNA followed the formation of a relaxation complex termed the relaxosome. The multi-functional VirD2 protein attached to the 5'-end of the displaced single-stranded T-DNA serves presumably to guide the T-DNA complex through the transmembrane channel and into the plant cell. The characteristics of this channel, the DNA transfer mechanism, including the formation of the *trans*-membrane channel, are not well understood (Winans et al., 1996; Christie, 1997). To gain further insight on the T-DNA transfer apparatus, our previous sequence homology studies revealed that the *virB* genes and their proteins were identical in gene organization and were highly homologous in nucleotide sequence to those genes involved in conjugative pilus formation of plasmid F in *Escherichia coli* (Shirasu and Kado, 1993a,b). Other groups also reported similarities between the sequence of *virB* genes and those of conjugative plasmids such as IncP plasmid RP4 (Motallebi-Veshareh et al., 1992; Lessl et al., 1992) and IncN plasmid pKM101 (Pohlman et al., 1994). Close examination of the *virB* genes revealed a striking sequence similarity between VirB2 of the Ti plasmid and

TraA of F plasmid. TraA is known to be the propilin protein that is processed from12.7 kDa into a 7.2 kDa protein which is the pilin subunit of the conjugative F pilus (Frost et al., 1994). Likewise, VirB2 is processed from a 12.3 kDa protein into a 7.2 kDa protein (Shirasu and Kado, 1993b; Jones et al., 1996). By virtue of the similarities between VirB2 and TraA protein sequence and processing, the *virB2* gene as encoding a pilus appeared to be needed for the transfer of the T-DNA (Kado, 1994b), and indeed *virB*-specific pili were observed (Kado, 1994a, Fullner et al., 1996). VirB-specific pili, whose production also depends on the VirD4 protein, are produced in abundance at 19°C (Fullner et al., 1996). Definitive studies demonstrated that the *virB2* gene indeed encodes the pilus, which is composed of processed VirB2 protein (Lai and Kado, 1998). The *virB2*-specific pilus was designated heretofore as the "T-pilus" (Lai and Kado, 1998). The T-pilus is a long filamentous structure with a diameter of 10 nm. The T-pilus may serve as a protective tube or conduit through which the T-DNA is transferred from the *Agrobacterium* cell into the plant cell. Hence, we propose herein that the T-pilus may be the apparatus used to deliver the single-stranded T-DNA into the plant cell where the single-stranded DNA binding protein VirE2 encapsulates the DNA.

Materials and Methods

The flagella-free strain NT1REB (Chesnokova et al., 1997) containing the appropriate plasmid was used in the mating studies and for visualization of the T-pilus. The plasmids were as follows: pUCD2614, a replicon containing only the *vir* regulon was used as the helper plasmid (Rogowsky et al., 1990); pUCD2335 containing the right border sequence and selectable markers for transformation (Zyprian and Kado, 1990). *Streptomyces lividans* TK60 was kindly provided by Stanley Cohen. Induction medium (I-medium) was described by Rogowsky et al. (1987). Inductions were performed using 200 μM of acetosyringone (Aldrich). *S. lividans* was grown in R2 medium (Hopwood et al., 1985).

T-pilus was purified as described previously (Lai and Kado, 1998) and further refined by cesium chloride density gradient centrifugation (Eisenbrandt et al., 1999). Electroporation was carried out as described by Cooley et al.(1991). Thin sections were prepared using epoxy (50 % in acetone) infiltrated into the specimen for 2-4 h at room temperature. The specimen was then placed in epoxy resin overnight at room temperature. Sections of 1.5 μ were prepared from the solidified specimen and stained with a mixture consisting of a 1:1 mixture of 1 % hot methylene blue in 1 % Na borate and 1 % azure II. Sections were made with a RMC MT-2 ultra-microtome with a diamond knife and transferred to 200 mesh grids. The sections on the grids were stained with 2 % uranyl acetate and 2 % lead citrate. Transmission electron microscopy was performed using a Phillips model EM410 electron microscope at 80 kV under conditions described previously (Lai and Kado, 1998). Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF-MS) was performed with a Bruker Biflex III mass spectrometer (Bruker Deltonics, Billerica, MA) in the Facilities for Advanced Instrumentation, University of California, Davis.

Results and Discussion

VirB2 is the propilin of the T-pilus

Previous sequence analyses of the *virB* operon of both nopaline and octopine Ti plasmids revealed a high degree of sequence conservation of the genes of this operon. Studies were subsequently initiated to identify the functions associated with each *virB* gene product. Our

comparative analyses revealed a striking homology of the VirB2 protein to that of TraA which is the propilin of F plasmid in *E. coli* (Shirasu and Kado, 1993). Like TraA, VirB2 is processed from 12.3 kDa to a 7.2 kDa protein which localizes mainly on the inner membrane. Because TraA is the propilin of the conjugative F pilus, we reasoned that VirB2 may be the propilin of a pilus in *A. tumefaciens*. This hypothesis was confirmed by the visualization of a VirB2-specific pilus that was subsequently purified and characterized (Lai and Kado, 1998). No other VirB proteins were found associated with the purified pilus, designated as the "T-pilus" (Lai and Kado, 1998). This was confirmed by mass spectrometry (below).

Construction and use of a bald strain of A. tumefaciens *for filament-free analyses*

Visualization of the T-pilus was made clearer with a bald strain than when wild type, flagella-bearing strains of *A. tumefaciens* were used. The bald strain was constructed by first identifying the genes involved in flagella biogenesis followed by their removal by deletion mutagenesis (Chesnokova et al., 1997). The bald strain designated NT1REB proved to be useful in studies dealing with the visualization of the interconnecting element between *A. tumefaciens* and the host cell.

Visualization of the T-pilus interconnection between A. tumefaciens *and* Streptomyces

The large size differential between the bacterial cell and the plant cell reduced the probability of obtaining a serial thin-section in the same plane as the interconnecting element and the *Agrobacterium* and plant cell. Thus, we recruited the use of *Streptomyces lividans* as the recipient since there is a clear distinction in the morphology of this organism with that of *A. tumefaciens*, and since their sizes were closer than that of *Agrobacterium* cells associated with plant cells (Figure 1).

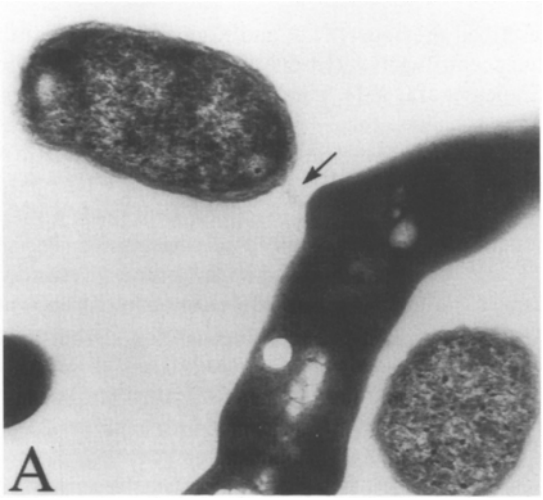

Figure 1. Attachment of *A. tumefaciens* to *S. lividans* hyphae via a supercoiled T-pilus (arrow).

Strain NT1REB harboring the T-vector pUCD2335 was induced with acetosyringone and mixed with *S. lividans* hyphae. The resulting transconjugants were selected on agar plates containing the appropriate antibiotics. Serial sections were made of a series of matings to

visualize any potential mating "bridges". As shown in figure 1, a single rod-like interconnection was observed between *A. tumefaciens* cells and the hyphae of *S. lividans*. Such interconnections were never observed when either *A. tumefaciens* was not induced with acetosyringone, or lacked the Ti plasmid or T-vector; or contained a polar and non-polar mutations in *virB* genes.

The interconnecting element is therefore *virB* gene-specific and is dependent on *virB* gene induction for its production. It is reasonable to assume that the products of the *virB* gene are needed for the assembly and function of the trans-membrane pore, and that a mutation in a *virB* gene causes a non-functional trans-membrane apparatus including the absence of T-pilus formation. The interconnecting element may therefore be the T-pilus since the element has a diameter similar in size to that of the T-pilus and is dependent on the *virB2* gene.

Characterization of the T-pilus

Studies were undertaken on the purified T-pilus to determine its physical characteristics. The T-pilus filaments were subjected various environmental factors, including elevated temperature, pH extremes, and enzyme treatments. For example, treatment with Triton X-100 caused the T-pilus to dissociate leaving a donut shaped structure of about 30 nm in diameter and a central hole of 6 nm. The lumen of the T-pilus was made distinctly visible by treatment with 0.1 % SDS (Figure 2). The lumen, which extends the length of the T-pilus, is sufficient in diameter (2-3 nm) to accommodate the T-DNA terminally complex with the VirD2 protein.

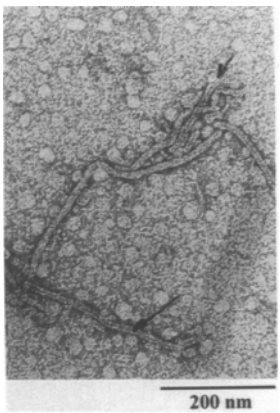

Figure 2. Purified T-pilin treated with 0.1 % SDS exposing its lengthwise lumen.

Characterization of the T-pilin

Purified T-pilin was analyzed by MALDI-TOF mass spectrometry at 7 to 10 K resolutions. As shown in figure 3, the T-pilin has a mass of 7184.2 Da. This value is less than the predicted mass of 7202.2 Da based on amino acid composition. The difference between the two masses is 18 kDa, a value equal to that of one water molecule. Hydrolysis of the T-pilin can arise from the formation of an ester or peptide bond.

When the T-pilin was analyzed using an acidic matrix, which will normally cleave ester bonds, the peptide remained intact, indicating that the T-pilin formed a peptide bond. The only

plausible peptide bond formation will be due to a circularization of the T-pilin. This was verified by on-target cleavage of the T-pilin with trypsin and chymotrypsin, which generated specific peptides with the predicted masses and contained the peptide bonded site between the N-terminal glutamate and the C-terminal glycine as predicted. On target peptide sequence analyses verified that a head-to-tail linkage occurred at the signal peptide cleavage site glutamine-48 and the carboxy terminal glycine-121. Mass spectrometry of the T-pilin revealed no other protein associated with this element.

The formation of a cyclic peptide used as a structural subunit of an external appendage is novel in bacteria. There are, however, semi-cyclic biologically active peptides such as bacitracin A, which is synthesized by *Bacillus licheniformis*, and the syringomycin family of phytotoxins, which are produced by *Pseudomonas syringae* (Bender et al., 1999).

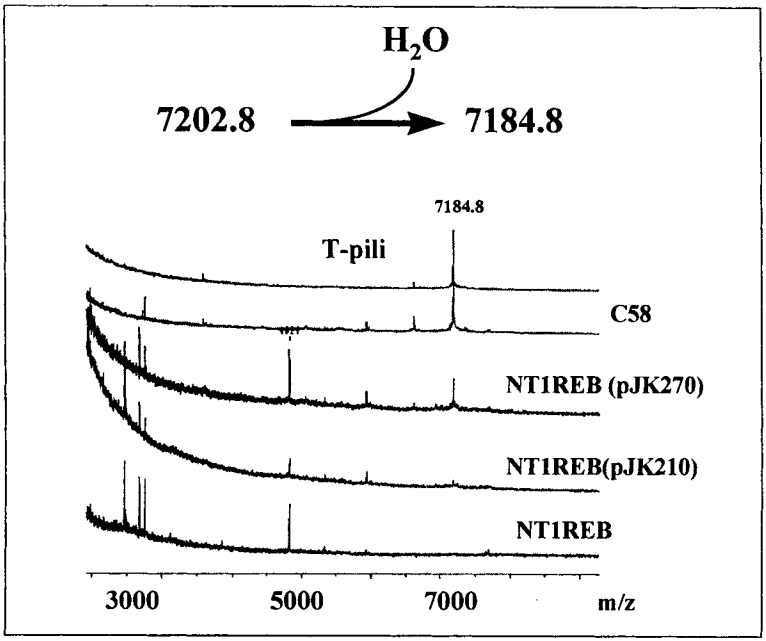

Figure 3. MALDI-TOF mass spectrograph of purified T-pili of *A. tumefaciens* C58 and compared to whole cell analyses of *A. tumefaciens* NT1REB harboring either Ti plasmid pJK270 or pJK210 containing a polar mutation in *virB10*, or no Ti plasmid.

From the results of these studies, we conclude that upon induction with acetosyringone, *A. tumefaciens* forms a filamentous polar appendage called the "T-pilus". This pilus is composed of a 7.2 kDa processed and cyclized VirB2 protein called the "T-pilin".

As shown as a hypothetical model in figure 4, the T-pilin makes up the entire T-pilus through which the single-stranded T-DNA passes into the plant cell.

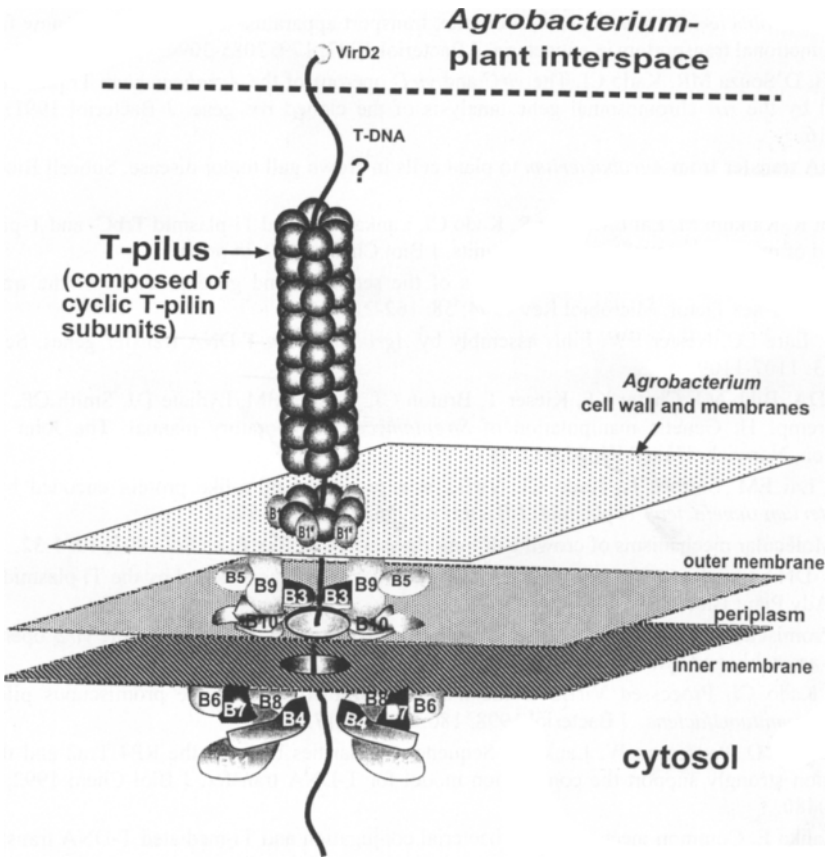

Figure 4. Hypothetical model of the T-pilus through which the single-stranded T-DNA is transmitted

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Plant Genetic Engineering: Towards the Third Millennium
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Sugarcane (*Saccharum hybrid*) Genetic Transformation Mediated by *Agrobacterium tumefaciens:* Production of Transgenic Plants Expressing Proteins with Agronomic and Industrial Value

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Introduction

Sugarcane is an important crop for many countries around the world. Investment in breeding efforts remains the best approach for maximizing productivity of sugarcane. However, due to the biological complexity of this crop, sugarcane breeding can benefit a lot from the use of non-conventional methods. In particular, genetic engineering can not only shorten the period of time and reduce costs to produce an improved sugarcane line, but it can also incorporate to sugarcane genome new important agronomic traits that are absent in the natural germplasm of this species such as, resistance to pests or herbicides. In some crops, resistance to fungal attack has been enhanced through the expression of antifungal proteins in transgenic plants (Cornelissen and Melchers, 1993). The industrial behavior of this crop could be also improved by gene engineering, increasing the quality of the plant as raw material for the production of sugar and/or other by-products. Moreover, the high biomass production capacity and low agronomical requirements make sugarcane an excellent candidate to be used as bioreactor in the synthesis of new products for medical or industrial applications. As interesting application, we approach the expression of *Acetobacter diazotrophicus* levansucrase (LsdA) in transgenic plants. This enzyme acts on sucrose yielding high amounts of 1-Kestose (Tambara et al., 1999), a fructooligosaccharide (FOS) which is commercially used for a healthy nutrition of humans and animals (Yun, 1996). The bacterium *Agrobacterium tumefaciens* has been widely used as a vehicle for the genetic transformation of dicotyledoneous plants. However, *Agrobacterium*-mediated gene transfer into monocotyledonous plants was not possible until recently, when reproducible and efficient methodologies were successfully established for rice (Hiei et al. 1994). Genetic transformation mediated by *Agrobacterium* has advantages over direct transformation methods in aspects such as: lower copy number of the transgen, leading to fewer problems with co-suppression events and genes instability. In this study, we use an antinecrotic solution during *Agrobacterium*-sugarcane tissue interactions in order to increase the cellular viability.

Materials and Methods

Plant material

Conditions for aseptic culture of sugarcane cv. Ja60-5 and B4362 in MS basal medium (Murashige and Skoog, 1962) were established essentially as described by Ho and Vasil, (1983). The compositions of culture media used in this work are described in table 1.

Table 1. Composition of the culture media for *Agrobacterium*-mediated genetic transformation of sugarcane c.v. Ja60-5 and B4362.

Medium	Composition
P^{+5}	MS Salts; 1 mg/L nicotinic acid; 0.8 mg/L vitamin B_1; 0.5 mg/L vitamin B_6; 100mg/L myo-inositol; 20 g/L sucrose; 500 mg/L casein hydrolysate; 5mg/L 2,4 D; 8g/L Phytoagar
$P^{+5}Ao$	P+5 supplemented with 15 mg/L ascorbic acid; 40 mg/L cystein; 2 mg/L silver nitrate
$P^{+5}CR$	P+5 supplemented with 50mg/L of chlorophenol red
P^{-}	MS Salts; 1 mg/L nicotinic acid; 0.8 mg/L vitamin B_1; 0.5 mg/L vitamin B_6; 100mg/L myo-inositol; 20 g/L sucrose; 7g/L Phytoagar
$P^{+5}Hyg$	P^{+5} supplemented with 120mg/L Hygromycin
$P^{-}Hyg$	P^{-} supplemented with 30mg/L Hygromycin

For genetic transformation assays, spindle sections were taken from field-grown 6 month-old sugarcane plants and sterilized by immersion in a 1.5 % NaClO solution during 20 minutes. The sensitivity of meristematic explants of Ja60-5 and B4362 obtained from *in vitro* and field-grown plants to different concentrations of PPT and hygromycin agents were determined during different culture phases.

Bacterial strain and plasmids

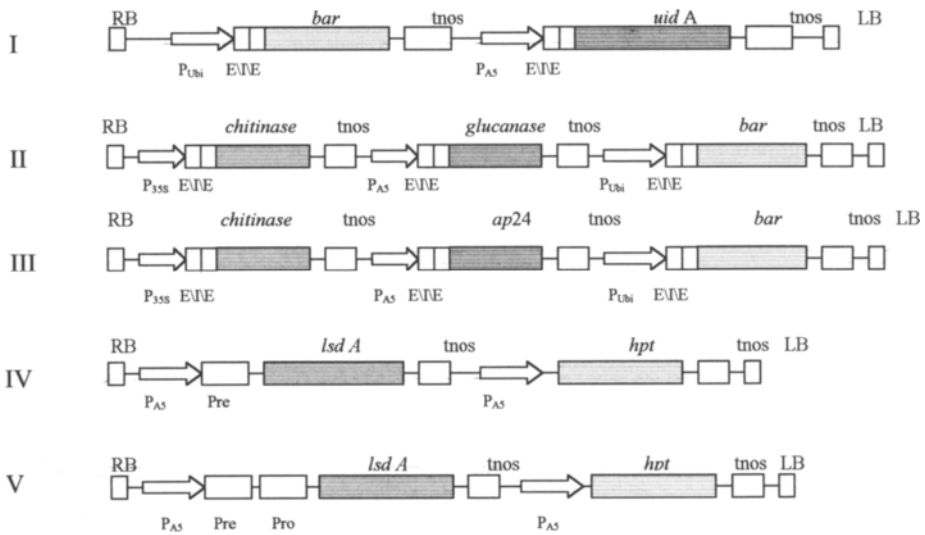

Figure 1. Integrations cassettes of genetic constructions used for sugarcane transformation. Abbreviations: RB and LB, right and left borders; P_{Ubi}, ubiquitin promoter; E\|I\E, exon-intron-exon; tnos, terminator of nopaline synthase; P_{A5}, promoter system composed of a 18-bp enhancer from the octopine synthase promoter (18ocs), the 35S CaMV promoter; P_{35S}, 35S CaMV promoter. I. pGT GUSBAR: carrying *bar* and *uid* A genes. II;.III. pHCG59 and pHCA58 carrying chitinase, glucanase.and Ap24 genes. IV. pHES82: carrying Pre, sporamine signal peptide coding sequence; *lsdA*, levansucrase from *A. diazotrophicus* and *hpt*, hygromycin phosphotransferase genes. V. pHES83: carrying Pre, sporamine signal peptide coding sequence; Pro, sporamin vacuolar-targeting signal coding sequence; *lsdA*, levansucrase from *A. diazotrophicus* and *hpt*.

Agrobacterium tumefaciens strain At2260 was used throughout this work. The bacterium was grown on YEB medium supplemented with rifampicin (50 mg/L), carbenicillin (50mg/L), ampicillin (100mg/L), streptomycin (100mg/L) and spectinomycin (100mg/L) as needed.

The plasmid pGT GUSBAR bearing *uid* A and *bar* genes was used for establishment of a genetic transformation protocol (Enríquez et al., 1997). Additional plasmids were constructed for the expression of the levansucrase from *Acetobacter diazotrophicus* (LsdA) (pHES82 and pHES83), and co-expression of glucanase and Ap24 (pHGA58) or chitinase and glucanase (pHCG59) (see results and discussion). Plasmids were introduced into *A. tumefaciens* by direct transformation protocol (Holster et al. 1978).

Sugarcane genetic transformation

The effect of three antioxidant (Ao) compounds on cellular viability of sugarcane meristem and *Agrobacterium* growth were evaluated at different incubation intervals. Optimal conditions for calli culture in the presence of these compounds were established as ascorbic acid (15mg/L), cystein (40mg/L) and silver nitrate (2mg/L).

A. tumefaciens harboring the binary plasmid pGT GUSBAR was grown in YEB medium to an OD_{620} of 0.6. The cells were collected by centrifugation and resuspended in the initial volume of P^{+5} medium supplemented with antioxidants ($LP^{+5}Ao$). Genetic transformation was performed as depicted in the flowchart in figure 2, according to protocol described by Enríquez et al. (1998). The same procedure was also repeated with the inclusion of acetosiringone 20 mg/L in $LP^{+5}Ao$ medium.

Isolation of meristematic sections from field- or *in vitro*-grown sugarcane plants, cv. Ja60-5 and B4362.

⇓

Incubation of meristematic explants in liquid $P^{+5}Ao$ medium for 6 hours in the darkness.

⇓

Add 1 ml of *At* culture to the $LP^{+5}Ao$ medium (in proportion 1:25), and incubate the explants at 28 °C for 10 min.

⇓

Transfer the explants to P^{+5} medium for 3 days.

⇓

Transfer explants to P^{+5} medium supplied with cefotaxime (500 mg/L) for 10 days at 28°C in the darkness.

⇓

Selection on $P^{+5}Sel$ for 5 weeks and multiplication on the same medium.

⇓

Plant regeneration on solid P^{-} medium in illuminated room at 25°C.

⇓

Soil adaptation of regenerated plants in the greenhouse.

Figure 2: Flow chart describing the procedure enabling for meristems genetic transformation of two sugarcane commercial cultivars (Enriquez et al., 1998).

Transformed PPT-resistant calli were cut and subcultured every month on medium P^{+5}Sel during three months. PPT resistance of small calli was confirmed by the chlorophenol red method (Kramer et al., 1993). Calli able to grow in PPT containing medium, produce a yellow halo up to 24 hours of incubation. Regeneration was carried out on P^- medium according to standard procedures (Ho and Vasil, 1983). Greenhouse trials of transgenic plants were performed on flowerpot-grown plants. Application of the herbicide BASTA in a 2.5g/L solution was performed by foliar spray 60 days after planting. Resistance was evaluated qualitatively. Only plants without any visible foliar damage due to chemical toxicity were submitted to field trials. DNA from field-grown BASTA-resistant sugarcane plants was purified according to Dellaporta et al. (1983). Southern Blot analysis was performed as described by Sambrook and Fritsh (1989) using as probe a 1.2 kb DNA fragment containing the *bar* gen ^{32}P-labelled with a Prime-a-Gene system (Promega) (Enríquez et al., 1998).

Hygromycin resistant calli transformed with pHES82 and pHES83 were regenerated in medium P^-Hyg (Table 1). Hygromycin resistant plants were adapted to greenhouse and assayed by PCR for the presence of transgenes.

Results and Discussion

Meristematic sugarcane explants isolation from *in vitro*- or field-cultured plants is usually accompanied by rather large tissue necrosis, visualized as browning phenomena. These symptoms are enhanced during *Agrobacterium*-meristematic tissue interactions, especially in explants from field-grown conditions. In order to reduce necrogenesis, explants of Ja60-5 and B4362 were treated with a combination of antioxidants compounds. Cell viability was evaluated with Evans Blue staining method (Evans et al., 1983). Sugarcane meristem responded positively to these treatments with a significant reduction of necrogenesis and calli death (Enríquez et al., 1997; 1998). The antioxidant mix of ascorbic acid, cystein and silver nitrate did not affect either *Agrobacterium* growth nor sugarcane callus quality.

Genetic transformation

The inhibitory effect of PPT on meristematic tissue in different culture phases was studied. Meristems from both, *in vitro*- and field-grown plants stopped their growth at 4mg/L of PPT and died. In some species, setting up transgenic plant selection conditions using PPT resistance is difficult (D'Halluin et al., 1995). We determined that the best concentration of PPT for selection is 4 mg/L and that it is very important the use of this selective agent only ten days after co-culture. Application of PPT selection immediately after co-cultivation caused a reduction of GUS activity (Jefferson, 1987) at the seventh day from 29 to 5 and 40 to 14 spots for Ja60-5 and B4362, respectively (Table 2), probably due to a reduction of cellular activity. By contrast, incorporation of PPT in the culture medium at the tenth day after co-cultivation resulted in an enhancement of GUS activity from 3 to 26 and 7 to 49 spots for Ja60-5 and B4362 respectively (Table 2). These results might be explained by a presence of a higher proportion of transformed cells in the explant. No enhancement of transformation efficiency resulted from the use of acetosiringone.

Most of the cells colonies recovered after the first month of PPT selection proliferated when subcultured to fresh selective medium. Plants were readily regenerated when resistant tissue was transferred to a regeneration medium without PPT. A large number of these plants

were resistant to BASTA herbicide in green house conditions (Enríquez *et al.* 1998). Genomic Southern blot on BASTA resistant plants confirmed transgene integration.

The previous protocol was used to obtain transgenic plants from B4362 transformed with the genetic constructions pHCA58 and pHCG59. PPT resistant calli derived from infection gave rise to plants when in regeneration medium (Table 3). The number of resistant calli and the total number of plants regenerated using pHCG59 were higher when compared with those from pHCA58. While plants regenerated per callus ratio was very similar in both cases (Table 3). All plants obtained using pHCA58 plasmid (151 plants) and pHCG59 plasmid (227 plants) were adapted to greenhouse and at the height of 50 cm transferred to field conditions. Additionally ten non-transformed plants were used as controls. Evaluation of transgenic plants resistance to natural infection by the fungal pathogen *Puccinia melanocephala* is in progress.

Table 2. Influence of the PPT selection on the transformation efficiency by GUS activity in Ja60-5 and B4362, cultured at different times with the selection agent.

Sugarcane meristem	Co-culture	7 Days		14 Days	
		+PPT[1]	-PPT	+PPT[2]	-PPT
Ja60-5	65	5	29	26	3
B4362	53	14	40	49	7

1. PPT was added after co-culture. 2. PPT was added at 10 days after co-culture.

Table 3. Efficiency of transformation in sugarcane B4362 with two antifungal genes combinations.

Constructs	PPT-resistant calli	Shoots/callus	Numbers of plants evaluated	Plants adapted to greenhouse (%)
pHCA58	91	8	151	151 (100)
pHCG59	192	5	230	227 (98.6)
Control	-	6	12	12 (100)

Hygromicin has been reported as an effective selective agent for monocots species (Hiei et al., 1994; Arencibia et al., 1998). In this report a concentration of 120 mg/L of this antibiotic was needed to inhibit sugarcane meristematic growth during callus formation. Transformation using plasmids pHES82 and pHES83 was carried out as described above. Transformed cells formed calli clearly separated from the necrosed tissue. At the regeneration stage, hygromicin at 30 mg/L was enough to kill non-transformed tissue. Hygromycin resistant plants were transferred to greenhouse and the transgenes were detected by PCR. Plasmids pHES82 and pHES83 were designed for expression of the enzyme and localization in the apoplast and vacuolar, respectively, with the aim of converting sugarcane into a source of fructooligosaccharides.

The present work suggests that difficulties in the *Agrobacterium*-mediated gene transfer to monocot plants be related with the poor survival rate of the target cells. By using an antinecrotic pretreatment of the meristematic explants, we have achieved good transformation efficiencies with an ordinary *A. tumefaciens* strain, a conventional binary genetic vector and without the use of chemical inducers (acetosyringone).

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Plant Genetic Engineering: Towards the Third Millennium
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Progress in *Agrobacterium*-mediated Maize Transformation at the Plant Transformation Facility of Iowa State University

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Introduction

Biolistic transformation for the recovery of fertile transgenic plants (Gordon-Kamm et al., 1990) is now a routine procedure in maize transgenic research. For example, in our laboratory, averages of 10 transgenic events are recovered from selection for every 100 callus pieces or immature zygotic embryos bombarded (Frame et al., submitted). Recent publications have indicated that *Agrobacterium*-mediated gene transfer to maize may offer some advantages to biolistic transformation (Ishida et al., 1996; Zhao et al., 1998). In addition to being highly efficient (Ishida et al., 1996 and Zhao et al., 1998), this method produced a higher proportion of low or single copy transgenic events than did comparable bombardment experiments (Zhao et al., 1998). To take advantage of these potential benefits, our laboratory undertook to implement a routine protocol for *Agrobacterium*-mediated transformation of maize. Our initial research goal was to transform maize using the super binary vector system and immature zygotic embryos of the maize inbred line A188, as described by Ishida et al. (1996). Here we demonstrate the production, at low efficiency, of transgenic plants and progeny from maize A188 immature zygotic embryos using *Agrobacterium*-mediated gene transfer.

Materials and Methods

Agrobacterium plasmid and strain

The super-binary vector system (PTF3) used in our study was similar to pSB131 described in Ishida et al. (1996). However, the 35S-promoter-*bar* and 35S promoter-*gus* in pSB131 were replaced with a fragment containing the *bar* and *gus* genes both driven by the maize ubiquitin promoter (Christensen and Quail, 1996). *Agrobacterium tumefaciens* LBA4404 (PTF3) was maintained on solid AB medium (Lichternstein and Draper, 1986) supplemented with 100 mg/L spectinomycin. For infection, bacteria from a 3 day-old plate culture were suspended with a spatula or platinum loop in infection to achieve 2×10^9 cfu ($\cong OD_{600}$ of 0.8).

Plant material

Immature zygotic embryos (1.0-2.0 mm) from growth-chamber grown ears of the maize inbred genotype A188 were dissected 8-10 days after pollination for infection.

Media

LS-infection medium (LSI), LS co-cultivation medium (LSC) and LS selection medium (LSS) were as described by Ishida et al. (1996). Revised versions of these media were also used for

comparison in most experiments. Revised LS-infection medium (LSIrev) differed from LSI as follows: 0.5 g/L MES was added, glucose was reduced to 10 g/L, sucrose was reduced to 20 g/L, acetosyringone concentration used was 200 μM and the pH was raised to pH 5.4. Revised LS co-cultivation medium (LSCrev) was modified by using 200 μM acetosyringone and 0.9 % Gelrite and revised LS selection medium (LSSrev) was modified by using 1.25 % Gelrite. Selection pressure was reduced in all experiments compared to the levels reported by Ishida et al. (1996).

Infection and co-cultivation

For infection, immature zygotic embryos were dissected to LSI or LSIrev medium in eppendorf tubes. In some experiments, embryos were first dissected to LSCrev and then transferred to liquid infection medium in eppendorf tubes. Washing and infection procedures were as describe in Ishida et al. (1996). *Agrobacterium* suspension, washing and infection were carried out in LSIrev medium exclusively or in side by side comparisons with the unrevised media. Embryos were co-cultivated (25°C) for 3 or 4 days, embryo axis side down on LSC or LSCrev medium. Petri-plates were wrapped with vent tape during co-cultivation.

Selection and regeneration

Selection was begun immediately after co-cultivation on LSS or LSSrev medium containing 250 mg/L cefotaxime and 1 or 3 mg/L glufosinate. If embryos did not appear swollen and vigorous after co-cultivation, they were transferred first to LSS or LSSrev without glufosinate for 3 days and then taken to selection. Two to 3 weeks later, selection was enhanced from 1 or 3 mg/L glufosinate to 5 mg/L glufosinate. Callus pieces were subcultured every two weeks on 5 mg/L thereafter. Putatively transformed events were identified 8 weeks after infection based on their prolific growth habit. They were bulked up and subcultured for several months on 8 mg/L glufosinate (LSS or LSSrev). For regeneration, embryogenic Type I calli were first transferred to MS medium (Murashige and Skoog, 1962) containing no hormones and 6 % sucrose (Armstrong and Green, 1985) supplemented with 250 mg/L cefotaxime and 3 mg/L glufosinate. The medium was solidified with 1.5 % Gelrite. Petri-plates were wrapped with vent tape and cultured for 2-3 weeks in the dark (25°C). Mature somatic embryos were transferred to the light on the MS medium as described but with the sucrose content reduced to 3 %, where roots and shoots emerged.

R_0 plantlet and R_1 progeny screening

A glufosinate spray test to screen for *bar* gene expression in putatively transformed R_0 plantets and R_1 progeny was conducted in the greenhouse. Plants were sprayed twice (4 days apart) with a freshly prepared solution of 250 mg/L glufosinate and 0.1 % Tween in water (Brettschneider et al., 1997) using a hand-held sprayer. R_0 plants were first sprayed about 10 days after transplant to soil and seed-derived progeny were first sprayed 9 days after planting. In all spray tests, A188 seed-derived plants were used as negative controls. For chi-square (χ^2) segregation analysis, the number of glufosinate resistant and glufosinate susceptible (dead) plants were assessed 2 weeks later.

Molecular analysis

Histochemical analysis of expression of the *gus* gene in Type I callus and leaf tissue of primary transformants or R_1 progeny plants was done according to Jefferson, (1987).

Transient GUS assays were carried out 6 days after infection (after 2-3 days on 250 mg/L cefotaxime). Southern blot analysis on leaf tissue of primary transformants and progeny plants was done as described by Southern, (1975). Genomic DNA from transgenic plants was digested with Hind III that cut only once in the T-DNA and probed with the *bar* gene.

Results and Discussion

Putative transformants were recovered from two experiments, as outlined in table 1. In Expt 11/12/98 embryos from the same 5 ears were applied to each treatment. Selection was delayed for 3 days after co-cultivation and initiated on 1 mg/L glufosinate. In Expt 4/30/99 embryos from the same 2 ears were applied to each treatment and selection was begun on 3 mg/L glufosinate selection immediately after co-cultivation. Both putative events were recovered from treatments in which LSIrev was used during infection. The Type I callus response varied considerably between treatments with putative events arising from the highest (100 %) and the lowest (25 %) responding treatments represented (Table 1). Table 2 outlines the analysis we have performed on the R_0 primary transformants and the R_1 progeny of these two events.

Table 1. Description of transformation experiments that produced putative events.

Expt	Infection Medium[1]	Co-cultiv. medium	Embryo size (mm)	#embryos infected	% embryos responding Type I[2]	# putative events recovered	Event ID
11/12/98	LSI	LSC	1.0-1.2	14	50	0	
	LSIrev	LSCrev	1.0-1.2	22	100	1	PTF3-1
4/30/99	LSI	LSC	1.0-1.2	24	42	0	
	LSIrev	LSC	1.0-1.2	105	25	1	PTF3-2

[1] See Materials and Methods. [2] Type I callus response assessed on selection medium 10-14 days after infection.

Table 2. Molecular and expression analysis of two putative transformation events.

Event	R_0 callus and plants				R_1 progeny plants			
	GUS		# resist. plants / # sprayed[1]	Southern pos (*bar*)	#resist. plants/# sprayed		GUS Leaf[4]	Southern pos (*bar*)[5]
	Callus	Leaf			(female)[2]	(male)[3]		
PTF3-1	pos	pos	7/8	degraded	79/141	12/27	11/11	11/11
PTF3-2	neg	ND	4/4	4/4	ND	ND	ND	ND

[1] Number of regenerated plants that survived glufosinate leaf spray (Materials and Methods).
[2, 3] Number of progeny plants (outcross with transgenic as female[2], or male[3]) which survived glufosinate leaf spray. All others plants, along with sixty non-transformed A188 plants sprayed as negative controls, died. See Results and Discussion for chi-square analysis (using Yates continuity correction).
[4, 5] Histochemical analysis[4] and southern analysis[5] on subset of surviving progeny plants from glufosinate leaf spray test

Callus and all regenerated plants from PTF3-1 tested assayed positively for the *gus* gene (Table 2). Pollen produced by two of these R_0 plants also segregated for *gus* expression (data not shown). PTF3-1 progeny plants (subset of 11 that survived herbicide spray) all expressed

the *gus* marker gene in leaf tissue. Levels of *gus* expression in progeny plants were higher than those visualized in the R_0 regenerated sibling plants. Callus of PTF3-2 was non-expressing in histochemical GUS assays.

One of the 8 plants regenerated from PTF3-1 callus died in the glufosinate leaf spray test (Table 2). This plant was vigorous in regeneration under glufosinate selection. Non-transformed A188 callus regenerated through the same pathway, as was putatively transformed callus never produced a regenerated plant, suggesting that *in vitro* selection was tight. No GUS assay or other molecular analysis was performed on this particular plant. It was not clear whether the transgene in this plant was suppressed during development or it was simply an escape. All 4 R_0 regenerated plants of PTF3-2 survived the glufosinate spray test.

Chi-square analysis was done on the PTF3-1 progeny segregation ratios for *bar* gene activity reported in table 2. For inheritance of a single, dominant gene, a 1:1 ratio of glufosinate resistant to glufosinate sensitive plants was expected, with a critical χ^2 (1 d.f, P=0.10) of 2.71. Resulting χ^2 values of 1.8 (transgenic outcrossed as the female) and 0.14 (transgenic outcrossed as the male) do not exceed the critical value, confirming that the *bar* gene was transmitted through the male and female zygote in typical Mendelian fashion.

Southern analysis was performed on R_0 regenerated plants of PTF3-1 and PTF3-2 and on a subset (11 plants) of PTF3-1 R_1 progeny plants which survived the glufosinate progeny screen. Results of this analysis are shown in figure 1.

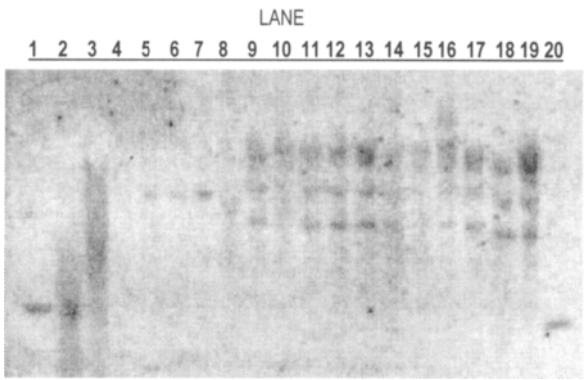

Figure 1. Southern Blot analysis of R_0 and R_1 leaf tissue. Hybridizing but degraded genomic DNA of two PTF3-1 R_0 plants (lanes 2-3). Untransformed A188 leaf (lane 4). Four R_0 plants of PTF3-2 (lanes 5-8). *Bar* resistant, R_1 progeny plants of PTF3-1 outcrossed as male (lanes 9-11) and female (lanes 12-19). Lanes 1 and 20 are the 1 kb ladder showing the 1.6 kb band.

DNA samples for the two PTF3-1 R_0 plants tested were degraded and show no clear banding pattern (lanes 2 and 3). Lane 4 was the non-transformed control. DNA-banding patterns for the 11 PTF3-1 progeny were identical whether the transgenic was used as the male (lanes 9-11) or the female (lanes 12-19) in the outcross. The 3 hybridizing bands for PTF3-1 indicate a more complex integration pattern than a single copy insertion. By comparison, the single band DNA-banding pattern shown for the four primary transformants of PTF3-2 (lanes 5-8) suggests that it, instead, may be a single-copy event.

These results confirm that we have successfully obtained fertile, transgenic maize plants using *Agrobacterium*-mediated transformation as was previously reported (Ishida et al., 1996). However, our efficiency of clone recovery is currently too low to draw conclusions about treatment effects responsible for this limited success. While table 1 outlines the particular treatment parameters from which these events were recovered, it is by no means an exhaustive description of the experiments we have undertaken. In fact, over 900 additional A188 immature zygotic embryos infected with *Agrobacterium* under similar conditions in 9 other experiments yielded no stable clones.

In these experiments we consistently observed that, for any treatment applied prior to or during infection which increased the transient *gus* expression in the embryos, the % Type I callus response was, in turn, decreased (data not shown). In agreement with Ishida et al. (1996), optimizing transient *gus* expression alone appears to be an ineffective way of optimizing infection parameters using this protocol. Like Lupotto et al. (1998) we rarely saw transient GUS staining on scutellar cells (from which callus initiation begins). The two stable events discussed here were recovered from treatments that showed low transient expression primarily localized on the under side (embryo axis side) of the embryo. Our efforts are currently focussed on improving growth conditions for donor plants and testing parameters that favor higher rates % Type I callus formation from infected embryos.

Summary

In conclusion, we have demonstrated stable transformation of maize A188 immature zygotic embryos using *Agrobacterium*-mediated gene transfer as previously described (Ishida et al., 1996). We have confirmed that the selectable and non-selectable marker genes were both transmitted to, and are expressing in, R_1 progeny plants. Efforts are underway to identify treatment parameters that will enable the routine and efficient implementation of this protocol.

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Assessment of Conditions Affecting *Agrobacterium*-mediated Soybean Transformation and Routine Recovery of Transgenic Soybean

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Introduction

Soybean [*Glycine max* (L) *Merr.*] has been used as a model plant species for studies on plant physiology, biochemistry, and molecular biology. Introduction of foreign genes of interest will benefit these studies and increase soybean values, if transgenic soybean can be developed routinely.The successful transformation of soybean, however, has been limited (Hinchee et al., 1988; Chee et al., 1989; Padgette et al., 1995; Di et al., 1996). Recently, the use of *bar* gene as a selectable marker coupled with glufosinate as a selective agent has been exploited for a routine transformation of this crop via *Agrobacterium*-mediated cotyledonary node system (Zhang et al., 1999). We showed here the assessment of several conditions affecting this cotyledonary node transformation system. The system is now being used for a routine recovery of transgenic soybean and to provide genetic transformation service for soybean researchers.

Materials and Methods

Agrobacterium *strains and binary vectors*

Agrobacterium tumefaciens strains EHA101 and EHA105 (Hood et al., 1986, 1993), each carrying binary vectors pPTN140 and pPTN101 (Zhang et al., 1999) or pPTN102, respectively, were used throughout experiments. The vector pPTN140 and pPTN101 contain *bar* gene as a selectable marker conferring resistance to glufosinate. The vector pPTN102 was constructed in the same way as pPTN101 except that a hygromycin resistance *hpt* gene is in the replacement of the *bar* gene.

Plant transformation and regeneration

Our routine plant regeneration and transformation procedures have been described previously (Zhang et al., 1999) with some modifications. Briefly, sterilized soybean Asgrow3237 seeds were germinated in B5 basal medium (Gamborg et al., 1968) for 5 days and target explants were prepared by manually wounding cotyledonary nodal region. Three days after cocultivation with the *Agrobacterium* strains, explants were transferred to B5 medium supplemented with 1.67 mg/L BA and various levels of glufosinate for shoot initiation during a 4-week period. Explants were subcultured every two weeks. Shoot elongation was initiated by transferring the explants to MS/B5 medium containing 1 mg/L zeatin-riboside, 0.5 mg/L GA3, 0.1 mg/L IAA, and reduced levels of glufosinate. Elongated shoots were transferred to a

MS/B5 rooting medium amended with IBA without further selection. Each rooted shoot was transferred to soil in a Magenta tissue culture box for acclimatization and later transferred to greenhouse. *In vitro* cultures of all stages were in Percival biological incubators. For specific experiments to assess infection conditions, however, procedures are specified accordingly.

Histochemical GUS staining and Southern blot analysis

Histochemical GUS staining assay (Jefferson et al., 1987) was used to screen the marker *gus* gene throughout experiments. The sacrificed explants and excised portions of putatively transformed shoots/plants were stained using X-Gluc and recovered plants were further analyzed by leaf-painting assay (Zhang et al., 1999) and Southern blot (Southern, 1975).

PCR

PCR was used to amplify a 438-bp DNA fragment within the *bar* ORF using sense primer GCGGTCTGCACCATCGTCAA and antisense primer GCTGCCAGAAACCCACGTCA. The cycle parameters included 5 min at 94°C hot start, 1 min at 94°C denature, 1 min at 60°C annealing, 1 min at 72°C extension, and 35 cycles.

Results and Discussion

Assessment of conditions affecting soybean transformation

We have established a routine transformation system for soybean using *Agrobacterium*-mediated cotyledonary node system. To improve the efficiency, we assessed conditions affecting the transformation. Our routine method for assessment of transformation and regeneration was as follows. Soybean cotyledonary node explants were inoculated with *Agrobacterium* strain carrying selectable marker gene *bar* and screenable marker gene *gus*. After 3-days of co-cultivation, explants were transferred to B5 medium containing herbicide glufosinate for two weeks (1st shoot initiation). Explants were then subcultured to the same medium for additional two weeks (2nd shoot initiation). *In vitro* regeneration responses were evaluated at the end of both 1st and 2nd shoot initiation stages, whereas the infection was assessed at the end of 2nd shoot initiation stage by sacrificing explants for GUS assay. The regenerated explant is defined as the explant showing multiple buds or shoots formation. Two categories of infection were examined: GUS^{+} bud/shoot (either clonal or chimeric) that referred to GUS staining on regenerating buds or shoots seen at the cotyledonary node region and thus were likely to further develop to plantlets; GUS^{+} sectors that referred to GUS staining on non-differentiation tissues of the explant and therefore had no potential for regeneration. The rate of infection (%) is defined as the total number of GUS^{+} explants (including GUS^{+} bud/shoot and GUS^{+} sectors) over the total number of explants inoculated (Zhang et al., 1997).

Effect of Agrobacterium growth phases on soybean transformation

We have observed that the infection on soybean varied when *Agrobacterium* used for inoculation was collected at different growth phases. Most of transgenic plants were obtained when incubated with *Agrobacterium* collected at its log phase (data not shown). The impact of *Agrobacterium* growth phases on soybean transformation was evaluated. *Agrobacterium* cells were harvested at different growth phases as indicated by optical density at OD_{650}. The cells

were then resuspended in inoculation medium and adjusted to the same cell density before inoculation. Table 1a,b summarized data from two separate experiments.

Table 1a. Effect of *Agrobacterium* (EHA105) growth phases on the infection*.

OD_{650}	# explants	Regenerated and sacrificed explants	GUS^+ bud/shoot	(%) GUS bud/shoot	Total GUS^+ explant	(%) infection
0.6	47	43	5	10.6	9	19.1
0.9	87	82	5	5.8	18	20.7
1.2	70	65	5	4.3	22	31.4

* *Agrobacterium* cells were collected at different growth phases and resuspended in inoculation medium and adjusted to the same cell density (OD_{650} = 0.6).

Table 1b. Effect of *Agrobacterium* (EHA105) growth phases on the infection*.

OD650	# explants	Reg. and sacrificed explants	GUS^+ bud/shoot	(%) GUS^+bud/shoot	Total GUS^+explant	(%) infection
0.15	30	10	1	3.3	10	33.3
0.3	30	10	3	10.0	10	33.3
0.6	30	5	2	6.7	5	16.7
0.9	30	9	1	3.3	9	30.0

* *Agrobacterium* cells were collected at different growth phases and resuspended in inoculation medium and adjusted to the same cell density (OD_{650} = 0.35).

In table 1a, the highest rate of "effective" infection indicated by (%) GUS^+ bud/shoot was from the explants inoculated with *Agrobacterium* cells collected at OD650 = 0.6. Similarly, *Agrobacterium* cultures of OD650 = 0.3 and 0.6 resulted in the highest infection rates on soybean in replicate 2 (Table 1b). It suggested that *Agrobacterium* culture at its log phase gave most effective infection to soybean cotyledonary node explants.

Length of inoculation

Various lengths of inoculation time may affect the infection (Table 2), simply because it takes at least 15 min for the *Agrobacterium* cells to complete the attachment process (Kado, 1991).

Table 2. Effect of inoculation time on infection.

Inoculation time (min)	Total explants inoculated	Explants regenerated	(%) regeneration	GUS^+ bud/shoot	(%) GUS^+ bud/shoot	Total GUS^+ explant	(%) infection
30	75	65	86.7	6	8.0	18	24.0
60	77	58	75.3	2	2.6	18	23.4
90	75	55	73.3	3	4.0	9	12.0
120	81	55	67.9	2	2.5	12	14.8

Data were from duplicated experiments

To determine what time period was optimal for the inoculation, excised cotyledonary node explants were inoculated with *Agrobacterium* by submerging the explants in freshly prepared *Agrobacterium* suspension culture (OD650 = 0.6) at the room temperature for different periods of time. Inoculation plates were agitated every 30 minutes.

The data suggested that the prolonged inoculation time prior to co-cultivation affected both infection and *in vitro* regeneration negatively.

Genotype evaluation

Soybean line A3237 was used to generate transgenic soybean plants successfully when the bar gene was used as selectable marker (Zhang et al, 1999). We further selected three public soybean genotypes, Jack, Peking, and William 82, to compare with A3237 using *Agrobacterium* strain EHA105. Since we have observed different genotype responses towards the glufosinate selection (data not shown), we used 3 mg/L glufosinate at shoot initiation stage for A3237, Peking and William 82, and 5 mg/L for Jack. The regeneration response and infectionability of these genotypes are shown in table 3.

Our result suggested that Peking displayed the highest response in regeneration, whereas William 82 scored the lowest in our culture system. Both A3237 and Peking had most total GUS^+ explants, indicating their high susceptibility to *Agrobacterium*. Jack, on the other hand, had most counts on GUS^+ bud/shoot, indicating a potential cultivar for transformation.

Table 3. Regeneration responses and infectionability of four soybean gentypes.

Genotypes	Total explants	(%) regeneration	Explants sacrificed	GUS^+ bud/shoot	(%) GUS^+ bud/shoot	Total GUS^+ explants	(%) infection
A3237	227	63.9	43	1	2.3	25	58.1
Jack	112	81.3	56	4	7.1	19	33.9
Peking	72	98.6	44	2	4.6	23	52.2
William 82	256	58.2	60	1	1.7	17	28.3

Data were from duplicated experiments

Selectable markers and selective agents

Selectable markers such as *nptII* and *hpt* genes have been used for soybean transformation (Hinchee et al., 1988; Parrott et al., 1994). To ascertain the best selectable marker coupled with selective agent for efficient transformation, we tested the *nptII, hpt*, as well as *bar* genes in both sacrifice and stable transformation experiments. No transgenic soybean plants have been recovered using geneticin (G418), paramamycin, or hygromycin despite various levels of antibiotics (0-200 mg/L) used.

In spite of good infection of the strain carrying *hpt* gene construct, no transgenic soybean plant was recovered with any of the above selection schemes were using this selectable marker. Instead, transgenic plants were obtained from the experiments using the *bar* gene combined with glufosinate as a selective agent (see below section; also see Zhang et al., 1999).

Hygromycin B has been used as a more favorable selective agent than kanamycin for transformation of a number of crop species including peanuts and soybean, in a somatic embryogenesis regeneration system (Ozias-Akins et al., 1993; Parrott et al., 1994). We

noticed, however, that both hygromycin B and kanamycin seemed less efficient than glufosinate to remove the epical dominance of nontransformed buds or shoots in our culture system that is based on a direct shoot organogenesis. This was obvious when we compared bud/shoot morphorgenesis of soybean cultures selected from different selective agents under microscopy. This may contribute to the difficult recovery of transgenic soybean using antibiotics such as hygromycin for selection in this organogenesis system. Table 4 illustrated such experiments from a pooled data comparing hygromycin B and glufosinate.

Table 4. Selection schemes for hygromycin and glufosinates (mg/L).

Selection Scheme*	Total explants	Regenerated explants	(%) regeneration	(%) GUS+ bud/shoot	(%) infection
Hygromycin					
0-0	80	43	53.8	2.3	74.4
5-5	65	40	61.5	12.5	70.0
10-10	56	35	62.5	14.3	51.4
20-5	67	7	32.8	13.7	27.3
Glufosinate					
0-3.3	88	44	50	0	19
4.3-4.3	72	36	50	1	13
5.3-5.3	61	25	41	2	15
6.3-6.3	68	25	36.8	0	13
7.3-3.3	56	34	60.7	1	14

* 0-0, 5-5, , 7.3-3.3 indicate levels (mg/L) of selective agents at 1st and 2nd shoot initiation stages, respectively.

Optimal glufosinate selection scheme for a routine recovery of transgenic soybean

Glufosinate selection schemes

The selection schemes were further defined using various concentrations of glufosinate ranging from 0 to 7 mg/L for shoot initiation in combinations with 2 mg/L for the shoot elongation (Table 5).

Table 5. Glufosinate selection schemes (mg/L)*.

Selection schemes**	# Explants inoculated	# Southern+ R0 plants	Transformation efficiency (%)
0-2	142	1	0.7
3-2	450	2	0.4
4-2	468	3	0.6
5-2	609	6	1.0
6-2	326	1	0.3
7-2	175	0	0.0

*: Data were from pooled experiments.
** 0-2, ... 7-2 indicates levels of glufosinate used in shoot induction (SI) and shoot elongation (SE).

Putatively transformed plants recovered from these experiments were analyzed by histochemical GUS assay, herbicide leaf-painting assay and Southern blot analysis (data not shown). The best selection schemes under our current culture conditions were the concentration of glufosinate at 5 mg/L during shoot initiation and 2 mg/L during shoot elongation, respectively. All 13 primary transformants (R0) obtained from these experiments were GUS positive and herbicide resistance (data not shown).

Progeny analysis

Seeds from primary transformants (R0) were analyzed using histochemical GUS staining, leaf-painting assay, Southern blot or PCR.

All tested events showed segregation of both *gus* gene and *bar* gene among the progeny (data not shown). Table 6 illustrates in details the result of such progeny analysis on one event (line 1-2). Among 15 seeds germinated, 12 were tested positive for the *gus* gene. The segregation ratio for the *gus* gene was 3:1 as expected. Nevertheless, a discrepancy exited among the progeny, 1-2-5, 1-2-10, and 1-2-11 in herbicide leaf-painting assay. While these plants were GUS positive, they were sensitive to the herbicide. PCR analysis indicated that the *bar* gene was present in the genome. The suppression of herbicide tolerance in these progeny may result from gene silencing of the *bar* gene.

Table 6. Progeny analysis of transgenic line 1-2.

Plant code	Histochemical GUS assay (+/-)	Leaf-painting assay (R/S)	PCR (+/-)
1-2 (R_0)	+	R	Southern positive
1-2-1	-	S	-
1-2-2	-	S	-
1-2-3	-	S	-
1-2-4	+	R	+
1-2-5	+	S	+
1-2-6	+	R	+
1-2-7	+	R	+
1-2-8	+	R	+
1-2-9	+	R	+
1-2-10	+	S	Weak positive
1-2-11	+	S	Weak positive
1-2-12	+	R	+
1-2-13	+	R	N/T
1-2-14	+	R	N/T
1-2-15	+	R	N/T
Ctrl, -	-	-	-

N/T: Not tested.

Summary

We have assessed several conditions affecting *Agrobacterium*-mediated transformation of soybean. Sacrificing explants 4 weeks after co-cultivation for GUS assay allowed us to

evaluate various parameters at early stage. It is essential to optimize conditions influencing regeneration as well as the transformation of differentiation tissue on the cotyledonary node region. Our results suggested that soybean genotypes, *Agrobacterium* growth phases, inoculation time, selective agents and selection stringency had effects on the infection and transformation efficiency. Furthermore, we showed here a routine transformation system for soybean via *Agrobacterium*-mediated cotyledonary node system using the *bar* gene as a selectable marker. The transgenic plants tested to date have transmitted both *bar* and *gus* genes to their progeny faithfully.

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Genetic Engineering of Cuban Rice Cultivars: Present and Perspectives

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Introduction

Rice is by far the most important crop in the developing world in terms of production and consumption and one of three most important crops all over the world. Just Indica-type rice varieties feed more than 2 billion people, predominantly in developing countries. This plant is well adapted to hot and humid environments, and is grown widely in both tropical and temperate regions.

In Cuba, rice is the second crop in importance in cultivated area, just behind the sugarcane, although the domestic rice demand is largely unsatisfied by a local production of less than 300 000 TM. The per capita consumption of rice in Cuba is more than 40 Kg/year.

Rice has been -and is nowadays- the basic source of calories in the Cuban people diet. It's usually eaten every day, and for most of the population it's common to have it twice a day. By-products from rice industrial processing are used in animal feeding and alternative sources of energy.

Many constraints that use to limit rice production around the world are now being approached by genetic engineering. Among these, alien genes for resistance to insect pests is the one on which more effort have been dedicated, but also relevant are virus resistance, fungi resistance, herbicides resistance, and starch content modification. Some others, like abiotic stresses resistance, are not at the same research stage, but will be gaining importance as the basic studies involved in their under-standing become more advanced, providing the ways for more ap-plied investigations.

Research on the manipulation of regulatory sequences dealing with the expression of the engineered genes just in desired plant tissues and only during required developmental periods of the plants have been reported in recent years, which will effectively help to avoid some of the present concerns on genetically engineered plants.

These approaches also offer an alternative to the plant breeder who searches for genes very difficult or impossible of being bred by conventional genetics, because it often takes many years, if possible, for the incorporation of one of the mentioned genes into an agronomic important genetic background.

Features and Constraints of Rice Production in Cuba

Most of the rice production in Cuba is obtained in large state-run enterprises where the crop is cultivated in two seasons. Cuban cultivars –Indica-type all of them obtained in a wide breeding program conducted from the beginning of the 60's by local breeders with the

assistance of IRRI (International Rice Research Institute) and other foreign experts are grown in these areas. Although many of these cultivars yield over 6.0 TM/ha in experimental conditions, the average yield in production areas hardly surpass 3.2 TM/ha at present, mainly because of the lack of funding and human resources needed for cultivation under high input conditions.

Private sector –the vast majority composed of small-scale farmers- account for more than one third of the national rice production. These farmers cultivate rice in low input conditions, mainly during the rainy season.

Among biological factors limiting rice production in Cuba, competition of weeds and red rice are of outstanding importance. Control of weeds occurring in rice growing areas demands a significant budget for chemical control in public enterprises. For the relevant part of the private sector that grows rice in rained culture and have no funding for chemicals, weed control by mechanical means suppose a big effort and a time consuming task.

Fungal diseases are also an important limitation for rice production in Cuba. The blast disease caused by *Pyricularia oryzae*, the sheath blight caused by *Rhizoctonia*, and more recently the sheath rot caused by *Sarocladium oryzae*, are permanent threats for rice fields.

Insect pests attacking rice in Cuba are leaded by the rice water weevil (RWW) *Lissorhoptrus brevirostris*. Other pests of importance for rice are the stinkbug *Oebalus insularis*, the armyworm *Spodoptera frugiperda*, and the Homopteran *Togasodes oryzicola*. In addition, during the last few years the mite *Steneotarsonemus spinki* was found to be closely related to outbreaks of the sheath rot disease.

Since about 10 % of the rice production take place in salt affected areas, salinity is one of the main abiotic factors limiting rice yields in Cuba. Droughts also significantly lower yields, mainly for farmers depending on rains for rice production.

Occasional temperatures under 15°C occurring in Cuba during the winter, specially if they coincide with the anthesis stage, reduces pollen viability and can remarkably reduce yield.

Hybrid technology for rice production has not been applied in Cuba because of lack of labor force.

Goals and Results

A few years ago, biotechnology research on rice in Cuba was restricted to some attempts for anther culture, and selection of mutants from somaclonal variation or irradiated calli. When at the Center for Genetic Engineering of Biotechnology of Sancti Spiritus we decided to start studying the potential of genetic engineering for approaching genetic improvement of local rice varieties, we found that almost no basic research on cell and tissue culture of this specie was done previously. Our primary goal was then to set up procedures for *in vitro* manipulation of the Cuban rice varieties.

First, procedures for calli induction from mature seeds and maintenance, as well as for plant regeneration in the main local rice cultivars were established. From an initial 25 % of calli regenerating plants and about 1 average plant per callus obtained in the beginning, protocols were refined in such a way allowing some times 80 % of efficiency and more than 3 plants per callus, as reported in Coll et al. (1996). Additionally, we created conditions for induction of calli from rice coleoptiles as an alternative source of explants (Coll et al., 1997).

A very versatile regeneration medium for Cuban rice cultivars, including kinetin, BAP, and NAA in MS medium (Murashige and Skoog, 1968) was found to work efficiently for the cultivars in study (Coll, et al., 1998).

Procedures for the isolation of physiologically active protoplasts from the Cuban cultivar Perla using etiolated leaves and roots were established. Transformation of protoplasts from both sources, as well as determination of transient GUS expression has been routinely performed in our laboratory. In parallel, studies to explore potential insecticidal molecules against two key pests of rice in Cuba have been performed in our laboratory. The digestive activity of the armyworm *Spodoptera frugiperda* was first characterized (Alfonso et al., 1996).

S. frugiperda α-amylase showed maximal activity at pH 8.5-9.5 in Tris buffer (Alfonso et al., 1997). *S. frugiperda* α-amylase activity in non-denaturing PAGE was observed during larval life, and no difference among the 5 different isoenzymes was detected. Proteinaceous inhibitors from wheat and barley were studied. The tetrameric inhibitor inhibits 25 and 60 % respectively of the total α-amylase activity at 7 and 11 days of larval life cycle. Inhibition with dimeric and monomeric inhibitors was not significant. Protease activity of the trypsin type in the larvae midgut was also detected. It was inhibited at 64 % by 5 μg of CMe proteins from barley cv. Bomi. We have also characterized the digestive protease activity of the rice water weevil as the first step for identifying proteins with inhibitory potential for plant protection strategies. Azocasein quantitative enzymatic assays (Table 1) and gelatinolytic activity on SDS-PAGE showed the existence of a complex proteolytic system in the RWW gut fluid, in which cysteine proteinase activity clearly predominates.

Table 1. Response of RWW digestive proteinases to various specific proteinase inhibitors.

Inhibitors	Concentration	Inhibition (%)[a] ± SE	ProteinaseSpecificity
E-64 (1)	280 μM	78.1 ± 1.1	Cysteine
Kunitz trypsin inhibitor	100 μg/ml	5.3 + 0.1 [b]	Serine
Leupeptin (2)	200 μM	75.3 ± 1.8	Cysteine/Serine
Aprotinin	5.1 μM	6.9 ± 0.8	Serine
PMSF (3)	2 μM	6.5 ± 1.0	Serine
EDTA (4)	10 μM	1.5 ± 0.5	Metallo
Pepstatin A (5)	26.4 μM	17.7 ± 3.8	Aspartyl
Egg chickencystatin (6)	10 μg/ml	49.1 ± 0.7	Cysteine
1 + 2 + 3 + 4 + 5 + 6	[c]	95.0 ± 1.3	All

a : Data are expressed as percent of inhibition activity, calculated according to the formulae : (1 - SAi/SAc) x 100, where SAi represents the specific activity in presence of inhibitor, and SAc represents the control specific activity to which no inhibitor was added. Each datum is the mean of four determinations ± SE. b: Activation instead of inhibition. c : The same concentration each as when individually used.

We concluded that the tetrameric and CMe trypsin inhibitors might be taken into account in genetic engineering strategies for protecting rice plants against *S. frugiperda* attack. Furthermore, several *Bacillus thuringiensis* strains were screened for toxicity against *S.*

frugiperda reared in artificial diet. A *B. thuringiensis* strain showing high toxicity was selected (Alfonso et al., 1994) and the gene coding for its δ-endotoxin was isolated and cloned in expression vectors. The recombinant δ-endotoxin protein expressed in *Escherichia coli* was compared with the natural protein in feeding experiments, demonstrating its toxic potential.

Transformation of Cuban rice cultivars have been attempted both particle acceleration and *Agrobacterium tumefaciens*. Although conditions were optimized for efficient transient expression in calli (Menéndez, et al., 1999), no plants have been recovered from bombarded tissues. We identified a promoter driving strong expression of the GUS gene in bombarded immature rice embryos using the gene gun device.

However, *Agrobacterium tumefaciens* have proven to be an efficient vehicle to deliver foreign DNA into cells from Cuban rice cultivars. We have developed a procedure allowing so far recovering of transgenic rice plants from 3 cultivars. These plants, transformed with the GUS gene, were selected for hygromicin resistance. Molecular characterization of the transformed plants showed that most of the plants carry the introduced genes and express the active GUS protein.

Transformation experiments aimed to introduce genes coding for antifungal proteins are in process. Chitinase, glucanase and PRs genes are combined in binary expression vectors in order to constitutively express antifungal proteins in transgenic rice plants from local cultivars. Transgenic rice plants will be tested for resistance to fungal diseases caused by *Rhizoctonia, Pyricularia*, and *Sarocladium.*

A *Bacillus thuringiensis* gene will be also introduced shortly into rice to study its insecticidal potential against the rice water weevil (*Lissorhoptrus brevirostris)* larvae. At present, several recombinant cysteine proteinase inhibitors are being evaluated to test their ability to interfere with the digestive activity from the RWW.

Future Prospects

A strategy for the search of other *Bacillus thuringiensis* strains carrying novel δ-endotoxin genes was designed in our laboratory. Introduction of δ-endotoxin genes coding for insecticidal proteins differing in their RWW midgut receptor might both enhance efficiency of control and delay appearing of insect resistance. The introduction into rice of insecticidal genes with different mode of action, such as protease inhibitors or lectins, might also contribute to this purpose.

Since some pests of economical importance for rice in Cuba are not of the chewing type, eg. the stinkbug *Oebalus insularis*, the Homopteran *Togasodes oryzicola*, and the mite *Steneotarsonemus spinki*, the potential of insecticidal molecules different from δ-endotoxins of *B. thuringiensis* need to be explored.

Besides chitinase, glucanase and PRs genes, other antifungal genes are envisaged to be introduced into rice for ensuring a longer lasting effectiveness of transgenic plants in the field. Among these, genes coding for systemic acquired resistance (SAR) would be of outstanding importance.

The development of expression systems for specific expression of the desired proteins in particular tissues, organs, cell compartments, and plant developmental stages will be necessary to cope with the expectations created around transgenic rice plants.

In particular, it would be very convenient to set up systems capable of diminish the risk of gene escaping through the pollen from transgenic plants to sexually compatible wild or relative rice species, and/or controlling the viability of potential undesired hybrids. These is a technical development specially appropriated when creating transgenic plants carrying genes and could confer competitive advantage if unintentionally bred into natural populations.

Transformation of local rice cultivars with genes targeted to improve the defense of the plant against the main environmental stresses limiting rice yields in Cuba (cold, drought and salinity) would enable biotechnology to help breeders to complement rice breeding for attaining genetic goals not feasible using conventional techniques.

The introduction of genes capable of simplifying the technology for production of hybrid seed or providing rice cultivars with the uniqueness of perpetuating the advantages of hybrids will means a breakthrough for rice production in Cuba.

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Histological and Ultrastructural Analysis of *A. rhizogenes*-mediated Root Formation in Walnut Cuttings

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Introduction

The induction of adventitious roots is crucial for vegetative propagation in plant or micropropagation *in vitro* of many woody species, yet many woody species are totally recalcitrant to rooting (Altamura 1996). This recalcitrance may be associated with various histologically detectable factors, such as the inability of the explant cells, after stimulation, to organize root meristemoids (Altamura 1996). In English walnut (*J. regia* L.), the propagation by grafting on seedling rootstocks is an expensive process, yet the alternative practice of grafting on black walnut seedling rootstocks makes trees vulnerable to the lethal blackline disease. *In vitro*, root formation may be a response to wounding *per se* or it may be associated with the presence of root inducers, such as auxin (Altamura, 1996). However, for recalcitrant woody species, auxin may not induce root formation. As an alternative or complementary strategy, localized infection with *Agrobacterium rhizogenes* may be used and has recently been adopted for walnut microcuttings (Caboni et al., 1996).

The potential of *A. rhizogenes* to induce rooting has been attributed to the insertion and stable integration of a portion (T-DNA) of the bacterium's root-inducing (Ri) plasmid into the plant genome. Four loci involved in root formation have been identified ("rol A, B, C and D") (Vilaine et al., 1987). In particular, rolB seems to play an important role in inducing meristemoid formation, at least in herbaceous model systems (Altamura, 1996).

The present study investigated histological and ultrastructural changes occurring in the agroinfected cuttings of a recalcitrant walnut genotype cultured under conditions inductive for rooting, with the aim of determining how agrobacteria trigger root formation in the stem of a recalcitrant woody cutting, their migration in the explant, and the cytological events resulting from the combined presence of infection and exogenous auxin.

Materials and Methods

In vitro cultures of a seedling of *J.regia* L. (cv. Sorrento) were multiplied as suggested by Caboni et al. (1996). Microcuttings (2 cm in length, consisting of the 3 most apical internodes and the apex) were isolated from the shoot clusters at the end of the multiplication phase and used for the rooting experiments. The rooting medium contained the same salts and organics of the multiplication medium (Caboni et al., 1996) and was kept for 30 days under 16 h light per day either in hormone-free conditions (HF treatment) or with 10 μM IBA (IBA treatment) or 10 μM IAA (IAA treatment). The growth, inoculation, and co-cultivation of *A.rhizogenes* Conn. (wild type 1855 NCPPB) were carried out according to Caboni et al. (1996). The

infected microcuttings were then transferred onto the same rooting medium used for non-infected explants, containing 0.52 mM cefotaxime to inhibit further bacterial growth, and either 10 μM IBA (IBA Ar treatment), 10 μM IAA (IAA Ar treatment), or no hormones (HF Ar treatment), for 30 days, under 16h light per day. At 0, 10, 15, 20 and 30 days, the stem portion (15 mm in length starting from the basal end) of five microcuttings per hormonal treatment was fixed in FAA. (90 ml ethanol, 5 ml acetic acid, 5 ml formaldehyde), dehydrated, embedded in paraffin, sectioned at 10 μm and stained with safranine-fast green (Jensen, 1962) or toluidine blue-0 (O'Brien, 1965) for the histochemical detection of polyphenols.The presence of bacteria in the vessels showing polyphenol deposition was also detected in sections stained with safranine-fast green observed with a Zeiss-LSM3 Confocal Scanning Laser Microscope. Microcuttings taken on the same sampling days were alternatively post-fixed in 1 % osmium tetroxide and then embedded in Spurr's low viscosity resin at 70°C for 8h. Ultrathin sections (80 nm thick) of the samples were stained with citric acid lead (II) salt trihydrate and then observed with a Zeiss EM 10/C transmission electron microscope.

Results and Discussion

At culture end (day 30), rooted explants were sporadic in the absence of infection, whereas in the presence of infection a conspicuous response was obtained even for HF treatment (about 40 % of the cultured explants showed roots). In the presence of IBA, a very high response was obtained (about 60 %), whereas in the presence of IAA the response was very low (about 20 %). In comparison with day 0 (Figure 1a), an increase in xylem development occurred in all the cuttings starting from day 10, and especially in those of IAA Ar treatment (Table 1).

Polyphenols, absent at day 0, were present at day 10 in both the primary and secondary xylem (Figure 1b), as well as in the secretor cavities of the cortex. Parenchyma cells, flanking some vessels, produced these compounds, which were then secreted into the vessels (Figure 1b). Polyphenol deposition increased during the culture, and mainly in the combined presence of infection and exogenous IAA (Table 1).

Table 1. Histological events occurring in infected (Ar) and non-infected walnut cuttings throughout the culture period. (+, low; ++, medium; +++, high; and ++++, very high occurrence of the event. -, event not observed).

Treatment	Xylem development			Polyphenol deposition			Root meristemoids			Root primordia			
	10	15	20	10	15	20	10	15	20	10	15	20	30
	days			days			days			days			
HF Ar	+	+	+	+	++	++	+	+++	++++	-	+	+	+++
HF	+	+	+	+	+	+	-	-	-	-	-	-	-
IAA Ar	++	++	+++	+	+++	+++	+	+	+	-	-	-	++
IAA	+	++	++	-	+	+	-	-	++	-	-	-	-
IBA Ar	+	+	+	+	++	+	+	++++	++	-	+	+++	++++
IBA	+	+	+	+	+	+	+	+	++	-	-	-	+

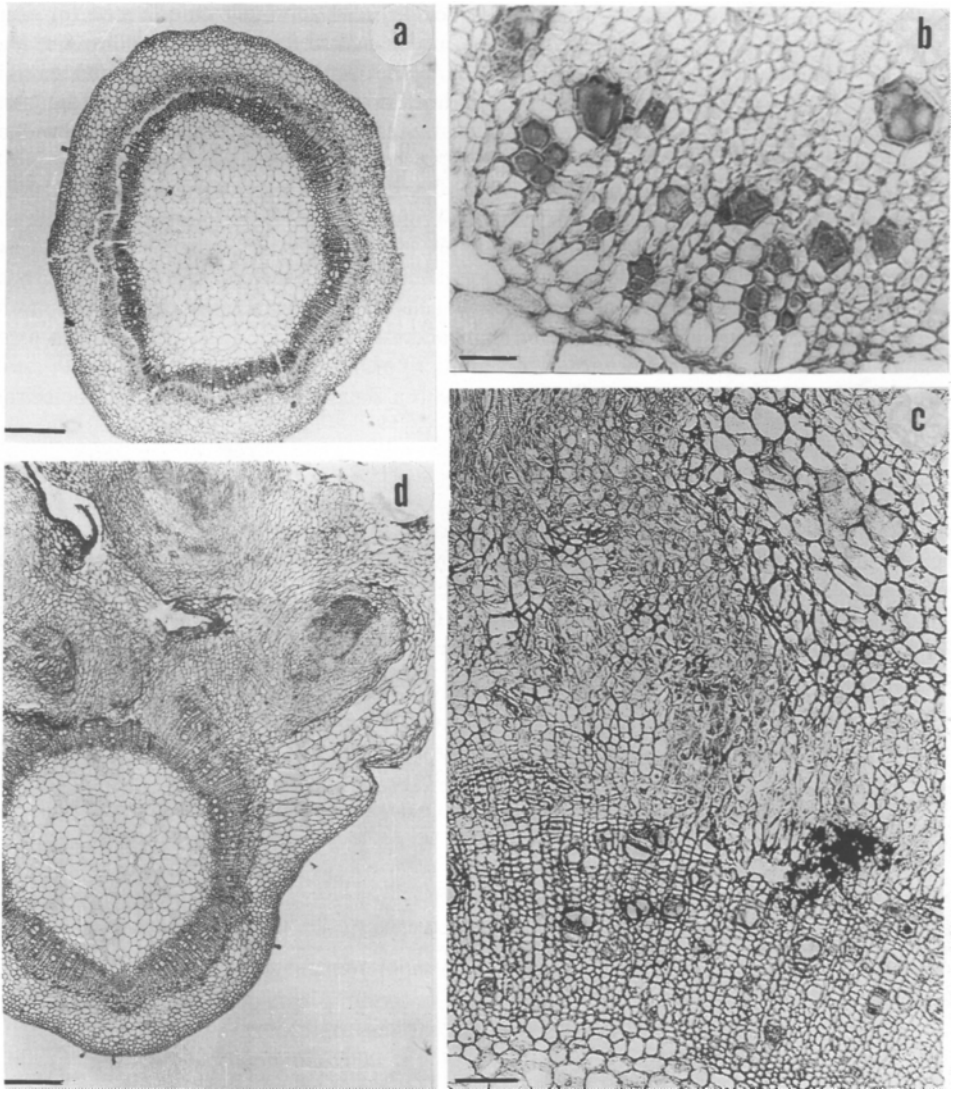

Figure. 1. Histological events of adventitious root formamation in walnut microcuttings.[Transection under light microscopy. Bars= 300μm (a,d); 50μm (b); 10μm (c)]. a. Stem showing the secondary vascular structure at culture onset (day 0). b. Polyphenol deposition in primary and secondary xylem (IAA Ar-treated explant, day 15). c. Meristemoid in close proximity to the cambium (direct genesis) (IBA Ar-treated explant, day 10). d. Dome of a root primordium form in the cortical callus (indirect genesis) (IBA Ar-treated explant, day 20).

In the infected cuttings, the bacteria were localized in the secretor cavities of the cortex and in the nearby cells (Figure 2a), and especially in the tracheary elements of the xylem.

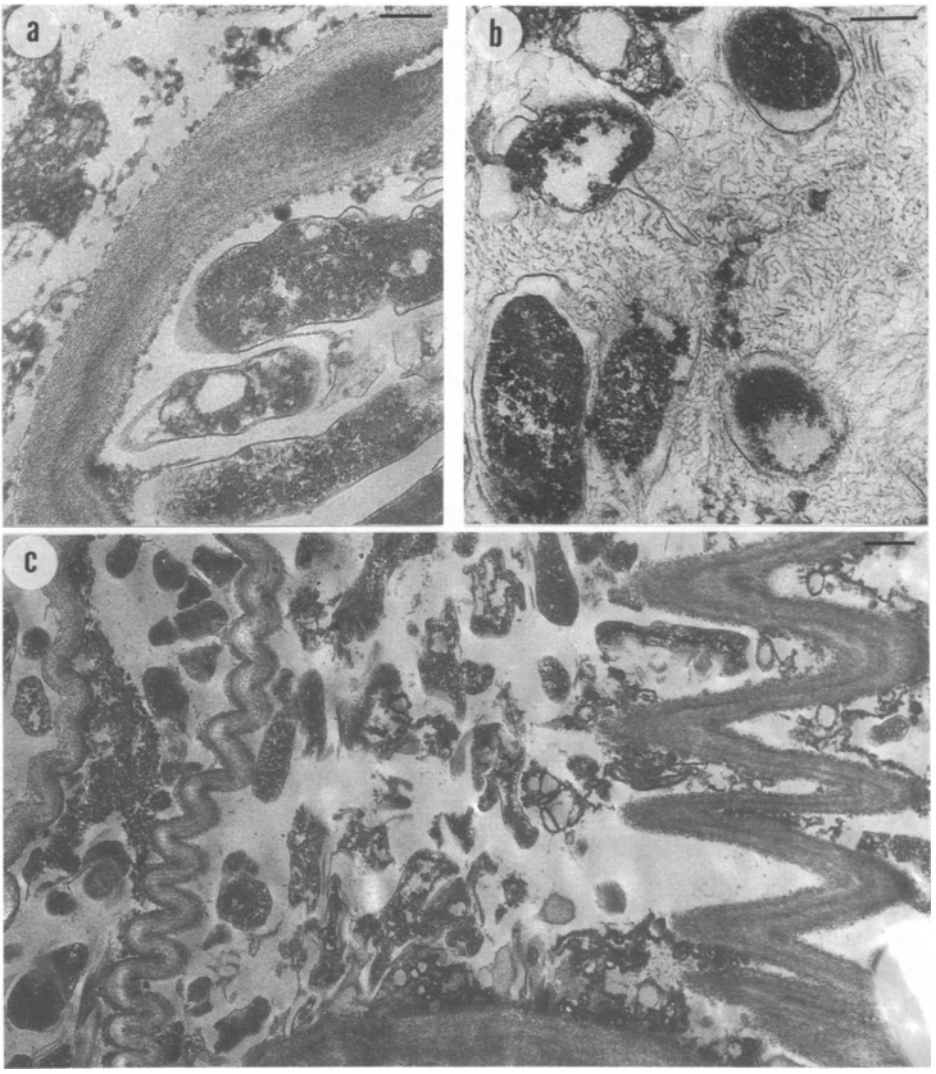

Figure. 2. Presence of *A.rhizogenes* in the infected cuttings after 10 days of culture in rooting media. [Thin longitudinal sections observed under TEM. Bars= 250nm (a); 260nm (b); 1μm (c)]. a. Detail of a degenerating cortical cell showing irregularly shaped and plasmolysed bacterioids (IBA Ar-treated cutting). b. Detail of a xylem parenchyma cell showing bacterioids immersed in a fibril material (HF Ar-treated cutting). c. Wavy walls of cortical parenchyma cells (IAA Ar-treated cutting).

The bacteria were frequently plasmolysed and irregular in shape (Figure 2a-b). Bacteria were present not only in the vessels but also in the xylem parenchyma cells surrounding them (Figure 2b) and, less frequently, in the pith ray cells and the cambial cells. Agrobacteria were also observed in the intercellular spaces and between the plasmamembrane and the wall. The parenchyma cells lost their structural integrity when they became filled with bacteria (Figure 2a-b). Although these cells continued to show a quite regular wall texture (Figure 2a), their walls frequently became wavy (Figure 2c), and fibril material accumulated in the protoplast, surrounding the bacteria (Figure 2b). This material greatly differed from the material observed in the vessels colonized by the bacteria and located at the borders of the secondary wall thickenings. In the cells containing high amounts of polyphenols, a conspicuous presence of bacteria was detected. Neither bacteria nor fibril materials were present in the non-infected cuttings.

Another event observed starting from day 10 in infected explants of all treatments, and in the non-infected explants treated with IBA only, was the formation of meristemoids (Table 1), with a preferential location in close proximity to the cambium (Figure 1c) (direct genesis, Altamura, 1996).

In the treatments with IBA, meristemoids were also present in the cortical callus (indirect genesis; Altamura, 1996). At day 15, a very high number of meristemoids was observed in the infected cuttings treated with IBA, and, to a lesser extent, in those of the HF treatment, whereas the quantity of meristemoids did not significantly increase in the IAA Ar treatment in comparison with day 10 (Table 1). Furthermore, in the cuttings of the IBA Ar treatment, the first root primordia were also present and showed either indirect or direct genesis. At day 20, the quantity of meristemoids (all with a strictly basal location in the stem) greatly increased in the HF Ar treatment, whereas it decreased in the IBA Ar treatment, in which there was instead an increase in root primordia formation (Table 1). In general, the number of root primordia progressively increased up to day 30 (Figure 1d, Table 1).

Starting from day 15, in the IAA-treated explants, especially in the infected cuttings, there was significant xylem development (Table 1). At day 20, the radial extension of xylem was significantly greater (by about 33%) in the infected cuttings, compared to the non-infected ones treated with the same hormone. Furthermore, in the infected cuttings, vessels showing high polyphenol deposition (Table 1), frequently associated with bacteria were observed and were extended to the entire radius of the xylem.

This study demonstrates that in a recalcitrant walnut, a specific IBA treatment and the infection with *A. rhizogenes* synergistically induce a very precocious and abundant formation of direct and indirect meristemoids, resulting in a high macroscopic rooting response. In fact, infection *per se* is also able to trigger a rather consistent rooting response, yet meristemoids are produced later and in lower quantities than those obtained when IBA is added to the medium, and their location in the stem is strictly basal. It is known that endogenous auxin positively affects rooting, and that it accumulates at the stem base because of basipetal transport (Lomax et al., 1995). Thus, infection might trigger the rooting process in cells with a sufficiently high auxin content. The observation that in the infected cuttings treated with exogenous IBA the meristemoids are present along an extensive part of the stem supports the hypothesis that IBA treatment increases the population of cells with the endogenous auxin content necessary for rooting. It is not clear which aspect of infection (contamination or transformation) triggers rhizogenesis in walnut cuttings. It cannot be excluded that

contaminated cells, before their death, may produce diffusible signals for other, healthy cells, which become capable of initiating the rooting process. However, it is probable that, more than bacterial contamination, the transformation of the plant genome with the T-DNA genes of the root-inducing plasmid of *A. rhizogenes* plays the pivotal role in triggering rhizogenesis.

The ultrastructural analysis of the present paper shows that *A. rhizogenes* is present in the cutting up to the end of culture and that the bacterial foci are mainly located in the conducting elements of the xylem. *A. tumefaciens* has been reported to form microcolonies in the vascular tissue of *in vitro* shoot cultures of tobacco (Matzk et al., 1996), and *A. radiobacter* has been shown in damaged cells of the cortex and the pith and in the vessels of *Kalanchoë daigremontiana* (Bogers, 1972). Furthermore, two different types of fibril material are produced as the consequence of contamination. The fibril material near the pit cavities of the vessels resembles the adhesion fibril material, composed of polysaccharides, produced by other bacteria during various pathogeneses (Mount & Lacy, 1982). The other type of fibril material resembles that produced by the rice cells in the leaf blight disease incited by *Xanthomonas oryzae* (Horino, 1976). The waviness of the walls of the host cells may be interpreted as a stress-induced response to intracellular bacterial colonization. In fact, a similar wall alteration has been observed in tobacco cells subjected to the stress of polyamine biosynthesis inhibition (Berta et al., 1997).

In walnut microcuttings, IAA has a specific effect on the differentiation of the secondary xylem and not on rooting. It is possible that IAA triggers the sensitivity of the potential provascular cells of the cutting before activating the sensitivity of potential pre-rhizogenic cells, thus irreversibly conditioning the explant towards the xylary response. Differently from rhizogenesis, high levels of auxin are necessary for both the induction and maturation of the xylem (Fukuda, 1996). Our investigations demonstrate that polyphenol deposition is higher in the infected explants of the IAA treatment, and that when the bacteria are present in the vessels, they are preferentially located in those showing depositions of polyphenolic compounds. Assuming that polyphenol deposition is stimulated by bacteria, the "protective" role of such compounds on IAA (Lee et al., 1982) might contribute to prolonging xylem formation. Thus, in the infected cuttings treated with IAA, the positive interaction between xylem differentiation and polyphenol deposition might explain the failure of the rooting response.

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Genetic Improvement Program at the Institute of Plant Biotechnology

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Somaclonal Variation and *In Vitro* Mutation Induction

Somaclonal variation and *in vitro* mutation induction have been used in sugarcane, banana and potato, with the objective of improving specific characters, mainly the resistance to diseases and other defects that limit the use of some important commercial varieties.

Sugarcane was the first crop and the one where the most important results have been obtained. A complete selection scheme was established and it was demonstrated that, only after three vegetative multiplications in field, a selection of true mutants for the main quantitative characters could be carried out. Sixteen somaclones were obtained and 5 of them were approved for the extension and introduction in the production as commercial varietes. The selection process lasted from 5 to 7 years in total, these results demonstrated the efficiency of *in vitro* mutation in comparison with the conventional improvement for specific characters. This advantage has been outlined by several investigators, but not with practical results to date (Larkin and Scowcroft, 1981, Micke and Donini, 1994).

In potato, mutation induction and *in vitro* selection have been used in order to improve the resistance against *Alternaria solani*, in the variety Desiree that occupies around 80% of the cultivated area in the country. Four resistant somaclones were selected which are under field testing for agronomic traits. Bananas and plantains have two main phytopathologic problems: *Fusarium* and Black Sigatoka. For *Fusarium* resistance 17 000 somaclones from the variety Gross Michel were studied and after 5 years of selection in greehouses and field, 5 resistant somaclones have been selected. They are evaluated under production conditions. For Black Sigatoka, 50 000 somaclones from Grand Naine have been studied and no resistant individuals were selected. Although, with the development of an efficient regeneration system via somatic embriogenesis combined with *in vitro* selection better results are expected.

The results obtained with the use of somaclonal variation, mutation induction and *in vitro* selection have created the methodological bases for the handling and selection in plant populations obtained from tissue culture. The main problems for selection in such populations are associated with epigenetic and juvenile effects induced by *in vitro* conditions. These problems are also present in transformed plants.

This program not only has given knowledge, but it has also generated materials of great value for molecular biology and transformation projects. For instance, in sugarcane 3 rust (*Puccinia melanocephala*) resistant somaclones were obtained, as well as a clone selected from classical mutation in buds. These clones are used as resistant progenitors for hybridization and they also were crossed with the original variety to determine the inheritance of the resistance that was obtained by mutation induction. The final goal is to clone and

characterize the gene(s) involved in the resistance mechanism to be used in genetic transformation.

Plant Cell and Tissue Culture as a Basis for Genetic Transformation

Genetic improvement by mutation induction and/or genetic transformation requires efficient systems of plant regeneration in the target crops. In our case, priority has been given to the development of the regeneration via somatic embryogenesis.

Sugarcane

The first attempts for plant regeneration via somatic embryogenesis were started in this crop. A methodology for somatic embryo production was established from callus derived from leaf explants and afterwards the regeneration of plants in cell suspensions was developed (Freire, 1998). Field trials were conducted with plant derived from callus and cell suspensions during several vegetative multiplications in field. These plants showed as main characteristic an increase in the number of stalks and a reduction in stalk diameter. This methodology has been scaled up in bioreactors with productions of up to 150 000 somatic embryos per liter and a total of 23 200 plants were regenerated and planted in seed plots.

Papaya

Direct somatic embryogenesis was obtained using immature zigotic embryos as initial explants in 4 varietes and one hybrid of papaya. Repetitive multiplication of the somatic embryos in solid medium and their germination in Temporary Immersion Systems (TIS) was achieved ranging 92.4-96.8 % germination in 30 days. Acclimatization of the plants and their plantation in field are already a resolved problem, 3 200 plants were transplanted to open field. A distinctive characteristic of this process is that all the evaluated plants are hermaphrodite and they showed an improved vigor and more intense color of their leaves than plants derived from seeds and micropropagated ones.

Bananas and plantains

Calli with high frequency somatic embryogenesis (HFSE) were obtained using the methodology described by Escalant et al. (1994). Plant regeneration and cell suspensions were developed for several genotypes of bananas and plantains, including the new tetraploid hybrids: FHIA 03, 18 and 21. Embryo germination in temporary immersion systems ranged from 72.5-90.8 %, depending on the cultivar. Secondary multiplication of somatic embryos was carried out in bioreactors with an efficiency of 192 000 somatic embryos per liter. They have already been acclimatized and planted in field to study the genetic stability of the regenerated plants. A population of 500 plants from the cultivar Grand Naine and 1 000 plants from FHIA–18 are evaluated.

Coffee

A system for plant regeneration from embriogenic cell suspensions was established. Cell suspensions were initiated from HFSE calli derived from leaf sections (Jiménez et al, 1993). This system was scaled up in bioreactors, with an average production of 74 000 embryos per liter (Jiménez et al, 1995). Three different genotypes have been used: Caturra rojo and Catimor 9722 (*Coffea arabica*) and Robusta (*Coffea canephora*). Germination of the embryos was carried out in solid media under illuminated conditions, germination rates of 80 % were

achieved. Field trials with two years old plants are conducted and until now morphological variants have not been observed.

Genetic Engineering

Papaya

Somatic embryos of the variety Maradol rojo were transformed via *Agrobacterium tumefaciens* using the methodology proposed by Pons et al. (1998). *A.t.* strain EHA-101 was used, carrying the plasmid pDBCPRV-32, which contained the gene that codes for the coat protein of the *Papaya Rin Spot Virus* (CP-PRSV) (Meneses et al., 1998). Somatic embryos were cultivated in a selective media containing 15 mg/l BASTA, regenerated plants were acclimatized in plastic bags and transplanted to field under natural infection conditions at the Field Experimental Station of IBP. The presence of the CP-PRSV gene in the transformed plants was screened by PCR and, the integration of the gene by Southern-blot analysis using a non radioactive labeling kit. ELISA test using a polyclonal antibody was used to detect the presence of the virus in transformed plants. From 17 lines tested, 5 were positive by PCR and Southern blot, they have incorporated between two and three copies of the T-DNA in their genome. The transgenic plants showed no visual symptoms of PRSV infection after 10 months cultured in natural infection conditions and no level of the virus was detected by ELISA test.

Bananas and plantains

Embryogenic cell suspensions of the cultivars FHIA-18 (AAAB) and Grand Naine (AAA) were transformed using the methodologies developed by Sagi et al. (1995) and Pérez et al. (1998) for banana transformation by particle bombardment and *Agrobacterium tumefaciens*, respectivelly. For *A.t.* transformation experiments, strains EHA-101 and At 2260 were employed. plasmids pHCG58 and 59 (from Plant Division, Center for Genetic Engineering, Havana, Cuba), carrying two antifungal proteins: quitinase class I from bean and glucanase from tobacco in the first case and quitinase plus Ap24 in the second, were used. After bombardment or *A.t.* infection the cells were plated in selective media with 6 mg/L BASTA. Selection was performed during two subcultures. Resulting embryos were germinated in a solid medium without selective agent. At the present time, there are 74 and 3 transgenic lines of the cultivars FHIA-18 and Grand Naine, respectively. They will be tested for the resistance against *Micosphaerella fijiensis*.

Coffee

The objective of improvement in this crop is insect resistance, specifically against leaf mining (*Leucoptera coffeella* Guerr.). Biological tests were carried out to determine the relative effectiveness of three recombinant deltaendotoxins from *B.t.* against larvae of this insect. The highest larvicidal activity was obtained with the toxin CryIA(c) (Ocaña et al., 1995). Transformation experiments were carried out using embryogenic cell suspenions of the cultivars Caturra rojo (*Coffea arabica* L.) and Robusta (*Coffea canephora* Pierre). Direct transformation methods, electroporación of intact cells and gene gun, as well as *A.t.* mediated transformation have been used (Barbón, 1997; Pérez et al, 1998). The highest levels of GUS transient expression, 140 blue points per 100 mg of biomass, have been obtained when infecting embryogenic cell suspensions with the strain LBA 4404, carrying the plasmid

pGSulfGUSINT. There are several cell lines growing in selective media with 30 mg/l sulfadiazin and the transformation experiments with the construction that carries the gene of the toxin CryIA(c) of *B.t* have begun.

Conclusions

We conceive the genetic improvement like a unique strategy in which biotechniques should be combined with the classic methods of hybridization. One example is the papaya, where the character virus resistance should be combined with high yield and quality of the fruits. These characteristics are given by a great number of genes, mainly with heterotic effects. Hence, we started a program to obtain hybrids and the first step was to select the best combinations on the base of specific combinatory aptitudes. Hybrids were selected which increases in 4 units the brix content in the fruits and with 30 % increase in yield. These hybrids are transformed with the CP-PRSV gene in order to confer resistance against the Papaya Ring Spot Virus.

The obtainment of hybrids transformed with genes of agronomic interest and propagated via somatic embryogenesis is the fundamental objective of the genetic improvement program at the Institute of Plant Biotechnology.

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Sweet Potato (*Ipomoea batatas* L.) Regeneration and Transformation Technology to Provide Weevil (*Cylas formicarius*) Resistance. Field Trial Results

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Introduction

Although sweet potato is one of the most important plants around the world (FAO, 1989), biotechnological work on it has lagged behind. In the last years few groups have reported their experiences in sweet potato molecular biology manipulation to increase its nutritional quality (López et al., 1996) and to give it pest resistance (Newel et al., 1995). Others have been working on protoplast isolation and regeneration (Sihachakr and Ducreux, 1987), and regeneration and transformation from roots, petioles, stems and leaves (Carswell and Locy, 1984; Gosukonda et al., 1995). It has been established that one of the most important factors affecting the evolution of the *in vitro* culture, regeneration and transformation responses of sweet potato is the genotype worked on (Gosukonda et al., 1995).

The sweet potato weevil (*Cylas* spp.) is the major biological antagonist of sweet potato worldwide. The improved cultivars delivered by traditional breeding do not show a stable performance for different ecological conditions; most of them depend on soil structure or the physiological and botanical features of the cultivar rather than on the genetic production of chemical defenses against the pest (Sutherland, 1986). The lack of any strong conventional genetic improvement programs to obtain pest resistance make sweet potato an important target to be modified by biotechnological tools. Here, we show our experiences on sweet potato biotechnology and our strategies to improve this important crop.

Materials and Methods

Regeneration and transformation conditions

Leaves and stems were taken from young plants, from Jewel cultivar, grown in glass culture tubes containing 7 ml of MS medium (Murashige and Skoog, 1962). The *in vitro* plants are kept during six weeks at 25°C in a 12/12 photoperiod, 66 % relative humidity. We have studied more than 151 combinations of growth regulators including: Indol-3-acetic acid (0 mgl^{-1}, 0.25 mgl^{-1}, 0.5 mgl^{-1}, 0.75 mgl^{-1}, 1.0 mgl^{-1}, 1.25 mgl^{-1}, 2.0 mgl^{-1}); Naftalenacetic acid (NAA) at 0.1 mgl^{-1}; 0.5 mgl^{-1}; 1.0 mgl^{-1}; 2.0 mgl^{-1}; Zeatin riboside (0.11 mgl^{-1}, 0.22 mgl^{-1}, 0.5 mgl^{-1}, 0.75 mgl^{-1}, 1.0 mgl^{-1}); Kinetin (0.1 mgl^{-1}, 0.5 mgl^{-1}, 1.0 mgl^{-1}, 2.0 mgl^{-1}); 6-benzilaminopurine (BAP) at 0.25 mgl^{-1}, 0.5 mgl^{-1}, 1.0 mgl^{-1}, 2.0 mgl^{-1} and 3.0 mgl^{-1}; Paclobutrazol (PPP) at 0.1 mgl^{-1}; 0.5 mgl^{-1}; 1.0 mgl^{-1}; 2.0 mgl^{-1} and PPP at 0.0 mgl^{-1}; 1.0 mgl^{-1}; 2.0 mgl^{-1}; 3.0 mgl^{-1}.

We calculated the direct shoots regeneration efficiency as follows: RE= (RS/ TNE) x 100. Were RE, represents the regeneration efficiency of direct shoots in percent (%); BR, total number of regenerated shoots; and TNE total number of tested explants. The term regeneration frequency was introduced to determine the functional shoot or root emission per explant. It was calculated as follows: RF= NRP/ TNE. Were RF, is the regeneration frequency per explant; NRP, is the number of rooted functional shoots; and TNE, represent the total number of assayed explants.

Agrobacterium *mediated transformation procedure*

Agrobacterium tumefaciens strain C58C1-pGV2260 (Str^r; Spc^r; Rif^r; Amp^r) was used for plant transformation containing the pDEBTT binary plasmid carrying both the *Bacillus thuringiensis* var. *tenebrionis* delta-endotoxin and the *npt II* genes.

Several factors affecting the transformation efficiency were evaluated in our studies such as: co-cultivation medium, co-culture time, use of *vir* gene stimulators, co-culture conditions and regeneration medium.

All the regeneration media tested were supplemented with kanamycin at 45 mg l^{-1} as selectable. The frequency of transformation (TF), also termed % of kanamycin resistant plants, was determined as the number of shoots that rooted on the propagation medium containing kanamycin (KRS) per the total number of explants experiment (TNE). It is expressed as follows: TF=(KRS/ TNE).

Biological tests

Some of the kanamycin resistant clones and one untransformed were selected to perform the first biological test for sweet potato weevil resistance. Under controlled environmental conditions, 12 plants per clone were planted in an earth chamber. Established parameters for a good tuber yield were provided. Between each plant, highly infected tubers were planted, in order to guarantee pest population as inoculum.

Damages were evaluated 120 days after planting, considering the parameters: infestation percent (I), and pondered degree of infestation (PDI). Both of them, following a traditional methodology established in cuban agriculture by using the procedure of Thousend and Heuberge, (1948).

They were calculated as follows.

$I=\Sigma(a.b).100/D\ (n)$

$PDI=\Sigma(a/n)\ (b)$

where:

a= Values of scale of damages. (From 1 to 4).

b= Number of tubers per value of the scale.

N= Total number of tubers.

D= 4 (Maximum value of the scale)

In the experiment under controlled conditions, results of parameters I and PDI, were statistically compared using Duncan test for variance analysis both between treatments (clones and control plants) and replicates.

A second experiment for biological activity under field conditions following a random block design with three replicates per clone was carried out. Three transgenic clones

previously selected both by PCR/Southern hybridization and by their behavior in an experiment under environmentally controlled condition were tested against sweet potato weevil attack under field conditions. The experiment was performed at experimental areas of the INIVIT, Santo Domingo, Cuba, and it fulfilled the requirements demanded for works with genetically modified organisms. Seeds were planted according to the established design in lands highly infested by sweet potato weevil. Damages were evaluated 120 days after planting. One hundred percent of tubers were analyzed and the weight of commercial tubers was measured. The evaluation criteria were the same established for the experiment under controlled conditions.

Results and Discussion

The stem explants were able to form non-embryogenic calli on the cut side of the explants but early organogenic shoots appeared first than the calli and they were always regenerated from the meristematic area placed in the nodal section. However, the best results were obtained for leaf explants were the regeneration area was placed on the top of a petiole piece remained on the leaf and petiole junction. On this area an organogenic response was obtained giving functional aerial shoots and organogenic roots; both of them were obtained by indirect organogenesis because they were able to be induced from a green and compact callus formed on the cut tissue of the petiole. The regenerated root was able to form aerial shoots both when they were individualized to growth regulators free MS medium or when they were kept on the regeneration medium for long time periods. These secondary shoots appeared as a red point on the regenerated root, were increasing its organization and finally shot like a bud giving functional plants able to rooting once individualized to the propagation medium. Newell et al in 1995, reported the regeneration from meristematic bud like centers (MBLCs) existing in the tuberous roots and there might be a coincidence between those structures reported by this group and that ones which were able to form aerial shoots in the regenerated roots of our experiments.

The more effcient shoot emission for the Jewel cultivar was obtained on the MPM medium, but the above described pattern for organogenic regeneration was similar for all the combinations that works efficiently. Remarkably, auxin influence to induce direct organogenesis was observed (table 1) to be greater than that of cytokinins both alone or combined with auxins.

Table 1. Regeneration efficiency on different regeneration media for Jewel cultivar. BN-3 medium, MS + Sucrose 3 % + BAP (0.1 mgl^{-1}) + NAA (1.0 mgl^{-1}); PN-2 medium, MS + Sucrose 3 % + PPP (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}); MPM medium, MS + Sucrose 3 % + IAA (0.5 mgl^{-1}).

MEDIUM	Direct shoots (%)	Indirect shoots	Regeneration frequency
BN-3	68.9	100	0.7
PN-2	85.00	100	0.85
MPM	200.00	100	3

It was usual to find more stimulation for non embryogenic calli formation when the explants were treated with cytokinins (even at low concentrations) than with auxins. In some cases shoots were achieved by giving moderated auxin treatments (IAA 0.5 mgl^{-1}; NAA 0.1

mgl^{-1}) and the highest regeneration frequencies were obtained on these treatments. There is not any previous reference of using auxin based stimulation alone to induce plant regeneration on sweet potato and we believed that it must be because the endogenous growth regulator balance of sweet potato. A high regeneration frequency was obtained on several combinations of NAA and Paclobutrazol. However, there was a inhibitory effect on the normal shoot development when Paclobutrazol concentration was over 1.5 mgl^{-1}.

Taken into account that leaves behaved better than stem in all our regeneration experiments we chosen this explant source as target tissue for the next transformation. Long term cultures in liquid medium very much affected the physiological and morphological stage of the explant during the transformation step. Although, it was proved the liquid medium gives more possibilities for the full interaction between the target explant and the bacterium it was better to co-cultivate the explants not for long periods in liquid medium. When the explants were co-culture in liquid medium for more than 36 hours, 100 percent of them died after planting on the regeneration medium supplemented with kanamycin at 45 mg l^{-1}. The optimum time for co-cultivation in liquid medium was established for 24 hours; for this period of co-cultivation the 100 percent of the explants kept green and formed kanamycin resistant roots. Long term culture of the explants with the bacterium was possible on solid medium but and some few transgenic roots and shoots were obtained (1 % and 3 % for the best treatment). It must be possibly due to the limited interaction between the more potential area for regeneration placed on the top of petiole section retained and the *Agrobacterium tumefaciens* which growth and colonize only in the base of the leaf that is fully extended on the medium.

The regeneration efficiency was a very important factor influencing the transformation of sweet potato. As it was shown in the table 2, there is a coincidence between the behavior of the regeneration media during the regeneration studies and during the transformation step. The transformation efficiency on the different media fits very well with their regeneration efficiency; still, the MPM medium offered the highest transformation frequency (table 2).

Table 2. Transformation frequency on three different media.

Medium	Km^r shoots (%)	Km^r roots (%)	TF
BN-3	30.2	76.8	93.25
MPM	55.3	100	100
PN-2	45.3	82.3	100

Biological test results

According to parameters I and PDI, two transgenic clones, C27 and C1, have shown the least weevil infection (table 3).

Table 3. Evaluation of transgenics sweet potato clones for resistance to sweet potato weevil (*Cylas formicarius sp. elegantulus*) under controlled conditions, 120 days after planted. C: Untransformed control plants belonging to Jewel

Clone	I	PDI.
C-27	2.59	0.104
C-1	8.01	0.319
C	14.09	0.591

In this experiment, the damages in tubers of C27 and C1 were at least 5 times and almost twice lower than untransformed plants, respectively. As it could be noted in both experiments, transgenic clone called C27 exhibited lower average values of I than untransformed control, thus less weevil affectation (table 4). Average values of parameter I was 5 and 1.89 times higher in control plants than in plants belonging to the clone C27 in the experiments. However, these differences were not statistically significant by the Duncan test performed. According to the parameter I, clone C1 behaved variable. In the experiment 2, it showed the lowest I value, even a little lower than the clone C27. In the experiment 1, the average I value for C1 was almost the same of C27. Plants belonging to the clone C27 rendered 1.12 times more commercial tubers than untransformed plants.

Table 4. Evaluation of transgenic sweet potato plants carrying the *cryIIIA* gene of *Bt.t.* under field conditions. I: Percent of infestation. c.t: weight of commercial tubers in Kg.

Replicates	I		II		III		IV		Average	
Clones	I	c.t.	I	c.t.	I	c.t.	I	c.t.	I	c.t.
C1	2.1	23	0.6	35	2.9	31	8.5	20	3.53	27,25
C101	3.5	28	0.9	34	7.3	26	12.4	19	6.03	26,75
C27	1.8	33	1.9	36	3.5	30	8.1	24	3.83	30,75
Control	8.8	25	3.5	35	10.5	28	6.1	22	7.25	27,5

By Dellaporta's protocol for plant DNA extraction (Dellaporta et al., 1983), a high quality material was obtained. In some kanamycin resistant clones, bands at the same size of the positive control were amplified by PCR. Similar results were obtained after hybridization of PCR products with the selected probe. This fact confirmed the successful transformation with the *Btt.* toxin gene. Neither by PCR, nor by PCR product hybridization, the bands were detected in untransformed control plants. Genomic Southern blot analysis confirmed the integration of the *cryIIIA* gene into the sweet potato genome as a single copy.

Western blot, performed with proteins from the clones C27 and C1, showed that CryIIIA toxin expression was detected on the expected molecular weight in both, and there was not any band in the lane of untransformed control plants. Despite these results, the toxin expression was visually estimated no higher than 0.005 -0% of the total protein content, even for the clone C27 that exhibited higher levels.

There is coincidence between the expression levels of the CryIIIA toxin and the mRNA detection. Regarding the low toxin expression detected, some papers have described the weak expression of the *B.t.* endotoxins in transformed plants carrying the native bacterial genes. Numerous factors could affect the expression of heterologous genes in transgenic plants, such as the codon usage, the A+T content, the molecular context at the translational start, mRNA sequence and structure and some others (Ely, 1993).

Conclusions

Our progress and results on sweet potato biotechnology and molecular biology to confer pests' resistance have demonstrated that biotechnological manipulation of this tropical crop is a suitable tool to improve its agronomic performance. Nowadays, we are strongly working in

building a synthetic deltaendotoxin gene to increase the expression levels in the plant tissues and to grow up the resistance against weevils. Transgenic plants with the novel synthetic genes are almost to be generated and will be tested under field condition on the second half of 1999.

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Regulation of Transgene Expression: Progress Towards Practical Development in Sugarcane, and Implications for Other Plant Species

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AlbD Level Determines Leaf Scald Disease Resistance in Transgenic Sugarcane

Leaf scald disease of sugarcane is a finely balanced host-pathogen interaction, with prolonged latent infection often preceding the development of damaging chronic symptoms or the devastating acute phase of the disease (Ricaud et al., 1989). Albicidin toxins produced by the systemic vascular pathogen, *Xanthomonas albilineans*, cause the characteristic chlorotic symptoms by blocking plastid DNA replication, and may be also a key factor in systemic invasion or the unpredictable transition from latent infection to disease (Birch and Patil, 1983; 1987a,b). We recently cloned a gene (*albD*) for albicidin detoxification, from a bacterium that provides biocontrol against this disease (Zhang and Birch, 1996; 1997). Transgenic *X. albilineans* strains expressing *albD* is attenuated in pathogenicity (Zhang and Birch, 1997). Preliminary results summarised below show that expression in transgenic sugarcane of the albicidin detoxification gene *albD* can confer resistance to leaf scald disease caused by *X. albilineans*. The level of resistance depends on the level of activity of the introduced gene product.

The coding region of *albD* was cloned between the Ubi promoter from the maize *ubi-1* gene (Christensen and Quail, 1996) and the *Agrobacterium nos* terminator (Bevan et al., 1983) to drive expression in sugarcane cells. Leaf scald susceptible sugarcane cultivars Q63, Q87 and 85S797 were transformed by co-precipitation of the Ubi-*albD* construct (pU3Zald) and an *aphA* construct (pEmuKN) onto tungsten microprojectiles, bombardment into embryogenic callus, selection for resistance to antibiotic G-418 (geneticin®), and regeneration of transgenic plants. There was a high co-expression frequency for the unselected *albD* gene, consistent with previous results using reporter genes in the highly efficient sugarcane transformation system (Bower et al., 1996).

Plants were challenged by application of the pathogen to the freshly cut surface of plants decapitated above the apical meristem. This decapitation method simulates the spread of this mechanically transmitted pathogen in the field, and is widely used in testing sugarcane varieties for resistance to the disease (Ricaud et al., 1989). Inoculated leaves emerging from the cut spindle typically show characteristic 'white pencil line' symptoms, resulting from blocked plastid differentiation in parenchyma cells surrounding invaded vascular bundles (Birch and Patil, 1983).

One month after inoculation, AlbD enzyme activity was measured in extracts from leaf and stem tissues of uninoculated plants of each line, based on the amount of albicidin inactivated in the assay described previously (Zhang and Birch, 1996). The results (presented as ng

albicidin detoxified / mg soluble protein in the plant tissue extracts) accurately rank samples for AlbD enzyme activity.

In glasshouse trials two weeks after inoculation, all plants of 22 tested *albD* control lines of sugarcane cultivars Q63 and Q87 showed typical white pencil line symptoms. In contrast, 44% (Q63) to 56% (Q87) of 61 independently transformed lines regenerated following particle bombardment to introduce *albD* showed no symptoms in inoculated leaves. There was a strong negative correlation between *albD* activity in the youngest fully emerged leaf and the number of white pencil lines developed in inoculated leaves (Spearman r = -0.48, P<0.0001). Plant lines with high AlbD activity (>200 ng albicidin detoxified / mg soluble protein) showed few or no white pencil lines. Severely diseased plant lines (>5 white pencil lines per plant) had little or no AlbD activity (Figure 1A).

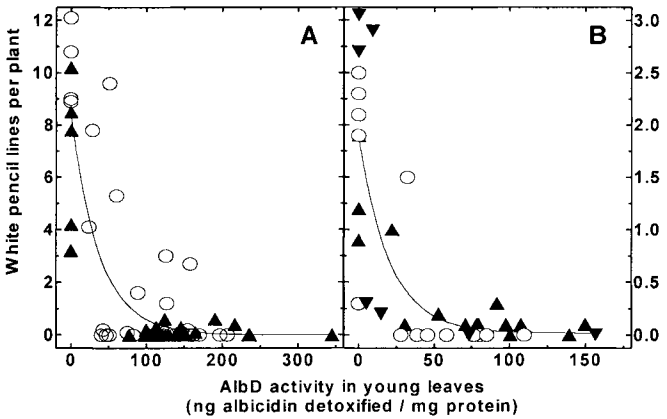

Figure 1. Relationship between albicidin detoxifying activity and leaf scald disease severity in inoculated leaves of cvs. Q63 (O), Q87 (▲), and 85S797 (▼), under glasshouse (A) and field (B) conditions.

The most resistant plant lines under glasshouse conditions were not necessarily highly resistant under field conditions. However, the strong negative correlation between AlbD activity and disease severity was maintained in the inoculated field plots (Spearman r = –0.74, P<0.0001) (Figure 1B). These results indicate that leaf scald resistance is strongly dependent on the level and pattern of *albD* expression, which can vary between the glasshouse and the field.

Albicidins are produced as a family of secondary metabolites, which may differ in relative antibacterial and phytotoxic activities, but all of which appear to be inactivated by AlbD enzymatic activity (Zhang and Birch, 1997). *Xanthmonas albilineans* strains engineered to express *albD* do not cause chlorosis in leaf-scald susceptible sugarcane, and sugarcane engineered to express *albD* does not develop chlorosis following inoculation with wild type, toxigenic *X. albilineans*. It can be concluded that albicidins are the pathogenesis factor responsible for the damaging chlorotic symptoms of sugarcane leaf scald disease. Although chlorisis is a spectacular visible manifestation of albicidin action in the leaves, the consequences of blocked plastid division and differentiation in other tissues exposed to the toxin during systemic colonisation of the xylem by *X. albilineans* may be of greater relevance

in disease development. Plastids are the site of essentially all fatty acid biosynthesis, nitrite reductase activity, essential amino acid biosynthesis, and production of essential precursors of defence-related aromatic compounds in plants. It follows that albicidin-mediated disruption of plastid division and development could facilitate systemic invasion and disease development, by weakening plant defence mechanisms. We are currently testing this possibility by comparing multiplication of the pathogen in inoculated control and *albD* expressing sugarcane lines.

The AlbD enzyme activity in the sugarcane lines with the highest activity characterised here corresponds to 1-3 ng AlbD / mg soluble protein in cell extracts from the stem apex region, and 5-6 ng / mg from fully expanded leaves (based on reconstruction experiments with purified AlbD protein). This low level of an introduced gene product is not expected to impose any substantial metabolic load. Genetic transformation to obtain clones with an appropriate pattern of *albD* expression is therefore a promising approach to rescue agronomically outstanding cultivars that would otherwise be discarded because of leaf-scald susceptibility. This can be accomplished at present by screening large numbers of transformants for favourable transformation events. An improved understanding of the most effective pattern of *albD* expression, and improved control of transgene expression through tailored promoters and genomic integration, are likely to be key developments for more efficient use of the strategy.

The Maize Ubi Promoter Drives Sustained But Environmentally Responsive Expression in Field-Grown Sugarcane

In sugarcane, as in all other plants, independent transformants with the same promoter-reporter gene construct can show very different expression strengths and patterns of tissue specificity. This can be attributed in part to the influence of different sites of integration into the plant genome, and possibly to interactions between multiple integrated copies of the introduced DNA, some of which may show various sequence truncations or rearrangements. These effects reduce the useful transformation frequency, and necessitate detailed characterisation of multiple independent transformants to identify commercially useful transformation events (Birch, 1997). In addition, the Ubi promoter is known to be environmentally responsive in maize (Christensen et al., 1992), and it responds to a range of biotic and abiotic stresses in transgenic sugarcane. This stress responsiveness may account for the substantial variation observed in transgene activity levels between adjacent stalks or stools of the same transformed plant line under field conditions.

For practical application of most potentially useful new genes, stability or predictability of transgene expression over time will be a vital consideration. We followed transgene expression over several years under glasshouse and field conditions in ten independently transformed lines of sugarcane cv. Q117 containing the *luc* gene (Ow et al., 1986) under the control of the maize Ubi promoter, or the artificial Osa or Emu promoters (Bower et al., 1996). Classification of transgenic lines into broad classes of foliar transgene expression strength gave approximately the same rankings in the first vegetative generation in the glasshouse, as in the field trial. All lines showed substantial variation (22 - fold on average) in transgene activity over time. There was no progressive decrease in expression and the pattern of decrease and increase in activity over the sampling period was consistent across lines. This

could arise due to broad seasonal influences operating on all of these "constitutive" promoters or on the overall metabolic activity in sugarcane at the sampling times.

The practical significance of this variation will depend on the specific pattern of transgene product accumulation necessary to fulfil the intended agronomic function of each new gene. We are currently exploring sequence alterations that may reduce such inducible variation or increase the constitutive expression level.

Transgene in Sugarcane is Promoter-Dependent and Copy Number Independent

High copy number and complex integration patterns are commonly cited as contributing to instability of transgene expression (Matzke and Matzke, 1995). We set out to determine whether this was true for sugarcane, and to test the effect of DNA concentration in the microprojectile precipitation mix on the copy number of transformants generated by microprojectile bombardment.

Transformation efficiency declined with reduced concentration of the selected marker plasmid pEmuKN in the precipitation mix, and coexpression frequency declined with reduced concentration of the non-selected reporter plasmid. By reducing DNA concentration 100-fold from the routine concentration of 1mg/ml in the precipitation mix, it is possible to achieve a 4-fold decrease in the number of transgene integration sites at the cost of a 15-fold reduction in transformation efficiency.

Ten of eleven tested transgenic lines with either one or two genomic Ubi-*luc* integration sites showed no detectable LUC activity. It is possible that the *luc* integration sites in these lines are in chromosomal regions inaccessible for transcription, or that they contain truncated insertions. In eleven tested lines with detectable *luc* expression, there was no correlation between LUC activity in transgenic plant lines and the number of integration sites of the Ubi-*luc* gene. There was no evidence of homology-dependent silencing of the Ubi-*luc* gene in sugarcane, even at high copy numbers (>10 integration sites). Other genes including *uidA* and *albD* also show no relationship between copy number and expression strength or silencing when driven by the Ubi promoter in trangenic sugarcane (Figure 2).

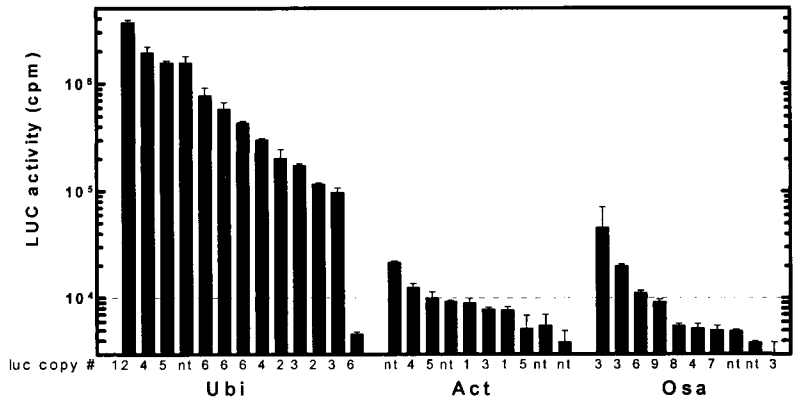

Figure 2. Luciferase activity in leaves, 5-8 months after plant regeneration from LUC positive sugarcane callus lines transformed with various promoter-luc fusions. Approximate copy number is shown for lines tested by Southern analysis.

In contrast to the results with Ubi, the *luc* gene driven by the rice actin promoter (McElroy et al., 1991) or the artificial Osa promoter (Bower et al., 1996) is silenced upon plant regeneration in a high proportion of sugarcane transformants, independent of copy number. These are strong promoters in transient expression assays, and in transgenic callus before plant regeneration. We have subsequently observed the same effect with the rice RCg2 and sugarcane Rsg promoters. This indicates that the transgene silencing mechanism is promoter sequence dependent and copy number independent. In the case of the Ubi promoter, the mean number of around six integration sites generated under routine bombardment conditions does not result in adverse effects on transgene expression in sugarcane. However, the capacity to reduce the number of integration sites may be advantageous in some circumstances, for example in promoter tagging experiments or to reduce the probability of insertional inactivation of sugarcane genes.

Transgene Silencing in Sugarcane Involves Multiple Mechanisms, Including DNA Methylation and Post-Transcriptional Effects

Methylation of transgene and endogenous sequences is often associated with gene silencing, and can sometimes be relieved by treatment with the demethylating agent 5-azacytidine (Kilby and Leyser, 1992). Silencing can involve reduced transcription, sometimes associated with promoter sequence methylation and perhaps altered chromatin configuration (Park et al., 1996) or post-transcriptional mechanisms such as mRNA degradation, sometimes associated with methylation of coding regions (Baulcombe and English, 1996). Most models postulate that transcriptional silencing is triggered by recognition of DNA as foreign (for example because of inappropriate base composition) or by ectopic pairing of homologous sequences. Post-transcriptional silencing is thought to be triggered by RNA threshold effects, production of antisense RNA, or DNA-DNA pairing effects leading to production of aberrant RNA (Matzke and Matzke, 1995; Baulcombe and English, 1996). We investigated ten transgenic lines of cv. Q155 in which the *luc* gene was either silenced or substantially down regulated in regenerated plants, relative to the expression levels in the transformed callus.

Callus was reinitiated from each silenced line and incubated on medium containing 0, 100 or 300 μM azacytidine for 3 weeks, then assayed for LUC activity. Callus was then placed on regeneration medium containing the same concentration of azacytidine, and regenerated plantlets were assayed for LUC activity after 4 wks. LUC activity was substantially increased in azacytidine-treated callus/and or plantlets from 2/5 Osa-*luc* lines and the single tested Act-*luc* line. LUC activity was not increased in three silenced Ubi-*luc* lines, but it was increased in a strongly expressing Ubi-*luc* line included as a control. DNA methylation is implicated in silencing in azacytidine-responsive lines, but lack of a response does not preclude methylation effects in other lines, as azacytidine treatment results in only partial demethylation of genomic sequences.

Northern analysis showed no detectable *luc* mRNA in silenced lines, but runoff transcription experiments revealed strong transcription. No transcriptionally silenced lines were detected. This is a surprising result, because the observed promoter sequence dependence and developmental onset of *luc* transgene silencing appears more consistent with the postulated triggers for transcriptional silencing.

Implications for Other Plants of Efficient Transgene Silencing in Sugarcane

We have shown LUC activity in 90 % of tested Ubi-*luc* lines but only 10-20 % of transformants with *luc* driven by other 'strong constitutive' promoters. This is the highest efficiency of transgene silencing recorded in plants. However the system may be more remarkable for the clarity of silencing due to early onset and completeness, rather than the frequency of silencing which may be matched through patchy and progressive onset in other species.

Several models and mechanisms of transgene silencing have been proposed, based largely on analysis of model dicotyledonous plants (Matzke and Matzke, 1995; Baulcombe and English, 1996; Park et al., 1996; Stam et al., 1997; Vaucheret et al., 1998). Our results in sugarcane exclude a simple dependence on transgene copy number, arrangement, fragmentation, or integration sites, because these parameters were common to unsilenced (Ubi, Emu) and silenced (Act, Osa) populations. The expression levels in transient assays do not support a simple expression threshold for silencing. We can not exclude the possibility of production of aberrant RNA from certain constructs, for example by defective splicing of the Act intron, but any such defect must be species-dependent because the same promoter is not efficiently silenced in maize and rice (Zhang et al., 1991; Zhong et al., 1996).

Sugarcane is a crop of exceptional genetic complexity, with 100-130 chromosomes in cultivars that are highly heterozygous, polyploid (auto-octaploid for some chromosome sets), aneuploid, interspecific hybrids between *Saccharum* species (Grivet et al., 1996). This could be accompanied by an exceptional elaboration of gene silencing as a mechanism for control of polyploid gene expression (Leitch and Bennett, 1997), but the insensitivity of Ubi indicates that the capacity to recognize a gene for silencing, or to maintain at least one copy unsilenced, must be sequence specific.

Each of the tested constructs used the same reporter gene (*luc*) in closely related (pUC-derived) vector sequences. Silencing was unrelated to the source of the 3' polyA signal sequence (*ocs* for Osa-*luc*, and *nos* for Act-*luc* and Ubi-*luc*). Silencing was promoter sequence specific. The term promoter in this context is applied to the region 5' of the coding region, and common to expression cassettes intended to drive various useful transgenes. These promoter regions include not only RNA polymerase binding and transcriptional enhancer domains, but also different untranslated leader sequences, including introns in the case of Ubi, Act and Emu. It has been speculated that gene silencing in dicots could be partly an artifact of the wide use of the viral 35S promoter (Matzke and Matzke, 1995), but this possibility has not been experimentally tested. Our results in sugarcane show that silencing can be promoter-sequence specific, and not dependent on any viral sequence in the promoter.

Efficient, 5' sequence-specific, and developmentally-regulated transgene silencing in sugarcane has broad implications, because most major crops are polyploid (Leitch and Bennett, 1997) and endoreduplication results in high ploidy levels in differentiated vegetative tissues of most plant species (Traas et al., 1998). The first evidence for an effect of ploidy on silencing was provided by Scheid et al. (1996), who observed that one simple and one complex 35S-*hpt* locus in *Arabidopsis* were both more susceptible to silencing in the hemizygous state in triploid and tetraploid genomes than in a diploid genome. Progressive or patchy silencing of transgenes, readily observed using visual markers in many plants, may be associated with increasing endopolyploidy and polysomaty (cells with different ploidy levels) in maturing tissues. We speculate that silencing is manifested more sharply in sugarcane

because of the complex polyploid genome. If differential silencing is a natural control mechanism in the exploitation of developmental polyploidy in plants a clearer understanding of sequences susceptible or resistant to the effect is of profound importance in plant molecular improvement. The efficient transformation and regeneration procedure for sugarcane (Bower et al., 1996) and the sharply contrasting effects on Ubi, Act, Osa and Emu sequences described here, provides a tractable experimental system with which to address these questions.

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Plant Genetic Engineering: Towards the Third Millennium
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Polycistronic Translation in Plants. What Can we Learn from Viruses

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Transgenes are generally used to endow plants with various types of pest resistance, improved nutritional value, commodities and others. While so far mainly mono-genomic traits were handled, the demand for employing also multi-genomic traits is increasing, especially, e.g., to achieve fungus resistance through enzymes producing anti-fungal secondary metabolites ("agrobiotics") (Hammer et al., 1997) or to supplement crops with substances like vitamin A (Burkhardt et al., 1997). The introduction of a series of genes required for such a multi-genomic trait provides some challenge, since it is difficult to foresee the expression efficiency of each individual transgene. One has to consider that the concentration of the desired end product is limited by the weakest link of the chain and that the possibility of loosing the trait through gene silencing is potentiated with the number of genes involved, especially if the same promoter is used for several genes (Matzke et al., 1996). It would therefore be desirable that all genes required for an engineered multi-genomic trait are located on a single mRNA. This, however, would require the availability of polycistronic expression units. Polycistronic translation is generally not used in eukaryotes, since normally 40S ribosomes and initiation factors assemble at the 5'-cap structure, scan then along the RNA to find an appropriate start codon, join there with the 60S ribosome, translate the ORF and then fall off. On the other hand, polycistronic translation is common in prokaryotes, since ribosomes enter the RNA internally at a "Shine-Dalgarno Sequence" and several of these sequences, each preceding a coding region, can be accommodated per RNA molecule. Since chloroplasts are of prokaryotic origin, they can use the prokaryotic translation mechanism (Staub et al., 1995). Thus they could be used for expressing polycistronic RNA as soon as chloroplast transformation technology is available. So far this is the case only for tobacco (Zoubenko et al., 1994).

Viruses, due to the pressure to optimize compactness of their genome, have developed alternative mechanisms to cope with polycistronic translation in the main cytoplasm and we can learn from them to employ these mechanisms with transgenes. Caulimoviruses code for a multifunctional transactivator (TransActivator,Viral; TAV; Bonneville et al., 1989), which also acts as a "translation reinitiation factor". This factor binds to RNA, to a ribosomal protein and to eukaryotic translation initiation factor 3 (eIF 3; Park et al., in prep.). It directs translation from cauliflower mosaic virus (CaMV) polycistronic RNA by promoting reinitiation after short intercistronic regions (Figure 1a, Fütterer et al., 1991; Zijlstra and Hohn, 1992).

An obstacle in using the TAV system in transgenic plants relies on the intracellular toxicity of the TAV transgene (Zjilstra et al., 1996). This problem might be overcome by using mutants of CaMV TAV or exploring the TAV genes of other caulimoviruses.

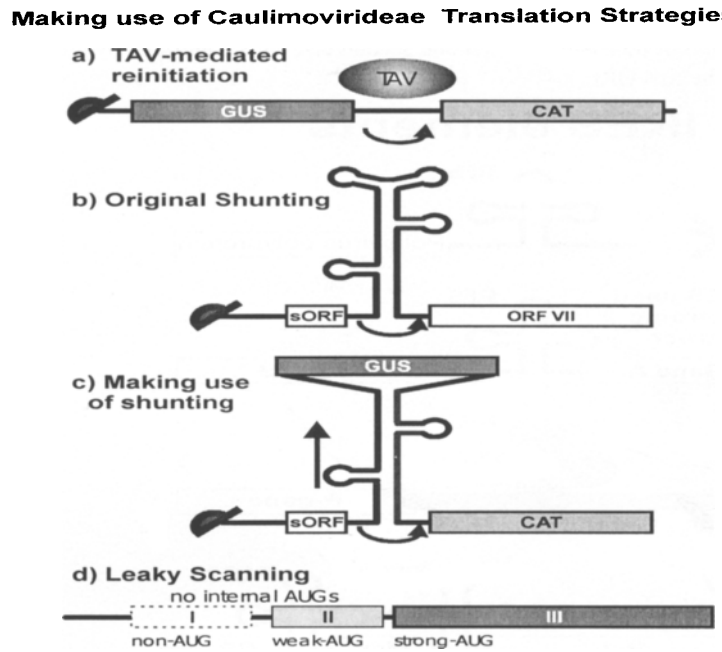

Figure 1. Translation mechanisms derived from Caulimovirideae

Caulimoviruses and badnaviruses have long and highly structured leaders. A combination of a small open reading frame (sORF) (Pooggin et al., 1999) and a strong stem structure (Dominguez et al., 1998; Hemmings-Mieszczak et al., 1998) divert scanning ribosomes from a 5'-proximal region to the end of the leader in a process called "shunting" (Figure 1b, Fütterer et al., 1993, 1996; Schärer-Hernández and Hohn, 1997). This process, however, is not tight and a sub-population continues scanning to the center of the leader (Ryabova et al., 1999). Expression units can be constructed based on this leader which have one ORF in its center and one of at its end (Figure 1c, Fütterer et al., 1993). Depending on whether a ribosome scans or shunts, either the first or the second ORF is translated. The ratio of expression of the two ORFs could be manipulated by the strength of the stem structure and the position and length of the small open reading frame.

Badnaviruses also have polycistronic RNAs. In the case of rice tungro bacilliform virus (RTBV), the first two ORFs have leaky start codons (non-AUG start codon and weak-context-AUG start codon) that are recognized by some ribosomes, while others ignore them and continue scanning for the second and third one (Figure 1d, Fütterer et al., 1997). Since within the first two ORFs no internal start codon exists, a total of three ORFs can be translated from RTBV RNA. Cap- and eIF4A-independent translation was first observed with animal picornaviruses. These have internal ribosome entry sites (IRES) located on their RNA (Figure 2, Jackson et al., 1995).

Although the picornavirus RNAs are originally monocistronic, their IRESes can be used for polycistronic translation if they are positioned between two ORFs of a capped mRNA. We recently have indications that another animal virus, hepatitis B virus, uses an IRES in a

dicistronic context to translate the capsid protein (first ORF) and the polymerase (second ORF). In this case the ratio of translation of first and second cistron is controlled by an sORF overlapping the start of the first ORF.

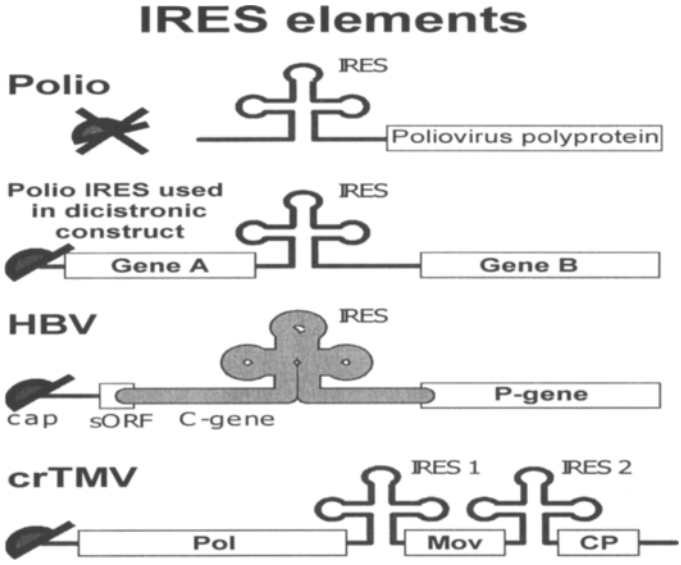

Figure 2. Internal initiation.

Most of the animal IRES elements do not function in plants. We found that the HBV IRES mentioned above is an exception, since it can be used in wheat germ extracts. Furthermore, recently IRES elements have been found in a plant virus, namely in front of the movement protein and coat protein genes of crucifer tobacco mosaic virus (crTMV) RNA (Ivanow et al., 1997; Skulachev et al., 1999). In this case the full-length virus RNA itself is polycistronic. The exact structure of the crTMV IRES and the mode of action are under our study in the frame of a project supported by INTAS.

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Towards Plantibody-Mediated Resistance to Plant Parasitic Nematodes

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Plant Parasitic Nematodes

Plant-parasitic nematodes have developed diverse adaptations enabling them to parasitise roots. This has involved the development of a protrusible stylet (to penetrate cell walls and feed on cell contents) and marked morphological and physiological adaptations of the oesophagus. The oesophagus gland cells have enlarged and the nature and function of their secretions changed when nematodes adapted to parasitism. The majority of the physiological functions of these secretions are not understood. Nematode parasitism is a complex and dynamic interaction. It involves hatching stimuli, attraction to the host, penetration of host tissue, recognition of tissues suitable for feeding site formation, modification of host tissue and an active response from the host plant.

Sedentary endoparasitic nematodes (e.g. *Globodera, Heterodera* and *Meloidogyne spp.*) have developed highly specialized manners of parasitising host roots. After root penetration they migrate to the stele where they induce a multinucleated feeding site (Golinowski et al., 1996). The most intriguing phenomenon is the way sedentary endoparasitic nematodes manipulate their host cells such that a transition takes place into a feeding cell. Insight is emerging into the molecular basis of this part of the pathogenesis process (Fenoll et al., 1997). One effect is the re-entry of young feeding cells into the cell-cycle (Niebel et al., 1996). Another effect is the increase in turgor pressure in these cells. In concert with this phenomenon, genes such as the Lea-like gene, which encode for proteins thought to be involved in osmoprotection, and a tobacco root-specific gene encoding a putative water channel have been cloned (Opperman et al., 1994;Van der Eycken et al., 1996). Also, an increase in extensin expression has been observed and this protein may have a role in fortifying the cell wall (Niebel et al., 1993). In addition to proteins whose expression levels are upregulated in feeding cells, several pathogenesis related proteins have been found in other tissues (Niebel et al., 1995).

During parasitism the functions of the oesophageal glands change. The subventral glands are assumed to be active mainly in the initial stages of parasitism, during root penetration, root migration and feeding cell induction. These glands are packed with secretory granules in preparasitic and parasitic second stage juveniles, but not in the later stages of nematode development. In contrast, the dorsal gland contains only a few granules in the earlier stages. This gland fills up with secretory granules at the end of migration and during the initial stages of feeding cell induction. It is generally accepted that the compounds used to form a feeding tube arise from this gland. Feeding tubes that function as molecular sieves are formed prior to withdrawal of cell contents. It is, as yet, unclear what the functions of the subventral glands

are, once the nematode has become immobile or which other functions the dorsal gland has during this stage.

Plantibodies

Knowledge of the molecular basis of nematode parasitism can be used to design antibody-mediated resistance traits. Antibodies offer a good methodology to obtain resistance. They can have a profound effect on the biological activity of proteins upon binding (Schots et al., 1992). This was shown *in vitro* for several proteins as well as *in vivo* in animal cells and plant cells upon heterologous expression of antibody genes (plantibodies). The expression plantibodies inhibits replication of artichoke mottled crinkle virus (ACMV, Tavladoraki et al., 1993) and tobacco mosaic virus (Voss et al., 1995). Physiological processes may also be influenced. Plant expression of anti-phytochrome antibodies resulted in aberrant phytochrome-dependent germination (Owen et al., 1992). Seed-specific immunomodulation of abscisic acid (ABA) by an anti-ABA antibody resulted in a switch from the seed maturation program to a germination program (Phillips et al., 1997). These examples clearly demonstrate the feasibility of immunomodulation in plants.

Isolation and Characterisation of Targets

Plantibody-mediated resistance against sedentary plant parasitic nematodes is only possible if suitable targets are available. However, the molecular basis of nematode parasitism is poorly understood. Little is known about those molecules produced by the nematode which allow it to parasitise host roots. Only one has recently been characterized and is described below. It is necessary to isolate, possibly clone and characterize more of these nematode molecules prior to raising inhibitory plantibodies.

The isolation of target molecules was approached by raising monoclonal antibodies against the contents of the oesophageal glands (Davis et al., 1992; Davis et al., 1994; Goverse et al., 1994; Hussey et al., 1994; De-Boer et al., 1996a; De-Boer et al., 1996b). Initially shot gun approaches were used. Crude nematode homogenates were used to immunise mice and monoclonal antibodies were selected after a hybridoma cell fusion. This resulted in the production of monoclonal antibodies specific for the subventral or dorsal glands of *Heterodera glycines, Globodera rostochiensis* and *Meloidogyne spp.*

New strategies were developed to raise monoclonal antibodies binding to the secretory proteins of sedentary endoparasites. Davis et al. (1992) used homogenates of the anterior regions and stylet secretions of *M. incognita* adult females as the immunogen. This resulted monoclonal antibodies binding to the secretory granules in the dorsal gland and the subventral glands. In our lab we decided to an alternative strategy based on the partial purification of nematode proteins (De Boer et al., 1996b). Homogenized second stage juveniles of *G. rostochiensis* were separated by preparative continuous flow electrophoresis in the presence of sodium dodecyl sulphate (SDS) into 50 protein fractions. The average molecular mass of these proteins ranged from 30 kDa to 52 kDa. The fractions were pooled to form a set of 16 samples of increasing molecular mass. These were used to immunise 16 individual mice. After the second immunisation, antisera were collected and screened with immunofluorescence for binding to the oesophageal glands of second stage juveniles of *G. rostochiensis*. The sera of the eight mice immunised with the successive protein fractions from 36 kDa to 52 kDa showed a strong reaction with the subventral oesophageal gland cells. One mouse, immunised

with the 38 kDa to 40.5 kDa protein sample was chosen for monoclonal antibody production. Ten monoclonal antibodies were selected which bound to the entire contents of the subventral gland cells including the gland extensions. Western blotting revealed that these monoclonal antibodies bound one or more of four proteins with molecular mass of 30 kDa (svp30), 31 kDa (svp31), 39 kDa (svp39) and 49 kDa (svp 49). They almost exclusively bound to members of the genus *Globodera*. Only monoclonal antibody MGR48, which binds to all four svps, showed cross reactivity by binding to *Heterodera glycines* and *H. schachtii*. Electron microscopy using MGR 48 as the probe revealed that the protein antigens localise mainly in the electron dense core of the secretory granules indicating that the svps are indeed secreted.

When second stage juveniles of *G. rostochiensis* were hatched in potato root diffusate, and after extensive washing were incubated in standard pore water (SPW; a water solution mimicking ground water) the antigens were secreted into the SPW (Smant et al., 1997). Juveniles that hatch spontaneously in tap water do not secrete these proteins. A one-dimensional Western blot revealed three of the svp's (31 kDa, 39 kDa and 49 kDa) to be in SPW, with svp31 predominating.

Developmental regulation of svp's throughout the nematode's life cycle was determined by Western blot analysis. Maximum levels of svp's were detected in homogenates of preparasitic hatched juveniles. The levels decreased in parasitic second stage juveniles. In later developmental stages the svp's could not be detected except for low levels of svp 49 in adult males. So far it was not possible to detect the presence of svp's in plant tissue. However, their presence was assessed in the lumen of the oesophagus of parasitic second stage juveniles.

Cloning of Targets

Svp's are actively secreted from the subventral oesophageal glands of *G. rostochiensis*. To assess the function of these proteins we decided to clone their genes (Smant et al., 1998). Protein homogenates of *G. rostochiensis* and *H. glycines* were separated by preparative continuous flow electrophoresis. Those fractions showing a reaction with MGR 48 on a Western blot were used for further protein purification by immunoaffinity chromatography. The N-terminal amino acid sequences of the purified proteins were then determined. Sequences were obtained for svp39 of *G. rostochiensis* and svp49 of *H. glycines*. Based on these sequences, primers were designed and used to amplify the genes from cDNA libraries by PCR. Two PCR products were obtained for each of the two nematode species. These genes were cloned and their DNA sequences determined (Table 1).

Table 1. Summary of the cDNA clones obtained, their sizes and molecular mass of the putative protein.

Species	Clone	cDNA	Open reading frame in obtained clone	Molecular mass of putative protein (Da)
G. rostochiensis	GR-eng-1	1546	1416	49700
G. rostochiensis	GR-eng-2	1300	1179	42000
H. glycines	HG-eng-1	1615	1428	49800
H. glycines	HG-eng-2	1191	957	34700

The nematode proteins have significant homologies to β-1,4-endoglucanases (EGases) of various bacterial origins. The proteins GR-ENG-1 and HG-ENG-1 are comprised of distinct

domains. Each has a cellulolytic domain at the N-terminus connected by a typical linker sequence to a type II cellulose binding domain at the C-terminus.

The question that remained was whether or not the cloned genes indeed encode β-1,4-endoglucanases. This was investigated using an EGase activity assay. Proteins from homogenates were separated by SDS polyacrylamide gel electrophoresis using a reduced SDS concentration. Enzyme activity was then assessed by overlaying the gel on an agarose bed containing carboxymethylcellulose (CMC). In the *G. rostochiensis* homogenate a protein of approximately 50 kDa showed CMC hydrolase activity, whereas in *H. glycines* a slightly smaller protein hydrolysed CMC. Both apparent EGases aligned with one of the subventral gland specific antigens of approximately 49 kDa recognised by MGR48.

To localise EGase transcripts whole mount *in situ* hybridisation experiments were done. Anti-sense RNA probes transcribed from HG-eng-2- cDNA bound specifically within the subventral glands of preparasitic second stage juveniles of *H. glycines.*

Prospects

Cyst nematodes migrate intracellularly towards the stele to a procambium cell. Originally, this migration was thought to be facilitated by mechanical means only. However, the cloning of β-1,4-endoglucanase indicates that the intracellular migration through the cell walls of host roots involves a combination of mechanical piercing and enzymatic softening of the cell walls. The β-1,4-endoglucanases from *G. rostochiensis* and *H. glycines* are the first pathogenicity factors ever cloned from a plant parasitic nematode. In addition to cellulose as a possible substrate, EGases may also be involved in the hydrolysis of xyloglucan. In addition to cellulose and xyloglucan, the plant cell wall consists of substantial amounts of pectin, pectin-like structures and other proteins. It is, therefore, likely that sedentary endoparasitic nematodes also produce other cell wall degrading enzymes.

Because sedentary endoparasitic nematodes use enzymes as well as mechanical means for their migration within host roots, inhibition of these enzymes would be likely to hinder them reaching their destination. During penetration and migration the juveniles rely for their energy supply completely on their lipid reserves. When raising a panel of monoclonal antibodies against an enzyme usually a large percentage of antibodies will substantially inhibit the enzyme. Therefore, plantibodies may be well suited to inhibiting *in situ* cell wall degrading enzymes.

The technology to express plantibodies in the apoplast has been well developed. Full size plantibodies can be expressed without difficulty to high levels equalling 1 % or more of total root protein (Van Engelen et al., 1994). So, plantibodies may be useful for obtaining resistance against intercellularly migrating nematodes (e.g. *Meloidogyne* spp.) and intracellularly migrating nematodes (e.g. cyst nematodes). Also, intracellular expression levels of single chain antibody fragments (scFv's) can reach levels of 1 % or higher (Schouten et al., 1996). Such symplastic expression may also be useful for intracellularly migrating nematodes as they can directly bind to proteins secreted from the oesophageal glands during parasitism. In addition, those molecules secreted by sedentary nematodes for inducing or maintaining a feeding site, may be targeted by antibodies expressed intracellularly.

The expression of antibodies against an oesophageal gland protein of *M. incognita* in plant cells has been tried. Rosso et al. (1996) constructed a scFv from the IgM monoclonal antibody

6D4 (Hussey et al., 1990). This antibody recognizes a large molecular weight glycoprotein (>212 kDa). The scFv was stable upon transient expression in tobacco protoplasts. Independent *Agrobacterium*-mediated transformation of tobacco with the 6D4 heavy and light chain genes resulted in transgenic plants producing either the heavy or the light chain (Baum et al.,1996). These plants were crossed resulting in progeny producing functional antibodies as was revealed in ELISA and immunofluorescence. However, the molecular weight of the heavy chains produced by the plants is smaller than those produced in hybridomas. The antibodies did not affect root-knot nematode parasitism. Either the antibody had no effect on any actions of the antigen, or this glycoprotein has no pivotal role in parasitism.

Research concerning antibody-mediated resistance against plant parasitic nematodes has so far focused on sedentary endoparasites. The question is whether or not this approach can be applied to obtain resistance against other nematodes as well. Migratory endoparasites such as *Pratylenchus* spp. and *Radopholus* spp. penetrate host roots and disrupt a large number of root cells. It has to be expected that these nematodes also use cell wall degrading enzymes. Hence, suitable targets may be available. Migratory ectoparasites which feed on cells below the epidermis genera (e.g. *Xiphinema, Longidorus, Helicotylenchus* spp.) are a third category for engineering this type of resistance. Many of these species also invoke a kind of feeding cell often accompanied by typical swelling, due to a hyperplastic response around the penetration site of the root (Sijmons et al., 1981). Furthermore, these nematodes often feed for prolonged periods. For instance, *Xiphinema* spp. induces so-called coenocytes. These are cells that are formed by repeated mitosis without cell division. Feeding on these cells can last up to two weeks. These two groups exemplify the idea that plantibody-mediated resistance may be used to engineer resistance against a wide range of nematodes, which use different strategies to parasitise host roots.

Conclusion

In conclusion, the plantibody strategy is a valuable method to obtain transgenic resistance against various groups of plant parasitic nematodes. The fact that many species are polyphagous, hinders the application of natural resistance. This makes the use of plantibodies a worthwhile alternative. High level expression in plants, and the possibility of inhibiting the function of proteins and other compounds involved in nematode pathogenesis render it a promising approach for obtaining transgenic resistance.

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Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Field and Molecular Evaluation of Insect-Resistant Transgenic Poplar (*Populus nigra* L.) Trees

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Introduction

Poplar plantations all around the world frequently experience infestation by leaf-eating insects. Considerable damage, with substantial economic loss, is particularly caused by *Lymantria dispar* L., *Apochemia cinerarius* Erscheff and *Orthosia incerta* Hufnagel. Their pupae survive in the soil thus assuring cycles of infestation.

To face the problem, in 1992-93, transgenic poplar (*Populus nigra* L.) plants have been produced by infecting leaves with *Agrobacterium tumefaciens* carrying a *Bacillus thuringiensis* (*B.t.*) toxin gene under a duplicated CaMV 35S promoter (Tian et al., 1993). Selected transgenic plants were propagated by cuttings at two experimental farms in China (in Beijing and in Manas in the Xingjiang province, China). Bioassays on insecticide activity in the laboratory and greenhouse evaluation of putative transgenic plants for traits such as insect resistance, presence and expression of the *B.t.*-toxin gene and somaclonal variation (Wang et al., 1996) provided the bases for field evaluation. This was started in 1994, when plants selected on the base of insect-tolerance and good silvicultural traits were cloned and established in the experimental field in Manas, Xinjiang province, China. The site was selected because it offered optimal temperate climate for *P. nigra* and suffered from heavy infestations by both *A. cineraria* and *O. incerta*. This report describes the results of field and molecular evaluations obtained since then in the field trials.

Materials and Methods

Plant material

The experimental field set up in Manas (Xinjiang Ulygur Autonomous region, China) in 1994 is shown in figure. 1. The transgenic poplar clones were those numbered 12, 110, 141, 153, 172, 192, 197, 254 in Wang et al. (1996) and others coming from further selections (numbered 13, 139, 162, 208, 209, 222). Two control non-transgenic *P. nigra* clones (ck2 and ck3) and one *P. x euramericana* cv. Robusta clone were also used. Protection lines outside the transgenic plantation were composed of *Ulmus pumila*, *P. popularis* and *P. x euramericana* cv. Robusta.

Evaluation of insect- toxicity within the transgenic population

During a peak period of *A. cinerarius* infestation in April-May 1997 insect-toxicity was evaluated by determining: (i) density of larvae on the leaves present on one-meter branches

placed in corresponding positions on the trees. Results are expressed as the average of 5 counts of number of larvae/1 m twig. (ii) number of damaged leaves. One hundred leaves were collected from individual trees and evaluated for insect damage according to the categories of Table 2. (iii) density of pupae in the soil. This was calculated at the base of the trees (in a square metre for a depth of 0.3 m) after the larvae of *L. dispar*, and *O. incerta* has moved into the soil and developed into pupae. Results are the average of 30 counts at random soil locations.

Populus (Section Leuce Duby)

Protection line(one line)

Ulmus pumila(two lines)

Protection line(one line)

			V						VI		
153	Ck2	222	Ck1	162	172		Ck1	172	Ck2	162	172	153
172	153	Ck2	222	Ck1	162		153	Ck1	172	Ck2	162	172
162	172	153	Ck2	222	Ck1		172	153	Ck1	172	Ck2	162
Ck1	162	172	153	Ck2	222		162	172	153	Ck1	172	Ck2
222	Ck1	162	172	153	Ck2		Ck2	162	172	153	Ck1	172
Ck2	222	Ck1	162	172	153		172	Ck2	162	172	153	Ck1
			III							IV		
13	Ck3	254	208	197	209		208	13	254	Ck1	197	Ck3
209	13	Ck3	254	208	197		Ck3	208	13	254	209	197
197	209	13	Ck3	254	208		197	Ck3	208	13	254	209
208	197	209	13	Ck3	254		209	197	Ck3	208	13	254
254	208	197	209	13	Ck3		254	209	197	Ck3	208	13
Ck3	254	208	197	209	13		13	254	209	197	Ck3	208
			I							II		
139	Ck1	110	12	141	192		110	12	Ck2	141	139	Ck1
192	139	Ck1	110	12	141		Ck1	110	12	Ck2	141	139
141	192	139	Ck1	110	12		139	Ck1	110	12	Ck2	141
12	141	192	139	Ck1	110		141	139	Ck1	110	12	Ck2
110	12	141	192	139	Ck1		Ck2	141	139	Ck1	110	12
Ck1	110	12	141	192	139		12	Ck2	141	139	Ck1	110

Protection line (one line)

Populus x euramericana (plantation)

Protection line(three lines)

Populus x popularis(plantation)

Figure. 1. Map of the *P. nigra* transgenic plantation set up in the spring 1994 in Manas, China. Plant spacing (rectangles) is 3 x 4. Underlined plants were assessed dead in 1998. Protection lines are made up of a mixed population of transgenic poplar plants. Control non-transgenic plants: ck2 and ck3, clones of *P. nigra*; *P. x euramericana* cv. Robusta; *P. popularis*, *Ulmus pumila*.

DNA extraction, purification and analysis

DNA was extracted from young leaves (1-2 g fresh weight) and purified as described (Wang et al., 1996). Detection of the *B.t.*-toxin and *nptII* genes by PCR amplification and RAPD analysis were as described (Wang et al., 1996).

Results

In order to analyse the field performance of those transgenic poplar clones (a total of 14) previously selected for best insect-tolerance and silvicultural traits within the transgenic population described by Wang et al. (1996), in the Spring of 1994 a plantation was set up in the experimental farm in Manas (Xinjiang Ulygur Autonomous region, China). The site was selected since it offers optimal environmental conditions for the growth of *P. nigra*. Containment conditions were those requested for field trials of transgenic plants. In 1997-98, DNA was extracted from the leaves of some of these plants and used to verify the presence of the transgene and genomic changes correlated with somaclonal variation.

Presence of the B.t.*-toxin and of the* nptII *genes in the genome of transgenic plants*

Table 1 shows the results obtained when DNA was analysed for the presence of the two genes (the *B.t.*-toxin and the *nptII* gene). A comparison with the previously produced data (Wang et al., 1996) show that the transgenes are inherited in all tested clonally propagated transgenic plants. They were not found only in clones 254 and 13, that had already been shown to be false transgenics (Wang et al., 1996), and in control plants.

Table 1. Presence (+) or absence (-) of the *B.t*-toxin or of the *nptII* gene fragments in the genome of transgenic clones and controls

n°	Clone/ plant	*B.t.*	*nptII*	n°	Clone/ plant	*B.t*	*nptII*	n°	Clone/ plant	*B.t*	*nptII*
1	12/5	+	+	13	162/1	+	+	25	153/3	+	+
2	12/6	+	+	14	162/2	+	+	26	153/6	+	+
3	192/2	+	+	15	254/2	-	-	27	172/1	+	+
4	192/3	+	+	16	254/4	-	-	27	172/4	+	+
5	13/1	-	-	17	197/1	+	+	29	ck2/2	-	-
6	13/3	-	-	18	197/2	+	+	30	ck2/3	-	-
7	139/5	+	+	19	141/3	+	+	31	ck3/4	-	-
8	139/6	+	+	20	141/5	+	+	32	*P.popul.*	-	-
9	209/2	+	+	21	208/1	+	+	33	Ulmus/1	-	-
10	209/6	+	+	22	208/2	+	+	34	Ulmus/2	-	-
11	110/1	+	+	23	222/4	+	+	35	*P.x eur.*	-	-
12	110/2	+	+	24	222/6	+	+				

Somaclonal variation in the transgenic plants

Variation in leaf shape was the most evident somaclonal variant trait described in the primary transformants (Wang et al., 1996). Survey of the transgenic plantation showed that in all cases the clonally propagated plants retained the variant leaf shape.

Genomic changes from the original *P. nigra* clone had been verified by Wang et al. (1996). Now, by RAPD analysis, we show that these changes are basically retained and that the transgenic population display essentially the same degree of genomic diversity (Figure 2) that was recorded in 1995 (Wang et al., 1996).

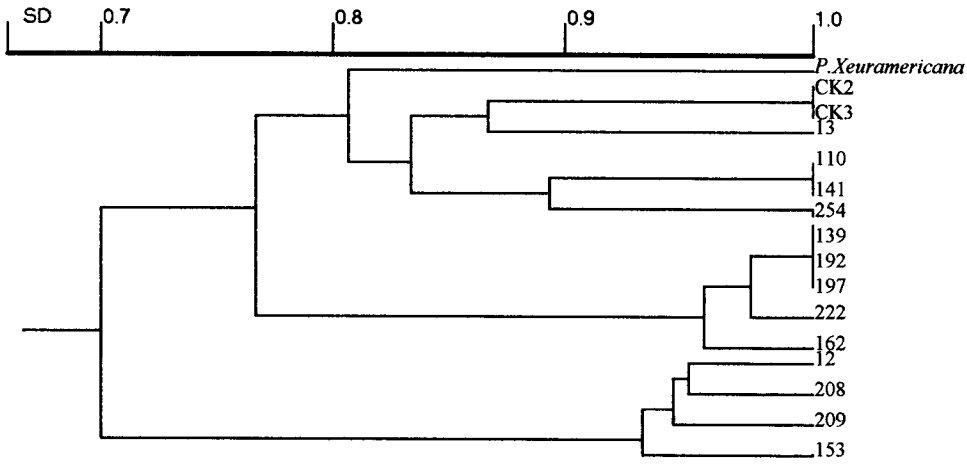

Figure 2. UPGMA dendrogram based on the Dice similarity index (SD) showing genomic similarity among the transgenic clones selected for the field trial and non-transgenic poplar (*P. nigra* ck2 and ck3 or *P. x euramericana* cv. Robusta).

Insecticide activity of transgenic plants

This was investigated by verifying density of larvae on twigs, damaged leaves and presence of insect pupae in the soil. Results are summarised as follows: (a) the density of larvae on young branches was found to be consistently lower than that on control plants. In fact, counts, during the peak of *Apochemia cinerarius* infection (April 1997), on transgenic trees numbered 12, 153, 172, 192 and on controls ck2 and ck3 gave values of 2.3, 4.0, 3.0, 2.7, 8.7 and 8.0, respectively. (b) The rate of leaf damage was significantly lower in transgenic plants than in controls. In fact, counts of damaged leaves done after heavy insect infestation (middle of May, 1997) showed an average damage rate which was consistently lover than that of control plants (Table 2). Results also lead to a further interesting observation: even non-transgenic plants present within a transgenic plantation are protected from insect-attack. (c) Field growth of transgenic plants reduced the pupae density in the soil far below the danger level. In fact, count of *L. dispar*, and *O. incerta* pupae in the soil at the base of 30 randomly selected trees after the larvae had moved into the soil and developed into pupae (middle of May, 1997) gave the following overall results: 547, 2631 and 2196 pupae, respectively for transgenic plantation, for *P. nigra* and for *P. euramerica*na. The latter two were plantations situated at 4 km from the experimental field. Thus, pupae concentration in the soil of transgenic plants (with an average of 18 per square metre) was 4-5 times lower than that in the soil of non-transgenic plants.

Table 2. Number of leaves damaged by insect-attack, as counted after a heavy insect infestation in May 1997.

Number	Poplar clone	Number of trees	1	2	3	4	5
1	*P. x eur*	23	15	14	21	26	24
2	ck2	17	11	15	21	28	25
3	ck3	12	7	12	19	30	32
4	12	12	12	14	22	31	22
5	13	11	9	14	21	27	29
6	110	11	8	13	18	27	35
7	139	11	9	11	20	30	30
8	141	11	7	12	19	30	32
9	153	12	14	17	23	30	16
10	162	12	9	15	21	29	26
11	172	16	11	16	23	27	22
12	192	6	5	13	22	31	29
13	197	11	7	13	20	30	29
14	208	10	13	16	24	27	20
15	209	10	11	14	21	29	25
16	222	6	6	12	20	36	26
17	252	12	19	19	21	22	18
Total		203	171	139	358	490	442
Average			10	14	21	29	26
18		*P.nigra**	90	8	2	-	-
19		*P. x eur**	80	15	5	-	-

* Plantations at 4 Km from the transgenic plants. Numbers 1 to 5 refer to leaves that are: 1- 100 % damaged; 2- 50 % damaged; 3- 25 % damaged; 4- less than 25 % damaged; 5- undamaged. AVERAGES refer to values for plants 1-17.

Discussion

Results of this research are relevant for a number of basic questions in the field of transgenic trees. In fact, they show that the foreign gene, as well as its expression, is retained in adult, clonally propagated plants and that the trees in the experimental plantation are endowed with a convenient level of insecticide activity. While confirming previous bioassays on insecticide activity performed by confining young transgenic leaves into glass tubes containing first-instar larvae of *A. cinerarius* and *L. dispar* (Wang et al., 1996), the result of the field tests revealed a general reduction in the number of damaged leaves, of larvae density on the leaves and of number of pupae in the plantation soil. Results also show that transgenic plants not only have the ability to control damages to their own leaves, but also protect insect-sensitive plants cultivated in the same plantation. The obvious explanation of this is that the insecticide activity reduces larva density in the field thus providing protection to all plants in the plantation. The phenomenon of cross-protection is relevant in practice but has an obvious

drawback when field trials are used to identify insect-resistant clones within a mixed transgenic population.

On the bases of previous experience on pest protection in the Manas region, heavy insect pest damage will occur widely in the coming year if the number of pupae in the soil is over 36 heads per square meter. On average, only 18 heads per square meter were found in the soil of our transgenic plantation, far below the point of concern.

We had already shown that the transgenic poplar plants of this work have undergone extensive somaclonal variation (Wang et al., 1996). Some of the poplar plants selected for good silvicultural traits have modified leaf shape and genome constitution. A direct correlation between morpho-physiological modifications and genomic changes in transgenic plants has not yet been established (Sala et al., in press). However, it is clear that both types of changes may be found in transgenic plants and that these do not impair field performance and superior quality in the poplar clones selected for field trial.

The transgenic trees of this work have, that were originally produced using a female *P. nigra* clone, reached sexual maturity and are now flowering. We shall now verify if this sex determination is retained and if any of the transgenic plants show abnormal sexual development or sterility. The latter would be of great interest for practical exploitation of insect-tolerant clones. T1 progenies will also be evaluated for segregation of the transgene as well for somaclonal variation.

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Insect-resistant Tropical Plants and New Assessment About Cry Proteins

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Expression of Delta-endotoxin *cry* Genes in Plants

The cloning and expression of *Bacillus thuringiensis* δ-endotoxin genes in transgenic plants have been used to protect the crops from insect attack (Vaeck et al., 1987; Delannay et al., 1989).. Increased expression of the insecticidal *cry* genes in plants has been critical to the development of genetically transformed plants with agronomically acceptable levels of insect resistance (Adang, 1991). Low expression levels of such genes has also an environmentally implication: the release of low expressing insect tolerant transgenic plants may result in the rapid appearance of resistance to Cry toxin in target insect. The problem of the expression of *B. thuringiensis cry* genes is due to the expression of bacterial prokaryotic genes in higher plants or in any other eukaryotic organism. Fully modified genes can express up to 100-fold higher level of insecticidal toxin compared to those levels obtained when typical bacterial gene is expressed. The most important aspects present in the bacterial wild-type *cry* genes, affecting their expression in transgenic plants were described (Adang, 1991). The analysis includes aspects of transcriptional regulation, mRNA stability, and preferences in codon use and translational efficiency. According to exposed considerations modified *cry* genes has been reconstructed allowing increasing the expression levels in transgenic plants (Perlack et al., 1990, 1991, 1993; Koziel et al., 1993; Adang et al., 1990, 1993).

Plant Transformation Methods

The improvement in transformation methods made many plant species previously inaccessible for genetic manipulation, able to be transformed. Particularly important is the extension of this single-cell transformation methodology to monocotyledonous plants. This advance has biological and practical implications. Firstly, because of advances of *Agrobacterium tumefaciens*-mediated gene transfer over the direct transformation methods, which where the only way for genetic manipulation of economically important crops as cereals and legumes. Second, it has been demonstrated that T-DNA is transferred to dicot and monocot plants by an identical molecular mechanism. This confirmation implies that any plant can potentially be transformed by this method if a suitable transformation protocol is established. *A. tumefaciens*, a plant pathogen causing tumorous crowngalls on infected dicotyledonous plants, naturally does not infect monocotyledonous and for this reasons many economically important plants such as cereals, remained inaccessible for genetic engineering manipulation during long time (Herrera-Estrella et al., 1984; Horsch et al., 1985; de la Riva et al., 1998). For those cases alternative direct transformation methods has been developed (Shillito et al., 1985), such as polyethyleneglycol-mediated transfer (Uchimiya et al., 1986), microinjection (de la Pena et al., 1987), protoplast and intact cell electroporation (Fromm et al., 1985,1986; Lörz et al.,

1985; Potrikus, 1989; Arencibia et al., 1995) and particle bombardment technology or gene gun (Sanford, 1988). The gene gun technology and the intact cell electroporation allow the direct delivery of DNA into plant cells avoiding the difficulties in the regeneration of transgenic plants from transformed protoplasts, however significant part of the regenerated plants are not whole-transformed but mosaic-transgenic plants. To avoid this problem the development of high efficiency *Agrobacterium*-mediated transformation of monocot plants is necessary. Some results in *Agrobacterium*-mediated transformation of monocot plants has been reported (Graves and Goldman, 1986; Hooykaas et al., 1984; Rainieri et al., 1990) and the most important groups increase the efforts in this direction. The CIGB successfully established the *Agrobacterium*-mediated transformation of sugarcane (Enríquez et al., 1998; Arencibia et al., 1998)

Tropical Crops Resistant to Insect Attack

Cuba is a tropical country and its most important crops suffer great losses by insect attack. The most important pests for Cuban crops are *Diatraea saccharalis* (sugarcane stem borer) *Spodoptera frugiperda* (maize worm), *Heliothis virescens* (tobacco budworm), *Leucoptera coffeella* (coffee leave miners), *Cylas formicarus* (sweetpotato weevil) among others.

In our institution, sugarcane was used as a model to develop insect-resistant transgenic plants. Sugarcane is the most economically important crop in Cuba. Transgenic sugarcane plants expressing a truncated version of the BTK HD-1 *cryIA(b)* gene driven by the CaMV 35S promoter were obtained by a transformation procedure where intact plant cell are electroporated (Arencibia et al., 1997). We suggest that this approach may be useful in any direct transformation method. Sugarcane (*Saccharum officinarum* L.) is a monocot plant widely spread and economically important in many regions around the world. The stem borer (*Diatraea saccharalis* F., Lepidoptera, SCSB) is the most important pest of this crop, causing extraordinary agricultural and industrial losses annually (Bennett, 1977). Control of SCSB is very difficult and expensive due to the typical feeding behavior of the larvae into the sugarcane stem. This fact brings about inaccessibility of the conventional pesticides to the target insect. For many years, entomophages and entomopathogens like *Trichogramma* sp., *Beauveria basiana* have been unsuccessfully tried to control the SCSB in field conditions (King et al., 1975). However, despite the fact that at least 5 lines of transgenic sugarcane plants were completely self-pesticide under small parcel conditions, the low expression level of the unmodified truncated *cryIA(b)* gene version may result in rising of CryIA(b)-resistant SCSB lines in a natural insect population under real field conditions (Vazquez et al., 1996). New transgenic sugarcane lines with high t*cryIA(b)* gene expression are being developed in our laboratory. A more effective promoter system and an mRNA stabilizing region, both with remarkable activity in monocots, as well as gene modifications in order to get a better codon usage and them eliminating destabilizing sequences, are being tried to enhance the transgenic expression (Arencibia et al., 1997).

The possibilities for the development of resistance to *B. thuringiensis* δ-endotoxin in genetically modified plants are polemic topic subject to permanent and active discussion. The fact that different *B. thuringiensis* δ-endotoxin can bind different proteins could help to prevent the development of insect-resistance. In laboratory conditions we can study the development of resistance to one δ-endotoxin and know the susceptibility of resistant insect to other ones. These tests let us suggest the combination of different toxin genes in plants or the

alternation of transgenic plants expressing different toxins. Another strategy consists in the limitation of gene expression only to the economically important organs of the plant but this requires an extensive work for the construction and use of efficient tissue specific promoters. The determination of the molecular aspects of the mode of action of δ-endotoxins, including the structure and function of larval mid gut receptor will be essential in order to design strategies to control the resistance development in insects.

The screening projects could lead to the discovery of *B. thuringiensis* strains with novel entomocidal specificity, which may be the source of novel δ-endotoxin genes potentially suitable for expression in transgenic plants (Feiltelson et al., 1992). Various groups to search for *B. thuringiensis* with such a new insecticidal spectra are carrying out extensive screening programs. The production of transgenic sugarcane plant lines resistant or tolerant to insect attack is not the answer for all insect problems in agriculture, it's an alternative method to be integrated in the current pest management strategies. Some other methods for the manipulation and use of *B. thuringiensis cry* genes, including the development of genetic engineered strains with improved δ-endotoxin production and novel entomocidal specificity are very promising.

Interaction of Cry Toxins with Vertebrate: New Assessments and Promising Applications.

Although, Cry toxins are safety to the environment and vertebrates, the increasing spread of insecticidal Cry toxins and the use of transgenic plants, made necessary perform new toxicology experiment in order to demonstrate its innocuousness at molecular levels. Recently we have reported unusual inmmunological properties for Cry1Ac protein (Vázquez et al., 1998). This polypeptide is a strong immunogen when applied to mice by mucosal and systemic routes. Moreover adyuvanticity studied used Cry1Ac, both protoxin and toxin, stimulated the humoral immune response induced against the Hepatitis B surfaces antigen via intranasal and intermuscular. Cry1Ac was able to bind the mouse intestinal surface *in situ* experiments. These result evidence an interaction of Cry1Ac protein with the mouse physiological system, that be demonstrated the Cry1Ac harmless properties with immunotoxicological test, could be used in edible vaccine derived from transgenic plants (Vazquez et al., 1999a, b).

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Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Inserting the Nucleoprotein Gene of Tomato Spotted Wilt Virus in Different Plant Species, and Screening for Virus Resistance

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The number of viruses that can infect plants is very high and increases every day, but fortunately only a few are really important pests. Among these is Tomato spotted wilt virus (TSWV), a member of the genus Tospovirus. There are two main reasons for its success over the last two decades. First, the prevalence of its vectors: TSWV is transmitted mainly by, the Western flower thrips *Frankliniella occidentalis*, which has increased its area of distribution, and is itself a pest. Second, the virus can infect several hundred-plant species belonging to both monocotyledonous and dicotyledonous families. The consequence is that today TSWV is found in most countries with a temperate or subtropical climate, in both hemispheres. Crops severely affected include horticultural and ornamental plants, grown in the open and under protection. Several other tospoviruses (Table 1), all vectored by thrips species, are spreading and may increase in importance in the near future.

Table 1. Members of the genus Tospovirus (Fauquet & Mayo, 1999).

Chrysanthemum stem necrosis virus	CSNV
Groundnut bud necrosis virus	GBNV
Groundnut chlorotic fan-spot virus	GCFSV
Groundnut ringspot virus	GRSV
Groundnut yellow spot virus	GYSV
Impatiens necrotic spot virus	INSV
Iris yellow spot virus	IYSV
Physalis severe mottle virus	PhySMV
Tomato chlorotic spot virus	TCSV
Tomato spotted wilt virus	TSWV
Watermelon bud necrosis virus	WBNV
Watermelon silver mottle virus	WSMoV
Zucchini lethal chlorosis virus	ZLCV

It is not surprising that TSWV has been attracting more and more attention from plant breeders, who have been looking for natural sources of resistance in the varieties of a given species and in related compatible species (Roselló et al., 1996). One example for tomato is the SW5 gene, introgressed from *Lycopersicon peruvianum* in *L. esculentum* (Stevens et al., 1994). However, suitable sources of natural resistance are quite limited and the creation of a

resistant variety requires a big effort over several years. Moreover, when working with many highly heterozygous, partially sterile, and vegetatively propagated ornamentals, traditional breeding strategies can sometimes be ineffective.

For all these reasons, in recent years plant genetic engineering has been used as an alternative to conventional breeding for introducing resistance to TSWV. Based on the principle of "pathogen-derived resistance" (Sanford and Johnston, 1985), different parts of the complex TSWV genome have been introduced and expressed in plants, generally by *Agrobacterium*-mediated transformation. Only sequences of two TSWV genes have proved to induce resistance: the N (nucleoprotein) and the NSm (non-structural middle) genes (Prins et al., 1996). However, plants expressing transgenic NSm protein showed growth aberrations (Prins et al., 1997), and only N gene has been used extensively in several laboratories.

In 1991 transformation of tobacco with the TSWV N gene provided the first example of genetically engineered resistance to a negative-strand virus (Gielen et al., 1991). The chimeric gene cassette used comprised the 35S promoter, a short leader sequence (from TSWV itself, or from TMV), the complete N gene, and the nos terminator. This "sense" cassette was inserted into the binary vector pBin19 and introduced into tobacco via *Agrobacterium*-mediated transformation. The progeny of 15 self-pollinated primary transformants were challenged with TSWV by mechanical inoculation and monitored for the appearance of systemic symptoms. Progeny plants were then divided in four classes, according to the level of protection. Some lines showed good protection, but no correlation could be found between the amount of N protein accumulating in transgenic plants and level of protection. Moreover protection was obtained when an untranslatable version of the same construct was used (de Haan et al., 1992), and therefore a RNA-mediated mechanism was proposed. Most of the research conducted later used similar constructs and resistance analysis of progeny. The TSWV-resistant tobacco lines carrying the untranslatable construct were challenged with two other tospoviruses, TCSV and GRSV, but no resistance was found.

The "antisense" approach was tested on tobacco (Pang et al., 1992; Kim et al., 1994), but resistance of practical value was not observed. Pang et al. (1992), analyzed 190 single plants, progeny of "sense" lines, for their level of N protein expression, and then challenged them with TSWV (3 isolates), INSV and GRSV. High protein expressors showed a certain degree of resistance to different isolates of TSWV and to INSV, while low protein expressors resistant to TSWV were infected by INSV. In no case protection against GRSV was observed. These results indicated a possible role of the transgenic protein, at least when expressed at high levels. In order to study the role of transgenic RNA expression in resistance, the same authors tested an untranslatable version of the construct previously used, as well as promoterless and antisense versions (Pang et al., 1993). No protection was observed against INSV and GRSV. When TSWV isolates were tested, no resistance was observed with the promoterless construct and the antisense construct had a very limited effect. Plants carrying the untranslatable construct could be divided in high RNA expressors (susceptible) and low RNA expressors (resistant). Comparisons with previous results on transgenic plants expressing the intact N gene suggest that resistance to homologous and closely related TSWV isolates in plants that express low levels of the translatable N gene is due to the presence of the N gene RNA transcript and not the N protein itself. In contrast, resistance to other tospoviruses is due to accumulation of high levels of the N protein, and not due to the presence of the N gene transcript.

Beside tobacco, another laboratory plant has also been used in transformation experiments. This is *Nicotiana benthamiana*, which has the advantage of being infected by INSV systemically rather than locally. Transgenic plants were obtained with a translatable version of the N gene construct (Pang et al., 1994; Vaira et al., 1995), and analyzed for transgene expression (RNA and protein) and resistance to infection with TSWV and other tospoviruses. Pang et al. (1994) selected primary transformants with different levels of N protein expression, then challenged the self-pollinated progeny with the homologous TSWV isolate, a non-homologous one, INSV and GRSV. As they previously observed on tobacco, low levels of N protein (measured in each plant) correlated with high resistance to TSWV and susceptibility to INSV and GRSV, while high expression correlated with various levels of protection against TSWV and INSV, but not GRSV. The level of protection in high expressors was dependent on inoculum concentration, as already reported in tobacco with a similar construct by MacKenzie and Ellis (1992). Forty-five primary transformant lines were analyzed by Vaira et al. (1995). Level of N protein expression was determined, and vegetatively propagated plantlets were challenged with TSWV, INSV, GRSV and GBNV. Resistance to all TSWV isolates tested was found in two lines. The expression of the transgene was lower in these than in any of the susceptible lines. The resistant lines were susceptible to the other tospoviruses tested, but they developed symptoms milder than controls when inoculated with GRSV. Some of the lines producing high levels of N protein showed partial protection (2-3 weeks delay in symptom expression) with one or more TSWV isolates and with INSV and/or GRSV. All lines were susceptible to GBNV.

In conclusion, experiments on *N. benthamiana* confirmed and extended the results on tobacco, and suggest that two resistance mechanisms are acting in plants transformed with intact N gene: one is RNA-mediated, requiring low transgene expression (measured as steady-state level), and is very active with TSWV but inactive with other tospoviruses; the second, protein-mediated, which requires high protein expression, and is active also against INSV, but can be overcome by high doses of inoculum.

The N gene of TSWV has been introduced into a number of crops, with a certain success. These include tomato, lettuce, tobacco, peanut, *Eustoma*, *Osteospermum*, chrysanthemum, New Guinea impatiens. In most of these species constructs with the intact N gene were used.

In 1994 it was reported that expression of the N gene in tomato can induce resistance to TSWV (Kim et al., 1994). A further step was the use of inbred lines for the transformation; Ultzen et al. (1995) and Cirillo et al. (1998) transformed inbred fresh market tomato lines and screened the self-pollinated progeny for resistance to TSWV by mechanical inoculation. A few lines, showing both high resistance and a simple integration pattern of the transgene, were selfed to obtain homozygous progeny, which was then used in crosses with other parental lines to obtain hybrid seeds. Tests on these hybrids showed that resistance is maintained even when the transgene is hemizygous. Different hybrids derived from one transgenic line were recently tested under field conditions in the presence of viruliferous thrips vectors (Nervo et al., unpublished) and no transgenic plant became infected.

Transgenic lettuce lines have been produced and used for studying mechanisms of resistance (Pang et al., 1996). Partial protection (symptom delay or local infection) was found in lines expressing high levels of N protein, while one line accumulating low levels of N protein was highly resistant. It was shown that this resistance is due to post-transcriptional gene silencing. This kind of mechanism could explain several previous reports of high

resistance in plants that were low expressors of the transgene. A high rate of transgene transcription is thought to result in the activation of an RNA degradation mechanism resulting in low levels of accumulation of transgene message. As a result, the RNA of the challenging virus is also targeted for degradation, and a highly resistant phenotype is observed.

Tobacco is a rare case where stability of resistance has been studied for several generations (up to R6) in greenhouse and field conditions (Stoeva et al., 1998). Transgenic lines were obtained from commercial tobacco cultivars, tested first by mechanical inoculation, then by thrips-mediated virus transmission in the field. Extensive tests proved that the resistance to TSWV is stable in different environments and is a stable inherited trait.

Peanut has also been transformed with the N gene (Li et al., 1997). Progeny of transgenic plants expressing the gene showed a 10-15 day delay in symptom development after mechanical inoculation with the homologous isolate of TSWV.

When considering genetic engineering of ornamental species, transformation efficiency and/or regeneration from transgenic tissue are often very important limiting factors. With *Eustoma*, for example, transformation and stable integration of the N gene was achieved, but only a very small number of calli could be regenerated into plantlets (Semeria et al., 1996). On the other hand, tissue culture techniques and transformation protocols are available for engineering *Osteospermum* (Allavena et al., 1998), species for which resistance to TSWV has been reported after introduction of the N gene. Two other species recently transformed for TSWV resistance's are chrysanthemum (Sherman et al., 1998) and New Guinea impatiens (Daub et al., 1999). In *Osteospermum* and chrysanthemum the best performing lines had multiple transgene copies and expressed low levels of transcript, suggesting that resistance may be the result of post-transcriptional gene silencing.

Resistance assays, particularly with ornamental plants, are not always easy to perform, and mechanical or thrips-mediated inoculations do not necessarily produce the same results. *Osteospermum* could not be efficiently mechanically inoculated, and use of the natural vector proved necessary for appropriate screening of transgenic lines (Vaira et al., unpublished). On the contrary, for rigorous resistance tests mechanical inoculation was necessary on chrysanthemum lines (Sherman et al., 1998).

In conclusion, N gene-mediated resistance against TSWV operates both in laboratory models and in economically important plants, and results obtained in laboratory plants like tobacco and *N. benthamiana* can probably be extended to many species, provided a sufficiently high number of transformants are obtained and analyzed. Thus, it is likely that the degree of resistance against other tospoviruses, tested so far only in laboratory plants (de Haan et al., 1992; Pang et al., 1992, 1993, 1994; Vaira et al., 1995), will be similar in other species. While resistance against homologous and heterologous TSWV isolates is more than satisfactory, it is clear that the TSWV N gene will not be sufficient to protect plants against other tospoviruses in the field. A strategy to obtain plants resistant to three tospoviruses has been tested on tobacco (Prins et al., 1995); a transformation vector expressing the N genes of TSWV, TCSV and GRSV was used to obtain one transgenic line that was highly resistant to all three viruses. Another strategy was successfully used in tomato, where Cucumber mosaic virus (CMV) is a serious pest; a TSWV-resistant transgenic line was crossed with a CMV-resistant transgenic line (Gonsalves et al., 1996), giving progeny resistant to both viruses.

Work done with different constructs and in different plant species has shown that highest levels of resistance to TSWV are achieved by inducing a condition of (trans)gene silencing in plants; what still is not perfectly clear is how this condition can be stabilized during plant development (Pang et al., 1996) and through vegetative propagation and sexual reproduction (Accotto et al., unpublished). One advantage of the strategy is that it does not require production of any transgenic protein, and so may present less of a problem of public acceptance for edible crops. Another feature of RNA-mediated resistance has recently been described (Pang et al., 1997): the length of transgene having homology with the incoming virus can be appreciably reduced, thus raising the possibility of developing a simple new strategy for engineering multiple resistance in transgenic plants.

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Plant Genetic Engineering: Towards the Third Millennium
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Advances in Potato Improvement Through Genetic Engineering

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Efficiency of Transformation and Phenotypic Variation in Transgenic Plants

The basic premise of exploiting genetic manipulation is that the ensuing material will have improved properties compared with the parents and that the plants that are regenerated maintain the key characteristics of the original cultivar. This is not always the case and the highest potential for deviation from typical performance usually occurs in yield and tuber size gradings. The frequency of off-types can vary between 15 % and 80 %, depending on cultivar, but off-types are not always apparent until grown in the field. Glasshouse trials are no substitute for extensive field trials (at least for commercial attributes) and clearly transformation can be highly unpredictable with regard to gene integration and expression.

Pest and Disease Resistance

Viruses

Viral genes: Whilst the search for the identity of new sources of resistance continues to be a major challenge, the success of genetic engineering for virus resistance must still represent one of the major success stories in plant biotechnology (Davies 1996 and references therein). New sources of antiviral transgenes that give resistance continue to be discovered. One of the best-documented approaches is coat protein (CP)-mediated resistance, now widely effective against PVX, PVY, PLRV and mop top. There have been many large-scale field trials of transgenic potato with CP-mediated resistance, which confirm the durability of the trait in true-to-type lines. Other approaches involve modifying the expression of viral movement proteins, viral proteases involved in processing polyprotein gene products, and viral RNA-dependent polymerase or "replicase" proteins. Replicase-mediated resistance against PLRV in Russet Burbank holds up well in field trials (although the approach apparently has limitations due to restricted specificity).

Plant derived genes: As far as plant-derived genes are concerned potato has been successfully engineered to express high levels of pokeweed antiviral protein (PAV) which conferred resistance to PVX and PVY when mechanically inoculated. PAV is an example of a gene encoding antiviral proteins, also known as ribosome inactivating proteins (RIPs), which can modify ribosomal RNA and interfere with translation. Other groups have engineered plants to degrade endogenous salicylic acid - a compound which, when applied to plants, induces a subset of pathogenesis-related (PR) proteins-normally induced following pathogen recognition. The transgenic plants are unable to establish systemic acquired resistance (SAR), adding weight to the argument that induced expression of PR genes forms an integral part of the SAR response.

Non-plant, non-pathogen-derived transgenes: The expression of a gene encoding mammalian oligonucleotide synthetase provides protection against PVX in field-grown plants. The synthetase, activated by double-stranded RNA replicate intermediates formed during viral replication, polymerises ATP into an oligomeric form - thereby activating ribonuclease (RNaseL). This in turn degrades viral RNA. The development of mini- or single-chain antibodies, containing only critical functional domains necessary for antigen recognition and binding can also be effective against viral coat protein.

Natural host resistance genes: Currently, there are significant efforts to clone the potato *Rx* gene using MAP-based cloning strategies. The *Rx* gene confers extreme resistance or immunity to PVX. Once induced by the virus the mechanism can suppress replication of completely unrelated viruses. One longer-term objective is to broaden the recognition specificity of *Rx* whilst retaining the capacity for non-specific suppression of virus replication.

Bacteria and fungi

Some of the most successful strategies arise from the use of genes coding for lytic enzymes derived from bacteria or insects. For example, expression of bacteriophage T4 lysozyme in potato cell walls reduces the extent of tissue maceration when plants are challenged with *Erwinia carotovora*. Genes encoding non-toxic cecropin peptides (a potent group of antibacterial factors found in the cecropia hemolymph) are also effective at delaying symptoms, disease severity and mortality in transgenic potatoes challenged with *Pseudomonas solanacearum*. Alternative approaches exploit the fact that plant defence responses to pathogens involve the production of active oxygen species including hydrogen peroxide (H_2O_2). Transgenic potato expressing an *Aspergillus niger* glucose oxidase gene shows improved disease resistance against *Erwinia carotovora* and *Phytophthora infestans*. Many groups are attempting to generate fungal resistance using antifungal protein genes (AFPs) isolated from several sources. Durable resistance is likely to depend on over-expression of a suite of effective genes.

Nematodes and insects

Research is focused on the search for promoters to drive expression at feeding sites only and a few have been reported. Otherwise emphasis is placed on the expression of specific lectin genes e.g. Concanavalin A to bind nematode sensory apparatus and inhibit feeding. Approaches involving expression of cytotoxic genes and enzyme inhibitors (similar to those described above for bacteria and fungi) are also in vogue. At present information on the outcome of these approaches using potato is scarce. The expression of genes encoding the *Bacillus thuringiensis* (*Bt*) toxin is now well known to protect against insects such as tuber moth and Colorado beetle and need not be expanded upon here. Resistance management strategies, including the establishment of refuges, may prolong the effectiveness of these genes and are likely to be obligatory under new European legislation.

Developmental Processes and Responses to Stress

Tuberisation

A gene encoding S-adenosylmethionine decarboxylase (SAMDC) has been isolated from tuberising stolon tips of potato. SAMDC is a key enzyme in the biosynthesis of the polyamines spermine and spermidine. Polyamines are ubiquitous in all living organisms and

appear to be essential for normal growth and development. Potato plants containing an antisense SAMDC construct under the control of the 35S CaMV promoter showed a range of stunted phenotypes with highly branched stems, short internodes, small leaves and inhibited root growth. More recently, SAMDC has been over expressed and antisensed using the tuber-specific patatin promoter. Over expressing lines show a significant shift in tuber size distribution with larger numbers of smaller tubers but no overall change in tuber yield (unpublished data). The ability to modify tuber numbers and sizes without adversely affecting other parameters has distinct commercial value for several market sectors.

Stress physiology

Potatoes are grown in a range of climates throughout the world. Even within a relatively localized geographical area seasonal extremes in weather patterns will modify crop performance, affecting efficiency of production, yield and quality. Whilst resistance to (or tolerance of) drought, salt, high and low temperature stresses are likely to be examples of polygenic traits, evidence is accumulating that inroads into alleviation of specific stresses can be made through the application of transgenic biology. Expression of Type 1 antifreeze proteins (AFPs) from winter flounder (*Pseudopleuronectes americanus*) in potato results in a substantial decrease in electrolyte leakage from leaves following a pre-treatment at –20°C for 2 hours, indicating reduced membrane damage at freezing temperatures (Guerra, pers comm). Expression of tomato genes encoding cytosolic and chloroplastidic forms of Cu and Zn dependent superoxide dismutases in potato induces paraquat tolerance (tolerance to oxidative stress). The approach offers a route to protection against photo-oxidative damage and, as yet, undefined stresses.

Tuber Quality and Composition

Carbohydrates dominate tuber composition and the potato has, for many, become the model system for research into the control of primary carbohydrate metabolism. The literature is extensive and cannot be fully covered in this mini-review. Reviews covering work on carbon partitioning and metabolism in transgenic potato has been published by Sonnewald et al. (1994); Frommer and Sonnewald (1995); Davies (1998). Some examples are provided.

Starch quality and content

The ratio of amylopectin:amylose is important in determining starch uses. The highly branched amylopectin give starch its thickening properties. One of the classical success stories in plant biotechnology in recent years has been the isolation of a waxy mutant of potato (amf), the identification of the genetic lesion as a mutated granule bound starch synthase (GBSS1) gene and the subsequent generation of high amylopectin starch (with no apparent yield penalty) by antisense silencing of the GBSS1 gene. There are also real environmental benefits from the use of this starch compared with native potato starch, which requires chemical derivatisation. The first product to be produced from the transgenic waxy potato starch is Quicksolan SPS, used in airjet looms to reduce abrasion damage in textile machinery. Loom efficiency is increased from 82 % to 92 %. Other groups have shown that antisense inhibition of a novel potato gene, designated R1, reduces the phosphate content of starch, drastically influencing its pasting properties and offering the potential for novel polymer production. There are a number of other examples in the literature, which show that the expression of heterologous genes encoding bacterial glucan synthases and branching enzymes can also

modify starch structure significantly. The challenge now is to define how these 'novel' starches can be best exploited in food and non-food industries. The Monsanto group has shown that it is possible to increase the starch content of tubers by over-expressing a mutated *E. coli* ADP-glucose pyrophosphorylase. This provides advantages to the processing sector.

Sugar balance

The processing of tubers directly out of cold store to produce high quality products (French fries, chips) is an important goal for the potato processing industry. Several groups have successfully manipulated carbohydrate metabolism in tubers to restrict hexose accumulation and hence improve fry colour. These include modifications to the expression of genes involved in starch breakdown, starch synthesis, sucrose biosynthesis and sucrose breakdown (see Davies 1998 and references therein).

Bruising and enzymic browning

Blackspot bruising of potato results when mechanical damage to the tuber (caused by cutting or rough handling for example) initiates enzymic browning, resulting in the production of black, brown and red pigments. The reaction leading to pigment production is catalysed by polyphenol oxidase (PPO). A number of PPO genes have been cloned from potato and PPO activity has been reduced by gene silencing (KEYGENE, Monsanto and other groups). Visible symptoms of bruise have been alleviated substantially. More detailed information on the performance of these transgenics is awaited.

Molecular "Pharming", Alternative Uses and Added Value

Significant interest has developed in the production and storage of novel compounds, (including pharmaceuticals) and metabolites (e.g. high value fructans) in transgenic plants. Plants, as a renewable energy source, may offer an attractive alternative to production in fermenters, for example, but only if product yields and efficiency/ease of extraction are economically viable. A number of modified forms of human serum albumen have been produced in GM potato with yields of up to 0.2 g per kg of total protein in all plant parts. This may be a viable approach since protein is yielded in the liquid fraction following potato starch extraction. A gene encoding a heat labile enterotoxin (LT-B) subunit (from *E. coli*) fused to a microsomal retention signal has also been expressed in potato which, when fed to mice at very low concentrations, provides successful immunisation against hepatitis B. This opens up a potential route to the production of edible vaccines.

The Future

Future challenges include the use of new promoters and enhancer sequences to drive high levels of expression in the correct tissues at the most relevant stages of development. A wider range of more 'acceptable' selectable marker i.e. not based on antibiotic resistance may be required. New vectors and selectable markers (or routine protocols for marker elimination) will be important if traits are to be modified by over-expressing or antisensing several genes sequentially or simultaneously. New sources of genes for resistance and quality traits must be found. Scientists must also begin to address the problems of selecting commercially viable transgenics under glasshouse conditions, since this does not appear to be a substitute for selection in the field. The effects of environment on transgene expression and trait development and stability will also need to be addressed.

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Plant Genetic Engineering: Towards the Third Millennium
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Agriculture for Marginal Lands: Transgenic Plants Towards the Third Millennium

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Introduction

Recent projections of world food supply and demand indicate that plant production will have to be doubled within the two next generations. These estimations show that an additional 446 million hectares of land must be put into cultivation beginning the next century just to maintain the food security of the increasing human population.

Several studies indicate that the greatest potential for agricultural expansion is marginal soils, in which crop production has been absent and where there is still available land for cultivation. In this respect, the acid soils of the humid tropics and the alkaline calcareous soils of semiarid climates, which have been rejected for their limiting plant growth conditions, constitute a promising alternative for the establishment of productive plantations. In this review we will summarize the recent findings concerning the plant adaptation to marginal soils with particular emphasis to acidic and alkaline soils. We highlight the preponderant role of organic acids in aluminum (Al) tolerance and phosphorus (P) nutrition in crops and we show how the modification of citrate synthesis in transgenic plants could be used to obtain novel plant varieties more adapted to grow under adverse soil conditions.

Marginal Lands

Acid Soils

Nearly 4000 million hectares of the global land is composed of acid soils. This is approximately 30 % of the total ice-free land area that account for about 40 % of the arable land worldwide. Acid soils are particularly abundant in tropical and subtropical regions (e.g. 850 million ha in tropical America) in which the water (the principal element for cultivation) is not limiting. Historically, acid soils have resisted agricultural use principally for the high level of toxic aluminum and their high phosphorus fixation capacity (Von Uexkull and Mutert, 1995).

Alkaline soils

Alkaline soils, which are very common in semiarid and arid climates cover more than 25 % of the earth's surface. These soils are typically highly porous, freely draining and saturated with calcium carbonate. The abundance of Ca^{2+} in the soil solution limits P-solubility by forming sparingly soluble Ca-P compounds. In developing countries, calcareous soils sustain traditional rain-feed cultivation despite the intrinsic nutrient (P, Fe and Co) unavailability,

which is imposed by the high pH and cation abundance. However, high cost P fertilizers must be added to maintain a sustainable agriculture (Marschner, 1995).

Organic Acids in Plant Adaptation to Soil Stress

Over the past 20 years some of the mechanisms for what plant adapts to marginal soils became to be clear. In this interesting picture, the production of reducing molecules and chelating compounds by the stressed plant root as an active response has attracted the attention of plant physiologist, ecologist and molecular biologist as potential biotechnological targets. This has been the particular case of organic acids biosynthesis, their mobilization in the plant and their exudation by roots in response to soil stress.

Organic acids are low-molecular weight compounds ubiquitous to all organisms that are characterized by the possession of one or more carboxyl groups. The negative charges of these carboxylic groups make organic acids strong cation chelators (such as malate and citrate) and efficient solubilizers of anions in solution. Several lines of evidence have implicated organic acid in important processes operating in the soil, including the detoxification of heavy metals by plants (e.g. aluminum), the mobilization and assimilation of essential nutrients (e.g. phosphorus and iron) and promoting the proliferation of microorganisms in the rhizosphere (Jones, 1998; Herrera-Estrella et al., in press).

Aluminum tolerance in acid soils

Plant species and cultivars vary widely in their resistance to Al toxicity, which is the major factor limiting plant production on acid soils. The phytotoxicity of Al varies with its chemical species. Many studies have shown that the chelated forms of Al (as those forming in the presence of organic acids) are less toxic to plant growth than the ionic forms (Al^{3+}). The fact that certain organic acids were present in higher amounts in the rhizosphere of Al-tolerant plant species led researchers to propose a presumptive role of organic acid exudation in Al tolerance. The first suggestive evidence was presented by Miyasaka et al. (1991) who found that citric acid was released by roots of an Al-resistant snapbean in response to Al stress. Later, Delhaize et al. (1993) found that Al-resistant genotypes of wheat excreted 5 to 10 fold more malic acid than Al-sensitive genotypes. Pellet et al. (1995) found the rapid Al-induced excretion of citric acid in Al-resistant maize. More recently, Ma et al. (1997) described the same responsive mechanism in *Cassia tora*.

The production of organic acids in aluminum tolerant plants could also account for a more efficient use of fixed-P or Al-P compounds presents in acidic soils. Although consistent evidence is now lacking, this response may be part of a more general adaptive mechanism to increase the performance of plant species to grow in acid soils.

Phosphorus uptake

Phosphorus is an essential macronutrient for plant growth and the second mineral element (after nitrogen) limiting agricultural production worldwide. Due to its insolubility and high sorption capacity, P availability is a major problem for crop cultivation in acidic and calcareous soils.

Since the 50's decade, it has been well documented that by cause of its high affinity for di- and tri-valent cations, citrate and other organic acids can displace P from insoluble complexes

making it more available for plant uptake (Struters and Sieling, 1950; Bradley and Sieling, 1953).

In acidic controlled conditions, a citrate overproducing carrot cell line selected to be tolerant to aluminum was evaluated for P uptake. When insoluble Al-phosphate (2.0 mM) was supplied as a sole source of phosphate, the selected cell line, but not wild type cells, grew as normally as cells supplied with Na-phosphate in the absence of Al. This work indicates that citrate exudation may confer advantages to grow in acid soils by conferring tolerance to Al as well as enhancing insoluble Al-P use (Koyama et al., 1988).

In an interesting work made by ecologists, the importance of organic acids in plant acquisition of P was examined. They investigated organic acid exudation in 10 alkaline soil adapted (calcicoles) versus non adapted plant species (calcifuges). In calcicoles, the level of organic acid released was higher in several orders of magnitude when compared with calcifuges. The authors conclude that the ability of calcicoles to grow in alkaline soils is due to their capacity to extract phosphate and iron through the exudation of organic acids (Tyler and Strom, 1995).

The exudation of organic acids in response to P deficiency has been well documented in some dicotyledonous plants and especially non-mycorrhizal species such as *Lupinus albus* and *Brassica napus* (Table 1). Malate and citrate appear to be the major organic acids released by the roots of these plants under P deficiency (Gardner et al., 1983; Johnson et al. 1996; Dinkelaker et al., 1989; Hoffland et al., 1989).

Table 1. Organic acid exudation in P-deficient plants.

Plant species	Organic acid	Report
Lupinus albus	Citric	Gardner et al. (1983)
Medicago sativa	Citric	Lipton et al. (1987)
Brassica napus	Citric	Hoffland et al. (1992)
Banksia integrifolia	Citric	Grierson (1992)
Raghanus sativus	Tartaric	Zhang et al. (1997)
Brassica chinensis	Citric	Zhang et al. (1997)

In *Lupinus albus* and the *Proteaceae* family of plants the formation of lateral short-branched roots (proteoid or "cluster" roots) has been widely described. These specialised roots are directly responsible for the 13-40-fold increase in the citrate and malate exudation (Dinkelaker et al., 1995).

It has been reported that organic acids excretion by plants constitutes a drain of 5-25 % of the total fixed C, however, this does not appear to significantly affect dry matter production. This fact has also been confirmed in transgenic plants that constitutively overproduce citrate (Dinkelaker et al., 1989; Johnson et al., 1996; López-Bucio et al., submitted).

Alteration of citrate biosynthesis in transgenic plants

Despite the suggested picture of organic acids as an adaptive trait, until recently, there had been little direct evidence to support this hypothesis. In order to gain a more precise knowledge in the role of organic acids in plant adaptation to soil stress, we decided to modify

citrate biosynthesis in transgenic plants by expressing a bacterial citrate synthase in the cytoplasm of plant cells.

We decided to overexpress a bacterial citrate synthase for two reasons. First, to direct citrate biosynthesis in the cytoplasm and second, to avoid the plant regulatory system that often turns off the expression of transgenes. Then, we fused the citrate synthase gene from *Pseudomonas aeruginosa* to the strong and constitutive 35S CaMV promoter. This construct was used to transform tobacco by *Agrobacterium tumefaciens* and papaya plants by particle bombardment.

The plants carrying the citrate synthase gene secreted five to six times more citrate from their roots than controls, demonstrating that it is possible to increase organic acid excretion both in monocots and dicots by Genetic Engineering (de la Fuente et al., 1997).

Aluminum tolerance in CS transgenic plants

It has been proposed that the principal site of Al toxicity is the root tip, where it affects many cellular processes that lead the inhibition of root and plant growth (Kochian, 1995).

The first step we made in characterizing the novel transgenic lines was to study whether these plants were tolerant to aluminum. We carried out several experiments monitoring the growth of transgenic and control plants in nutrient media containing toxic concentrations of Al. It was found that citrate overproducing plants could grow well in Al concentration 10-fold higher than those tolerated by control plants (de la Fuente et al., 1997).

In order to examine whether citrate overproduction also protects the root, we germinated seeds from the CS and control lines on media containing toxic levels of this metal. At concentrations higher than 300 μM, control seeds germinated but did not develop a root system, and at low Al concentration (50-75 μM) root growth was only slightly affected, but root hair development was severely impaired. In all cases, CS transgenic lines were more resistant to these effects.

The penetration of Al inside the roots of transgenic plants was tested by hematoxylin staining (that specifically detects the location of Al in plant tissue). The CS lines showed less staining than did controls when exposed to high concentrations of Al, indicating that lower amounts of Al penetrated the root tip and root hairs of the transgenic plants (de la Fuente et al., 1997).

Taken together these results demonstrate that organic acids excretion is indeed a mechanism of Al tolerance in higher plants that acts maintaining the toxic Al outside the root (de la Fuente et al., 1997).

Enhancing P Uptake by Citrate Overproduction

With the objective of elucidate the direct role of organic acid exudation in P uptake, we compared the growth and productivity of transgenic and control lines in a natural alkaline soil (pH 8.4) with low P soluble content. In this soil, the growth of control plants was severely restricted and after 6 months they failed to flower and to fructify, whereas transgenic plants were able to grow and reproduce. The inability of control plants to complete their life cycle in this calcareous soil is probably a reflection of calcifuge behavior in which the inability to mobilize P is of central importance (Tyler, 1992).

To confirm the relationship between P deficiency and plant growth, we evaluate the growth of the plants in the same alkaline soil under different sources and treatments of P. At high levels of soluble P application (NaH_2PO_4 108 ppm) no differences in size, shoot and capsule dry weight were detected between transgenic and control plants. However, at sub-optimal levels of soluble P (10 and 22 ppm), the transgenic plants had more shoot and capsule biomass and accumulated more P in their tissues than did controls. In addition, transgenic plants produced significantly higher amounts of capsule biomass when supplied with sparingly Ca-P sources (hydroxiapatite) (López-Bucio et al., submitted).

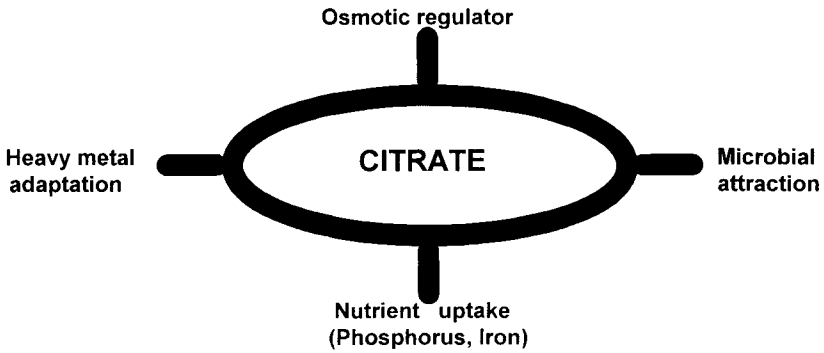
The fact that transgenic plants grow efficiently in alkaline soils with low P soluble content, the better use of Ca-P compounds and the higher accumulation of P in their shoots demonstrate the importance of citrate exudation as adaptive trait of plants to survive under P limiting soil conditions (López-Bucio et al., submitted).

Concluding Remarks

The plant roots are frequently exposed to adverse soil conditions that limit plant growth and agricultural production. Soil stress induces a response in several biological forms of life to exclude toxic mineral elements or to make nutrients available for uptake, transport and use in essential biochemical reactions and cell construction.

With the use of transgenic plants we have demonstrated that organic acid excretion (in particular citric acid) is an important mechanism of tolerance to aluminum in plants and account for an efficient use of different P sources as those Ca-P compounds present in alkaline soils. These results enforce the novel view of organic acids not only as energetic intermediates, but also versatile molecules with a direct role in plant adaptation to soil stress (Figure 1).

Figure 1. Citrate exudation in plant responses to soil stress.



Highly significant is the fact that by Genetic Engineering, this trait could be improved in economically important crops to enhance plant production at low monetary and environmental cost. This alternative is just an example of the potentiality of transgenic agriculture for marginal lands. We believe that by combining this biotechnology with traditional breeding and controlled fertilizer use, in an integrative crop management will help us to satisfy food demand towards the third millennium.

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Commercialization of Genetically Engineered Potato Plants Resistant to Disease

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Introduction

Potato (*Solanum tuberosum* ssp. *tuberosum* L.), one of the most important food crops in the world, is particularly subject to virus diseases because vegetative propagation allows pathogens several years to accumulate over successive generations. It is therefore critical that the introduction, accumulation and transmission of viruses in vegetatively propagated crops be prevented or attenuated. Strategies for genetic engineering of resistance to plant viruses increase as our understanding of virus infection and host-pathogen interactions improves. The potential targets for disruption of the virus infection process include entry, disassembly, translation, replication, encapsidation, movement, and transmission. Several mechanisms that are not necessarily mutually exclusive have been proposed to explain the resistance observed with the various strategies. This review focuses on the development and commercialization of genetically engineered disease resistance designed to control luteovirus infection.

Strategies

Coat Protein

Genetically engineered plant virus resistance was first reported by Powell et al. (1986). A cDNA clone of the tobacco mosaic tobamovirus (TMV) coat protein gene was constitutively expressed in *Nicotiana tabacum* and plants that accumulated coat protein developed no disease symptoms or showed delayed symptom development when inoculated with TMV. The level of resistance conferred by coat protein genes is variable and depends on the virus and host.

Variability of resistance has also been observed within individual transformed lines since the random insertion of the coat protein gene into the genome can influence expression levels. Reduced symptoms, movement and titres were observed in potato or tobacco plants expressing the coat protein gene of potato leafroll luteovirus (PLRV) (Kawchuk et al., 1990, 1991, 1997; van der Wilk et al., 1991, Barker et al., 1992, 1993, 1994; Presting et al., 1995, Derrick and Barker, 1997, Graham et al., 1997). Results suggest a mechanism independent of virus disassembly since coat protein concentration does not correlate with PLRV resistance and resistance was not influenced by increasing inoculum levels (Kawchuk et al., 1991). Various levels of specificity have been reported for the resistance conferred by coat protein genes but the highest resistance is usually to the virus from which the coat protein gene was derived.

Replicase

Evidence that the replicase may provide resistance was first observed in transgenic plants expressing the putative 54 kDa derivative of the TMV replicase were completely resistant to TMV, even at inoculation concentrations 1000-fold greater than those that overcame resistance with the coat protein gene (Golemboski et al., 1990). Several different versions of the PLRV replicase, including the full-length native protein, were reported to be highly virus resistant (Kaniewski et al., 1995). Resistance levels are frequently higher than that observed using coat protein genes but can be extremely specific for the virus or isolate from which the replicase was derived. For example, potato plants transformed with the PVYO replicase gene were highly resistant to the homologous PVYO strain but not PVYN (Audy et al., 1994). Resistance obtained with a replicase gene has been reported to involve two independent mechanisms influencing virus replication and movement (Hellwald and Palukaitis, 1995; Nguyen et al. 1996).

Movement protein

Cell to cell movement of viruses can involve a virus encoded movement protein that interacts with the plasmodesmata to increase the size exclusion limit (Wolf et al., 1989; Deom et al., 1992). Expression of an incompatible or defective movement protein can interfere with virus movement. Tobacco plants transformed with a defective TMV movement protein gene or with the movement protein gene from BMV, a virus that cannot move within tobacco, are resistant to TMV (Lapidot et al., 1993; Malyshenko et al., 1993). Similarly, Tacke et al. (1996) expressed modified movement proteins of PLRV in potato and observed broad-spectrum resistance to PLRV and unrelated PVX and PVY. The broad-spectrum resistance induced by movement protein manipulation may be a consequence of defective or heterologous movement proteins competing with functional movement proteins for a limited number of target sites within the plasmodesmata.

Untranslatable virus sequences

Antisense RNA and untranslatable sense RNA sequences derived from different regions of the luteovirus genome, including the coat protein gene (Kawchuk et al., 1991, van der Wilk et al., 1991, Palucha et al., 1997), movement protein (Tacke et al., 1996), or replicase gene (Kaniewski et al., 1995) have been exploited in engineering resistance. The resistance is believed to involve an interaction of complementary virus and transgene sequences that disrupt translation, replication, stability, or access of host molecules. Resistance levels differ depending on the sequence and virus being examined.

Antiviral proteins

Resistance to PLRV, PVX, and PVY was reported in potato and tobacco expressing a pokeweed ribosome-inhibiting antiviral protein (Lodge et al., 1993). Expression of the ribosome-inhibiting protein and the virus involved in the primary infection were confined with a transactivatated ACMV promoter (Hong et al., 1996). Heterologous proteins represent a diverse strategy with enormous potential for virus control that may be specific to a particular virus or provide resistance to several unrelated viruses.

Future Developments

Strategies for engineering virus resistance have increased rapidly since the initial report of transformation with the TMV coat protein. New strategies for engineering resistance to potato viruses will evolve from the characterization of pathogen- and host-derived genes, virus genome organization and expression, and the process of virus infection including disassembly, replication, encapsidation, movement, and transmission. For example, recent advances have identified within PLRV (Figure 1) a second subgenomic RNA (Ashoub et al., 1998) and a 25 kDa nucleic acid binding protein derived from the C-terminus of P1 (Prüfer et al., 1999). These efforts should be greatly assisted by the production of full-length infectious luteovirus clones developed to characterize gene function (Prüfer et al., 1995; Prüfer et al., 1997).

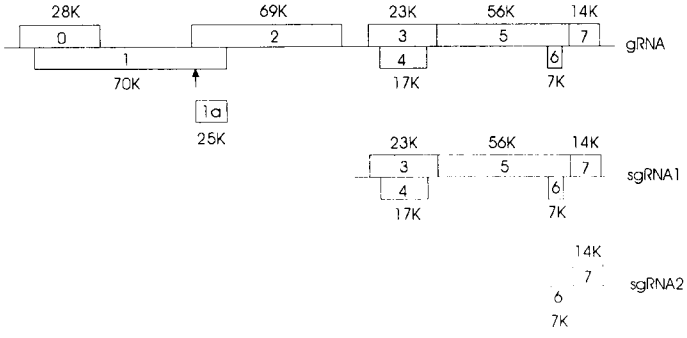

Figure 1. Schematic diagram of the PLRV genome showing the various open reading frames and products. Arrow indicates putative proteolytic cleavage site.

Strategies are not necessarily independent of one another and it is possible that resistance involves protein, nucleic acid and other interactions. For example, homology-dependent silencing is a relatively recent mechanism that may also operate in many virus control strategies (Baulcombe, 1996). This post-transcriptional mechanism causes suppression of transgene expression, possibly by methylation, and only requires sequence similarity between the transgene and virus to produce resistance (English et al., 1996). Several strategies may also be combined to increase the level and number of viruses that the host is resistant. Barker et al. (1994) transformed PLRV resistant germplasm with the PLRV coat protein gene and reported that the resistances were additive. Progress has also been made in combining the coat protein gene of more than one virus in a single transformed plant. Potato plants transformed with the coat protein genes of PVX and PVY were resistant to both viruses (Lawson et al., 1990).

Several concerns have been expressed regarding the production of transgenic plants resistant to viruses. One concern is the possibility of recombination between a virus and virus-derived genes within the plant, creating a virus with new characterisitics. Such recombinations have been reported to occur between viruses and transgenes of the cowpea chlorotic mottle bromovirus (CCMV) (Greene and Allison, 1994) and ACMV (Frischmuth and Stanley, 1998) but similar opportunities occur in mixed infections. Another concern is the potential for transformed potato plants to outcross with weedy species. However, many potato-growing

areas do not have weedy species that hybridize with potato and where such species coexist the incorporation of resistance in a weedy species could reduce virus reservoirs. Risk associated with a particular strategy depends on the gene and each transformant must be evaluated individually. Overall, most examples to date have considerable merit, minimal risk, and represent a nucleic acid or protein equivalency in that the molecule occurs naturally.

An increasing number of plants genetically engineered for virus resistance is being released for commercialization. In addition to potentially improving yield and quality, engineered virus resistance may reduce transmission by vectors and thereby reduce the use of pesticides. The USDA has granted regulatory approval for the commercials release of papaya resistant to papaya ringspot potyvirus (PRSV) (Fitch et al., 1992) and squash resistant to CMV, WMV2 and ZYMV (Tricoli et al., 1995). Potato plants resistant to PLRV, PVS, PVX, and PVY are available to the seed industry and should be available early in the new millennium. Presently, the greatest impediments to the commercialization of genetically engineered disease resistance are business rather than science related.

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Potato Transgenic Plants Expressing Mammalian Double Stranded RNA-Dependent Protein Kinase (mPKR)

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Introduction

Protein phosphorylation is a major strategy in the regulation of cellular processes. In mammals, the interferon system provides such a universal antiviral response. Interferons induce the synthesis of additional protein that directly led to the inhibition of virus multiplication. One of these is the 2'-5' oligoadenylates (2-5A), which activates a latent endoribonuclease (RNAse L) that degrades viral and cellular RNAs and is itself degraded by a cellular 2'-phosphodiesterase (Samuel, 1991).

Devash et al. in 1982 found inhibition of tobacco mosaic virus (TMV) multiplication in tobacco leaves disks by 2-5A oligoadenylate and recently Truve et al. (1993) and Mitra et al. (1996) reported the mammalian 2-5A system functions as an antiviral pathway in transgenic plant. We think in another enzyme of the interferon-induced antiviral systems: the double-stranded RNA-dependent protein kinase.

In mammalian cells an analogous dsRNA-dependent kinase (mPKR) has been identified and characterized (Hovanessian, 1989). Interferon treatments increase the level of this kinase, which is dependent upon dsRNA for initial autophosphorylation reaction. The phosphorylated mPKR is activated and capable of subsequent phosphotransferase activity. The α-subunit of eIF-2 is an important substrate for phosphorylation. PKR activity is directly linked to cellular antiviral responses, apoptosis (Lee and Esteban, 1994) and cell differentiation (Koromilas et al., 1992; Meurs et al., 1993).

Plant virus and viroid infection stimulate the *in vitro* and *in vivo* phosphorylation of an endogenous serine/threonine protein kinase (pPKR) in plant cells (Crum et al., 1988; Hiddinga et al., 1988; Roth and He, 1994). Increased pPKR phosphorylation occurs during early events in pathogenesis and is correlated with viral replication events (Hu and Roth, 1991). Catalytic domains appear to be conserved between plant and animal kinases, although regulatory regions are significantly more diverse (Lawton et al., 1989).

In the present work we showed transgenic potato plants expressing mammalian double-stranded RNA-dependent protein kinase like a strategy to protect crops against viruses.

Materials and Methods.

General DNA methods

All nucleic acid manipulations throughout this study were performed according to standard methods (Sambrook et al., 1989).

Isolation and cloning of mPKR gene

Dr Brian Williams of the Clinical Foundation of Cleveland, USA, supplied us the cDNA clone BS-PKR. Primers used for polymerize chain reaction (PCR) were the No-3782 (5' cgggatccatggctggtgatctttcagc 3') and No-3783 (5' gctctagactaacatgtgtgtcgttc 3') corresponds to the 5' and 3' end sequence of the mPKR gene. For PCR amplification, samples of 25 µl containing 200 µM of each dNTP, 10 pmoles of each primer, 10 ng of BS-PKR, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 5U of Taq DNA polimerase were subjected to 20 cycles of amplification. One cycle corresponded to 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Amplified cDNA was isolated from LGT (low gelling temperature) agarose gel 0.8 %, digested *BamHI-XbaI* and ligated to the same sites of the plasmid pUC18, generating the plasmid pUC-PKR.

Construction of plant vectors

PKR DNA fragment was excised from pUC-PKR by *BamHI-XbaI* digestion and ligated to those sites of the vector pBPFΩ8 under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the Ω untranslate region of TMV and nos terminator and introduced into the binary vector pDE1001. The recombinant plasmid pDE-PKR was used for *Agrobacterium tumefaciens* transformation. Competent *Agrobacterium* AT2260 cells were transformed with 1 µg of pDE-PKR plasmid.

Transformation of potato plants

Agrobacterium-mediated transformation of potato leaves was done according to the method improved by Enriquez et al. (1996). Plantlets rooting well on kanamycin containing medium were transferred to the greenhouse for further evaluations.

Screening by PCR

Genomic DNA was isolated from kanamycin resistant clones and non-transformed plants growing under greenhouse conditions. PCR reactions was carried out by mixing, 1 µg of total DNA as template, 100 pmoles of each primer (3782 and 3783), 200 µM of each dNTP, 5U of Taq DNA polimerase, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 1.5 mM MgCl2 in 20 µl final volume. The amplification was done using the conditions described above, but with 35 cycles of amplification. The amplified product was electrophoresed and transferred to hybond-N membrane for Southern blot hybridization, (Southern, 1975). The *BamHI-XbaI* fragment (1.67 kb) from pUC-PKR, was used as probe. Hybridization was done at 42°C in the presence of 50 % formamide. The membrane was washed thrice during 10 min in SSC 1X, 0.1 % SDS and exposed to X-ray film overnight at –70°C.

The expression of mPKR in potato transgenic plants

The expression was analyzed for Western blot. Total proteins were extracted from leaves using 2 % SDS, 12.5 % glycerol and 61 mM Tris-HCl pH 6.9. The protein extracts were diluted with buffer sample, heated for 5 min at 100°C and loaded onto a 12.5 % NaDodSO4-polyacrylamide gel. The electrophoresed proteins were transferred to a nitrocellulose membrane by using a transfer chamber in Tris-glycine buffer containing 20 % methanol at 300 mA for 3 h. The membrane was then treated with a blocking agent and anti-PKR-IgG as first antibody following by anti-rabbit IgG alkaline phosphatase as second antibody.

Infection of potato plants with PVY

For the greenhouse infection experiment, we chose four of the best clones previously selected from a preliminary experiment, in which, 50 clones had been infected with sap from PVY-infected tobacco leaves. Potato leaves were homogenized to completion in PBS-Tween containing 2 % of PVP 1:10 (W/V). The virus concentration in infected plant leaves was analyzed using the Agdia PVY enzyme immunoassay kit. A purified PVY-virus preparation was used as standard for virus quantification. Statistical analysis of the infection data was carried out using ANOVA design.

Results and Discussion

Integration and expression of mPKR gene in Solanum tuberosum *L.*

The mPKR gene was cloned under the control of CaMV 35S promoter and *nptII* gene (conferring resistance to kanamycin) as a selection marker for potato plants transformation.

The genomic integration of the transgene in potato plants was analyzed by PCR. All the assayed kanamycin resistant clones amplified a clear band with a molecular weight of 1.63 kb corresponding with the size of the mPKR cDNA. This finding suggest that the transgene was integrated into the genome of the transformed plants. Sequences homologous to mPKR were not found in nontransformed potato plants. Southern blotting of PCR products showed that the probe hybridized strongly with the 1.63 kb amplified fragment. No hybridization signals were found in lines corresponding to the control potato plants. Although other authors have reported the presence of endogenous pPKR gen in barley plants using mouse and human cDNA probes establishing homology between the mPKR enzymes and pPKR (Langland et al., 1995), we did not find homologue sequences to mPKR in potato plant.

The expression and accumulation of mPKR was detected by probing Western blot. The immuno blot showed a single protein band of about 68 kDa in protein extracts from transgenic plants. This molecular weight corresponds well with the predicted size of the protein. These data indicate that introduction of the mPKR cDNA into transgenic plant led to production of a functional protein. In control potato plants did not appear any homologue proteins to mPKR

Proteins of potato transgenic plants were separated by SDS-PAGE, transferred and probed with policlonal antibody anti-PKR. Primary antibody bound was detected by the addition of goat anti-rabbit IgG alkaline phosphatase and colored substrate reaction. The molecular mass of mPKR (68 kDa) is shown. Lane 1-4: Extract of proteins of transgenic clones 1, 3, 7 and 9. Lane 5: Proteins extracts of nontransformed plant. Numbers at left are size markers in kDa

Biological activity

Transgenic clones (1, 3, 7 and 9), resistant to kanamycin and morphologically normal plants had been selected in a preliminary greenhouse experiments. The four clones selected (8 plants/clone) were transplanted to greenhouse and had fifteen days old at the time of inoculation (Figure 1A). Untransformed potato plants (16 plants) were used as controls. The plants were inoculated with the sap from PVY-infected tobacco leaves and PVY concentrations in the clones analyzed 14 and 24 days post infection (DPI). At 14 DPI although virus concentration in the clones were lower than in controls (Figure 3A) there were between an 12-25% of infected plants, it was still significant at 24 DPI where the infection percent was more than 70% of the plants (Figure 1B).

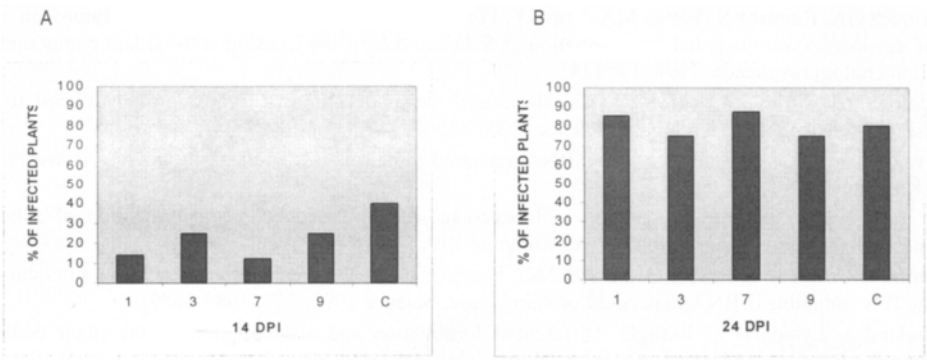

Figure 1. Percentages of PVY infected potato transgenic clones expressing mPKR (1, 3, 7 and 9) and in nontransgenic controls (C).

This was expected as PVY infection symptoms increase after 15 DPI. Anova statistical analyses showed that there aren't significant differences among the clones (treatment) (data not shown). We have shown that protection against PVY infection in potato plants can not be achieved by the expression of a mammalian double-stranded RNA-dependent protein kinase in plants under greenhouse conditions. Both mPKR and pPKR have been identified as being associated with ribosome and free in the cytosol and the subcellular forms of mPKR are distinctly different (Langland and Jacobs, 1992; Langland et al., 1995). Langland et al in 1998 found that TMV infection results in the increase in protein levels of the plant encoded dsRNA dependent protein kinase in cytosolic fractions. It is conceivable that the functional significance of pPKR with respect to pathogenesis differs between these fractions. In the regulation of protein synthesis has been implicated pPKR in ribosome associated fractions, the role and form of cytosolic pPKR is unclear at this time. Under the control of constitutive CaMV 35S promoter the mPKR accumulation will be in cytosolic fractions for absence of a signal peptide to any specific subcellular localization. Although the overexpression of mPKR occurs efficiently the virus infection is not controlling because mPKR cytosolic fraction is not involvement in protein synthesis regulations during key events in gene expression like ribosomal mPKR.

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Genetic Engineering of Potato for Tolerance to Biotic and Abiotic Stress

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Potato was introduced into Europe from the Andean region of South America in the sixteenth century, but its economic importance started to increase only 200 years later. Its cultivation in the temperate zones of Europe has since been facing severe constraints, mainly due to its sensitivity to pests or diseases caused by viruses and viroids as well as by bacterial and fungal pathogens. It is in this context that we apply molecular breeding to potato by the pathogen-derived protection (PDP) strategy (Sanford and Johnston, 1985). In doing so we focus on potato leafroll virus (PLRV) by firstly studying its molecular biology. Insights into PLRV RNA transcription, gene expression strategies and host/pathogen interactions may then provide a basis for attempts to genetically engineer new traits into the host plant potato.

PLRV belongs to the polerovirus group (previously luteovirus subgroup 2) of plant viruses. Its 5.8 kb single-stranded positive-sense genomic RNA (gRNA) contains 8 major open reading frames (ORFs; Figure 1). The 5′ gene cluster of ORFs 0, 1 and 2 is expressed from gRNA. At least two subgenomic RNAs, sgRNA1 (encoding ORFs 3, 4 and 5, Tacke et al., 1990) and sgRNA2 (encoding ORFs 6 and 7, Ashoub et al., 1998), serve for the expression of downstream located PLRV genes. In addition to these transcriptional analyses, studies on protein translation during PLRV gene expression have identified noncanonical translational mechanisms such as internal initiation, -1 ribosomal frameshifting, amber stop codon suppression and initiation at non-AUG codons as well as proteolytic processing (Prüfer et al., 1999). Functions have been attributed to some of the PLRV proteins on the basis of several lines of evidence (reviewed by Miller et al., 1995; Mayo and Ziegler-Graff, 1996).

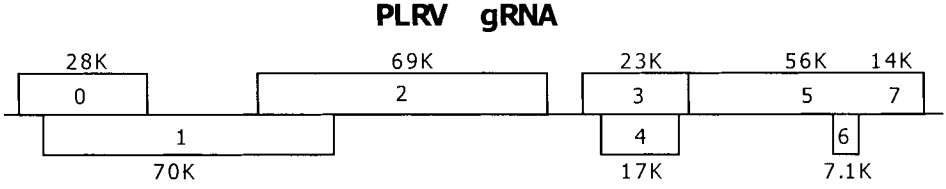

Figure 1. Schematic presentation of the PLRV RNA genome

Among these proteins, the 17 kDa PLRV movement protein P4 (prl7) has attracted much of our interest, both from a theoretical and an applied point of view. It is a nucleic acid-

binding protein with particular biochemical features residing in N- and C-terminal domains (dimerization via an amphipathic α-helix in the N-terminal domain; nucleic acid-binding by the basic C-terminal half; Tacke et al., 1993). The C-terminal half of PLRV pr17 is phosphorylated by a membrane-bound kinase with protein kinase C-like properties (Sokolova et al., 1997), and it shows high affinity for plasmodesmata of the sieve element/companion cell complex both in PLRV-infected as well as in pr17-transgenic potato plants (Schmitz et al., 1997).

Based on these molecular and biochemical studies, we have utilized the PLRV pr17 gene in the genetic engineering of potato for tolerance to biotic and abiotic stress. Expression of mutant pr17 proteins in transgenic potato lines induced broad-spectrum resistance to the potato viruses PLRV, PVY and PVX (Tacke et al., 1996). Evidence was presented that at least the induced protection against PVY depended on the presence of the mutant protein in transgenic plants, since potato plants that did not accumulate pr17 protein, but only its mRNA, were not resistant to virus infection.

As in the case of the tobacco mosaic virus 30K movement protein and its effect on carbohydrate metabolism in transgenic plants (Lucas et al., 1993), expression of PLRV pr17 affects phloem transport phenomena in transgenic plants leading to an altered partitioning of carbohydrates (sugars, starch). In tobacco, constitutive pr17 expression resulted in a severely stunted phenotype with the development of chlorotic and partially necrotic lower leaves and a reduced photosynthetic capacity in source leaves (Herbers et al., 1997). While a reduction in the rate of photosynthesis is also observed in potato plants transformed by the identical constructs, the plants display a close to normal phenotype with an only marginal growth reduction, although sugars and starch accumulate also in these potato lines (unpublished data).

The overexpression in transgenic plants of genes that will result in an increased production of osmolytes like sugars or sugar alcohols is one of the strategies to induce tolerance to abiotic stress conditions like drought, extreme temperatures or salinity (Holmberg and Bülow, 1998). As obvious from table 1, pr17-transgenic potato plants show indeed an increased tolerance to drought conditions.

Table 1. Performance of transgenic potato lines L4, L6, L7 and L8 and of the progenitor variety "Linda" under drought conditions (8 weeks).

	No. survivors / No. tested plants	
Genotypes	Exp. 1	Exp. 2
Linda	1*/5	1*/5
L4	4/5	5/5
L6	5/5	5/5
L7	5/5	5/5
L8	5/5	4/5

Note. Plants were kept for 8 weeks under water stress (drought conditions) with a single watering at 3 and 6 weeks, respectively. * The surviving plant developed a new shoot from the tuber, all original shoots died.

Therefore, the pr17-transgenic potato lines displaying broad-spectrum resistance to viruses (Tacke et al., 1996) were tested for tolerance to abiotic stress such as drought and salinity. Furthermore, while salt concentrations in the range of 10-200 mM NaCl for 5 weeks resulted

in the death of lower leaves and severe stem necrosis in the non-transgenic potato plants, all 4 pr17-transgenic lines (Table 1) maintained its normal stem anatomy, and only localized necrotic lesions became visible on the lower leaves (Figure 2).

In conclusion, a single transformation event in potato by the PDP strategy using a mutant form of the PLRV pr17 movement protein increases the general fitness of the potato plant in that it induces tolerance to both biotic (virus) and abiotic stress conditions (drought, salinity). Thus it is envisioned that in potato pr17 gene expression may provide the basis for molecular breeding by the induction of general stress resistance, while pyramidizing other transgenes will lead to additional, more specific traits.

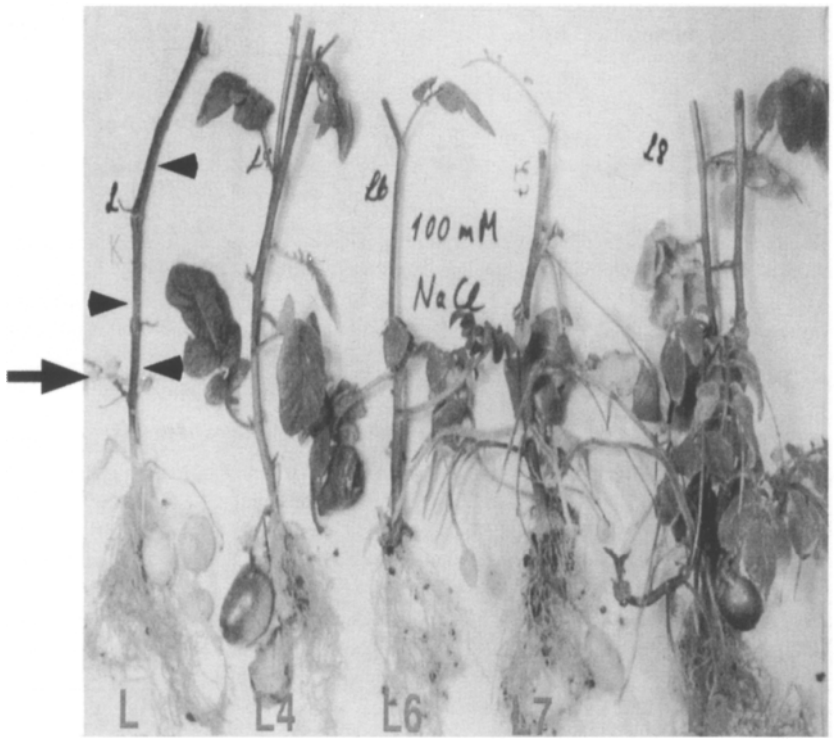

Figure 2. Salt tolerance test with pr17-transgenic potato lines 5 plants of each of the transgenic lines L4, L6, L7 and L8 and of the non-transgenic variety Linda (L) were watered with 100 mM NaCl for 5 weeks. As indicated by the arrowheads, the internodes of the control L developed severe necrosis. The arrow points to residual dead leaf tissue in the L plants, while most lower leaves have already decayed.

Such genetic engineering of more than one transgene may in the long term depend on the application of regulatory elements that warrant the simultaneous expression of the genes of interest. This could be accomplished for example by the transcription of a single mRNA functioning as a di- or polycistronic mRNA provided that internal ribosome entry sites (IRES) are present (Mountford and Smith, 1995). In fact, such regulatory sequences have been identified on the PLRV genome apart from the sequences for proteins of relevance to genetic engineering of potato (see above). A PLRV internal ribosome entry site of highly unusual

structure which allows the construction of di- or polycistronic mRNAs has recently been characterized (Jaag et al., submitted) and demonstrated to guide internal ribosome entry *in planta*. Furthermore, the phloem-specific suppression of the UAG stop codon separating ORFs 3 and 5 (Figure 1) opens the possibility to regulate tissue-specific gene expression at the translational level, although at low efficiency (Tacke et al., 1990). This latter strategy may, however, be sufficient for proteins that accumulate in transgenic plants by virtue of their biochemical properties, as is the case with PLRV pr17 (stabilization by protein/protein interactions and membrane association).

These few examples demonstrate that both coding and regulatory sequences of the PLRV genome show a high potential for the genetic engineering of potato and other crop plants for tolerance to biotic and abiotic stress by the PDP strategy.

Acknowledgements

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Metabolic Engineering of Brassica Seeds Oils: Improvement of Oil Quality and Quantity and Alteration of Carbon Flux

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Introduction

The general pathway for synthesis of various fatty acids and the steps normally involved in the Kennedy pathway to produce seed storage lipids (triacylglycerols; TAGs), are depicted in figure 1. These pathways have been reviewed extensively elsewhere (Stymme and Stobar, 1987; Ohlrogge and Browse, 1995).

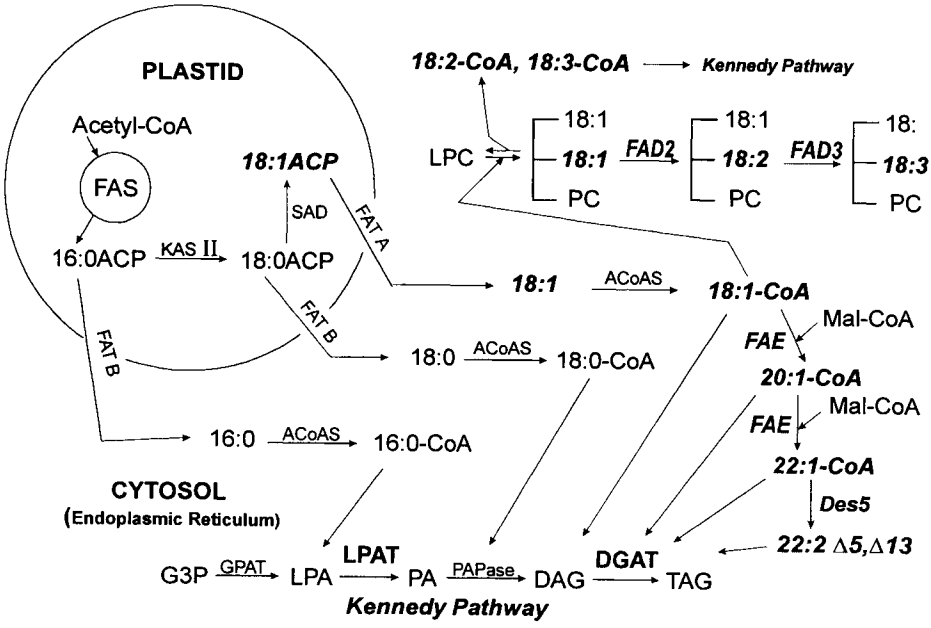

Figure 1. Schematic of storage lipid bioassembly in developing seeds of *Brassicaceae*. Not shown: PC can be reversibly converted to DAG via DAG: choline phosphotransferase.

Fatty acids up to and including oleic acid (18:1) are made in the plastid via FAS and their further modification (e.g. desaturation, elongation, hydroxylation etc.) occurs in the cytosol. These fatty acyl groups (as acyl-CoA thioesters) then act as donors in acyltransferase reactions of the Kennedy pathway, and become esterified to the glycerol backbone.

One of the strategic goals of our research is to modify high erucic acid rapeseed (HEAR) germplasm to increase the content of erucic acid (22:1 Δ13) and other strategic very long-chain fatty acids (VLCFAs) in the seed oil for industrial niche market needs. Erucic acid and its derivatives are feedstocks in manufacturing surfactants, plasticizers, nylon, and surface coatings; more than 1000 relevant patents have been issued (Sonntag NOV, 1993). The current market for high erucate oils exceeds $100 million US/annum. Worldwide erucic acid demand is predicted to increase from about 40 million pounds (M pds) in 1990 to about 75 M pds by the year 2010. Similarly, demand for the derivative, behenic acid, is predicted to triple to about 102 M pds in 2010. In recent years, production has lagged behind market needs (Chapman, 1996). A *Brassica napus* cultivar containing erucic acid levels approaching 80 % would significantly reduce the cost of producing erucic acid and its derivatives and could meet the forecast demand for erucic and behenic acids as renewable, environmentally-friendly industrial feedstocks (Taylor et al., 1992; Sonntag NOV, 1993). There are also niche market applications being developed for using 22:2 (Δ5, Δ13) as a feedstock for generating estolides which can be used to synthesize hydroxy fatty acid feedstocks, and to produce dimer acids, esters and amides for use as lubricants, and slip-anti-block agents in plastic film manufacturing (Erhan et al., 1993). Several patents are pending for these applications.

Results

Acyltransferase studies

LPAT: As reviewed previously (Taylor et al., 1992), it is well established that in HEAR B. napus, the specificity of the *sn*-2 acyltransferase (lyso-phosphatidate acyltransferase; LPAT) of the Kennedy pathway restricts the incorporation of erucoyl-CoA into the middle position on the TAG molecule. Thus, while erucic acid is found in the *sn*-1 and to a greater extent, the *sn*-3 positions of HEAR TAGs, it is essentially absent from the *sn*-2 position. The primary approach to alter this trait has been to transfer to HEAR cultivars, novel genes encoding sn-2 acyltransferases (LPATs) capable of inserting 22:1-CoA at the *sn*-2 position during TAG bioassembly (Lassner et al., 1995; Brough et al., 1996; Zou et al., 1997).

We have transformed various *Brassicaceae* with a mutated *sn*-2 acyltransferase (SLC1-1) gene originally isolated from yeast (Nagiec et al., 1993). The gene was shown to be an LPAT capable of utilizing a range of acyl-CoA donors, including erucoyl-CoA. Transgenic *Arabidopsis thaliana*, *B. napus* and *Brassica carinata* expressing the yeast gene under the control of both constitutive and seed-specific promoters have exhibited significant increases in seed oil content, in average seed weight, and in the proportions of erucic acid (Zou et al., 1997). Such increases in oil and erucic acid contents as a result of a single manipulation constitute a world first, and the results with the yeast gene are distinctly improved over those obtained by transformation of HEAR germplasm with meadowfoam (*Limnanthes spp.*) LPAT genes (Lassner et al., 1995; Brough et al., 1996) (Table 1; Figure 2). While the *Limnanthes* transgenes have a more dramatic effect on the proportions of erucic acid at the *sn*-2 position, the overall contribution of the SLC1-1 transgene to the net yield of erucic acid is superior.

Table 1. Comparison of effects of sn-2 acyltransferase transgenes on HEAR cvs. Results are for greenhouse-grown, selfed material. Con = non-transformed or plasmid only controls.

HEAR cv./ (Reference)	Control/ Transg. Source	% *sn*-2 22:1	% overall 22:1	Oil Content (% DW)	Avg Seed Wt.
Hero/ (14)	Con	0.5	45	33	↑by 9-38%
	SLC1-1	3-5	49-56	37-42	
Reston/ (6)	Con	0-0.3	38-40	Not reported	Not reported
	L. alba	4-15	no change		
Ag. Seed Res./ (1)	Con	0	32	Not reported	Not reported
	L. douglasii	9-28	no change		

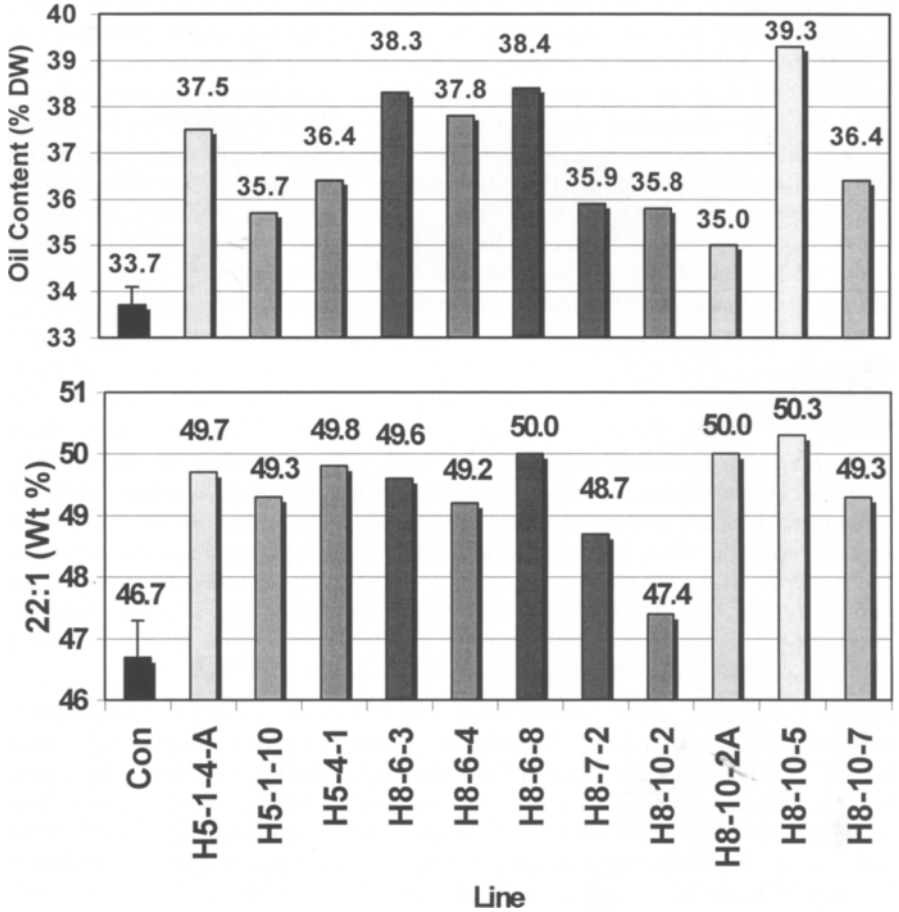

Figure 2. Seed oil and erucic acid content of *B. napus* cv Hero non-transformed controls (Con) and SLC1-1 transgenic (H#) T4 lines, field-tested in nursery rows at Rosthern SK, 1998. Average of 18 rows sampled for controls (± SD) and 2-6 rows sampled for transgenic lines. Analyses were conducted on open-pollinated samples.

Our data suggests that the plant LPAT reaction is tightly regulated, and that by introducing the heterologous SLC1-1 gene, we have perturbed flux through this key metabolic step. In transgenic *Brassicaceae*, the SLC1-1 gene or gene product (LPAT) may be free of regulation or regulated in a different way than the plant (meadowfoam) transgenes. In preliminary nursery row field trials, the SLC1-1 transgenic *B. napus* rapeseed lines containing the yeast LPAT gene show encouraging results, with 2-6 % increases in oil content on a DW basis (5-17 % overall), 2-4 wt % increases in erucic acid content, and average seed dry weight increases of 6-20 %. There is evidence from our work with a range of *Brassicaceae*, that in SLC1-1 transgenics, the overall proportion of the predominant fatty acid (erucic or oleic) is increased. Because the gene affects a pathway common to all oilseeds (Kennedy pathway), it is reasonable to expect that similar increases in oil content and proportions of erucic acid and other strategic fatty acids could be achieved in other oilseed crops. SLC1-1 utility is being evaluated in a number of these, including flax, soybean and corn.

DGAT: Another of our acyltransferase targets is the sn-3 acyltransferase, diacylglycerol acyltransferase (DGAT) (Figure 1). We have previously reported that an EMS mutant of *A. thaliana*, AS11, contains a lesion at a locus designated TAG1. The mutation results in a reduced DGAT activity, and increased DAG/TAG ratios during seed development (which is also delayed), and a reduced mature seed TAG content, and lower proportions of VLCFAs (Katavic et al., 1995). We are characterizing this mutant to assist in our isolation of a DGAT gene. Over-expression of a DGAT may enhance VLCFA and oil content (Jako et al., 1998).

Fatty acid modification studies

We have also initiated experiments to address the issue of enhancing the supply of 22:1 moieties to be incorporated into seed TAGs. Two transgenic approaches are being used: (1) modifying the expression of the condensing enzyme(s) (FAEs), the first in the four enzyme "elongase" complex, and (2) silencing the oleoyl-desaturase (FAD2) to allow more 18:1-CoA to be elongated.

FAE1: As an example, seed-specific over-expression of the *A. thaliana* condensing enzyme FAE1 has resulted in 10-12 % increases in VLCFAs in *A. thaliana* (Millar and Kunst, 1997). In our lab, we have performed similar experiments by transforming *B. napus* cv Hero with the napin:FAE1 construct and have achieved a 4-6.5 % increase in the 22:1 proportions in several T1 lines (Figure 3).

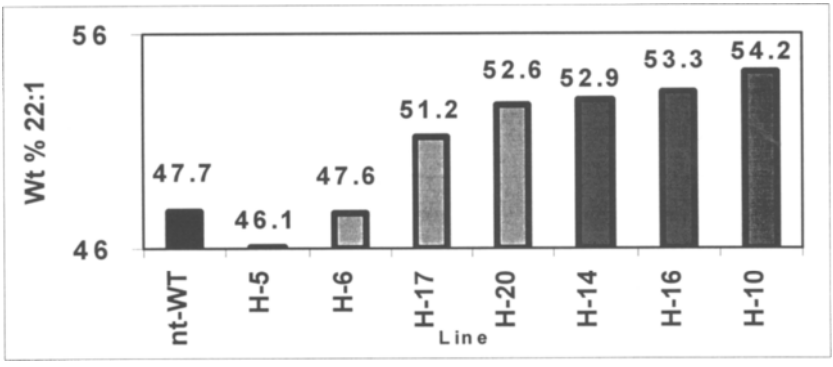

Figure 3. Proportions of erucic acid in *B. napus* cv Hero non-transformed wild type (nt-WT) and Napin: *FAE1* T1 transgenic (H-#) seed oils.

This suggests that the level of seed erucic acid is limited, in part, by the level of elongase activity. Work with this and other elongases (e.g. from yeast) continues. We hope to achieve further increases in erucic content by combining the heterologous SLC1-1 and FAE1 over-expression phenotypes.

FAD2: In attempting to alter the proportions of 18:1-CoA available for elongation, we have cloned the FAD2 cDNA from *B. carinata* (Marillia and Taylor, 1999) and have transformed breeding lines with both sense (co-suppression) and anti-sense napin:*FAD2* constructs. *B. carinata* seed oil contains about 45 % erucic acid but also more than 30 % of (18:2 + 18:3). By silencing *FAD2* in a seed-specific manner, we hope to reduce the proportions of 18:2 and 18:3 and re-direct the 18:1 to VLCFA production.

Des 5: There are a number of plant/conifer species that produce seed oils which accumulate unusual fatty acids with a $\Delta 5$ functionality, including *Limnanthes spp.* and white spruce (*Picea glauca*). A putative $\Delta 5$ desaturase isolated by random sequencing of *Limnanthes* cDNAs, has been inserted behind a seed specific promoter and expressed in *B. carinata*. We hope to produce a 22:2 ($\Delta 5$, $\Delta 13$) dienoic fatty acid. Another potential $\Delta 5$ desaturase candidate is a partial clone from a conifer cDNA library. The nucleotide sequence has some features which might be characteristic of a $\Delta 5$ desaturase gene. The full length clone, isolated by library screening, will be characterized and possibly transferred to *Brassica* species.

Studies on altering carbon flux

The mitochondrial pyruvate dehydrogenase complex (mtPDC), catalyzes the oxidative decarboxylation of pyruvate to give acetyl CoA, and is the primary entry point of carbohydrates into the Krebs cycle. The mtPDC links glycolytic carbon metabolism with the Krebs cycle, and, because of the irreversible nature of this reaction, the pyruvate dehydrogenase complex (PDC) is a particularly important site for regulation of carbon flux. Plants are unique in having PDH complexes in two isoforms, one located in the mitochondrial matrix as in other eukaryotic cells, and another located in the chloroplast or plastid stroma. Although both PDH complex isoforms are sensitive to product feedback regulation (by NADH and acetyl-CoA), only the mitochondrial PDC is regulated through inactivation/reactivation by reversible phosphorylation/dephosphorylation of the E1α subunit by PDC kinase (mtPDCK; Figure 4) and its dephosphorylation by PDC phosphatase.

For the first time in plants, we have isolated and characterized an A. thaliana mtPDHK gene (GenBank/EMBL Accession No. AJ007312). The PDHK gene product is able to catalyze the in vitro phosphorylation of co-expressed human E1α/E1β subunits of the pyruvate dehydrogenase complex (PDC), confirming its function as a protein kinase. The A. thaliana PDHK shares significant homology with known mammalian protein kinases. We have demonstrated that by modulating the level of PDCK expression, the activity of PDC can be genetically engineered. Gene repression by constitutive antisense PDHK expression in A. thaliana had significant effects on plant growth and development, and on seed oil content. Transgenic plants exhibited increased mitochondrial PDC and TCA cycle activity that was correlated with an earlier flowering and maturity phenotype Plants bolted earlier but developed normal fertile flowers which yielded mature seeds. Seed yield did not appear to be adversely affected and average seed weight and seed oil contents (Figure 5) were significantly increased. Thus, for the first time, there is strong evidence to indicate that mitochondrially-generated acetyl-CoA can contribute acetate moieties to plastidial fatty acid production in the developing seed.

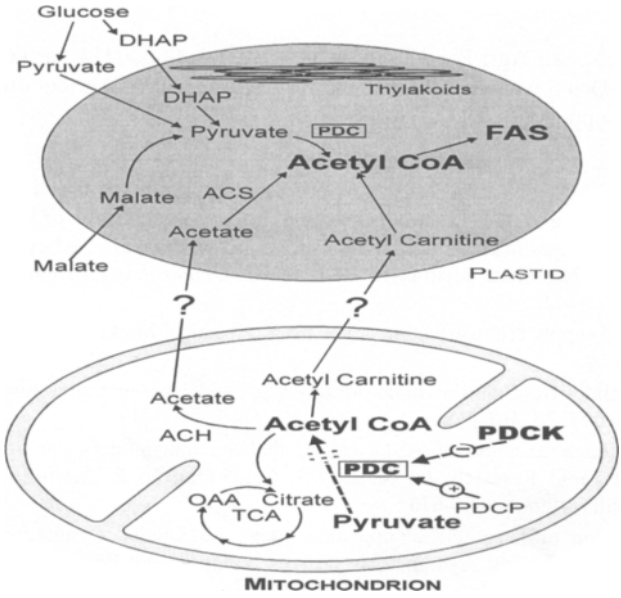

Figure 4. Schematic showing the critical role of mitochondrial PDCK in regulating PDC that controls the entry of acetyl-CoA into the TCA cycle.

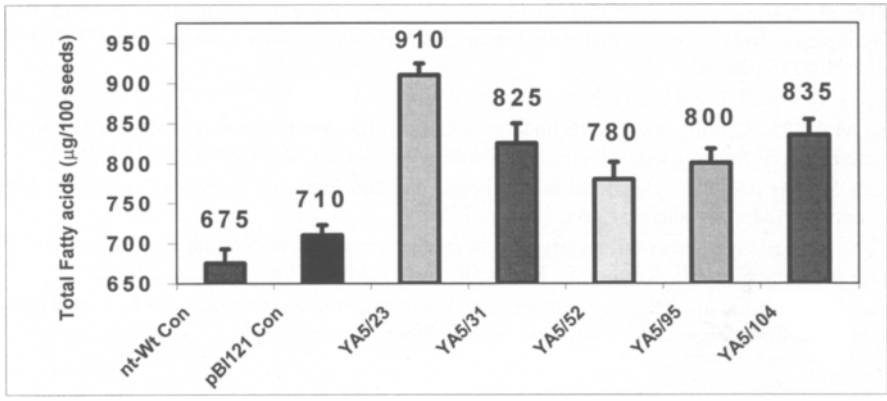

Figure 5. Oil content of non-transformed wildtype control (nt-WT Con) and plasmid only (pBI121 Con) and anti-sense PDHK transgenic (YA5) lines of *A. thaliana* T2 seed.

Acknowledgements

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Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Towards the Improvement of Sugarcane Bagasse as Raw Material for the Production of Paper Pulp and Animal Feed.

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Introduction

Sugarcane (*Saccharum officinarum* L.) is a member of the *Andropogoneae* tribe of the *Poaceae* (grass) family. Modern sugarcane cultivars are derived essentially from interspecific hybridisations involving different Saccharum species (Simmonds, 1976; Daniels and Roach, 1987). The *Saccharum* genus is complex and is characterised by high polyploidy and frequent aneuploidy (Bremer, 1961). Moreover, chromosomal mosaicism has been confirmed in sugarcane clones as a common phenomenon (Heinz et al., 1977). Sugarcane is one of the most efficient crops in terms of photosynthetic efficiency and biomass production.

Sugarcane ranks between the ten most planted crops all over the world. Almost one billion tons are yearly harvested worldwide (Ahlfeld, 1996). The importance of the sugarcane industry for tropical and subtropical countries is due not only to sugar production, which is the major export product for many developing economies, but also to its by-products. The by-products of the sugarcane industry and agriculture can be used to manufacture more than 50 first- and over 100 second-generation products. For instance, sugarcane by-products serve as raw materials for the generation of paper pulp, plywood boards, animal feed, wax, biofertilizers, alcohols and many other useful products (GEPLACEA, 1988).

The genetic improvement of sugarcane through traditional methods is extremely difficult due to the high genome complexity of the sugarcane, which generally has made it impossible to apply classical Mendelian genetics for the analysis of traits (Moore and Irvine, 1991). Nevertheless, traditional breeding has normally solved pathogen and productive problems in sugarcane agriculture. Breeding of sugarcane is time consuming. It takes 12 to 14 years to produce a few new elite clones from 100 000 or more seedlings at an estimated cost of 1 million dollars per clone (Birch, 1993). Moreover, sugarcane is not an ideal candidate for traditional genetic manipulations due to its characteristic non-synchronous flowering and low sexual seed viability. Additionally, as the first nobilised hybrids were derived from a very small number of parental clones, sugarcane breeding suffers from limited germplasm diversity and high genetic erosion (Arceneaux, 1965; Price, 1965; Harvey et al., 1994). Investment in breeding efforts remains the best approach for maximizing productivity in this crop. Because sugarcane has already a relatively high average production efficiency, there is a limited potential for further increasing the yield in response to increased agronomic inputs such as irrigation, fertilization and pest control (Moore, 1987). For the above-mentioned reasons, sugarcane breeding can benefit a lot from the use of non-conventional methods. In particular, genetic engineering can not only shorten the time and reduce the costs to produce an improved

sugarcane line, but it can also provide sugarcane with new important agronomical traits that are absent in the natural germplasm of this species, such as resistance to pests or herbicides. The high biomass production capacity and low agronomical requirements make sugarcane an excellent candidate to be used as bioreactor in the synthesis of new products for medical or industrial applications. Moreover, the industrial behavior of this crop could also be improved by genetic engineering, by increasing the quality of the canes as raw material for the production of sugar and/or other by-products.

Sugarcane Bagasse

The bagasse is the main residue of the sugarcane industry representing, by weight, almost 30 % (hundreds of millions of tons per year world-wide) of the sugarcane agricultural product, therefore bagasse utilization is important for both economical and environmental considerations. Sugarcane bagasse is a good low cost raw material for paper production or animal feed but, as for other plants, the cell wall structural polymer, lignin, rich in p-coumaryl subunits, has a negative effect on digestibility (Leng, 1991) and paper pulping properties (Chiang et al., 1988).

Lignins

Lignins are synthesized by the dehydrogenative polymerization of monolignols. The synthesis of monolignols is bound to the general metabolism of the phenylpropanoids in plants, having enzymes common with other processes (phenylalanine ammonia-lyase (PAL), caffeic acid 3-O-methyltransferase (COMT), ferulate-5-hydroxylase (F5H), caffeoyl-CoA-O-methyltransferase (CCoAOMT) and 4-coumarate: CoA ligase (4CL) for instance), as well as specific enzymes (cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD)) (Whetten and Sederoff, 1995).

Nowadays, great interest has emerged in the possibility of modifying the content and/or composition of the lignin polymer for improving the industrial value of many economically important crops. Using genetic engineering approaches the main attempts have been concentrated on down-regulating the levels of enzymes involved in the lignification process by means of sense or antisense expression of homologous or heterologous genes in transgenic plants (Baucher et al., 1998). Efforts have been mainly focused on the modification of expression levels of *comt*, *cad* and more recently, *ccr*, *f5h* and *ccoaomt* genes.

Genetic Engineering of Sugarcane Fiber

Within a project directed to improve the quality of sugarcane fiber for its use as raw material for paper and forage production, we report here the cloning, molecular characterization, phylogenetic relationships and the heterologous expression of the COMT, CCR and CAD cDNAs from sugarcane (Selman-Housein et al., 1999a,b). The work we are accomplishing to produce, by gene engineering, sugarcane clones with lower content or more extractable lignin, will be also discussed.

Cloning and molecular characterization of sugarcane cDNAs coding for enzymes involved in lignification

We performed the cloning of full-length cDNAs encoding the monolignol biosynthetic enzymes caffeic acid 3-O-methyltransferase (COMT), cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) from a sugarcane. Using total RNA from sugarcane roots, a cDNA library was constructed in the Lambda Uni-Zap XR vector and it was screened

with cDNA probes from maize (for COMT) and poplar (for CCR and CAD) to clone the corresponding sugarcane sequences.

The proteins encoded by the cloned cDNA fragments (39.6, 40.1 and 38.7 kDa for COMT, CCR and CAD respectively) were identified based on their sequence identities with the corresponding enzymes from other plant species. Pairwise comparisons of deduced amino acid sequences with known plant lignification proteins allowed the identification of important conserved domains and specific functional motifs within these enzymes.

Computer comparison between COMT and other methylases from different organism reveals a region of low similarity (positions 120–176 aa in the sugarcane sequence) which is extremely well conserved among COMTs. In this region we identified two sequence motifs (S1 and S2) highly conserved in all enzymes able to catalyse the methylation of hydroxycinnamic acids. The consensus sequence for S1 motif is GVS(V/M/I/L)(A/S)(P/A)(L/I)XLMN(Q/H)(D/G) and it is always located 13 amino acids before the S2 motif, which has the consensus (V/I)L(D/E)GG(I/V)PFNKAYGM. This region could have a biological significance, perhaps containing those residues responsible for the specificity of these enzymes to different hydroxycinnamic substrates. Indeed, the S1 and S2 motifs, as well as their spatial arrangement, are well conserved in loblolly pine O-methyltransferase AEOMT (ac. number U39301), a multifunctional enzyme with activity towards caffeic and 5-hydroxyferulic acids and low overall homology to COMTs. Based on this observation, it will be interesting to test the methylation activity on hydroxycinnamic acids for O-methyltransferases that contain regions of high homology to these new motifs but that actually are not classified as COMTs.

Phylogenetic analyses based on pairwise comparison between different COMTs, CCRs and CADs, clearly reflect the genetic divergence between gymnosperms, dicot angiosperms and monocots. Moreover, a very close evolutionary relationship between sugarcane and maize sequences can be seen.

The *comt*, *ccr* and *cad* transcripts appear to happen in a parallel way in different sugarcane tissues. The mRNA accumulation patterns suggest a transcriptional regulation of these genes dependent on their specific role in lignin synthesis. Nevertheless, quantitative experiments using internal controls of gene expression have to be done in order to corroborate this hypothesis.

The number of genes coding for these three enzymes in the sugarcane genome appears to be low, which is somewhat surprising if the complexity of the genome of this species is considered. Southern blot analyses are consistent with the presence of at least two copies of each studied gene in the sugarcane genome. Nevertheless whatever the explanation, the low variability existing in this species indicates that approaches such as antisense technologies may be applied although different alleles of the gene exist in the genome.

Heterologous expression of sugarcane genes in* E.coli *and production of polyclonal antibodies

A maize COMT and sugarcane CCR and CAD cDNAs, fused to a histidine-rich linker, were over-expressed in *E. coli* under the *trc* promoter. For each construction, the expression level reached almost the 20 % of total bacterial proteins four hours after the induction of the cultures with 2.5 mM IPTG. All recombinant proteins were purified by Immobilized Metal Affinity Chromatography (IMAC) under denaturing conditions using a Ni(II)-IDA-sepharose-

4B column. The expected size fused proteins (43kD for COMT, 43kD for CCR and 30kD for CAD) were obtained after elution with high recovery and more than 80 % purity by SDS-PAGE electrophoresis.

Using the purified recombinants proteins, polyclonal antibodies were produced in rabbits with a titer higher than 1/2000 for each one of the used antigens. These antibodies were successfully tested in western blots of different parts of sugarcane plants, detecting specifically the bands corresponding to lignification enzymes in those tissues where it was expected their expression at high levels.

Production of sugarcane transgenic plants

For sugarcane transformation by using *Agrobacterium tumefaciens*, four new binary vectors were constructed. These vectors are derivatives of pDE1001 binary plasmid in which the plant selection marker was removed by *Hind*III-*Nru*I digestion. The resulting fragment, containing the *bla*, *strep* and *spc* resistance genes for plasmid selection in bacteria and replication origins for *E. coli* and *Agrobacterium*, was further ligated to different plant expression cassettes containing combinations of BASTA and hygromycin resistance genes under different plant promoters (CaMV35S and maize poly-ubiquitin 1). This way, the vectors pDUBar, pDSBar, pDUHyg and pDSHyg were obtained. All these new genetic constructions contain many unique restriction sites for cloning within the TDNA region.

A DNA fragment from maize COMT was cloned in anti-sense orientation under the maize poly-ubiquitin 1 promoter in the binary vector pDSBar. CAD and CCR fragments were cloned in anti-sense orientation under a chimeric (4xOCS enhancer + CaMV 35S promoter + rice actin-1 intron-exon) promoter, and further introduced, alone or in combination, in the binary vector pDUBar to combine the expected positive results of downregulating CAD and CCR in transgenic sugarcane plants. All four gene constructs aim at reducing the lignin content or at mofifying the lignin composition in transgenic sugarcane.

To increase the extractability of sugarcane lignin by reducing the number of reactive sites in monolignols, we decided to test the sense overexpression of poplar CCoAOMT and maize COMT genes in transgenic plants. This would result in an increased degree of methoxyl content in sugarcane lignin and decreasing the number of reactive sites for the formation of residual lignin during the deslignification process.

The use of binary vectors that contain different selective markers for plant transformation gives us the possibility to combine both strategies for modification of lignin composition through the re-transformation of the most promising clones. All these binary constructs were further introduced into *Agrobacterium* and the transformation of sugarcane is now underway. Moreover, a new construction directed to over-express poplar *ccoaomt* and *f5h* genes in sugarcane is under construction. We consider that overexpression of a distantly related (poplar) genes will be more successful than overexpression of the sugarcane clones because of the reduced chance for co-suppression events.

Putative transgenic sugarcane clones transformed with an antisense-COMT construction have been planted in a field and we already started their molecular analysis. In addition, the putative transgenic calli obtained after sugarcane transformation with the double antisense-(CAD+CCR) and sense-(COMT+CCoAOMT) constructions are now at the stage of selection and/or regeneration. The cloning and characterization of sugarcane genes involved in lignification opens up the possibility of producing plants with lower and/or modified lignin by

genetic engineering. Moreover, the obtained cDNA fragments can also serve as probes in molecular marker-assisted breeding programmes for the production of new sugarcane varieties as sources of renewable fuel, paper pulp or forage.

Acknowledgments

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Strategies for Fructan Production in Transgenic Sugarcane (*Saccharmu spp* L.) and Sweet Potato (*Ipomoea batata* L.) Plants Expressing the *Acetobacter diazotrophicus* levansucrase

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Introduction

Fructans are polyfructose molecules produced by the action of fructosyltransferases on sucrose. Included in the diet, fructans function as fiber due to their non-digestibility. Those of low polymerization degree have a sweet-taste and can be used as low-calorie edulcorants. In addition, their ingestion promotes growth of beneficial bacteria in the gut improving humans and animals health (Yun, 1996). Other uses such as fat replacement, emulsifier, etc, are possible (Fuchs, 1993).

Difficulties in fructans purification from natural sources or their production through biotechnological methods hamper their potential applications. This problem could be overcome by the recombinant production and accumulation of these carbohydrates in economically important crops. The bacterial fructosyltransferase genes from *Bacillus subtilis*, *Streptococcus mutants*, *Bacillus amyloliquefaciens* or *Erwinia amylovora* have been successfully used to transform tobacco, potato and maize into fructan-producer crops (Ebskamp et al., 1994; Van der Meer et al., 1994; Caimi et al., 1996; Röber et al., 1996). Expression of the plant 1-sucrose:sucrose fructosyltransferase gene (*1-sst*) in transgenic potato and sugar beet resulted in the production of fructo-oligosaccharides (FOS) (Hellwege et al., 1997; Sévenier et al., 1998). Enhancement of stress resistance is another application that is envisioned for fructan production in plants. These compounds may be involved in resistance to drought and low temperatures (Hendry and Wallace, 1993; Pilon-Smith et al., 1995).

Marketed FOS consist essentially of 1-kestose, nystose and fructofuranosyl nystose produced from sucrose by microbial enzymes. *Acetobacter diazotrophicus* fructosyltransferase (LsdA) yields high levels of 1-kestose (Támbara et al., 1999), the commercially most attractive FOS (Yun et al., 1990). Herein, we focus on strategies followed for the recombinant expression of LsdA in two economically important crops: sugarcane (*Saccharum spp* L.) and sweet potato (*Ipomoea batatas* L.). Both cultivars show physiological features that could allow successful production and accumulation of fructans.

Recombinant LsdA is Produced Biologically Active in Eukariotic Hosts

The methylotrophic yeast *Pichia pastoris* was chosen as a model host to study LsdA expression in eukaryotic cells, before attempting the enzyme expression in the sucrose-rich plants sugarcane and sweet potato. According to this strategy, the product of a truncated fragment of the gene encoding levansucrase (lsdA) from *Acetobacter diazotroph*icus SRT4 was expressed and secreted in *P. pastoris*

by using the methanol-inducible promoter AOX1 and the yeast acid phosphatase signal peptide sequence. After cell fractionation, LsdA was recovered active from the periplasmic fraction (81 %) and the culture supernatant (18 %) to an overall yield of 1 % of total proteins. The cell-associated and extracellular forms of LsdA produced in yeast synthesized levan as a result of the fructosyl polymerase activity upon gel incubation in a sucrose-containing solution (Figure 1). Bands corresponding to both recombinant LsdA forms were fuzzy and migrated slower than the natural enzyme used as control.

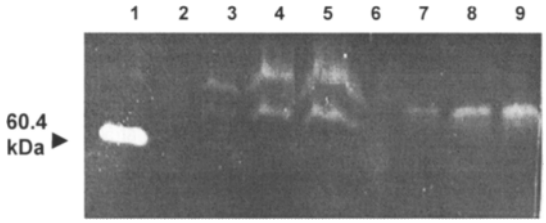

Figure 1: Levan synthesis by recombinant LsdA expressed in yeast. Culture supernatant and cells from 1 ml of fermentation broth were separated by centrifugation. The cells were resuspended in 1 ml of disruption buffer and milled with glass beads. The cellular debris was spun down. Proteins from culture supernatant and cell lysate were separated by 12.5% SDS-PAGE. The gel was rinsed in distilled water and incubated overnight in a solution of 10% sucrose, NaAc 100 mM, pH 5.2 at 42ºC. Levan formed appeared as white bands. Lane 1: *A. diazotrophicus* levansucrase (20 ng), lane 2: Wild type GS115 cell lysate (50 µg of total proteins); lanes 3,4,5: Recombinant yeast PP15 cell lysate (5, 25 and 50 µg of total proteins respectively); lane 6: 10 µl of GS115 culture supernatant; lanes 7,8,9: PP15 culture supernatant (1, 5 and 10 µl respectively).

The secreted enzyme migrated as a single band while the intracellular form appeared as a doublet suggesting the presence of different protein conformations. The higher apparent molecular mass of the yeast-expressed LsdA (expected mass 58 kDa) in comparison to that of the natural enzyme (60.4 kDa) used as control, probably resulted from glycosylation. Digestion of both recombinant LsdA forms with endoglycosidase H altered their electrophoretic mobility in denaturing SDS-PAGE as it was revealed by western blotting (Figure 2).

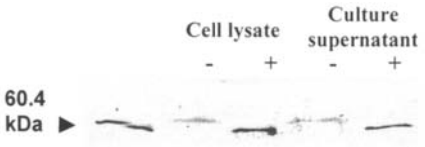

Figure 2: Glycosylation analysis of *P. pastoris*-expressed LsdA. Proteins (40 µg) from culture supernatant or cell lysate were denatured in 100 µl of 0.5% SDS, 1 % ß-mercaptoethanol at 100ºC for 10 minutes. After addition of 1/10 volumen of 1 M-sodium citrate buffer pH 5.5 at 25ºC, samples were reacted with Endoglycosidase H at 0.25 units μg^{-1} of protein at 37ºC for 10 h. Proteins (10 µg) were separated by 12.5 % SDS-PAGE and transferred to nitrocellulose membranes, then LsdA was inmunodetected. Lane 1: *A. diazotrophicus* levansucrase (100 ng), lane 2: PP15 cell lysate, lane 3: Endo H-treated PP15 cell lysate; lane 4: PP15 culture supernatant; lane 5: Endo H-treated PP15 culture supernatant.

Incubation of the secreted recombinant LsdA in the presence of sucrose (500 g l-1) yielded 1-kestose 50 % (w/w) from a maximum theoretical yield of 75 % (w/w) which represents a conversion efficiency over 67 % (Figure 3). These values are similar to those achieved by the natural LsdA (Támbara et al., 1999). Attempts to secrete the B. subtilis levansucrase in yeast by using different signal peptides were unsuccessful (Scotti et al., 1994, Scotti et al., 1996).

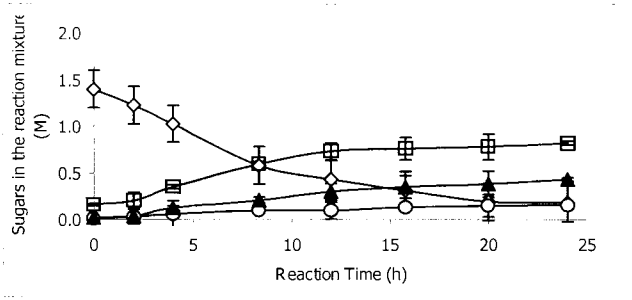

Figure 3: Time course of FOS production by secreted levansucrase produced in yeast. Sucrose at 500 g l^{-1} was reacted with extracellular recombinant LsdA at 10 units $gram^{-1}$ of sucrose in 100 mM sodium acetate pH 5.2 at 40°C with agitation at 200 rpm, for the indicated time. Products of the levansucrase transfructosylation reactions were analyzed by HPLC. Symbols represent: Sucrose (◇), glucose (□), 1-kestose (▲), fructose (O). Values represent the means of three different experiments.

Construction of Binary Vectors for Expression of the *lsdA* Gene in Transgenic Monocot and Dicot Plants

Sugarcane is a potential target crop for fructosyltransferase expression due to its high sucrose content in both, the vacuoles and apoplastic space. Sweet potato (*Ipomoea batata*) on the other hand, is a non-fructan producing plant considered one of the most important crops, around the world, for human and animal feeding, thus constitutes another promising candidate for the recombinant production of FOS. Sugar cane and sweet potato have already been transformed by *Agrobacterium tumefaciens* (Arencibia et al., 1998, Enriquez et al., 1998, 1999; García et al., 1998, 1999). Binary vectors were developed for expression of *lsdA* gene in transgenic monocot and dicot plants, and targeting of the enzyme to different cell compartments e.g. vacuoles and apoplastic space (Figure 4).

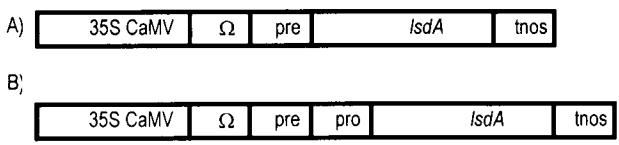

Figure 4: LsdA expression cassettes of the binary vectors used for plant transformation. Cassettes for LsdA expression and targeting to the apoplastic space (A) or vacuoles (B), respectively. 35S CaMV: 1.37 Kb long 35S promoter from Cauliflower Mosaic Virus (CaMV), Ω: synthetic Tobacco Mosaic Virus (TMV) untranslated leader, pre: sporamin signal peptide sequence, pro: sporamin vacuolar-targeting signal sequence, tnos: nopaline synthase polyadenylation sequence. These cassettes were inserted into binary vectors with kanamycin or hygromycyn resistance markers for dicot or monocot transformation, respectively.

The presence of an enzyme with fructosyltransferase activity towards sucrose should affect carbohydrate pools in the host, thus making interesting the study of the effect of LsdA accumulation in different plant locations. Since naturally occurring plant fructans accumulate in the vacuole, this cellular compartment constitutes an appropriate reservoir for the recombinant accumulation of these products. Expression of the *B. amyloliquefaciens* levansucrase fused to the targeting sequence of the sweet potato sporamin, resulted in 10 % fructan accumulation with no visible phenotype alterations (Caimi et al., 1996). Contrary,

expression of bacterial levansucrases in the cytosol of maize and tobacco cells resulted in visible plant damages (Ebskamp et al., 1994; Caimi et al., 1996).

Sugarcane transformation via *Agrobacterium tumefaciens* carrying the binary vectors designed for apoplastic or vacuolar targeting of LsdA, yielded hygromicin-resistant plants. The presence of the transgenes was detected by PCR (Figure 5). The transgenic plants, grown under greenhouse conditions, showed a normal phenotype. Sweet potato transformation experiments with constructions for LsdA expression are in progress. Rooted kanamycin-resistant plantlets have already been obtained.

Vacuolar and extracellular proteins are usually glycosylated in plants. The recombinant LsdA expression in yeast, althought glycosylated, yielded important levels of 1-kestose from sucrose. It is thus likely that LsdA targeting to vacuoles or apoplastic space of sucrose rich plants may result in a high conversion of sucrose to 1-kestose.

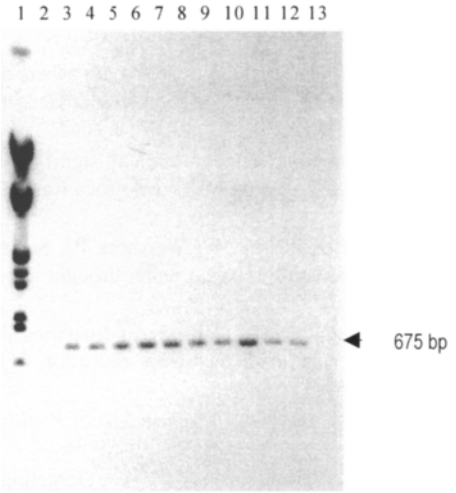

Figure 5: PCR analysis of hygromicin-resistant sugarcane plants. Lane 1) MWM, Lanes 2 and 13) Non-transgenic plants used as negative control. Lanes 3 to 8) Transgenic lines transformed with construct designed for apoplastic location of the enzyme. Lanes 9 to 12) Transgenic lines transformed with construct designed for vacuolar location of the enzyme. The amplified bands correspond to an internal 675 bp DNA fragment of the hygromicin resistance gene.

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Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Molecular Analysis of Plant Fructan Accumulation

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Introduction

Fructan (polyfructosylsucrose) is an important storage carbohydrate found in approximately 15 % of the flowering plant species. Among these plant species are major crops such as wheat and barley (Poales), chicory and Jerusalem artichoke (Asterales) and bulb-forming plants such as tulip and onion (Liliales) (Hendry and Wallace, 1993). Over the past decade the interest in using fructans in food and non-food applications has increased exponentially. A fructan rich diet has a major health promoting effect (Roberfroid, 1993). Fructans can not be digested by humans, but are efficiently used as carbon source by beneficial bifidobacteria in the colon (Gibson et al., 1995). Small fructans with degree of polymerisation (DP) of 3-6 are sweet tasting and therefore constitute natural low caloric sweeteners with a natural sugar taste. Higher DP fructans are currently mainly used in alimentary products where they function as a fat replacer. Emulsions of long chain fructans in water have organoleptic properties similar to fat. Due to their functionality such high DP fructans hold also great promise for uses in a variety of non-food applications (Fuchs, 1991, 1993).

Plant Fructan Metabolism

Based on their structure two major types of fructans can be found in plants: inulins and levans. Inulins consist of linear (2-1)-linked β-D-fructosyl units and levans have in addition linear (2-6)-linked β-D-fructosyl residues. Based on these basic structures more complex fructans are found with both glycosidic linkages (Housley an Pollock, 1993). The length of the fructosyl chain (DP, degree of polymerization) is dependent on the plant species and the physiological condition of the plant. The DP usually varies from 5 to 50. The most abundant fructan often has the lowest DP. An important characteristic of fructans is their solubility in water. Furthermore fructans are accumulated in the vacuole. Therefore fructans may be involved in osmotic regulation and are potentially involved in stress resistance (reviewed by Wiemken et al., 1995).

In inulin producing plants, like chicory, fructan synthesis involves at least two enzymes, sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan-fructan 1-fructosyltransferase (1-FFT). 1-SST catalyses the transfer of a fructose (F) residue of sucrose (G1-2F) to another sucrose molecule resulting in the formation of the trisaccharide 1-kestose (G1-2F1-2F) and glucose (G) (Figure 1). 1-FFT elongates the fructose chain by catalysing the transfer of a fructose residue of a fructan molecule to another fructan molecule. It can only use

trisaccharides or fructans with a higher DP as donor. The acceptor molecule is sucrose or a fructan with a higher DP (Koops and Jonker, 1996; Lüscher et al., 1996) (Figure 1). For synthesis of the more complex fructans additional enzymes are necessary. For example, in onion (*Allium cepa* L.) fructan-fructan 6-fructosyltransferase (6G-FFT) catalyses the transfer of a fructosyl residue of 1-kestose (G1-2F1-2F) to the carbon 6 of sucrose (G1-2F) resulting in the formation of neokestose (F2-6G1-2F) and sucrose (Vijn et al., 1997). In barley sucrose:fructan 6-fructosyltransferase (6-SFT) catalyses the formation of bifurcose (G1-2F1(6-2F)-2F) from sucrose and 1-kestose or 6-kestose when only sucrose is available as substrate (Sprenger et al., 1995)(Figure 1).

1-SST:	G1-2F + G1-2F	--> G1-2F1-2F + G
	sucrose + sucrose	--> 1-kestose + glucose
1-FFT:	G1-2F1-(2F)m + G1-2F1-(2F)n --> G1-2F1-(2F)m-1 + G1-2F1-(2F)n+1	
	Elongation of fructan chain	
6G-FFT:	G1-2F + G1-2F1-2F	--> F2-6G1-2F + G1-2F
	sucrose + 1-kestose	--> neokestose + sucrose
6-SFT:	G1-2F + G1-2F	--> G1-2F6-2F + G
	sucrose + sucrose	--> 6-kestose + glucose
	G1-2F + G1-2F1-2F	--> G1-2F1(6-2F)-2F +G
	sucrose + 1-kestose	--> bifurcose + glucose

Figure 1: Enzymatic activity of different fructosyltransferases.

At this moment, the only agronomically acceptable crop for fructan production is chicory. Chicory produces linear inulin. For the other types of fructan molecules, like the levan type and the branched fructans, no commercial source is available. Due to the limited availability of these fructan molecules the potential applicability of these fructans is still hampered.

Recently we succeeded in the isolation of the genes encoding 1-SST and 6G-FFT from onion (Vijn et al., 1997, 1998). Based on the known high sequence homology of fructosyltransferases the cDNA clones were isolated by screening an onion cDNA library with the cDNAs encoding tulip acid invertase or barley 6-SFT, respectively.

The introduction of the onion 6G-FFT into transgenic chicory resulted in a change of the type of fructan made by these transgenic chicory plants. Fructan of the inulin neoseries was made in addition to linear inulin (Vijn et al., 1997, 1998). From barley the gene encoding sucrose:fructan 6-fructosyltransferase (6-SFT) has been isolated (Sprenger et al., 1995). This enzyme is the key enzyme for biosynthesis of the branched type of fructan characteristic of the Poales. 6-SFT produces 6-kestose (G1-2F6-2F) when only sucrose is present as substrate, but the preferred reaction is the production of bifurcose (G1-2F1(6-2F)-2F) from sucrose and 1-kestose (Figure 1). Introduction of this gene into chicory resulted in an accumulation of branched fructans with β-2,6 and β-2,1 linkages in addition to linear inulin (only β-2,1) (Sprenger et al., 1997).

In Vitro Synthesis of Structurally Defined Fructan Molecules

The availability of the genes encoding enzymes with specific fructosyltransferase activities, like the barley 6-SFT, the onion 6G-FFT and 1-SST makes it possible to produce de novo

fructan molecules with different structures in agronomically important plants like sugarbeet (Koops et al., 1998).

For the *in vitro* synthesis of structurally defined fructan molecules, the various fructosyltransferases were transiently expressed in *Nicotiana plumbaginifolia* protoplasts (Goodall et al., 1990)(Figure 2).

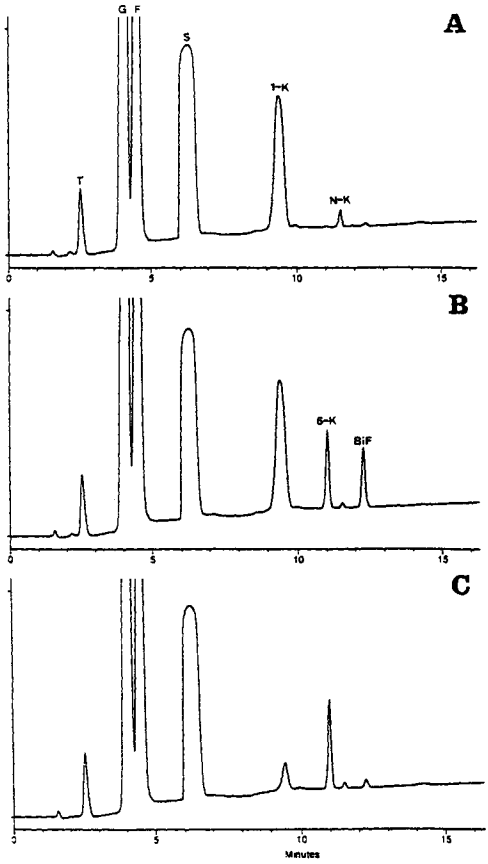

Figure 2. Analysis of the sugar products generated by either single or combined incubation of protein extracts of transformed protoplasts with different substrates. Sugar products were analyzed by HPLC after a 24 h incubation at 27 °C. (A) Combined incubation of protein extracts from protoplasts transformed with either the onion 1-SST or the onion 6G-FFT with 100 mM sucrose. (B) Combined incubation of protein extracts from protoplasts transformed with either the onion 1-SST or the barley 6-SFT with 100 mM sucrose and (C), single incubation of protein extract from protoplasts transformed with the barley 6-SFT with 100 mM sucrose. Peaks in the HPLC chromatograms: T, trehalose; G, glucose; F, fructose; S, sucrose (G1-2F); 1-K, 1-kestose (G1-2F1-2F);); N-K, neokestose (F2-6G1-2F); N, nystose (G1-2F1-2F1-2F), 6-K, 6-kestose (G1-2F6-2F)

For this purpose the genes were placed under control of the constitutive Cauliflower Mosaic Virus 35S RNA promoter and protein extracts of the transformed protoplasts were

incubated with different sugar substrates. Combined incubation of protein extracts from protoplasts transformed with onion 1-SST and 6G-FFT with 100 mM sucrose resulted in the formation 1-kestose and neokestose (Figure 2A). Incubation of only the protein extract from protoplasts transformed with 1-SST with 100 mM sucrose resulted only in the formation of 1-kestose (Figure 2B).

Incubation of only protein extract from protoplasts transformed with 6G-FFT with sucrose did not result in the production of any additional sugar products (data not shown). This shows that *in vitro* 1-SST and 6G-FFT together are capable to produce fructan of the inulin neoseries from sucrose (Vijn et al., 1998). Also a combination of protein extracts from protoplasts transformed with the onion 1-SST and the barley 6-SFT resulted in the formation of the expected products from sucrose, 6-kestose and bifurcose (Figure 2C). In contrast to onion 6G-FFT, barley 6-SFT is capable to synthesize additional sugar products from sucrose. The single incubation of the protein extract from 6-SFT transformed protoplasts with sucrose resulted in a slight 1-kestose production and in the formation of 6-kestose, but bifurcose is only produced in combination with 1-SST activity.

Expression of Bacterial Genes Encoding Fructosyltransferase in Plants

Introduction of a bacterial fructosyltransferases into tobacco, maize and potato resulted in the accumulation of fructan in these plants. Up to 20 % and 30 % of dry weight could be accumulated in transgenic tobacco and potato plants, respectively (Ebskamp et al., 1994; Van der Meer et al., 1994; Smeekens et al., 1996; Turk et al., 1997). In maize plants the level was less than 10 % of dry weight of the mature seeds (Caimi et al., 1996).

In both maize and tobacco plants the intracellular localization of fructan accumulation is important. Expression of the bacterial levansucrase into the cytosol resulted in both plant species into a changed visible phenotype. In maize the dry weight accumulation of transgenic seed was reduced to less than 10% compared to wild type seeds and fructan accumulation was low with approximately 1.6% of the dry weight (Caimi et al., 1996). Transgenic tobacco plants expressing bacterial levansucrase in the cytosol showed necrotic leaf lesions once fructans accumulated to sufficiently high levels (Ebskamp, 1994).

In fructan accumulation plants the fructans are synthesized in the vacuole. Targeting of the bacterial levansucrase to the vacuole in maize by the introduction of the vacuolar targeting sequence of the sporamin protein from Sweet potato (*Ipomoea batatas* L.) did result in a fructan accumulation up to 10 % of dry weight of the mature seeds and the fructan accumulating seeds did not show a changed visible phenotype. Transgenic tobacco plants expressing a vacuolar targeted bacterial levansucrase show complex results. Until now two different targeting signals have been used, the vacuolar targeting sequence of the yeast carboxypeptidase Y (CPY) and also the plant vacuolar targeting sequence of the sporamin protein from Sweet potato (*Ipomoea batatas* L.). Although transgenic tobacco plants harbouring the CPY targeted levansucrase accumulated fructan up to 10 %, there are now strong indications that the CPY-targeted levansucrase does not end up in the vacuole, but is retained somewhere in the endomembrane system (ER-Golgi) (Turk et al., 1997). However, these plants do not show a changed phenotype compared to wild type plants.

Although it has been shown that the vacuolar targeting sequence of sporamin is capable to translocate the ß-glucuronidase protein to the vacuole, the sporamin-levansucrase hybrid

protein is not translocated to the vacuole. The exact localization of the hybrid sporamin-levansucrase protein is not determined but it has been shown that the protein is glycosylated and is therefore probably retained somewhere in the endomembrane system (Turk et al., 1997). In contrast to the normal looking CPY-levansucrase transgenic plants the sporamin-levansucrase transgenic plants show stunted growth, bleaching of the leaves, reduced root growth and an accumulation of glucose, fructose, sucrose and starch. These results seem to be in contrast with the results in maize, as described above. However, it is not possible to compare the results obtained in maize and tobacco directly, since in maize an endosperm specific promoter is used and in tobacco the cauliflower mosaic virus (CaMV) 35S RNA promoter. Furthermore, the levansucrases used in the transformations were isolated from different bacterial strains. The *sacB* gene of *Bacillus amyloliquefaciens* was used for the maize transformation while for the tobacco transformation the *sacB* gene of *Bacillus subtilis* was used.

Up till now potato seems to be the best crop plant for fructan accumulation. The introduction of a CPY-levansucrase resulted in a accumulation up to 30 % of dry weight in the leaves and up to 13 % of dry weight in the tubers. The green tissue of the transgenic plants showed some stunted growth and tuber yield varied from 20 to 50 % compared to wild type and the tubers of the transgenic line with the highest fructan accumulation showed a brown phenotype. Upon storage at room temperature some of the tubers, which looked normal shortly after harvesting developed a brown phenotype, indicating that fructan accumulation might continue after harvesting at the expense of other carbohydrates. The use of tissue-specific promoters might overcome stunted growth and accumulation of fructan in the green tissues, resulting in an increase of fructan accumulation in the tubers.

Discussion

Introduction of the onion 6G-FFT or the barley 6-SFT into chicory resulted in the production of inulin of the neoseries and branched fructans, respectively, in addition to linear inulin (Vijn et al., 1997; Sprenger et al., 1995). This showed that the type of fructan made by a plant could be changed by introduction of a fructosyltransferase with a different specific activity than the endogenous fructosyltransferases. The *in vitro* experiments showed that it is possible to synthesize defined sets of structurally different fructan molecules from sucrose by combining fructosyltransferases with different enzymatic activities. Therefore introduction of specific sets of fructosyltransferases into plants will result in the accumulation of fructans with a structurally defined structure. As a result we are now not only able to change the type of fructan made by a plant but we will also be able to accumulate specific types of fructan molecules in plants that normally do not accumulate fructan. The applicability of fructan in the food and non-food industry has until now been hampered by a limited fructan production. This problem will be overcome by the introduction of fructan accumulation in important crops like sugar beet or potato. Bacterial genes can also be used for fructan accumulation but their proper intracellular targeting may pose problems and should be further investigated.

Acknowledgements

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Plant Genetic Engineering: Towards the Third Millennium
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Genetic Engineering of Fruits and Vegetables with the Ethylene Control Gene Encoding S-adenosylmethionine hydrolase (SAMase)

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Introduction

Fruits and vegetables continuously go through the processes of ripening and senescence during the postharvest period as evidenced by changes in color, texture, flavor nutrient content, and other qualitative attributes. It has been estimated that up to 80 % of some fresh fruits and vegetables are lost during the various stages of postharvest. The average loss reported for perishables is approximately 21 %.

In general, growers, packers, wholesalers and retailers alike routinely produce and/or receive overripe produce and are forced to sell it at a reduced price, if at all. In addition, the consumer often must consume the produce within a few days after it is purchased, since it may be overripe, and thus may not keep for prolonged periods.

Ethylene is generally referred as the ripening hormone of plants. It is responsible for the induction of genes involved in many aspects of plant physiology at very low levels. Among the diverse physiological effects, ethylene promotes ripening of fruits and vegetables, plant growth and senescence, leaf and fruit abscission, and flower fading and wilting.

The most typical examples of ethylene effects include rotting and bruising of tomatoes, apples and other fruits, discoloration of lettuce and broccoli, premature abscission of cotton bolls, short bloom/shelf life of cut flowers, and short shelf life of fresh fruits and vegetables.

Ethylene is synthesized in plants through a well-known pathway as shown schematically in figure 1 (see reviews by Theologis, 1992; Kende, 1993; Yang and Dong, 1993). In the ethylene synthesis pathway, S-adenosylmethionine (SAM) is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase. Subsequently, ACC is oxidized to ethylene by the ethylene-forming enzyme, ACC oxidase (ACO).

In addition to formation of ACC and subsequently to ethylene, ACC synthase also catalyses SAM into 5'-methylthioadenosine (MTA). These enzymatic reactions appear to be the rate-limiting step in the formation of ethylene. For example, wounding, bruising and fruit ripening induce the formation of ACC synthase and therefore, the conversion of SAM to ACC. The other reaction product, MTA, must be recycled back into the methionine pathway to provide an adequate supply of methionine as substrate for the continual production of ethylene (Figure 1).

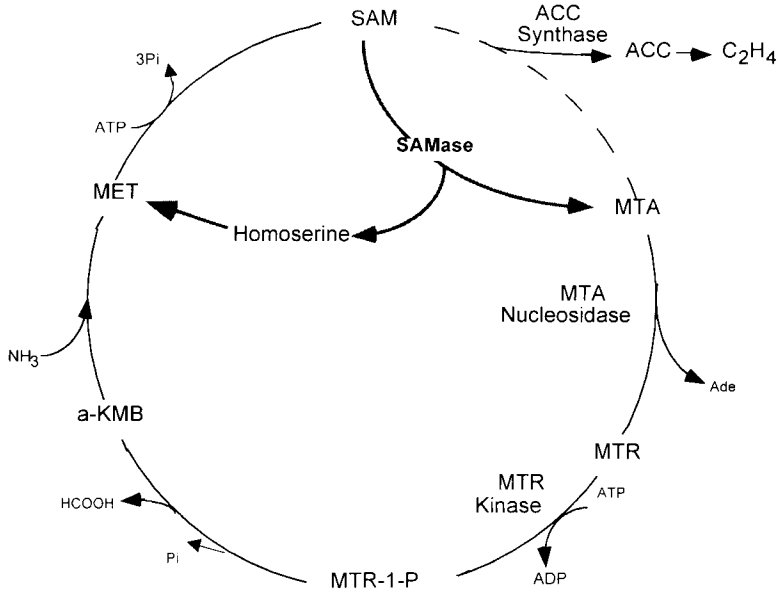

Figure 1: The methionine recycling and ethylene synthesis pathway in plants where expression of SAMase converts SAM into MTA and homoserine which re-enter the recycling pathway (shown with bold arrows) (Good et al., 1994). MET=methionine, SAM=S-adenosylmethionine, ACC=1-aminocyclopropane-1-carboxylic acid, MTA=5'-methylthioadenosine, MTR=5-methylthioribose, Ade=adenine, and a-KMB=a-ketomethylthiobutyric acid.

Biotechnological approaches to control ethylene production

Several groups have reported reduction or inhibition of ethylene in transgenic tomatoes using antisense ACO1 (Hamilton et al., 1990), antisense ACC synthase (Oeller et al., 1991), and ACC deaminase (Klee et al., 1991) methods. The transgenic fruits from these studies showed significant delays in ripening including the complete inhibition of ripening.

Most of these antisense approaches were not successful commercially because "on/off" methods of regulating gene expression may be useful only if complete gene inactivation is desirable. Complete inactivation of ethylene biosynthesis is not appropriate for the extension of shelf life in fruits and vegetables because production of ethylene is necessary for the development of plants and ripening of fruits. Furthermore, through antisense expression it is difficult to achieve intermediate levels of gene inactivation to control the ripening process in fruits and vegetables. Alternatively, the carefully modulated regulation of ethylene biosynthesis will essentially prolong but not eliminate the ripening processes and may, therefore, extend the shelf life of fruits and vegetables.

Agritope, Inc. uses an enzyme-based approach to control ethylene biosynthesis in plants (Good et al., 1994; Kramer et al., 1997) utilizing a gene (*sam*) from *E. coli* bacteriophage T3 that encodes the enzyme S-adenosylmethionine hydrolase (SAMase) (Hughes et al., 1987).

SAMase degrades SAM to homoserine and MTA, both of which re-enter the methionine cycling pathway as illustrated in figure.1 (bold arrows). SAM is the metabolic precursor of ACC and ultimately ethylene, the expression of SAMase reduces ethylene biosynthesis in plants. Because SAM plays an important role in various biosynthetic reactions, we utilize regulated gene expression in fruits and vegetables in such a way that SAMase expression is limited to specific developmental stages- or tissues- in fruits and vegetables.

Results and Discussion

Cantaloupe

Practically all cultivated forms of cantaloupe belong to the highly polymorphic species *Cucumis melo* L. that is grown for its sweet edible fruit (Purseglove, 1968). The term cantaloupe is an American usage that describes the netted melons commonly referred to as cantaloupe or muskmelon in US commerce (Everett, 1981). As a crop, cantaloupes are grown commercially wherever environmental conditions permit the production of an economically viable yield. In the United States, the principal fresh market cantaloupe growing regions are California, Arizona and Texas which produce approximately 96,000 acres out of a total annual acreage of more than 113,000 acres (USDA, 1998) that comprise approximately a $2.8 billion retail market. It has been estimated that postharvest losses, which are largely attributable to the effects of ethylene, can reach 30 % throughout the distribution chain. The gene encoding SAMase has been introduced into the *Cucumis melo* genome using standard *Agrobacterium* binary vectors. When expressed, SAMase alters the ethylene biosynthetic pathway and causes a modified fruit ripening phenotype in cantaloupe.

Non-transformed cantaloupe lines A and B are proprietary inbred lines developed by Harris Moran Seed Company, Inc, San Juan Bautista, California, USA. Both inbred lines produce fruit of the "Western Shipper" type; a round shape with a rough surface and minimal or no sutures. Fruits abscise, or "slip," when ripe, and the formation of the abscission zone is the primary harvest indicator. Transgenic line A is derived from a homozygous R1 progeny selected from an original R0 transformant. Transgenic line A was obtained through transformation of the parental inbred line A with a binary vector carrying the SAMase gene. Similarly, transgenic line B is derived from a homozygous R1 progeny selected from an original R0 transformant B that was obtained by transformation of the parental inbred line B.

There are many examples of fruit-specific and ripening-associated promoters from plants. For the expression of SAMase in cantaloupe, a novel ethylene responsive hybrid promoter was synthesized from elements of the tomato E8 and E4 promoters (Deikman et al., 1992; 1998; Xu et al., 1996; Clendennen et al., in press). The *sam-k* (modified form of *sam*) sequence was fused to the E8::E4 chimeric promoter and a nopaline synthase 3' termination sequence from *Agrobacterium tumefaciens* (Depicker et al., 1983). The SAMase expression construct also contains a selection cassette composed of the *kan*^{r} gene under transcriptional control of a proprietary constitutive promoter and the gene 7 termination sequence (Beck et al., 1982; Velten and Schell, 1985). The resulting binary expression vector was used in the transformation of the A and B parental lines, independently.

As determined by Southern blot analysis, transgenic line A contains two copies of the T-DNA insert, while line B contains one. In both lines the T-DNA insertions are at a single locus and have remained stable over three generations. Stable integration of the *sam-k*

transgene into the cantaloupe genome results in the production of a functional SAMase protein. The E8::E4:*sam-k* translational fusion present in lines A and B expresses SAMase in a fruit-specific and ethylene responsive manner, as determined by western blot analysis of total protein from different tissues of field-grown cantaloupe. Transgenic lines A and B initiate expression of the SAMase protein at varying levels in fruits that has begun to mature (full slip) and continue to express in fully mature (post slip) fruits.

Harris Moran Seed Company and Agritope, Inc. have conducted horticultural and physiological evaluations on lines A and B during the 1997 and 1998 field seasons in California, Oregon, Arizona and Texas. The parameters evaluated included harvest maturity (timing from anthesis to full slip), fruit size and weight, fruit firmness, mold susceptibility, external and internal color, soluble solids, and ethylene production in harvested fruit. Data collected from these trials, as well as from laboratory analyses demonstrate that SAMase-expressing cantaloupe lines A and B, except for the intended impact of SAMase expression on ethylene biosynthesis and related processes, do not differ from the non-transgenic parental varieties.

SAMase melons show significant reductions (up to 60 %) in ethylene biosynthesis, both as inbreds homozygous for the introduced SAMase transgene and as hybrids generated with non-transgenic lines. SAMase melons also show minimal delay in the time from pollination to maturity, and initial results indicate that the fruit of the transgenic plants may be characterized as ripening more uniformly in the field. Other horticultural traits and yield are minimally impacted by SAMase expression, with the transgenic fruit most often showing no statistical differences from the non-transformed controls.

It was frequently observed that the concentration of soluble sugars was significantly higher in the transgenic fruit compared with the controls. One possible explanation may be that full slip is achieved by the majority of transgenic fruit one to three days later than the average control fruit. The additional time on the vine may allow more sugar to accumulate in the fruit before it is harvested. These results are consistent with observations of melons exhibiting dramatically reduced ethylene biosynthesis as a result of antisense expression of ACC oxidase (ACO) (Guis et al., 1997). The ability to provide fruit with increased sweetness and prolonged shelf-life will benefit the consumer, while the uniform ripening characteristics and increased fruit firmness will benefit the grower and distributor, respectively.

Tomato

The tomato (*Lycopersicon esculentum*) is the second most important vegetable crop in the US, exceeded only by potato. In US, California is the leading state that harvests approximately 95 % of total processed tomatoes. The total US production in 1998 was 31.26 million carton weight harvested from 114500 acres with a farm value of $1.1 billion (USDA-ERS, Situation and Outlook Report, April, 1999). In US, the per capita usage of all tomatoes was estimated as 93 lbs. in which fresh and canned tomatoes share 17.4 and 75.6 lbs., respectively. In addition, the total US supermarket sales of tomato (tomatoes and tomato products) in 1997 accounted as $1.95 billion (USDA-ERS, Situation and Outlook Report, April 1999). Further more, it was estimated that US also imported a total of 1.8 billion lbs. tomatoes (with an estimated value of $0.7 billion) to meet the demands of 1998 domestic market.

Consumers for their superior flavor prefer tomatoes harvested just prior to natural abscission ("vine-ripened"). Because the vine-ripened tomatoes undergo extensive ethylene

induced post harvest damage within a few days, most fresh tomatoes are picked at the green or mature green stage and ripened by exposure to ethylene gas just prior to distribution. Unfortunately, tomatoes picked at the mature green stage stop synthesis of the compounds, which comprise the flavor, associated with a vine-ripened tomato. Fresh tomatoes harvested and ripened in this way are also lower in soluble solids and reducing sugars than vine ripened tomatoes. The only attributes of this harvest and distribution system is that the green tomatoes can withstand the pounding on the way to market and the artificial ethylene gas treatment can turn them red. An improved fresh market tomato could be picked at a more fully ripened stage and be resistant to postharvest damage through a reduced capacity to produce ethylene. Finding a way to slow down the ripening process by even 5-10 days would allow a more fully ripened tomato to be harvested and shipped over extended periods prior to sale thereby adding to the quality and consumer preference of that tomato.

A variety of fresh market tomato types including Cherry, Roma and large fruited varieties were transformed with *sam-k* using *A. tumefaciens*. However, in the present review, we have confined our discussion to the results of transgenic tomato lines in Large Red Cherry.

The *in vitro* germinated seedlings served as source of explants–cotyledon and hypocotyl for *in vitro* manipulation. The transformation method employed in Large Cherry was a modification of a protocol first described by Fillatti et al. (1987). Cotyledon tissues excised at both the tip and base were inoculated with EHA101 containing the binary vector of interest, grown in tissue culture and selected on medium containing (100μg/ml) kanamycin. Transgenic plants were generated with two pGA482-derived binary vectors, pAG5321 and pAG5420 and correspond to the 1.1kb tomato E8 promoter:*sam-k*, and 2.3kb E8:*sam-k* chimeric genes, respectively. The T0 generation of transgenic plants was grown at Agritope's greenhouse and the subsequent generations were grown at several locations both at greenhouse and field conditions. Tomatoes for RNA extraction were picked at different stages of fruit development.

The tomato E8 gene has been shown to be induced at the onset of ripening and by treatment of tomatoes with ethylene (Lincoln et al., 1987; Deikman and Fischer, 1988; Giovannoni et al., 1989; Deikman et al., 1992). Thirteen transgenic lines of pAG5321 were assayed for their ability to synthesize SAMase mRNA using an RNAase protection assay (RPA). Quantification of *sam-k* transcript was obtained after hybridization of <1.0 ng mRNA (isolated from different stages of ripening tomatoes) with radiolabeled antisense mRNA *sam-k* probe. Transgenic fruit tissues showed the presence of SAMase transcript whereas other tissues from these plants, including immature and mature leaves, flowers and stems, were negative for the presence of SAMase transcript. Although the SAMase transcript in these transgenic plants was detected in the post mature green fruit, it was repeatedly observed that the expression of SAMase mRNA was transient. An enzymatic assay for SAMase demonstrated the level of SAMase paralleled the mRNA expression pattern in extracts from the same tissues. Although there was a delay in the onset of ripening and in the number of days from the start of ethylene biosynthesis to peak ethylene production, ethylene evolution from pAG5321 transgenic tomatoes was not significantly reduced.

Extended growth and increased level of SAMase expression was obtained in plants transformed with pAG5420, where a longer 5' regulatory region from the native E8 promoter was used in place of the 1.1kb promoter in construct pAG5321. A total of three transgenic lines were obtained with pAG5420. It was clear from RPA results and quantitative

measurement of SAMase mRNA from these pAG5420 lines that the 2.3kb E8 promoter directs higher levels of mRNA expression at all three stages of fruit development in contrast to the 1.1kb E8 promoter. Ethylene evolution measurements were made from fruits picked at the breaker stage and analyzed daily in transgenic lines of pAG5420. Although the transgenic tomatoes had near normal levels of ethylene synthesis at the time of picking, the fruits were subsequently showed reduced synthesis of ethylene. The amount of ethylene produced is inversely proportional to SAMase gene expression. As SAMase gene expression is induced by the E8 promoter at the breaker and orange stage of ripening, the capacity of the tomatoes to produce ethylene is correspondingly reduced. Furthermore, the total amount of ethylene produced from these tomatoes was reduced by approximately 80 %. This pattern of ethylene biosynthesis is found to have a profound effect on the ripening physiology of these tomatoes. The time required for the fruit to develop their final ripened state was approximately two-fold longer, the level of lycopene production was reduced, and the fruit demonstrated increased firmness and a delay in senescence for as long as three months postharvest (Good et al., 1994).

Apple and pear

Among tree fruits, apple (*Malus* species) and pear (*Pyrus communis* L.) are important crops in United States with an annual production of $1.6 and 0.3 billion, respectively from approximately a total of 0.5 million harvested acres (Situation & Outlook Report of USDA-ERS, 1997). The economic benefit to tree fruit production from the use of biotechnology can be calculated from the losses experienced with the use of controlled atmosphere storage that are low at the beginning of storage period (<10 %) and increase towards the end of storage period (>30 %). Therefore, the ripening control technology has the potential for a substantial impact on the producers as well as distributors.

Recently, through genetic screening of various apple cultivars it was shown that the ripening-specific 1-aminocyclopropane-1-carboxylic acid synthase (ACS1) gene is responsible for ripening and softening of fruits (Sunako et al., 1999). Recognizing the economic value of ethylene control technology, we have recently initiated tree fruit research to introduce SAMase into commercially important cultivars of apple and pear. We have selected proprietary varieties of apple (cv. Gale Gala) and pear (cv. Bartlett) (obtained from Van Well Nursery, Wenatchee, WA) that have high qualitative and nutrient attributes but low shipability and postharvest properties due to their rapid ripening and softening of fruits.

Leaves from routinely micropropagated shoots were used for regeneration of apple and pear. Approximately 5-7 segments (horizontal to the leaf axis) were made from each leaf. The segments were placed on a shoot induction medium (modified N6 medium) supplemented with a combination of cytokinin and auxin, and incubated in the dark. Induction of callus was observed within five to six weeks of culture, and these cultures were transferred to light for differentiation of shoots.

Efficient and reproducible regeneration methods were developed in apple and pear. All of the leaves (and leaf segments i.e. 100 %) that were cultured showed induction of shoot regeneration. An average of 29 ± 13 shoots per leaf (with a range of 10 to 50 shoots per leaf) was obtained in apple cv. Gale Gala (approximately 6 shoots per leaf segment). Similar regeneration frequencies have been reported in different cultivars of apple. For example, 3.5 shoots per segment were reported in cv. McIntosh (Welander, 1988), up to 2.1 and 5.4 per leaf

were reported in cv. McIntosh and Triple Red Delicious, respectively (Fasolo et al., 1989), and a range of 1.5 to 4.5 shoots per segment were achieved in cv. Marshall McIntosh (Bolar et al, 1999).

In pear (cv. Bartlett), 96 % of the leaves (or 62 % of leaf segments) showed induction of shoot regeneration. An average of 18±9 shoots per leaf (with a range of 2 to 38 shoots per leaf) was obtained in pear cv. Bartlett (approximately 3 shoots per leaf segment). Similar results were reported in different cultivars of pear (Leblay et al., 1991). It was shown that an average of 1.2 to 3.7 shoots per leaf was recovered in different cultivars of pear.

We are currently transforming apple and pear with the SAMase gene using different fruit-specific promoters to regulate ethylene production during the climacteric stages and postharvest periods of fruit ripening. Initial experiments were carried out with two proprietary heterologous fruit-specific promoters, CH29 (from cherry) and *dru1.3* (from druplet of raspberry) to drive *sam-k* in pAG7102 and pAG4032 plasmids, respectively. Both plasmid constructs also contained the *nptII* marker gene driven by a constitutive promoter for isolating the transformed shoots through selection using the kanamycin resistance phenotype.

Our preliminary results with constructs pAG7102 and pAG4032 constructs showed a transformation frequency of 0.2 % (GG14 #1) and 0.8 % (GG30 #2), respectively in Gale Gala, and a frequency of 1 % (BA17 #1) to 3 % (BA30 #1), respectively in Bartlett. The transformed events were recovered through kanamycin selection at 50 mg/L and confirmed by ELISA for the NPTII protein.

Because of multiple shoots from a single explant (not separated as individual events), leaf samples from all recovered shoots in an explant were combined to carry out the NPTII protein assay and termed an event. Apple transformation events GG14 #1 and GG30 #2 showed 1.2 and 20.2 ng NPTII protein per mg total protein. Similarly, pear transformation events BA17 #1 and BA30 #1 showed 0.3 and 22.4 ng NPTII protein per mg total protein. We are multiplying these transformation events on propagation medium.

Because of the nature of shoot differentiation from callus, it is likely that most of the transformed shoots are chimeric in nature. Therefore, we are multiplying these transformed shoots on propagation medium supplemented with 40 mg/L kanamycin. Transformation events GG14 #1 and BA17 #1 appear more proliferative than events GG30 #2 and BA30 #1. These shoots will be transferred to rooting medium to recover plantlets, and finally transferred to the greenhouse and grafted onto mature trees. Based on the SAMase expression in cantaloupe and tomato, we are confident in harvesting the transgenic fruits with reduced levels of ethylene production to prolong the shelf life of apple and pear fruits.

Summary

Using a fruit-specific developmentally regulated SAM degradation strategy as a means to reduce ethylene biosynthesis in plants has a number of distinct advantages. The fruit-specific nature of gene expression targets only the SAM found in fruit and diverted to ACC for ethylene production. The fact that sam-k gene expression follows the normal pattern of expression of ethylene during the ripening process means that the SAMase protein is essentially transient and final concentrations in the ripe fruit are minimal. The use of an enzyme that degrades SAM may allow for the selection of a broad range of modified ripening phenotypes, which can be predictably determined by the level of SAMase protein, expressed.

We believe that these benefits will help to reduce production and handling related losses and produce a higher quality in the crop as a whole with the concomitant savings in labor and distribution costs. There also exists the potential to benefit consumers with a longer-lasting, higher-quality product

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Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Improvement of Wood Quality for the Pulp and Paper Industry by Genetic Modification of Lignin Biosynthesis in Poplar

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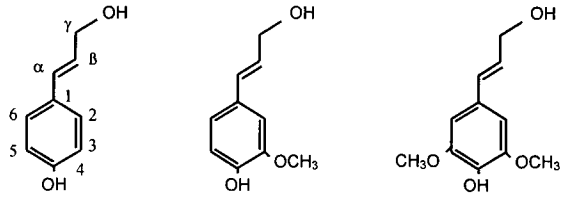
Introduction

One of the ways to meet the increased demand of wood and the worldwide concern to preserve the environment is to improve the productivity of plantation trees. A main usage of wood is for the production of pulp and paper. During chemical pulping, lignin has to be removed from cellulose. This process is toxic, energy consuming, and results in a low biomass utilization. For the pulp industry, it would be beneficial to process trees that have either less lignin, or a modified lignin that is easier to separate from cellulose. Lignin is a complex polymer deposited mainly in cell walls of supporting and conducting tissues. It is composed principally of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Figure 1). The proportion of these different units is spatio-temporally determined.



p-coumaryl alcohol (H unit) | coniferyl alcohol (G unit) | sinapyl alcohol (S unit)

Figure 1. Lignin precursors or monolignols.

Gymnosperm lignin consists essentially of G units and angiosperm lignin is mainly a mixture of G and S units. A lignin rich in G units has relatively more carbon-carbon linkages than a lignin that contains S units. This fact can be explained by the C5 position of G units that is free to make linkages whereas the C5 of S units is substituted with a methoxy group (Figure 1). The higher proportion of C-C linkages is considered to be the cause of the lower delignification rate of gymnosperm wood as compared to angiosperm wood upon chemical pulping (Boerjan et al., 1997). The lignin biosynthetic pathway derives from the amino acid

phenylalanine and follows several hydroxylation, methylation, activation and reduction steps to yield the three different lignin monomers or monolignols (Figure 2) (for a review see Boudet et al., 1995; Douglas, 1996; Baucher et al., 1998; Whetten et al., 1998).

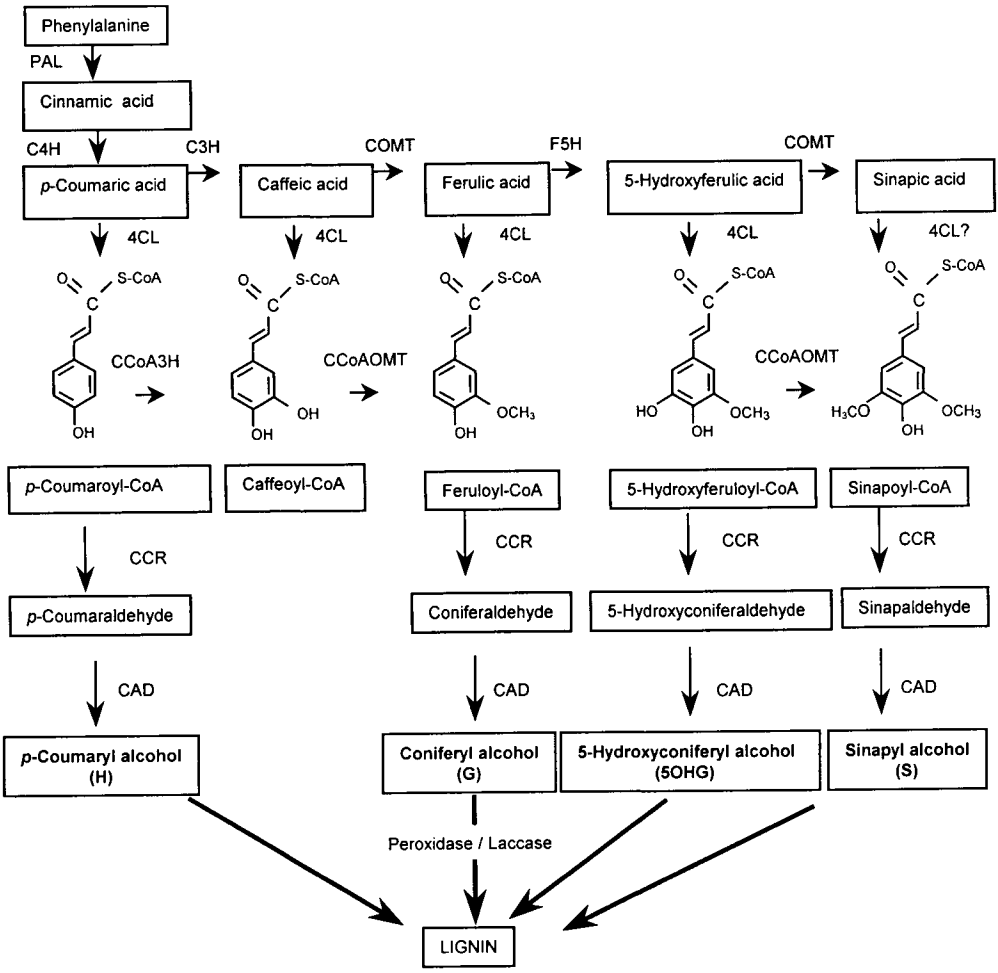


Figure 2. The lignin biosynthesis pathway. CAD, cinnamyl alcohol dehydrogenase; CCoA3H, coumaroyl-CoA 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; C3H, coumaric acid 3-hydroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid CoA ligase; COMT, bi-specific caffeic acid/5-hydroxyferulic acid O-methyltransferase; F5H, ferulic acid 5-hydroxylase; PAL, phenylalanine ammonia-lyase.

Although the different reactions occurring in the lignin biosynthesis pathway are well established, the precise order in which they take place is still unknown. Most of the genes involved in the lignin biosynthetic pathway have been cloned (Baucher et al., 1998;

Christensen et al., 1999a). Recently, the results obtained by altering the expression of several genes in the lignin biosynthesis pathway have evidenced the existence of alternative or parallel routes.

In the Department of Plant Genetics, we have cloned genes/cDNAs from poplar that encode enzymes playing crucial roles in lignin biosynthesis, i.e. caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT; Dumas et al., 1992), caffeoyl-CoA O-methyltransferase (CCoAOMT; Chen et al., 1998), cinnamoyl-CoA reductase (CCR; Leplé et al., 1998), cinnamyl alcohol dehydrogenase (CAD; Van Doorsselaere et al., 1995a) and putative lignin-specific peroxidase (POD; JH Christensen and W Boerjan, unpublished data). Using sense and antisense strategies, the expression of these genes has been altered by genetic engineering in poplar.

Here, we review the effect of these genetic modifications on lignin content and structure and on Kraft pulping. We have used poplar as a model tree. This genus has a fast growth, can be vegetatively propagated, is susceptible to *Agrobacterium tumefaciens* infection and can therefore be easily transformed. Another advantage is its small genome size that is only 4- to 5-fold the size of the *Arabidopsis thaliana* genome. In addition, poplar has a high economic value and is used in the pulp and paper industry. The model poplar line *Populus tre*mula x *P. alba* clone 717-1B4 was chosen for its efficient transformation procedure (Leplé et al., 1992).

Results and Discussion

Down-regulation of COMT

Through the antisense technology, genetically engineered trees with a 95 % reduction in COMT activity have been obtained (Van Doorsselaere et al., 1995b). No modification in the Klason lignin content was observed. However the lignin composition was strongly affected by this genetic transformation, the S/G ratio being drastically reduced because of a reduction in the S unit amount. Moreover, a significant amount of an unusual lignin monomer, the 5-hydroxyguaiacyl (5OHG), was detected in the lignin of the down-regulated COMT poplars (Figure 2). This compound is undetectable in wild type untransformed plants. Also, a lower yield in the thioacidolysis products was obtained, indicating a relatively lower frequency in ß-O-4 linkages and suggesting a higher proportion of C-C linkages. Indeed, enrichment in biphenyl (5-5') and in phenylcoumaran (ß-5) linkages was measured (Lapierre et al., 1999).

The wood of the transgenic trees was more difficult to delignify as compared to normal wood. This was indicated by the higher kappa number, reflecting the residual lignin present in the pulp after the pulping step, and the lower final brightness of the pulp of the transgenic lines (Lapierre et al., 1999; Petit-Conil et al., 1999). When the bark was removed, the wood of the down-regulated COMT lines showed a rose coloration as compared to the white color of the wild type wood. In a parallel study, Tsai et al. (1998) obtained transgenic poplars displaying a red coloration in the xylem, which was ascribed to an increased amount of coniferaldehyde, but the precise origin of the red color, is still under debate.

From these data we can conclude that a strong reduction in COMT activity does not significantly affect the quantity of lignin and that COMT plays a role in determining the incorporation of S units, but probably not that of G units in the lignin polymer. Moreover, these studies are in agreement with the hypothesis that a lignin relatively rich in G units is more difficult to extract in the Kraft pulping process.

Down-regulation of CCoAOMT

CCoAOMT is an enzyme involved in the methylation of caffeoyl-CoA into feruloyl-CoA and 5-hydroxyferuloyl-CoA into sinapoyl-CoA. This enzyme is thought to be responsible for the synthesis of G units because, as discussed in the previous paragraph, the lignin extracted from poplar down-regulated for COMT had a reduced level in S units but not in G units (see Figure 2). Transgenic poplar trees with a lower CCoAOMT expression have reduced levels of G and S units due to a decreased Klason lignin amount, and a slight increase in the S/G ratio. In addition, down-regulation of CCoAOMT reduces the kappa number whereas the yield of the pulp increases (Petit Conil et al., 1999; H Meyermans and W Boerjan, unpublished data). These results confirm the importance of CCoAOMT in the biosynthesis of lignin precursors.

Down-regulation of CCR

CCR catalyzes the first enzymatic step that is specific for the biosynthesis of the lignin precursors, that is the reduction of the hydroxycinnamoyl-CoA esters into their corresponding aldehydes. Several chimeric constructs consisting of either the full coding sequence or the N- or the C-terminal parts of the cDNAs under the control of the p70 promoter have been designed and introduced into poplar.

Some transgenic lines show an orange coloration, similarly to that observed in transgenic tobacco plants down regulated for CCR (Piquemal et al., 1998). The origin of the discoloration is still unknown but can probably be attributed to the incorporation of precursor molecules of cinnamyl alcohols in the lignin. No modification in the Klason lignin content and/or in the lignin composition was evidenced. However, preliminary-pulping analyses resulted in a significantly higher delignification rate (kappa number reduced by about 30 %) without any extended cellulose degradation (Petit-Conil et al., 1999).

Down-regulation of CAD

CAD catalyses the last step in the biosynthesis of the monolignols, which is the reduction of cinnamaldehydes into cinnamyl alcohols. Transgenic poplar trees with a reduced CAD enzymatic activity have been obtained (Baucher et al., 1996). Also the xylem of the CAD down-regulated lines displayed a red coloration. A slightly lower Klason lignin content was measured in these transgenic poplars (Lapierre et al., 1999). There was no modification in the S and G lignin unit composition but a higher content in free phenolic groups in S and G units was detected, an observation that may explain the higher solubility of the lignin (Lapierre et al., 1999).

The down-regulation of CAD in poplar has direct impact on lignin solubility and extractability. Moreover, the wood of down-regulated CAD plants was more easily delignified than control wood, which was shown by the lower kappa number of the pulp after chemical Kraft pulping (Baucher et al., 1996; Lapierre et al., 1999). Also, for a same kappa number, a higher pulp yield was obtained for the down-regulated CAD plants. This study shows that it is possible to engineer plants that can be delignified to a greater extent with lower chemical charges without any extended cellulose degradation (Petit-Conil et al., 1999), which could result in a lower utilization of chemicals in the paper industry.

Down-regulation of peroxidase

One of the possible functions of peroxidases is the polymerization of cinnamyl alcohols, although only evidence is still available. Two anionic peroxidase isozymes able to oxidize

syringaldazine, and therefore putatively correlated with lignification, were isolated from xylem tissue (Christensen et al., 1998). The corresponding cDNAs were cloned and several constructs with antibody tag sequences were made to transform poplar (Christensen et al., 1999b). The analysis of these transgenic plants as well as work aiming at altering the expression of this gene in poplar is currently underway. A summary of the results our research group obtained so far by genetic engineering of poplar is presented in Table 1.

Table 1. Genetic engineering of lignin in poplar

Gene	Enzyme activity or amount (% residual)	Lignin		Characteristics	References
		Content	Composition		
COMT	5	No changes	S/G decreased	Xylem pale rose	Van Doorsselaere et al. (1995b)
				Higher kappa	
			5OHG	Lower brightness	Lapierre et al. (1999)
CCoAOMT	10	Slight decrease	S/G slightly increased	Xylem red	H. Meyermans and W. Boerjan
				Lower kappa	
					Petit-Conil et al. (1999)
CAD	30	Slight decrease	More aldehydes	Xylem red	Baucher et al. (1996)
				Lower kappa	Lapierre et al. (1999)
				Higher pulp yield	Petit-Conil et al. (1999)
CCR	-	No changes	S/G not changed	Xylem orange	J.C. Leplé and W. Boerjan
				Lower kappa	
				Higher pulp yield	Petit-Conil et al. (1999)
POD	-	-	-	-	J.H. Christensen and W. Boerjan

S/G, syringyl/guaiacyl ratio; 5OHG, 5-hydroxyguaiacyl unit; -, not determined.

Conclusions

By modulating the enzymatic activity of several proteins of the lignin biosynthesis pathway we could obtain transgenic plants with significant changes in lignin structure. These results allow us to better understand the lignin biosynthesis pathway and the importance of lignin composition for the Kraft pulping industry. One of the major conclusions of this work is that the lignin composition can severely determine the pulping efficiency. In addition, these studies show that the lignin content does not need to be reduced to improve significantly the pulping which is beneficial when considering the plant growth. The transgenic poplars with modified COMT, CCoAOMT, CCR, CAD and peroxidase levels will be further analyzed for other characteristics such as disease resistance and calorific value. Field trials for antisense CAD, COMT, CCR and CCoAOMT plants are running. Several double transformants have also been produced and might yield transgenic plants with cumulative properties.

However more fundamental research is needed to obtain a more profound comprehension of the lignin formation and deposition. Indeed it is at present not possible to precisely predict the effect of a particular transformation on the wood properties. The epitope tagging method applied to xylem peroxidases may allow us to investigate the precise localization of these enzymes at the cellular and subcellular level.

The biotechnology applied to trees is still in its infancy but we are convinced that in the near future it will be possible to produce trees with a combination of improved properties such as wood quality, growth and disease resistance.

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Plant Genetic Engineering: Towards the Third Millennium
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Molecular Farming of Pharmaceutical and Veterinary Proteins from Transgenic Plants: CIGB Experience

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Introduction

Transgenic plants are emerging as an important system for the expression of a wide variety of foreign genes and offer the opportunity of large-scale protein production in agricultural systems. The production of foreign proteins in plants has several advantages: (1)- ease of genetic manipulation, (2)- efficiency of the transformation technology and speed of scale up, (3)- lack of potential contamination with human pathogens such as HIV, prions, hepatitis viruses, etc, (4)- conservation of eukaryotic cell machinery mediating protein modification, and (5)-low cost of biomass production.

Successful production of several proteins in plants, including human serum albumin (Sijmons et al., 1990), α-amylase (Pen et al., 1992), chymosin (Willmitzer et al., 1992), monoclonal antibodies (Table 1), vaccines (Table 2), erythropoietin (Matsumoto et al., 1995), and growth hormone (Bosch et al., 1994) has been reported. Tobacco has been used as our initial transgenic system for antibody production because *Agrobacterium*-mediated transformation is highly efficient, prolific seed production greatly facilitates biomass scale-up, and development of new "health-positive" uses for tobacco as Plantibodies. Sweet potato also has been used in our transgenic system using *Agrobacterium*-mediated transformation in order to produce animal vaccine.

I will describe briefly the progress that has been made by several groups in transgenic plants in the field of antibodies and vaccines, and a brief of some results obtained by us in these areas.

Expression in Transgenic Plants

Genetic transformation techniques are now applied routinely to a large number of plant species. The most convenient technique for introduce genes in plants is by *Agrobacterium tumefaciens* (Horsch et al., 1985) and biolistic (Sanford, 1998) mediated transformation. Majority groups have used the constitutive 35S promoter from cauliflower mosaic virus, a variety of cell types are expected to express the recombinant protein. There are many examples of plants that produced biologically active proteins as antibodies molecules for a wide spectrum of purposes as: in diagnosis, therapy, vaccines and purification of pharmaceutical recombinant proteins (Table 1) and vaccines propose (Table 2).

Table 1. Recombinant antibodies in transgenic plants: Plantibodies.

	Recombinant antibody	mAb form	Plants	Reference
1	Transition-state analogue	IgG (k)	Tobacco	Hiatt et al., 1989.
2	NP (4-hydroxy 3-nitro-phenyl)acetyl) (hapten)	IgM (λ)	Tobacco	During et al., 1990.
3	Substance P (neuropeptide)	single domain dAb	Tobacco	Benvenuto et al., 1991.
4	Anti-phytochrome	scFv	Tobacco	Owen et al., 1992 Firek et al., 1993.
5	Artichoke mottled crinkle virus	scFv	Tobacco	Tavladoraki et al., 1993.
6	Human creatine-kinase	Fab; IgG (k)	Tobacco Arabidopsis	De Neve et al., 1993.
7	Fungal cutinase	IgG (k)	Tobacco	Van Engelen et al., 1994.
8	Hapten oxazolone	scFv	Tobacco	Fiedler and Conrad, 1995.
9	p-azophenyl-arsonate	scFv	Tobacco	Wahl et al., 1995.
10	Abscisic acid	scFv	Tobacco	Artsaenko et al., 1995.
11	Streptococcus mutans adhesin	IgG (k) and IgA/G	Tobacco	Ma et al., 1995.
12	Beet necrotic yellow vein virus (BNYVV) coat protein (cp)	scFv	Tobacco	Fecker et al., 1996.
13	Root-knot nematode Meloidogyne incognita	scFv	Tobacco	Rosso et al., 1996.
14	Legumin B4 (LeB4)	scFv	Tobacco	Fiedler et al 1997.
15	Tobacco mosaic virus (TMV)-binds to virions (IgG)	scFv	Tobacco	Voss et al., 1995 Zimmermann et al., 1998.
16	Streptococcus mutans	IgA	Tobacco	Ma et al., 1998.
17	Anti-herpes simplex virus 2	scFv	Soybean	Zeitlin et al., 1998.
18	Anti membrana protein stolbur phytoplasma	scFv	Tobacco	Le Gall et al 1998.
19	Phyto-hormone abscisic acid (ABA)	scFv	Tobacco	Conrad et al., 1998.
20	Herbicides paraquat and atrazine	scFv	Tobacco	Longstaff et al., 1998.
21	Ubiquitous	scFv	Potato	Artsaenko et al., 1998.
22	Dihydroflavonol 4-reductase	scFv	Petunia	De Jaeger et al., 1999.

Table 2. Recombinant vaccines in transgenic plants.

	Recombinant antigen	Plants	Target	Reference
1	HBsAg	Tobacco	Hepatitis B	Mason et al., 1992. Thanavala et al., 1995.
2	Heat-labile enterotoxin B subunit (LT-B)	Tobacco Potato	*Escherichia coli*	Haq et al., 1995. Mason et al. 1998.
3	Glycoprotein (G-protein)	Tomato	Rabies virus	McGarvey et al., 1995.
4	Capsid protein	Potato and Tobacco.	Norwalk virus	Mason et al., 1996.
5	GAD		Diabetes	Ma et al. 1997.
6	Cholera toxin B	Potato	Cholera	Arakawa et al., 1997.
7	Insulin-CTB	Potato	Cholera	Arakawa et al. 1998.
8	Glycoprotein S	Arabidopsis	TGEV	Gomez et al. 1998.
9	VP1	Arabidopsis Alfalfa	FMDV,	Carrillo et al., 1998. Wigdorovitz et al, 1999.
10	VP60	Potato	RHD	Castañon et al., 1999

CIGB Experience

Plantibodies

We studied the secretion of antibodies in transgenic tobacco plants for this propose we cloned cDNA of a anti-hepatitis B specific scFv antibody, which binds to HBsAg, with hexa-histine tail attached to the amino terminus of the gene in order to facilitate the purification fused to signal sequences from sporamine store protein (Figure 1). A CaMV 35S promoter with double enhance and untralated sequence from TMV controlled the expression of the different scFvHb constructs in transgenic tobacco plants (Ω). Regenerated transgenic tobacco plants were analysed by, PCR, ELISA, RT-PCR, western blot and immunoelectronon microscopic, to assess expression and functionality of recombinant antibody fragment.

P35S-Ω-MASSHHHHHH-V_L-linker-V_H -- Nos C

P35S-Ω-**MKALTLALFLALSLYLLPNPAHS**MASSHHHHHH-V_L-linker-V_H- Nos_t .... AF

P35S-Ω-**MKALTLALFLALSLYLLPNPAHS**MASSHHHHHH-V_L-linker-V_H-KDEL-Nos_t .... ER

P35S-Ω-**MKALTLALFLALSLYLLPNPAHS***RFNPIRLPTTHEPAS*MASSHHHHHH-V_L-linker-V_H-Nos_t V

Figure 1. Forms of the fragment single chain antibody (scFv) anti-hepatitis B gene inserted into *Nicotiana tabacum*. The V_L and V_H domains are connected by a 14 amino acid linker peptide (underlined). The sporamine-prepeptide (bold) and sporamine-propeptide (italic) are located at 3' end of the peptide. Abbreviations are as follow: p35S CaMV, 35S promoter from cauliflower mosaic virus; Ω, nontranslate region from tobacco mosaic virus; Nost, nopaline synthetase terminator; C, cytoplasm; AF, Apoplastic Fluid; ER, Endoplasmatic Reticulum and V, Vacuole.

We verified the expression of scFv by western blotting (Figure. 2). The band at about 30 kDa corresponding with expected size of scFv gene product. Purified bacterially expressed scFv was used as a control. The amount of expression of scFv in The amount of the scFv in the three well expressing plants for each constructs (AF-12, ER-52 and V-20), was estimated about 200-300 μg/g of tissue for endoplasmic reticulum and 50-100 μg/g of tissue for apoplastic fluid and vacuole respectively. No scFv protein was detected in all plants containing the cytosol construct.The 6x His-tag fused to the single-chain Fv fragment are appropriate for purification of the recombinant scFv protein. The antibody purified was analyzed for the amount recovered its purity and biological activity. The biological activity of antibodies purified was confirmed by binding to the HBsAg. We could demonstrate that 6x His affinity chromatography is highly applicable for obtaining a biological active scFv protein from tobacco leave extracts.

The transgenic tobacco plants that expressed the scFv in apoplastic space were used for establishment a cells suspension. Immunoblot analysis showed that transformed plants accumulate high levels of scFv protein, accounting for up to 1 mg/L secreted to the medium and 5 mg/kg from intracellular. The identity of the scFv and the correct processing of the signal peptide were confirmed by N-terminal protein sequencing.

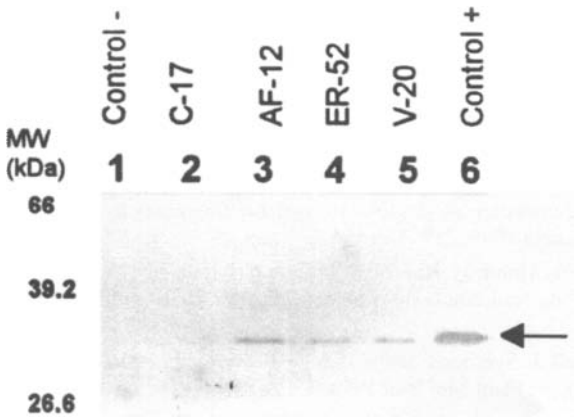

Figure 2. Western blot analysis of total soluble protein extracted from transgenic tobacco plants. The scFv antibodies were detected using the anti-Fab policlonal antibodies. The arrow indicates the 30 kDa scFv anti-Hep.B protein band.

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Plant Genetic Engineering: Towards the Third Millennium
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Toward Molecular Farming of Therapeutics in Plants

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The Exploitation of Stably Transformed Plants to Produce Recombinant Proteins by Molecular Farming

Here, we discuss the exploitation of stably transformed plants to produce recombinant proteins by molecular farming. This is an emerging technology that seems likely to have a significant impact on basic research and the pharmaceutical, agricultural and biotechnology industries. Plants have significant advantages in safety and cost over current expression systems and they produce high levels of fully functional mammalian proteins (Whitelam et al., 1993).

Bacteria have often been the expression system used for the production of recombinant proteins (Hannig and Makrides, 1998). However, plant cells are an attractive alternative to bacteria because they produce fully functional molecules with virtually identical properties to the original protein. As one example, recombinant antibodies produced in tobacco plants have the same specificity and affinity as monoclonal antibodies produced by the original hybridoma cell line (Voss et al., 1995).

Several advances have permitted the development of plant expression systems, with the convergence of recombinant DNA technology, plant transformation technology and antibody engineering at the forefront. This progress has made it possible to produce chimeric mouse-human therapeutic antibodies in plants in sufficient quantities for pre-clinical trials (Zeitlin et al., 1998; Vaquero et al., 1999). The ease with which plants can be genetically manipulated, and grown in single cell suspension culture or used for field scale production is a great advantage over the more commonly used microbial, mammalian cell culture and transgenic animal approaches.

We discuss the expression of therapeutic and diagnostic recombinant antibodies in plants and approaches for the downstream processing of recombinant antibodies (rAbs). We envisage the Molecular farming of plant based expression systems becoming one of the predominant methods for recombinant therapeutic and diagnostic protein production in the next decade.

Transgenic Plants as Bioreactors for Recombinant Protein Production

Monoclonal antibodies (mAbs) (Kohler and Milstein, 1975) are essential therapeutic and diagnostic tools used in medicine, human and animal health care, the life sciences and biotechnology. Antibody engineering has broadened the range of possible applications for recombinant antibodies, and made possible the production of novel molecules with desired

properties, such as molecules with reduced size or antibodies fused to biological response modifiers or toxins (Figure 1; Shin et al., 1993; Gerstmayer et al., 1997; Bookman, 1998).

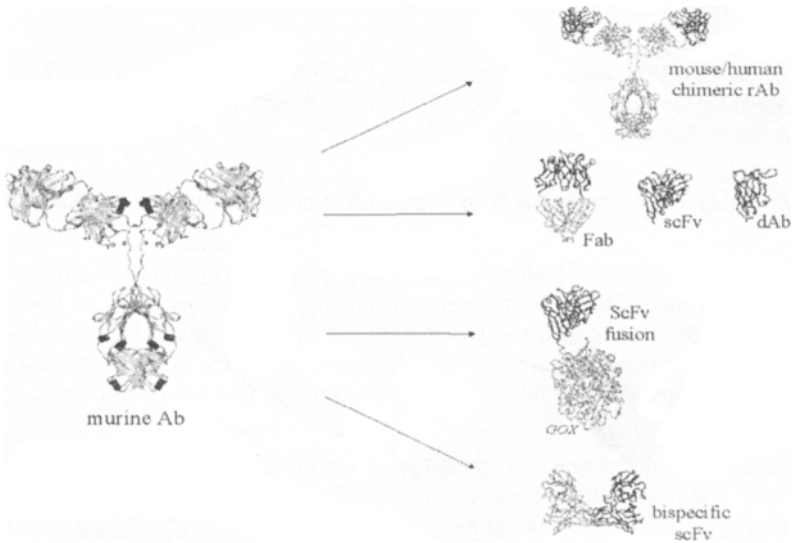

Figure 1: Examples of recombinant antibodies produced by antibody engineering. Ab: Antibody; rAb: recombinant antibody; Fab: fragment antigen binding; scFv: single chain antibody fragment; dAb: single domain antibody; scFv fusion: fusion protein with GOX (glucose oxidase).

The rapid development of phage display technologies now permits the isolation and expression of rAbs recognizing almost any target antigen and the fine-tuning of these rAbs toward desired properties, and their expression in microbes and heterologous hosts (Winter et al., 1994).

A critical development for the production of medicinal biomolecules in plants was the expression of functional antibodies in tobacco leaves in 1989 (Hiatt et al., 1989) and 1990 (Düring et al., 1990). Transgenic plants produce high levels of safe, functional recombinant proteins and can be cultivated on an agricultural scale (Whitelam et al., 1994; Whitelam and Garry, 1996). The molecular farming of recombinant proteins in plants requires only a virus-infected or a transgenic plant, water, mineral salts and sunlight. Current applications of the plant based expression systems in biotechnology include the production of recombinant antibodies (rAbs) (Ma and Hein, 1995a; Ma and Hein, 1995b), enzymes (Hogue et al., 1990; Verwoerd et al., 1995), hormones, cytokines, interleukins (Magnuson et al., 1998), plasma proteins (Sijmons et al., 1990) and vaccines (Mason and Arntzen, 1995). Chimeric plant viruses, produced in plants, are also used for the presentation of vaccines on the viral surface. Importantly, antibody expression itself can be used as a tool to modify the intrinsic properties of plants, increase pathogen resistance by the expression of anti-pathogen antibodies (Voss et al., 1995; Zimmermann et al., 1998) and alter metabolic or hormonally regulated pathways (Phillips et al., 1997).

Antibody engineering and production of monoclonal or recombinant antibodies are now standard techniques, but the ideal rAb expression system has yet to be developed. Plant based expression systems have several significant advantages over classical expression systems, such as bacteria, microbes and animal cells. Plants are easy to transform and grow and have similar pathways of protein synthesis, secretion, folding and post-translational modifications to animal cells, with only minor differences in protein glycosylation (Cabanes-Macheteau et al., 1999). Heterologous proteins accumulate to high levels in plant cells (Verwoerd et al., 1995) and plant-derived antibodies are functionally equivalent to those produced by hybridomas (Voss et al., 1995). A concern of antibody production in animal cell culture systems, the co-purification of blood-borne pathogens and oncogenic sequences, is entirely avoided using plant based expression systems and state of art purification methods. Classical methods are also expensive or require significant investment in rAb purification. Bacteria and mammalian cell cultures are the most well established methods, while yeast and baculovirus-infected insect cell systems play a minor role (Skerra, 1993; Taticek et al., 1994). Bacteria have drawbacks as an expression system since they do not produce glycosylated full-size antibodies or complex multimeric proteins (e.g. sIgA), and contaminating endotoxins are difficult to remove, recombinant proteins often form inclusion bodies, making labor- and cost-intensive *in vitro* refolding necessary. Mammalian cell cultivation can be difficult and requires expensive equipment and media supplements. In the classical expression systems, considerable care must be also taken during downstream processing of recombinant proteins to remove oncogenic sequences or viral contaminants for *in vivo* therapeutic applications. In addition, the use of transgenic animals (Echelard, 1996) as a source of recombinant antibodies is becoming limited by legal and ethical restraints.

Antibody Production in Transgenic Plants

Antibodies are the ideal model for the expression of therapeutic or diagnostically important proteins in plants (Hiatt, 1990; Hiatt, 1991; Hiatt et al., 1992; Hiatt and Ma, 1993). Expression studies have demonstrated that many forms of recombinant antibody fragments (Figure 1) can be functionally expressed and that the sub-cellular targeting of the protein is an important consideration for high level expression.

Functional full size rAbs were first expressed in transgenic plants in 1989 (Hiatt et al., 1989) and 1990 (Düring et al., 1990). Full-size antibodies (Ma et al., 1994; Voss et al., 1995; Baum et al., 1996; De Wilde et al., 1996), Fab fragments (De Neve et al., 1993) and single chain antibody fragments (scFvs) (Owen et al., 1992; Firek et al., 1993; Tavladoraki et al., 1993; Artsaenko et al., 1995; Fiedler and Conrad, 1995; Fecker et al., 1996; Schouten et al., 1996) can be expressed in leaves and seeds of plants without loss of binding specificity or affinity. However, there are technical considerations to bear in mind when planning recombinant protein expression in plants (Kusnadi et al., 1997). The pattern of codon usage in plants is different to that of animals but altering the composition of the heterologous cDNA to meet the plant pattern can increase the rate of translation (Kusnadi et al., 1997).

It is clear that the expression levels of recombinant antibodies in plants can be enhanced by exploiting the intrinsic protein sorting and trafficking mechanisms that plant cells use to target host proteins to sub-cellular compartments. Recombinant antibodies have been targeted to the following compartments of plant cells: the intercellular space, chloroplasts and endoplasmic reticulum (ER) (Düring et al., 1990; Firek et al., 1993; Ma et al., 1994; Artsaenko et al., 1995;

Voss et al., 1995; Baum et al., 1996; De Wilde et al., 1996; Schouten et al., 1996; Conrad and Fiedler, 1998). When antibodies are targeted to the secretory pathway instead of the cytosol, significant increases in recombinant antibody yield have been observed (Conrad and Fiedler, 1998). Targeting proteins for secretion to the intercellular space beneath the cell wall (apoplast) has advantages for downstream processing and also leads to significant levels of expression, however ER retention can give 10 to 100 fold higher yields (Conrad and Fiedler, 1998). Intracellular expression of rAbs in the cytoplasm has been achieved using scFv fragments (Owen et al., 1992; Tavladoraki et al., 1993; Schouten et al., 1996; Zimmermann et al., 1998), seemingly because scFv fragments require only minor post-translational processing. In the majority of transgenic plants expressing cytosolic scFvs, levels are very low or at the detection limit (Owen et al., 1992; Fecker et al., 1996; Schouten et al., 1996). There is a report where cytosolic scFvs have reached levels of up to 1.0 % of total soluble protein (De Jaeger et al., 1998), but this is still an exception. Recombinant antibody expression may be further enhanced in the future by the use of stronger and tissue specific promoters, improvement of transcript stability, translational enhancement with viral sequences (Gallie, 1998), and by using crop plants as expression systems.

Expression levels of different antibodies in stably transformed plants vary, with expression of full size IgG under the control of the 35S promoter ranging from 0.35 % (van Engelen et al., 1994) to 1.3 % of the total soluble protein (TSP) in tobacco leaves (Hiatt et al., 1989). This does not appear to be an upper limit, since transgenic plants have been identified with expression levels of scFvs in leaves reaching 6.8 % of the TSP (Fiedler et al., 1997) and levels of secretory IgA up to 500 μg per gram leaf material (Ma et al., 1995). Significantly, leaves from transgenic plants expressing ER retained scFvs can be dried and stored for more than three weeks without losses of scFv specificity or antigen binding activity (Fiedler et al., 1997). Seeds are storage organs that are often rich in protein and can be stored almost indefinitely (Fiedler and Conrad, 1995; Conrad and Fiedler, 1998; Conrad et al., 1998) and seeds can be exploited as storage containers for recombinant proteins, since single chain antibodies can reach up to 4.0 % of the TSP (Phillips et al., 1997). As with expression in leaves, ER retention gives increases in scFv accumulation and interestingly, scFvs can be stored for up to a year in seed at room temperature without losses. Potato tubers have also been used as storage containers with expression levels reaching 2 % TSP and cold storage for 18 months resulting in a 50 % loss of functional antibody (Artsaenko et al., 1998).

Expression in Stable Transformed Plants

Initial structural and functional characterization of a recombinant protein, such as an antibody fragment, can be performed by expression in *E. coli* or *Pichia pastoris*. However, when long-term production of complex molecules is anticipated, stable plant transformation is a pre-requisite. Although transient expression in plant organs or virally infected plants is prudent for checking the vectors, recombinant protein stability and protein function in plants, expression can be increased and optimized in stable transgenic plants, where the quantity of recombinant protein obtained will only be ultimately limited by the harvested area.

Plants have several key advantages over classical expression systems, as discussed earlier. High level accumulation of recombinant proteins in plants creates low production costs and some recombinant proteins already reach very high expression levels, for example, apoplast targeted recombinant phytase accumulates to ≈14 % total soluble protein (TSP) in tobacco

leaves (Verwoerd et al., 1995). Though average expression levels of recombinant antibodies in stably transformed plants are on the level of 1 to 2 % TSP, advances in promoter and protein expression technology seem likely to increase production levels in the near future. Protein synthesis, secretion, chaperonin-assisted protein folding together with the post-translational modifications (signal peptide cleavage, disulfide bond formation and initial glycosylation), are similar between plant and animal cells. To our knowledge only minor differences in further processing of complex glycans exist between animal and plant derived glycoproteins (Cabanes-Macheteau et al., 1999). Importantly, plants are the premier heterologous production system for secretory IgA antibodies.

The low costs of producing recombinant antibodies in plants are as great a benefit as the increased safety and authentic post-translational modification pathways. It has been estimated that proteins produced in plants are 10 to 50 fold less expensive than those made in *E. coli* (Larrick et al., 1998) and that these savings will be greater as production reaches agricultural cropping scales. These features justify the use of plants as an alternative, or better source for producing recombinant proteins at low cost while eliminating the disadvantages associated with microbial or animal cell systems.

Downstream Processing of Recombinant Antibodies from Transgenic Plants

Efficient purification schemes are a pre-requisite for the use of expressed recombinant antibodies for diagnostic or therapeutic uses (Baker and Harkonen, 1990; Mariani and Tarditi, 1992; Miele, 1997; Murano, 1997). Proteins must be highly purified to minimise or even eliminate any adverse clinical reactions against contaminants during clinical uses of the proteins. Compared to other expression systems, the major differences in purifying recombinant proteins from plant suspension cells arise in the very first steps of the procedure. If the protein of interest is contained in the culture supernatant, removal of cell material can be easily achieved by vacuum filtration followed by clarification of the filtrate before starting purification. However, if the target protein is located intracellularly, a suitable method for gentle and efficient cell disruption is essential. Mechanical cell disruption devices like bead-mills, although very efficient, give rise to problems related to heat generation, disruption of subcellular organelles, liberation of noxious chemicals (alkaloids, phenolics), and generation of fine cell debris, which can be difficult to remove. This can be overcome by using enzymatic methods to release antibodies from cells.

Although there are established protocols for purification of antibodies produced by animal or microbial sources, there is little data available on the purification of recombinant antibodies from plants, plant suspension culture cells, leaves or seeds (Moloney and Holbrook, 1997). We established a purification protocol for full-size rAbs produced in plant cell suspension cultures (Drossard et al., 1999). Our data demonstrate that full-size antibodies can be purified from plant cell extracts on protein-A and protein-G based affinity matrices in a similar manner to antibodies purified from animal sources. Our approach was to release antibodies secreted to the intercellular space of plant cells by partial enzymatic lysis of the cell wall and this was the superior method for isolation of functional antibodies. Integrating cross-flow filtration into the purification process significantly reduced time and labour for the clarification of a crude cell extract. Using affinity chromatography on a Protein-A matrix as the initial chromatographic step resulted in a very efficient removal of contaminants and a 100-fold concentration of the recombinant protein. Gel filtration served as a polishing step for the removal of rAb-dimers

and for exchange of the rAbs into a suitable storage buffer. Using this approach, more than 80 % of expressed full size IgG can be recovered from suspension cultured plant cells (Drossard et al., 1999). A promising approach for the isolation of recombinant proteins from plant extracts is the identification of synthetic ligands for protein isolation using phage or ribosome display (Ngo and Khatter, 1992; Clackson and Wells, 1994; Mattheakis et al., 1994).

Applications of Recombinant Antibodies Expressed in Plants

Plants are an ideal production system for therapeutic antibodies however, the applications of antibodies in plant biotechnology are wider than just using plant cells as a production system. Expressed antibodies can be used to modulate plant properties, increase resistance to pathogens, alter metabolic or developmental pathways and are being applied to increase the nutritional value of crops and remove environmental pollutants (Whitelam and Garry, 1996; Conrad and Fiedler, 1998; Fischer et al., 1998a; Fischer et al., 1998b; Longstaff et al., 1998).

Concluding Remarks

The key role for molecular farming in modern biotechnology is in its ability to increase the production levels of recombinant proteins to meet an almost unlimited demand. Transgenic crop plants offer advantages for pharmaceutical protein production since they can be grown on an agricultural scale and the recombinant macromolecule harvested from them. The most promising approach for protein expression and in field production is to target the protein to the ER and, if long-term storage is required, to target the protein for seed specific expression. Transformation of major crop plants is now becoming more straightforward. We foresee crop based expression systems (wheat, rice, corn, legumes) becoming heavily used because they have a lower content of toxic compounds than model species, like tobacco, and there is an existing infrastructure for crop cultivation, harvesting, distribution and processing.

The feasibility of using recombinant antibodies produced in plants for disease therapy and diagnosis is being evaluated because plants can produce safe, inexpensive and functional antibodies on a large scale with high yields. We speculate that it will be important to move from laboratory scale to pilot scale fermentation and toward expression of rAbs in the seeds of crop plants. Another important step will be the development of downstream processing technologies capable of large scale, fast purification of products, which will minimize processing times and proteolytic or oxidative degradation of recombinant proteins.

Molecular farming has the potential to make recombinant therapeutics as freely available as prescription drugs for the management and treatment of human and animal disease. This will have a significant economic impact. This goal may be best achieved by the combination of 4 cornerstone technologies: Protein Engineering, Expression Technologies, Molecular Farming and Downstream Processing. Protein engineering can generate novel, improved therapeutics and diagnostics and transgenic plants may serve as the ideal production system. The next decade will see more field trials of transgenic plants and clinical trials of the recombinant therapeutics and vaccines produced in them. With these advances, it seems likely that we will move towards Molecular Farming in plants becoming an economic and agricultural reality in the near future.

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Production of Autoantigens in Plant for Oral Immunotherapy of Autoimmune Diseases

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Introduction

In recent years it has become evident that plants are a valuable system for the expression of recombinant pharmaceutical proteins (Goddijn and Pen, 1995). In this regard, plants have several advantages over cell culture. Production of proteins in plants, unlike that in bacteria or mammalian cells, does not rely on expensive equipment or culture media, and therefore large amounts of proteins can be produced cheaply. Furthermore, plants, being higher eukaryotes, have a crucial advantage over bacteria in being able to properly fold, glycosylate and assemble proteins in a fashion similar to a mammalian system. This may be particularly important in producing plant recombinant protein antigens that have *in vivo* activities identical to their animal counterparts. In addition, the danger of transmitting pathogens from animal sources is avoided when plants are used, as plants do not serve as hosts for human pathogens such as HIV and hepatitis viruses. More importantly, in the case of oral vaccines, production in edible plants is very convenient. This not only avoid a costly purification as required by all other expression systems, but provide a minimally invasive method for delivery of the vaccine. Since the early work on the functional expression of a mouse monoclonal antibody in transgenic plants (Hiatt et al., 1989), efforts to develop plant systems as economical sources of pharmaceutical proteins have been steadily increasing. Today, plants are found suitable for the production, not only of antibodies, but of many other pharmaceutical proteins reviewed elsewhere (Miele, 1997; Ma and Vine, 1998).

More recently, autoantigens have been produced in plants, which may have an important impact on strategies for treating autoimmune disorders, including allergies and organ transplant rejection. Here, progress in production of autoantigens in transgenic plants will be reviewed. In addition, immunotherapy based on the induction of "oral tolerance" using transgenic plants will be discussed.

Immunological Basis of Oral Tolerance

The immune system has evolved mechanisms to distinguish foreign antigens such as bacteria and viruses from components belonging to the host's own body, which are called self-antigen. It responds specifically to foreign antigens to defend itself. T lymphocytes, specialized white cells that circulate in the blood, play a critical role in this discrimination. Activation of T cells by self antigens is under stringent control, as breakdown of tolerance to self antigens may lead to a whole array of disorders called autoimmune diseases (Kotb, 1995). For instance, in cell-mediated autoimmune disease such as insulin-dependent diabetes mellitus (IDDM), multiple

sclerosis (MS) and rheumatoid arthritis (RA), T cells active against self-tissue antigens (autoantigens) become activated and act against the host's own healthy tissue disturbing its function.

A goal in the treatment of autoimmune disease is to re-establish normal self-tolerance mechanisms. Oral administration of protein antigens, in addition to eliciting a local secretory immunoglobulin A (S-IgA) antibody response in mucosal tissues of the gastrointestinal tract, can induce a state of systemic antigen-specific unresponsiveness called oral tolerance. It is now clear that oral tolerance is an important physiological property of the immune system, whereby the organism avoids dangerous reactions such as delayed-type hypersensitivity (DTH) to ingested food proteins and other antigens, which produce inflammation and tissue damage (Weiner et al., 1994). Since mucosally induced immunological tolerance is exquisitely specific to the antigen initially ingested and thus, does not influence the development of systemic immune responses against other antigens, it is increasingly becoming an attractive alternative to drug therapy for controlling undesired immune responses associated with an autoimmune disease. The therapeutic efficacy of currently available immunosuppressive drugs is often limited by severe side effects such as toxicity and malignancy. As summarized in table 1, several experimental autoimmune diseases in animal models, including uveoretinitis, experimental autoimmune encephalomyelitis (EAE), rhumatoid arthritis (RA) and IDDM, have been shown to be inhibited by oral administration of autoantigens (Weiner et al., 1994; Weiner, 1997). In humans, oral tolerance has been used to treat patients with RA, uveitis and MS, and was found to be safe, without concomitant toxicity and no sigh of sensitization (i.e. increased antibody levels or increased proliferative responses to the fed antigens, which could potentially worsen the disease) (Weiner, 1997).

Table 1. Selected autoimmune diseases treated by oral tolerance

Animal model disease	Antigen fed
Experimental autoimmune	Myelin basic protein
Encephalomyelitis (EAE)	Proteolipid protein
Collagen-induced arthritis	Type II collagen
Uveitis	S-Antigen, Interphotoreceptor, retinoid-binding protein
Myasthenia gravis	Acetylcholine receptor
Diabetes (NOD mouse)	Insulin, Glutamic acid decarboxylase
Transplantation	Alloantigen, MHC peptide
Human disease	**Antigen fed**
Multiple sclerosis	Myelin basic protein
Rheumatoid arthritis	Chicken type II collagen
Uveitis	Bovine S-Antigen
Type I diabetes	Human insulin

The mechanisms by which oral tolerance is mediated are different depending on the amount of antigen administered. Low doses of antigen induce regulatory T cells, which migrate out of the gut to the lymphoid organs and into the general circulation system. Upon encountering the same or a similar antigen in the target (disease) tissue or organ, the regulatory T cells are stimulated to secrete suppressive cytokines such as transforming growth

factor beta (TGF-beta) to downregulate inflammatory immune responses induced by pathogenic T cells (Weiner et al., 1994). This process is known as active suppression. On the other hand, high doses of antigen induce clonal anergy. Anergy is defined as a state of T cell unresponsiveness characterized by absence of proliferation (Strobel and Mowat, 1998). Under this condition, pathogenic T cells are not deleted, but are rendered intrinsically incapable of responding, by proliferation, to a specific antigen. Therefore this mechanism will help eliminate inflammatory immune responses induced by pathogenic T-cells. Moreover, high doses of antigen also induce clonal deletion, resulting in elimination of antigen-specific T cells, which include both pathogenic T cells and regulatory T cells. The site of deletion has been located within the Peyer's patches of the gut (Weiner et al., 1994).

Depending on the type of immune response measured, induction of oral tolerance may also reflect a polarized activation of T helper 2 (Th2) cells with downregulation of T helper 1 (Th1)-dependent immune responses through the production of anti-inflammatory cytokines such as interleukine-4 (IL-4) and IL-10 (Liblau et al., 1995). It has been shown that the majority of autoimmune diseases studied (IDDM, MS and RA) appear to be mediated by interferon γ (IFNγ)- secreting Th1 cells, whereas protection and recovery from these autoimmune diseases is thought to be a result of prevailing Th2 responses (Liblau et al., 1995).

Selection of Candidate Autoantigens for Plant Expression

There are only a few published reports of candidate autoantigen (transplantation antigen included in this review) expression in transgenic plants. These have focused on three proteins: glutamic acid decarboxylase-67 (GAD-67) (Ma et al., 1997), insulin (Arakawa et al., 1998) and major histocompatibility complex (MHC) class II molecules (Ma and Jevnikar, 1999). The justification for utilizing these autoantigens is threefold. First, the roles of each autoantigen in autoimmunity, including their ability to downregulate or tolerate autoimmune responses when orally administered has been well defined in experimental animal models. Second, bioassays and animal models were available to allow for the convenient immunological characterization of plant-derived recombinant antigenic proteins, especially with regard to their original action as oral tolerogens in the induction of oral tolerance. Third, although many antigens hold promise for immunotherapy of human autoimmune disease, production cost may limit their clinical use. Each antigen, therefore, represents a significant opportunity for the development of new therapeutic agents.

Glutamic acid decarboxylase

Insulin-dependent diabetes mellitus (IDDM) is a common autoimmune disease in children and young adults (Atkinson and Maclaren, 1994). In this disorder, cells producing insulin (beta cells) within the pancreatic islets are specifically destroyed (Bosi and Botazzo, 1995). In the United States the lifetime risk for developing IDDM is approximately 1 in 300 individuals, with an average yearly incidence of approximately 15 per 100,000. In Canada, about 150,000 persons have diabetes. Clinical onset of IDDM is often associated with serious complications: blindness, cardiac and kidney failure.

The immunological mechanisms underlying human IDDM are still not fully understood, but studies in non-obese diabetic (NOD) mice, a widely accepted animal model of human IDDM, suggest that IDDM is the result of a genetically associated autoimmune process in

which the selective destruction of insulin-producing pancreatic beta cells is mediated by autoreactive T cells (Atkinson and Maclaren, 1994). The autoimmune response seems directed against a number of beta-cell components, of which glutamic acid decarboxylase (GAD) is one of the more important (Kaufman et al., 1993; Tisch et al., 1993). Antibodies to GAD can develop many years before the clinical onset of IDDM and are detected in most recent-onset IDDM patients (Hagopian et al., 1993). Experiments in NOD mice, which showed an early appearance of GAD-reactive antibodies and T cells (Kaufman et al., 1993; Tisch et al., 1993), and demonstrated protection of diabetes by early GAD treatment process (Elliott et al., 1994; Tian et al., 1996), suggest that GAD is a key antigen in the disease. From a therapeutic perspective, GAD may therefore be an appropriate agent for antigen-specific immunotherapy such as induction of oral tolerance to GAD. The role of oral GAD in the prevention of IDDM has not been tested clinically yet due to the inaccessibility of recombinant GAD in large enough quantities. Poor GAD protein solubility in bacteria and inadequate production capacity from mammalian cells has made it difficult to use these conventional expression systems for GAD production (Katarova et al., 1990). Moreover, recombinant GAD expressed in *E. coli* contains endotoxin, a potent inducer of T-cell proliferation, which interferes with the induction of oral tolerance. This has prompted us to express GAD antigen in transgenic plants.

Insulin

Insulin is a major constituent of the β cells in the pancreas and is itself another β-cell autoantigen that appears to have an important role in the pathogenesis of diabetes. Insulin antibodies are present in prediabetic and new-onset diabetic patients (Palmer, 1987). Insulin-specific T cells from NOD mice can mediate the adoptive transfer of diabetes (Daniel et al., 1995). Injection of NOD mice with insulin or insulin peptides was shown to inhibit the development of diabetes and insulitis (infiltration of the islet cells with inflammatory lymphocytes preceding the onset of diabetes) (Atkinson et al., 1990). Insulin therapy by injection has also been shown to delay IDDM in humans (Keller et al., 1993). Oral insulin therapy would have advantages of easy administration and lack of toxicity. In the United States, a major trial of oral insulin has recently been undertaken in newly diagnosed diabetic patients to determine whether induction of orally induced tolerance to islet cell antigens can prevent the clinical IDDM (Ramiya et al., 1997). The results of this study are not yet available. The expression of insulin in edible transgenic plants appears to offer an attractive alternative means of evaluating efficacy of oral insulin therapy in IDDM.

Major histocompatibility complex (MHC) class II antigen

Organ transplantation is increasingly becoming an attractive treatment in many clinical situations where end-stage organ failure is the result of disease. It is now physically possible to transplant organs such as kidney, heart, lung and liver. A difficulty in organ transplantation, however, is the necessity of drug-based broad immunosuppression to prevent allograft rejection (a graft of tissue from non-self donor of the same species). Potentially serious side effects of panimmunosuppression include opportunistic infection, drug toxicity and malignancy. The goal in clinical transplantation therefore remains the achievement of a state of tolerance without the need for long-term recipient immuno-suppression. Since the principal targets of the immune response to allografts are the major histocompatibility complex (MHC) molecules and T-cell recognition of allo-MHC is the primary and central event that initiates allograft rejection, induction of tolerance to donor-specific MHC antigens (alloantigens) would be an effective approach to prevent graft rejection. Indeed, several groups have

demonstrated that feeding cells expressing MHC class I and/or class II antigens induced antigen-specific tolerance to vascularized organ allografts in animal models (Craston et al., 1986; Madsen et al., 1988; Pouteil-Noble et al., 1991). Moreover, purified MHC class I antigen alone or a combination of both class I and II has been shown to be capable of inducing tolerance to renal allografts (Foster et al., 1992; Poutell-Noble et al., 1993). More recently, synthetic peptides of MHC class I and/or II molecules have been used to suppress graft rejection (Sayegh et al., 1992; Weiner, 1997). From a clinical perspective, however, the induction of oral tolerance to alloantigens using purified whole MHC molecules or its corresponding synthetic peptides may not be realistic. Intact MHC molecules are difficult to purify and the *in vitro* synthesis is hindered by the complexity of the molecule, while synthesis of many MHC-based candidate peptides in quantities sufficient for long term oral tolerance studies is often prohibitively expensive. We have therefore chosen transgenic plants as an alternative system for production of MHC antigens.

Expression of Autoantigenic Proteins in Plants and Testing for Oral Tolerance

Two basic strategies have been used to produce recombinant proteins in plants. One involves the generation of transgenic plants by stable genomic integration of a transgene by *Agrobacterium* T-DNA vectors (Zambryski, 1988). The other involves the use of plants for transient expression of transgenes carried by plant viruses (Porta and Lomonossoff, 1996). Each approach has its advantages and disadvantages. So far, all published reports on autoantigen production in plants have been focused on the *Agrobacterium* T-DNA vector system.

Glutamic Acid Decarboxylase 67 (GAD 67)

There are two isoforms of GAD in mammals, GAD65 and GAD67 (Erlander and Tobin, 1991). Whereas rat and human pancreatic islet express GAD65 predominantly (Hapopian et al., 1993), mouse islets express both GAD65 and GAD67, and GAD67 with GAD67 predominating (Kim et al., 1993). We therefore set out first to express GAD67 in plants to take advantage of excellent mouse model for testing. The expression of GAD67 at levels of up to 0.4% of total soluble protein in both potato and tobacco has been demonstrated (Ma et al., 1997). Western blot showed that the size of plant-derived GAD is no different from that of its mammalian counterpart. To assess whether the recombinant protein retained its ability to alter T-cell responses, plant tissue expressing GAD67 was added to the diet of NOD mice. After 4 weeks of diet supplementation, spleen T cells from treated mice were analysed for their capacity to proliferate in response to GAD67. As expected, proliferation of cells from mice fed plant GAD67 for 4 weeks was markedly reduced, while spleen T cells from control plant-fed mice retained capacity to proliferate when incubated with purified GAD67. The treated mice were also assessed for anti-GAD antibody responses. Although serum levels of total anti-GAD IgG antibodies changed little in either control mice or GAD67- treated mice, it was found that after challenge with purified GAD67 in the foot pad there was a twofold increase in anti-GAD IgG antibody in GAD67-fed mice compared with control mice. Further analysis on the isotypes of anti-GAD IgG antibody in treated mice revealed an increase in serum IgG1 anti-GAD antibody. No changes in IgG2a anti-GAD antibody were found in either GAD plant or the control plant-fed group. Moreover, analysis of cytokine in supernatant derived from spleen T cells activated *in vitro* in response to challenge with purified GAD67 showed that the concentration of IFN-γ was reduced but accompanied simultaneously with some increase of

IL-4 and IL-10 in the treated mice as compared to that in control mice. Taken together, these results suggest that feeding NOD mice with GAD67-expressing plant tissue can modulate immune responses towards the predominance of a protective Th2-type reactivity as characterized by selective induction of IgG1 GAD-specific antibodies and production of Th2 cytokines (IL-4 and IL-10).

To further determine the effects of feeding transgenic plant tissues producing GAD67 on suppression of diabetes, the diet of young female NOD mice were supplemented with GAD67-containing potato tuber or with GAD67-containing low-alkaloid tobacco leaf tissues from 5 weeks to 8 months of age. The amount of GAD consumed was calculated to be approximately 1mg per mouse daily. As shown in figure 1, ten out of twelve NOD mice fed either GAD-containing potato (n=6) or GAD-containing tobacco (n=6) remained free of disease (p=0.007 from controls). In contrast, in control experiments with plant lacking GAD 67, 8 out of 12 NOD mice (67 %) equally divided between tobacco and potato supplementation, developed diabetes. These results, which are comparable to those previously reported with other methods of GAD immunization (Elliott et al., 1994), suggest that oral plant GAD67 may offer an effective alternative approach to treatment of IDDM.

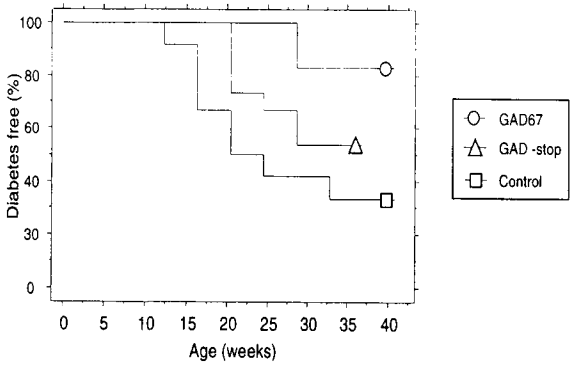

Figure 1. Oral administration of plant material expressing mouse GAD67 prevents development of diabetes in NOD mice. Female NOD mice (n=12/group) were fed plant GAD67 or control plant material from 5 week to 8 months of age. All mice were followed for the onset of hyperglycemia (defined as blood glucose > 16.7 mmol./l). Ten out of 12 NOD mice fed either low-alkaloid tobacco (n=6) or potato GAD67 (n=6) remained free of disease (p=0.007 from controls) with equivalent protection using tobacco or potato. In contrast, 8 out of 12 control plant-fed mice developed diabetes. In a separate experiment, NOD mice were given GAD67 plant material for about 2 months (8 week duration) followed by standard chow, they showed a delay in disease-onset, but the incidence level of diabetes eventually returned to that of control groups (shown as GAD-stop).

Insulin autoantigen

It had been previously reported that chemical coupling or genetic fusion of CTB with selected autoantigens can enhance oral tolerance by allowing the reduction of antigen dose and dosing schedule (frequency of antigen feeding) required for a similar level of tolerance induction by autoantigen alone (Sun et al., 1996; Bergerot et al., 1997). With this in mind, Arakawa et al. (1998) studied the expression of insulin as a form of fusion proteins with cholera toxin non-toxic (CTB) subunit in transgenic potato. Their goal was to use CTB as a carrier molecule for efficient mucosal delivery of the conjugated antigen. Immunoblot analysis of plant-derived

CTB-insulin fusion protein revealed a correct oligomeric form of the protein, a property determined by the CTB partner. In bacteria, CTB is synthesized as a pentamer, which is essential for optimal binding affinity to GM1-ganglioside on cell surfaces of the gut-associated lymphoid tissues (GALT). The plant-derived CTB-insulin fusion protein was found to retain its native immunogenicity to both CTB and insulin. Like plant-derived GAD antigen, feeding NOD mice with transgenic potato tuber tissue containing CTB-insulin fusion protein provided protection from diabetes.

MHC class II I-A^k antigen

MHC class II antigens are heterodimeric cell-surface glycoproteins consisting of one heavy (33-34 kd), or α, and one light (28-29 kd), or β, chain. The two chains are noncovalently associated, and both extend through the cell membrane (Germain and Malissen, 1986). In the mouse, MHC class II antigen exists as two non-allelic forms, I-A^k and I-E^k (Germain and Malissen, 1986). Recently we used the cDNA sequences encoding MHC class II I-A^k α, or β, or α and β chain polypeptides to transform low-alkaloid tobacco plants. Western blot analysis of transgenic tobacco leaf extracts showed the presence of unique protein bands recognized by antibodies specific for α or β chain protein (Ma and Jevnikar, 1999). The level of recombinant MHC class II protein accumulated in plant cells is relatively high, accounting for approximately 1% of total soluble protein. On the other hand, it appears that neither the α chain nor the β chain subunit is stable when separately expressed in tobacco, as in either case the amount of protein accumulated was calculated to be only about 0.005 % of total soluble protein. Evaluation of the oral immunosuppressive effects of the plant-derived MHC class II protein in a mouse kidney transplant model is under way.

Future Directions

The results discussed in this review clearly indicate that plants can be exploited as a new antigen source for an oral tolerance strategy to treat autoimmune disorders, including transplant rejection. The plant-based oral antigen therapy would have advantages of being simple, low-cost and minimally invasive. It is anticipated that these advantages will become more prominent when additional autoantigenic proteins are expressed in transgenic plants and assessed for induction of oral tolerance. It should however be noted that the encouraging results achieved in experimental animal models do not necessarily guarantee success in humans unless some modifications are made. Current protocols of mucosally induced tolerance have had limited success in suppressing an established or existing disease process, the most frequently encountered clinical case, where patients already have an overt autoimmune disease. By analogy with mucosal vaccines aimed at inducing immune responses to infectious pathogens, induction of immunological tolerance by mucosal administration of most antigens requires considerable amounts (grams to kilograms) of antigen/tolerogen and, unless the antigen/tolerogen is administered repeatedly over long periods, is of relatively short duration (Sun et al., 1996; Bergerot et al., 1997). It is therefore necessary to maximize the efficacy of oral tolerance to make the treatment clinically effective in patients with the disease and at the same time to minimize the doses in order to keep the cost low. In this regard, one feasible approach is probably to engineer plants for expression of chimeric autoantigens, which will consist of cholera toxin B subunit (CTB) coupled to antigens of interest. As discussed above, such a mucosal delivery system in which a soluble protein antigen is coupled to a mucosa-binding nontoxic carrier molecule may be advantageous for inducing oral

tolerance. A second useful approach will be to develop transgenic plants co-expressing autoantigen and Th2 cytokines such as IL-4 or IL-10. As one of the mechanisms of oral tolerance involves the generation of Th2-type regulatory cells, agents that promote the development of Th2 cell responses, hence of Th2 cytokine expression, may therefore enhance oral tolerance of the fed autoantigen. It has been well established that cytokines themselves play the most important roles in T helper cell differentiation and direct precursor Th0 cells towards Th1 or Th2 lineages (Mosmann and Coffman, 1989). Moreover, orally administered cytokines are immunoreactive while alleviating the severe side effects associated with their systemic administration (Rollwagen and Baqar, 1996). Expression of IL-4 in transgenic plants has recently been reported (Magnuson et al., 1998).

The prospects for application of plants in immunotherapy of autoimmune diseases are bright. Plants can provide not only an economical source of large quantities of autoantigenic proteins, but permit convenient oral delivery of these antigens as transgenic food stuff. It is hoped that in the near future clinical treatment of patients with an autoimmune disease will simply involve eating vegetables and fruits.

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Plant Genetic Engineering: Towards the Third Millennium
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Safety Assessments for Commercialization of Transgenic Crops and Results of Commercialization

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What is Plant Biotechnology?

Biotechnology is just what the term implies - the practical application (technology) of the science of life (bio). Genetic engineering is one form of biotechnology. An ancient example of biotechnology is fermentation in which yeast, a living organism, is used to produce leavened bread and beer. Through plant biotechnology, a selected gene (or genes) is inserted into the cells of a plant and all future generations of that plant will contain that gene. An extension of traditional plant breeding, biotechnology provides a more precise way to give crops specific, beneficial traits.

Through biotechnology, varieties of crop plants can be enhanced to enable farmers to produce more food at lower cost in sustainable ways and provide consumers with a more abundant, higher quality food supply. The initial crops first introduced in 1994, that have been modified through biotechnology have produced foods that are equivalent to the foods developed using traditional breeding, but in a more sustainable way. In the future, biotechnology also will be used to improve the quality, function or nutritional aspects of the crops.

Why is Plant Biotechnology Necessary?

Population is increasing worldwide, yet the amount of arable land available for the production of food is diminishing. In order to produce enough food, farmers everywhere will need crop plants that are higher yielding and require fewer inputs, such as insecticides, fertilizers and herbicides.

- By the year 2025, experts predict that Global population will increase from today's 5.8 billion to 8.5 billion people, with greatest growth in developing nations.
- Available, prime agricultural land will remain at about 1 percent of the world's landmass.
- The food gap - the difference between food production and demand - will more than double from 94 million tons in 1993 to 228 million tons.
- People in developing countries will consume twice as much meat.
- Life expectancy, worldwide, will rise from the current average of 68 years to 73 years.

Though traditional plant breeding and agricultural chemistry have increased yields remarkably since the 1960s and 1970s, new technologies that sustain the environment, reduce costs, increase yields and provide more nutritious food are needed. Because biotechnology

enables researchers to provide plants with new, beneficial traits not possible before, it can significantly increase the variety of plants farmers grow, while it reduces the cost of production and protects the land.

How Does Biotechnology Improve Crop Plants?

Plant biotechnology has been used to improve crop varieties by giving them built-in protection against a specific insect; built-in protection from a specific plant disease, or built-in tolerance to a specific herbicide (weed killer). By growing varieties with these traits, farmers can more effectively control the weeds, insects and diseases that reduce yields, and do so cost effectively.

Biotechnology also is being used to improve quality traits in crop plants to enhance their nutritional value; increase their protein, sugar or amino acid content, or decrease the level of saturated fat they contain. Improvements in flavor - such as tomatoes modified to ripen more slowly so they can stay on the vine longer-and improvements that make food easier to process are also among the benefits of biotechnology.

Herbicide-tolerant plants

In many parts of the world, such as North America and Europe, herbicides (chemical week killers) are used in almost all crop fields to control the weeds that would otherwise compete with the crops for sunlight, moisture and nutrients. In growing soybeans, for example, weeds are the greatest limiting factor for a successful crop. Consequently, American farmers traditionally have used herbicides in more than 95 percent of their soybean fields. Many herbicides must be worked into the soil or applied before the crop emerges from the soil because they can harm the soybeans as well as the weeds if they are applied over the top of the growing crop.

Herbicide-tolerant plants such as *Roundup Ready* canola, *Roundup Ready* corn, *Roundup Ready* soybeans and *Roundup Ready* cotton are developed by introducing a gene into the plant that enables the plant to continue growing even when a herbicide is applied that usually would harm it. With these plants, the farmer can apply the herbicide to weeds that are competing with the crop plants without destroying the crop.

Though there are other herbicide-tolerant plants available, *Roundup Ready* varieties enable farmers to use *Roundup* herbicide to control the weeds that compete with their crop.

This kind of built-in tolerance to a specific herbicide, such as *Roundup*, offers a number of environmental and agricultural benefits:

- Herbicides can be applied on an "only as needed" basis after weeds appears.
- Herbicide-tolerant plants fit well with and, in fact, encourage farmers to adopt no-till or conservation tillage systems, which help prevent soil erosion.
- In many cases, fewer herbicide applications or trips through the field are necessary.
- Weed control is improved, which leads to higher yields.

Herbicide-tolerant soybean, cotton, canola and corn plants now are being grown commercially in various parts of the world.

Disease-protected plants

Plant diseases caused by viruses or fungi can seriously reduce crop yield, so researchers have used biotechnology to give the plants defenses against disease. By transferring a non-disease-causing gene from the virus or fungus into the plant's genetic material, the plants receive built-in protection similar to the kind of protection people get through immunization. In some cases, farmers have used insecticides to control plant viruses by controlling the insects that transfer the virus from plant to plant, while fungicides are used to control fungi. Conventional breeding also has been used successfully in many cases to control disease in plants, but biotechnology is providing added, effective defenses.

The benefits of built-in disease protection include decreased farming costs and increased yields; less dependence on chemical disease control products; and higher quality vegetables and other food crops. Researchers have developed or are developing disease-protection in a wide variety of crop plants, including potatoes, squash, tomatoes, corn, canola, soybeans, grapes, cantaloupes, cucumbers and alfalfa.

Research Results

Virus-resistant potato research for Mexico

Potato production is a vital component of Mexico's agricultural economy, but annual yield losses due to a number of diseases are significant. Because these diseases cannot be controlled through chemical means, the development of virus-resistant potatoes through biotechnology is an important goal.

In 1991, the Mexican government and Monsanto began a joint research project with several leading Mexican scientific institutions to develop white potatoes that would naturally resist potato virus X (PVX), potato virus Y (PVY) and potato leaf roll virus (PLRV). The project focused initially on three potato varieties-Alpha, Nortena and Rosita-which account for a majority of Mexico's total potato production. Field trials are on going and farmer technology adoption could begin as early as 2000.

Indications are that potatoes modified to resist PVY and PVX will increase yields by 5 percent, with yield increases up to 22 percent when PLRV is added. These productivity increases will raise income levels among Mexican potato farmers, while they lower the cost of food for Mexican consumers. Perhaps more important, research in Mexico shows that the new technology which is incorporated in the potato seed can benefit both small-scale and large-scale farmers, helping to ensure equitable participation in the technology and its benefits.

Insect-protected plants

Plants that protect themselves against harmful insects such as *YieldGard* corn, *Bollgard* cotton, and *NewLeaf* potatoes reduce the environmental aspects of reliance on chemical insecticides in order to control pests. These plants benefit from an additional gene that enables the plant to produce a protein in its tissues, which controls specific harmful insects, such as the cotton bollworm or the European corn borer.

One protein used for this purpose comes from a common microbe called *Bacillus thuringiensis* (*B.t.*), which is naturally present in soil and known for its activity against certain insect pests. First discovered in 1902, *B.t.* has been studied for many years and has been found

to be harmless to humans, fish, wildlife and beneficial insects. *B.t.* has been used for decades as a foliar spray for the control of plant pests in a wide variety of crops. Adding the *B.t.* gene to crop plants provides them with continuous protection against certain insects that can reduce yield and increases the amount of income the farmer can make from his crop.

The benefits of built-in insect protection include: improved insect control; a reduction in the time, labor and money for use of chemical insecticides; and a sustainable, ecological approach to pest control. To date, cotton plants protected from bollworms (Table 1), corn plants protected from European corn borers and potato plants protected from Colorado potato beetles have been developed and commercialized in several countries.

Table 1. Bollgard performance by country per hectare.

Country	Insect Adv.	Yield Adv.	Total Adv.
Argentina	+$14	+256kg	+$99
South Africa	+$4	+300kg	+$126
Mexico	+$63	+434kg	+$185
United States	+$40	+8.4kg	+$100

Research Results:

Field trials illustrate* Bollgard's *benefits for China

Though India is the world's largest cotton growing nation in terms of number of hectares planted, China actually produces more cotton than any other country. Here, more than four million hectares of cotton are planted annually. Here, too, insect pressure is intense and farmers often spray their conventional cotton fields as many as 15 times a season to control Lepidopteran insects.

In 1997, Monsanto established on-farm demonstration trials for *Bollgard* insect-protected cotton in Hebel Province, one of China's major cotton growing regions. The results of these trials were truly impressive. Farmers who grew conventional cotton in Hebel sprayed their fields an average of 12 times during the 1997 growing season and realized a yield of 825 kg of cotton lint per hectare. In contrast, hectares planted with *Bollgard* required zero insecticide applications and yielded 1,125 kg of cotton lint per hectare.

In all, hectares planted with Monsanto's *Bollgard* provided farmers with about a 30 percent yield advantage over hectares planted in conventional cotton, while it eliminated the time, labor and money Hebel's farmers normally spend on insecticide applications. In an area of the world where profit margins are thin, *Bollgard* provides farmers with a significant advantage over conventional cotton systems.

Are Foods Developed Using Biotechnology Safe To Eat?

Years of research and testing have shown that commercially available foods developed through biotechnology are substantially equivalent to foods developed through traditional plant breeding and, therefore, are safe to eat. Scientists and regulatory agencies to say that the composition of these foods is basically the same as conventional foods and that the nutritional content is the same use the term "substantially equivalent".

In time, additional traits will become available through biotechnology that modify the specific qualities of food - such as higher nutritive value, lower saturated fat or higher starch. These products also will be rigorously tested, but they will not always be "substantially equivalent" to their traditional counterparts. As a result, products that contain different components than traditional ones -like oil higher in oleic acid- will be appropriately identified.

How are these foods determined to be as safe as existing food or feed products?. A number of regulatory and scientific agencies around the world, including those in the United States, the European Union, Japan and Canada, have asked and answered that question. All of the regulating agencies agree that a food product of biotechnology is as safe as it is traditional counterparts if its composition and characteristics are the same, and its nutrient, toxicant and allergen levels is comparable.

Each food product produced through biotechnology is subjected to stringent testing to determine that it is as safe as traditional foods. The process may vary from one country to another, but evidence required by all of them addresses the same technical issues. Nutritional value, composition, function and health-related aspects are all investigated before approval is given for the crop to be grown in a specific country or for the fruits of a crop to be marketed as food and feed.

Commonly-Asked Questions

In general, if the products of biotechnology are equivalent to their traditional counterparts, you won't know. Bulk foods, such as soybeans, wheat, canola and corn are traditionally co-mingled at the farm and throughout the distribution system, so bulk imports may contain food products improved through biotechnology mixed together with conventional food or feed. Products that are not the same, such as oilseeds modified to produce healthier oils, generally will be kept separate because they offer special additional value and are not equivalent.

The food produced through biotechnology is not likely to be more allergenic than conventional food, but assessment of allergenicity is part of the regulatory evaluation process. If a product is determined to contain an allergen, it will carry a label that identifies it as such. No product currently approved for planting and marketing contains such an allergen.

All products of biotechnology will complete proper regulatory reviews. Once they are approved, they can be processed and traded like other products.

Because biotechnology has benefits for both large and small farmers in many different kinds of crop plants, researchers around the world are modifying a variety of crop plants and developing varieties suited to specific locations. Field trials are underway in many parts of the world, which is a necessary step to be sure that the plants perform as expected in local climates and soils. Plants improved through biotechnology are likely to be available to farmers in many areas of the world in the near future.

In the United States

Applications of biotechnology to food and agriculture are the subject of extensive regulatory review by the US Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). This review process has been established to protect both food safety and the environment.

The USDA has the primary responsibility for field trials involving plants modified through biotechnology. Before a modified plant can be commercialized, the USDA determines whether it will grow in the field like traditional plants and carefully evaluates the environmental and ecological effects of the plant.

The FDA is primarily responsible for determining whether a food product is safe for human consumption or for use in animal feed (Table 2). Its assessment of safety focuses on the following considerations: does the food contain proper levels of nutrients; do the new proteins the plant produces meet safety and nutrition guidelines; are the oils and carbohydrates produced by the crop plant similar in identity, composition and nutritional value to conventional oils and carbohydrates; does the food product contain toxicants no different from those in conventional varieties; is the food free of common allergens; and does the food function the same when used in food and feed products?.

Table 2. Safety assessment summary *Roundup Ready* soybeans.

Component	Changed	Unchanged
Nutritional composition		X
Animal feed performance (rodents, fish, birds, chickens, cows)		X
Processing		X
Wildlife safety		X
Disease susceptibility		X
Allergenic potential		X
Tolerance to *Roundup* herbicide	X	
One additional protein/gene	X	

Technology developers are expected to provide evidence to the FDA that they have assessed the new food for each of the above considerations. Technology developers must also provide evidence that the new plant products are in compliance with the U.S. Food, Drug and Cosmetic Act. For example, if a technology developer introduces a potential allergen into a food, they must declare that fact on the food's label so those customers with allergies can avoid the product. This policy is applied to all foods produced in the United States whether they result from the application of biotechnology or not.

In other countries

The food products of biotechnology are similarly regulated in the European Union, Japan, Canada, Australia and Argentina, where the new plant varieties currently are being grown or imported. Each producing country also subjects these new crop plants to rigorous assessment by their own regulatory agencies before they can be grown their (Table 3).

Other agencies that have considered the safety of food developed through biotechnology include the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the International Life Sciences Institute (ILSI) and ILSI Europe, as well as the American Dietetic Association (ADA).

As a result, any food product from biotechnology that is imported into countries around the world has been thoroughly assessed, reviewed and approved by a variety of scientific and regulatory agencies throughout the producing and importing nations.

Table 3. Dozens of products have been approved around the world.

34+ Approvals in the U.S. (USDA, FDA, EPA)
20+ Approvals in Japan (MAFF, MHW)
30+ Approvals in Canada (AG Canada, Health Canada)
9 Approvals in the European Union
2 Approvals in Argentina
1 Approval in Australia
3 Approvals in Mexico
1 Approval in Brazil

Summary

Some of the food products developed through biotechnology will be indistinguishable, at least on the market shelf, from conventional kinds. Before long, others may have noticeable improvements, such as better flavor or higher nutritional value. In either case, ensuring the safety of these foods for people is an important concern to the producers of the new varieties, to the regulatory and scientific agencies in the countries where these foods are grown, and to the governments of countries that import food grown elsewhere.

Scientific, regulatory, medical and agricultural experts in many countries agree that plant biotechnology can significantly enhance the world's food supply while it helps farmers be more productive and, at the same time, sustains the environment. As a tool to augment traditional breeding, plant biotechnology will help make abundant, safe, high quality new foods available to people, worldwide.

Websites

United States: National Agricultural Library: Information services and publications on a variety of agricultural biotechnology topics. http://www.nal.usda.gov/bic

United States: Information Systems for Biotechnology: Information about agricultural and environmental biotechnology research, product development, regulatory issues and safety. http://www.nbiap.vt.edu

United States: Biotechnology Industry Organization: US and international information on biotechnology. http://www.bio.org

International: Biosafety Information Network and Advisory Service: A service of the United Nations Industrial Development Organization, which monitors global developments in biotechnology regulatory issues. http://binas.unido.or.at/binas/binas.html

Latin America: The Virtual Center of Biotechnology for the Americas. Information on issues affecting Latin America. http://www.ibr.unam.mx/virtual.cgi

Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Does Biotechnology Change the Research and Development Organizations?

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Humans have always been dependent on plants for the production of food, clothing and building materials. For millennia, generations of humans have used chemical substances produced in plants, substances that nowadays we classify as primary or secondary metabolites.

Within the last decade of second millennium, we have witnessed an unprecedented change in the way we produce food, agriculture crops and new substances for industrial applications by using plants or animals as 'bioreactors'.

New developments, often covered by the buzz word 'biotechnology', are about to bring very dramatic changes to the production and processing of important agricultural crops.

From everyday news, and scientific literature, we are aware of developments in the production of new agriculture crops. New GMO (Genetically Modified Organisms)-or "transgenic" microorganisms, plants and recently animals are getting a lot of publicity, and are appearing on the covers of major magazines. They create a real excitement in mass media, as their impact reach far beyond the scientific or technological aspects. The public safety, ethical and environmental issues, international trade barriers linked to the GMO are subjects of hot debates, violent demonstrations and governmental actions.

Biotechnology is a new interdisciplinary science, it will change many sectors of our economy, and attracts the attention of scientists, capitalists, manufacturers, lawyers and public-at-large.

The third millennium should benefit from developments already achieved in the production of agricultural crops and extend newly developed technologies to human health and nutrition. We will see medical applications of plant bioreactors for production of vaccines, drugs, as well further development of emerging categories cover by the general term "nutraceuticals" and consisting of dietary supplements, functional and medical foods.

Trends currently evident in agricultural biotechnology may be divided into several categories; the major ones being:

The growth potential in the agricultural biotechnology industry continues to be very promising. A recent study shows that amongst US biotechnology product sales, the agricultural sector will experience the fastest growth in the next decade (Table 1).

The global market for agriculture products and agriculture based value added products is undergoing change as the top players in agriculture and agricultural biotechnology face increased consolidation and ultimately form alliances in development, production and marketing.

Table 1. USA biotechnology product sales forecast (million US $).

SECTOR	BASE YEAR 1996	FORECAST		AVG. ANNUAL GROWTH RATE (%)
		2001	2006	
Human therapeutics	7,555	13,935	25,545	13
Human diagnostics	1,760	2,705	4,050	9
Agriculture	285	740	1,740	20
Specialties	275	690	1,600	19
Non-medical diagnostics	225	330	465	8
TOTALS	10,100	18,400	32,400	12

Source: Consulting Resources Corp.

Transgenic plants for human consumption and industrial applications are entering the marketplace. Novel, genetically engineered, plant based organisms (GMO) designed for resistance to herbicides, pesticides and environmental stress or for the production of valuable pharmaceuticals and vaccines are available. The increased power of plant genetics can be utilized to enhance crop production and quality, but also allows plants to be exploited as sophisticated bioreactors, thereby producing a wide array of industrial chemicals and intermediates, for example, plastics, flavors and colorants, pharmaceuticals, antibodies, vaccines, blood proteins, and enzymes for food, feed and industrial uses.

Green factories, i.e. plant bioreactors, will not only facilitate production of chemicals in an environmentally friendly manner by utilizing carbon dioxide and the sun's energy in their photosynthesis, but they will also produce a new generation of safer agrochemicals.

Globalisation of trade and the formation of several strong trading blocks: EU, NAFTA or Mercosur, allows swift capital flows between the block countries, taking advantage of lower labour costs and may contribute in the future to an even faster growth of contract research and development organizations.

The 1996 worldwide market for contract manufacturing of biopharmaceutical products was estimated at $ 200-300 million. Based on the current rate of growth, the market size is projected to reach $ 1 billion shortly after the year 2000. This is not a mere fashion trend, it is the reality of an industry under pressure to be creative in a fast moving and extremely competitive business opportunity environment.

Nutraceuticals represent a significant opportunity for biotechnology companies. Growing demand for foods and supplements that complement the use of traditional pharmaceuticals is driven by numbers of factors. Aging populations in developed countries, growing cost of medicare, change in lifestyle and "baby-boomers" desire to be in control of their own health created flourishing market for nutraceuticals. On the other hand, pharmaceutical and biotechnology companies are moving into nutraceutical arena because these products provide potentially rapid financial return on investment, nutraceuticals generally have insignificant

costs of clinical trials. Instead of 10 years and $ 250 million to bring a drug to market, a nutraceutical can take only a few years and a few million dollars. The nutraceutical market is estimated to be in the range of $100 - 250 billion in US and similar size in Europe, Japanese market is also significant and well established.

Major companies biotechnology and pharmaceutical have been investing over the past few years in nutraceuticals. For pharmaceutical companies, new functional and medical foods have the potential to bring in as high a margin as a drug. Biotechnology provided important tools for these new products. Notable examples include genetically modified DuPont's and Monsanto's canola and soybean oils designed for elimination trans fatty acids from human diet. Monsanto's acquiring Calgene technology for production gamma linolenic acid (GLA) and carotenoids in canola is entering nutraceutical market with its GMO products.

A growing demand for bioprocessing, test production, scale-up or providing data for registration and regulatory agencies has created new opportunities for contract research and development (CR&D) organizations. Biotechnology companies are supplementing their internal R&D with external resources to satisfy competing pressures. Contract R&D laboratories offer technical expertise, laboratories and specialized, state-of-the-art laboratory equipment that are not available to many biotechnology companies. Knowledge of regulatory agency expectations that is not always resident with biotech companies (especially for companies planning to register both inside and outside a particular country). The ability to meet extremely demanding timelines, that in many instances could not be met with the client's in-house capabilities, and compliance with GLP, GMP and ISO in testing and processing are additional factors favoring outsourcing. There is a visible trend - one of going from "bricks to brains", indicative of a change from high asset base, low R&D investment business to low asset base, high R&D investment operations. Tax incentives in some countries have created a favorable climate for this shift. In other countries, where labor is expensive and employment rights are strongly protected, it can be more attractive to buy the time and services of a contract company rather than to hire new or additional staff. Many companies are achieving savings in personnel cost by using CR&D organizations because fixed costs now become variable costs.

Outsourcing Requires New Management Strategies

There is a trend in the biotechnology industry to form new business relationships to enhance the research and developmental process. Virtual companies are turning over entire research programs to contract organizations and simply coordinating the research elements as they come together for decision making. With outsourcing becoming a basic strategy in the agricultural biotechnology, new approaches to effective managing of complex and strategic partnerships needs to be mastered. As a result of the rapid emergence of new technologies, clients require custom-tailored services, which results in the need for a close client/CR&D relationship. From the contracting company's perspective, the crucial issue is the makeup of the outsourcing team, which consists of specific expertise to make the partnership work effectively. It is extremely important to maintain efficient and effective communication channels with the CR&D company. This of course, can be readily facilitated by electronic communication where information, instruction and data can be easily exchanged, reviewed, commented upon and agreed to quickly and confidentially.

Some companies are merely seeking to achieve cost-driven benefits from collaborative partnerships, others are going beyond traditional subcontracting. Often strategic agreements are formed where sophisticated projects with predetermined relationships and financial terms and a more defined long-term set of strategic objectives are defined for both partners. This implies sharing certain aspects of intellectual property with the contract R&D organization. This may be technology or methodology, but very often it is largely the harmonization of regulatory aspects. In contract manufacturing cases, the CR&D on-site evaluation and audit is a first step to ensure GMP and often Good Laboratory Practice (GLP) compliance and validation of the facilities and related processes. In most cases, the contracting out partner provides standard operating procedures (SOPs) to ensure familiarity with their GLP/GMP requirements.

The up-front definition of the contract is critical. This includes a proposal that clarifies the technological requirements, the services the company wants to buy and their extent, the overall structure of the project and a clear understanding of intellectual property ownership. Setting clear expectations before initiating the project is often even more critical. The proposal should also address each partner's responsibilities in the event of project failure or deviation.

The risk of project failure may be reduced through sufficient testing and cross-company dialogue. The contract R&D partner can take additional steps to reduce the risk and build confidence in developing the new partnership. A feasibility study or bench-top scale investigation will cost less money than reprocessing or modification in a full-scale pilot plant run. To ensure that its own interests are secure, the CR&D quality assurance department needs to review and approve the project before execution.

The contract R&D organization must have in place well defined project management coordinating policies and procedures. The reporting structure will generally include the Directors of Research and Quality Assurance and the Principle Investigator or Study Director (or Project Leader). The flow of technical information between the two parties will be on a need to know basis but will need to be disseminated to the technical and operations staff in a manner that allows the orderly planning, execution and review of the contracted research program. Typically the senior management of the contract R&D company is not party to the specifics of the technical program, but only administers of the reporting structures and the financial aspects. During the execution of the research and testing programs, data collection, documentation, deviations and corrective actions must be handled appropriately, recorded and initialized according to protocols agreed upon between the parties. Generally these involve the use of accepted procedures recognized internationally under GMP or GLP guidelines. More recently, International players are requesting ISO 9000 series quality-assurance-certification. Auditing of the entire program by Quality Assurance at a level of about 10 % insures that the deliverables are met and that the basal requirements for the contracting partner's future validation, verification and regulatory needs are in place. The final products of the research program may be product or ingredient samples, analytical data, original copies of all raw processing data and an approved for release final report. Subsequently, the parties may explore additional support through production of materials for functionality testing and market evaluation using the same protocols that guarantee the validity of process methods, analyses and supporting documentation. Upon the completion of the contracted program the relationship can be terminated, respecting the contractual conditions for confidentiality and dissolution.

Conclusion: Third Millennium Crystal Ball

Following agriculture crops, genetically engineered plants with the ability to produce pharmaceuticals, such as vaccines and human proteins, hormones, and enzymes will enter commercial production.

In the near future, we can expect further dramatic changes in R&D landscape. Due to financial restrictions, time pressure, and fierce competition, biotechnology companies will focus their activity within their area of expertise. The market for contract research, especially in developmental bioprocessing will be growing considerably. Biotechnology companies will be out-sourcing major part of their developmental and engineering needs to professional and confidential, contract R&D facilities, as the pressure for the sales of their products and return of invested basic research dollars will be growing.

Major mergers and acquisitions have already occurred between seed and life science companies, as several leading biotechnology and chemical companies change themselves from purely chemical manufactures into life science companies. Monsanto, DuPont, Dow AgroSciences, Agrevo, Novartis, Aventis (Hoechst and Rhône-Poulenc) are the most important examples.

Globalization of research, manufacturing and marketing will pose new challenges for small and medium size research organizations, companies and national research programs. However, new opportunities will arise for well prepared, modern contract research facilities able to provide confidential and timely support for leading biotechnology companies.

The traditional model of publicly founded research and development, as well as the structure of technology transfer channels will undergo substantial evolution. Therefore, research organizations should be prepared for these challenges.

Further development and market introduction of new biotechnology products will depend on public acceptance of genetically modified organisms (plants, microorganisms and animals). Currently, there is a considerable difference in public opinion regarding this matter between North America and Europe.

Risk and Regulatory Issues.

Will the next millennium be the golden age of biotechnology? Will biotechnology provide an answer to the many problems the world is facing today? Or biotechnology will be another headache, unfulfilled dream or even a genetic Chernobyl?

Will biotechnology be able to contribute to elimination of currently know diseases, extend human life, make it better, and at which cost? For everyone, or only for selected and wealthiest?

What will happen to agricultural biotechnology? With evident erosion, shortage of irrigation water and about 80 million people added to the world's population each year the food production in many areas will decline. Does biotechnology have the potential to alleviate many of these problems?. The world food problems are daunting: the FAO estimates that, today 800 million people do not have sufficient food. New plant varieties may make it possible to improve food supply without increase in farmland, water or other resources.

It must be stressed, however, that new technologies alone are not sufficient. Other key issues are governments' regulations, international trade and corporate profits, sharing research and technology, and legally binding international biosafety protocol. Some countries, like the U.S. believe that a legally binding international protocol governing uses and releases of genetically modified organisms would interfere with research and the development of the biotechnology industry.

In contrast, some European countries are very conscious about the use of agricultural biotechnology, both at home and especially in developing countries. Many biotechnology companies have branches or joint ventures around the world and are ready to introduce transgenic plants globally. Transgenic plants can be a mixed blessing for developing countries. More productive plants could helps feed their population, but on the other hand, if genetically modified plants had not been adequately field-tested, they could have unexpected harmful effects, i.e. the spread of genes to wild relatives or the creation of new weeds.

Another issue is labeling and segregation of genetically engineered food. In U.S. under FDA'cs policy, most genetically engineered food will not be labeled. FDA has decided not to require labeling unless the food contains a known allergen or its composition is substantially different from the standard food. Canada in general, follows U.S. regulations.

In contrast, some European countries have introduced legislation requiring segregation and labeling of transgenic food. These differences in legislation might be an additional obstacle in international trade and will shape further development of biotechnology based industries.

Plant Genetic Engineering: Towards the Third Millennium
A.D. Arencibia (Editors)

Biological Aspects and Ethical Considerations for the Utilization of GMOs

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Introduction

The use of genetically modified organisms (GMOs) caused a lot of response from a range of different persons and organizations. These responses are partially based on facts and validated information, and partially on ethical opinions in the framework of natural resources management, philosophy or religion. Both objective and subjective aspects of the discussion on GMOs are disturbed by several factors. Scientists discussing facts and figures of genetic modification should be able to develop generally supported conclusions, but it appears that the rating of importance of certain arguments is individually different. It also should be recognized that ethical opinions are regularly presented as proven facts, sometimes without the notion that the argument put forward originates from an ethical viewpoint. The Harvard negotiation rules emphasis on several basic rules for discussions that should result in common agreements. These rules indicate principles such as the need to discuss matters and not personal preferences, to develop an open view for the arguments of the other participants instead of focusing on your own viewpoint, and to use a flexible and not a fixed attitude. After years of discussion on the use of GMOs it is extremely difficult to change the psychological load of a lot of arguments. Moreover, although the objective and subjective aspects of the utilization of GMOs are principally different from each other and should be recognized as such, the Harvard rules does not mean to say that there is no relation between these two aspects. It is not only the situation those subjective, ethical viewpoints are presented as objective arguments, but also scientific facts are used as basis for the development of subjective viewpoints. I will contribute to the discussion by highlighting some recently recognized evolutionary mechanisms that are linked to the transfer of genetic material between evolutionary not related species. This does not mean that ethical viewpoints as such are not important (Nuffield, 1999). Therefore these mechanisms will be placed in the broader framework of some ethical aspects relevant for the application of GMOs. The paper will be focused on the use of genetically modified plants.

Objective Aspects: Evolutionary Mechanisms

The view on evolutionary change as proposed by Darwin is predominantly based on selection of favorable characters, which originate in a species from an undetermined source. This process leads to adaptation of the species to local environmental circumstances. Since then, a lot of additional mechanisms have been discovered, some of them only recently. The mechanisms hybridization and introgression, horizontal gene transfer and DNA methylation are of primary importance for pinpointing genetic modification in a framework of natural occurring mechanisms. For each of these mechanisms two aspects are relevant. The natural occurrence and its frequency provide at first an indication whether the move forward made by

the development of biotransformation has a principal or gradual nature, and second this information provide knowledge about the level of risk that transgenes might give in the environment.

Hybridization and introgression

The classical mechanism involved in the interference of GMOs with wild relatives is hybridization. The rate of hybridization between domesticated plants and their wild relatives and of escape of domesticates has been estimated in the form of a so-called Dpdf code for 36 crop plants at the species level and for some genera (De Vries et al., 1992). The Dpdf code is a compilation of the chance of spontaneous gene dispersal by means of pollen (p), the chance of spontaneous dispersal by seed or other diaspores (d), and the distribution frequency of the crop in the wild (f). The higher the three values the higher the rate of hybridization and/or migration and, hence, the higher the risk after transgenic plant release could be. The Dpdf code values are rated in three classes to be indicated in terms of limited, medium and high exchange rates. In a statistical analysis of the three components p, d and f together with data on five additional parameters it appeared that the dispersal of pollen is the most important mechanism for the establishment of plants outside the range of their original population. This conclusion was supported by the high correlation between the frequencies of hybridization between crops and wild relatives, and establishment of crops in the wild (Van Raamdonk, 1995). The analysis generally supported the arrangement of crops in the three classes, but in detail some specific crops gave aberrant results. The estimation of exchange rate of De Vries et al. (1992) of crops like *Narcissus pseudonarcissus, Beta vulgaris, Avena sativa, Lactuca sativa* and *Brassica oleracea* should be reconsidered. Gregorius and Steiner, (1993) reached the same conclusion for *Narcissus pseudonarcissus* and *Lactuca sativa*. There is a strong correlation between estimated exchange rate and allogamy. *Lactuca sativa* is the only autogamous crop classified with an estimated high exchange rate.

The importance of pollen dispersal over seed dispersal and of hybridization is in concordance with literature studies (Ellstrand and Hoffman, 1990). Transfer by means of pollen can only be achieved to closely related plants (figure 1).

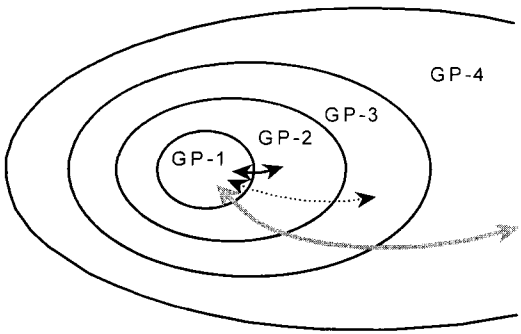

Figure 1. The genepool concept of Harlan & de Wet (1975). Gene Pool (GP-) -1 and GP-2 are connected by good or fair possibilities for hybridization and introgression (black arrow). Genetic exchange of GP-1 with GP-3 is limited or virtually impossible (shaded arrow) and with GP-4 impossible by sexual means. However, horizontal gene transfer (Grey arrow) allows the exchange of genetic information among all living organisms (GP-4).

In terms of the gene pool concept of Harlan and De Wet (1975), transfer to species belonging to Gene Pool (GP-) 3 of the crop is virtually impossible and transfer to all other organisms which can be considered as GP-4 is excluded.

Horizontal gene transfer

The transfer of genetic material by non-sexual means between systematically non-related organisms is called horizontal gene transfer (HGT). There is a lot of information on the transfer between microorganisms, where also the mechanism is known, and with respect to transposable elements (Capy et al., 1994). In contrast to transposable elements, the transfer of DNA of eukaryots by prokaryots is rarely documented (Wolf et al., 1999), whereas this specific situation of HGT is of particular interest for the discussion on the utilization of GMOs. Some years ago a list of nine documented cases of horizontal transfer was published (Syvanen, 1994). The evidence for HGT is usually poor or conflicting (Prins and Zadoks, 1994) or circumstantial by means of testing phylogenetic incongruencies between species trees and gene trees (Syvanen, 1994). Six of these nine cases refer to prokaryotic-eukaryotic tranfer. Further examples concern the resistance gene for the bacteria *Pseudomonas syringae f.* sp. tomato found in several non-related eukaryots (discussed in Van Raamsdonk, 1995) and the *torf13* gene from *A. rhizogenes* and several *Nicotiana* species (Fründt et al., 1998). A controlled experiment with *Acinetobacter calcoaceticus* cultured in pots with transgenic plants with a reporter gene did not yield a transformed bacterium strain (Nielsen et al., 1997). Notwithstanding the results of such experiments, HGT might have effect on an evolutionary time scale.

The mechanism of HGT is highly comparable to biotransformation: the transfer of DNA by non-sexual means between organisms that belong to GP-4 of each other (Figure 2), in a lot of cases by *A-tumefaciens* transformation.

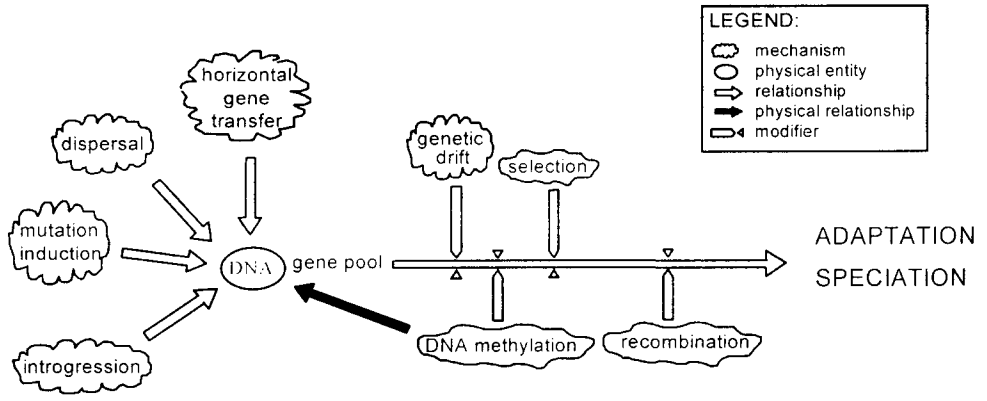

Figure 2. The framework of evolutionary mechanisms that influence DNA. The effect and relative importance of some of these mechanisms, such as horizontal gene transfer, introgression and DNA methylation should play a role in the discussion on the utilization of GMOs.

The comparability has two different aspects. A high natural frequency of HGT implies that modern biotechnology again is an extension of naturally occurring mechanisms, as was "classical" plant breeding by hybridization and selection. The other aspect is that a trait

introduced from another kingdom has a higher chance to be transferred unintentionally in nature by either vertical, genetic transfer to related species or by HGT to any other organism. The latter aspect might be less important, since HGT between pro- and eukaryots seems to be more frequent than among eukaryots. Anyway, increased knowledge of HGT may affect the discussion on biosafety of genetically modified organisms (van den Elzen, 1996).

DNA methylation

Methylation of DNA is a mechanism for gene regulation during the course of development of an individual organism or might play a role in non-mendelian inheritance of paternal or maternal copies of the same genes (imprinting; Bartolomei and Tilghman 1997; Spencer et al., 1999). It was suggested that DNA methylation evolved originally in prokaryotes as a defense system for foreign DNA. Invasive DNA sequences such as retroelements are usually highly methylated (Bennetzen, 1996), which indicates the protective function of DNA methylation besides its role in gene regulation. Transposable elements, retroviruses and transgenes are all examples of invasive DNA that are transferred by HGT. Although multiple copies of a transgene are usually required for methylation (Assaad et al., 1993), this is not always necessary (Meyer and Heidmann, 1994). Since the extent of silencing of invasive DNA is variable, the effect of genetic modifications and of transgenes has still to be established.

A new framework of evolutionary mechanisms

The most important classical mechanisms that are recognized for the process of evolution are mutation at several levels, hybridization, introgression, selection, genetic drift and recombination. These can be supplemented with more recently recognized mechanisms such as DNA methylation and horizontal gene transfer. A new framework of evolutionary mechanisms is presented in figure 2.

The recognition of and increasing knowledge on additional mechanisms allows a better positioning of biotechnological methods. A proper comparison of classical and biotechnological plant breeding will facilitate the discussion on the acceptance, since biological information is included in ethical viewpoints.

Subjective Aspects: Ethics and Personal Attitudes

The basic attitudes

Some years ago the Dutch philosophers Zweers and Kockelkoren developed the model of the four basic attitudes (see Schouten, 1997). The main aspect is the direction of the personal focus, either on nature's (ecocentric) interests or on human's (anthropocentric) interests (Figure 3). This does not mean that a person with a strong ecocentric focus does not have any feeling for human needs. The axis solely indicates the balance between the two extremes. Four different types of attitudes are placed on the axis (Figure 3).

For the ruler natural resources are available for the benefit of man. The steward wants to combine exploitation with preservation. In his opinion pure economic benefits might be less important. The partner considers human life and the other life forms as equally important. The integrity of life is an essential aspect of this attitude. The participator is part of nature and considers the living environment with its complexity as a whole. He must use natural products, but he will follow the natural processes. From these attitudes a support or rejection can be predicted for several types of GMOs (Figure 3).

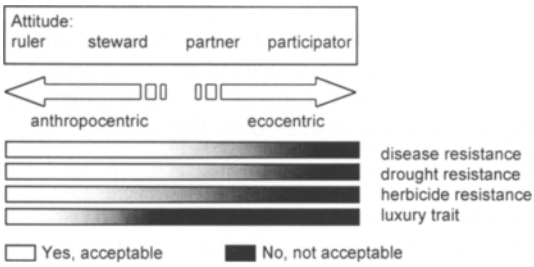

Figure 3. The four basic attitudes along the axis of human or nature interest. Grey scale bars indicate the acceptance of four different traits.

The ruler will accept the biotransformation for all kinds of improvements, including those gaining a luxury trait (blue roses, strawberry with cocoa taste, etc.), whereas the participator will reject any transformation. The partner might accept resistances against diseases, cold and drought, provided that regulations are designed and followed. The steward likes to have provisions but less strict.

The model of Zweers and Kockelkoren can be extended by a second axis that indicates the intensity of the attitudes. A person that has a strong desire to improve the food situation in developing countries together with a strong worry for the preservation of nature might feel to be a partner. In the same time another person without a positive feeling about the scientific progress and with a certain interest in the beauty of nature might finally be indicated as a partner as well. The difference is the intensity of the opinion, with might range from fundamentalism to opportunism. At least the extremes of the ruler and participator attitudes might be indicated as fundamentalism. It is not realistic to expect that a fundamental ruler and a fundamental participator will reach a consensus on the use of GMOs. An opportunistic steward or partner might accept biotransformation as methodology as far as they have nothing to fear, but an opportunistic viewpoint might have less impact on the discussion on acceptance of GMOs.

Reductionism and holism

In the framework of the discussed attitudes another spectrum has to be mentioned. The focus on the integrity of life and considering nature as one single entity can be indicated as holism. On the other hand, the process that the focus of science shifted to lower levels of organization (organisms, organs and tissues, biochemical processes, and finally DNA) led to reductionism. It is likely to indicate a ruler as a reductionist and especially a participator as a person with a holistic view, but the indications must be made with more nuances. Biotechnology is primarily reductionistic with regard to methodology, but the issue is that extrapolation has to be made to higher levels of organization.

It is vital for every participant in the discussion on the acceptance of GMOs to realize that viewpoints and arguments finally have a basis in basic attitudes and in the intensity of these. Differences between a holistic and a reductionistic view are only a part of this model.

Some Current Views and Approaches

Comparisons between classical plant breeding (i.e. breeding by selection of naturally occurring traits and/or by introducing them from naturally related species) and genetic modification have been made frequently (e.g. Regal, 1994). Aspects of these comparisons are the nature of the genetic transfer of DNA, the genetic stability of the newly constituted genome and the introduction in the field of new constitutes. For the first point, it has been argued that man has been moving around genetic material for centuries. This has been done by naturally acting mechanisms such as hybridization, between related organisms and with traits that naturally occur in those related organisms. Genetic modification allows the transfer of every piece of genetic material to every existing species (figure 1). That means that the genetic environment of the recipient genome is in theory not known, the interaction between the transferred gene(s) and the genome is at least difficult to predict, and escape to related wild species means an enrichment of the recipient wild gene pool. If not principal differences, these aspects indicate the different nature between classical breeding and genetic modification (Hill, 1999; Nuffield, 1999).

Another aspect is the comparison between the known cases of introduction and those regarding GMOs. Some effects of unwanted escape of traits that are part of classically bred crops is already known (Bartsch et al., 1993; Dale, 1994). Notwithstanding the existing cases of introgression and escape, it is still very difficult to predict the effect of escape of GMOs in the environment. A very useful approach for estimating possible effects is the use of simulation models, in which genetic as well dynamic population studies can be worked out (van Raamsdonk & Schouten, 1997). Recent studies indicate that the introduction of e.g. a resistance trait can have effect on the wild flora, but only after a high number of generations (Schouten, 1999).

With respect to food problems, recent studies focusing on reporter genes and the target product showed so far that no serious effects have been encountered. Nevertheless, lack of knowledge to predict the potential allergenicity of introduced proteins requires the application of a transgene centered approach, that focuses on the biochemical and epidemiological aspects of a particular protein (Gilissen et al., 1999). Recent political discussions seem to focus on the need to provide the consumers a freedom to choose between GMO-containing and GMO-free products (Nuffield, 1999). This situation requires two independent production chains, which means that from the consumers point of view there is a need for quality assurance, higher costs for the production and delivery of food supplies, and hence an existing willingness to pay.

Conclusions

The study of the effects of introduction of new traits, of the naturally occurring mechanisms for dispersal (horizontal gene transfer, introgression and seed dispersal) together with their frequencies of occurrence, and of the genetic effect of genetic modification by means of methylation or otherwise, would greatly support the discussion on the acceptance of GMOs. Nevertheless, it is made clear those objective facts and information must be founded on the basic attitude of every person involved. The progress and the quality of the discussion on acceptance of GMOs depend on the recognition of the principal importance of ethical attitudes.

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