Biotechnology in Agriculture and Forestry

Edited by T. Nagata
H. Lörz and J.M. Widholm

59 Transgenic Crops IV

Edited by E.C. Pua and M. R. Davey

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Transgenic Crops IV

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With 36 Figures, 8 in Color, and 33 Tables

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Dedicated to Linda Davey
for more than thirty-five years of unfailing support

Michael R. Davey

Preface

Exciting developments in crop biotechnology in recent years have prompted the necessity to update the first series of *Transgenic Crops I*, *II* and *III*, published in 1999 and 2001. In this current endeavor, 69 chapters have been compiled, contributed by a panel of experts in crop biotechnology from 26 countries. These chapters are grouped into three volumes, namely *Transgenic Crops IV*, *V* and *VI*. This new series not only reviews recent advances in cell and tissue culture and genetic transformation methodologies, but also presents aspects of the molecular genetics of target crops and the practical applications of transgenic plants. In addition, more than 30% of crop species that were not discussed previously are included in the present series.

This new series commences with the volume *Transgenic Crops IV*, consisting of 23 chapters that focus on cereals, vegetables, root crops, herbs and spices. Section I is an introductory chapter that places into perspective the impact of plant biotechnology in agriculture. Section II focuses on cereals (rice, wheat, maize, rye, pearl millet, barley, oats), while Section III is directed to vegetable crops (tomato, cucumber, eggplant, lettuce, chickpea, common beans and cowpeas, carrot, radish). Root crops (potato, cassava, sweet potato, sugar beet) are included in Section IV, with herbs and spices (sweet and hot peppers, onion, garlic and related species, mint) in Section V.

Transgenic Crops V also consists of 23 chapters in three sections devoted to fruit (Section I), trees (Section II) and beverage crops (Section III). Fruit crops target banana, citrus, mango, papaya, pineapple, watermelon, avocado, grape, melon, apple, *Prunus* spp, strawberry and kiwifruit, while trees include rubber, eucalyptus, legumes and conifers. Section III, on beverage crops, reports studies on coffee, cacao, tea and sugarcane.

As in volumes IV and V, *Transgenic Crops VI* has 23 chapters organized in five Sections. Section I targets oil and fiber crops (soybean, rapeseed, sunflower, oil palm, peanut, cotton, flax), followed by medicinally important plants (including ginseng, opium poppy, herbane, bellandonna, *Datura*, *Duboisia*, *Taxus*) in Section II. Ornamentals (roses, carnation, chrysanthemum, orchids, gladiolus, forsythia) are discussed in Section III, while Section IV involves forages and grains (alfalfa, clovers, tall fescue, ryegrasses, lupin). Section V has one chapter that discusses aspects of the freedom to commercialize transgenic plants, together with regulatory and intellectual property issues.

The editors express their sincere thanks to Maggie Yap Lan from Monash University, Malaysia, for her excellent secretarial and editorial assistance. She

forwarded to contributors timely reminders of deadlines, where appropriate, and assisted in editing the manuscripts for typographical errors and formatting.

This series will serve as a key reference for advanced students and researchers in crop sciences, genetics, horticulture, agronomy, cell and molecular biology, biotechnology and other disciplines in life sciences.

E.C. Pua and M.R. Davey

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Section I Plant Biotechnology in Agriculture

I.1 Impact of Plant Biotechnology in Agriculture

S.K. Datta[1]

1 Introduction

Ever since the advent of agriculture, there has been a need to improve crop plants for increased productivity, improved quality and to satisfy changing human preferences. This need is more acutely felt today and, particularly, in the developing world where the population is continuing to increase.

Genetic modification of plants probably began through selection of novel types about 10,000 years ago when human agricultural activities began and useful results were often a product of random or chance events. Through elucidation of the laws of genetics, molecular tools for understanding plant biology, plant breeding became a deliberate and predictable activity with the result that tailor-made crops are now in place (Table 1). Traditional plant-breeding methods have been very successful and have helped provide the volume of food required to allow the world population to grow to its present 6×10^9. Breeding efforts have provided remarkable diversity amongst various crop species and even some new crops, such as triticale, in addition to the introduction of new genes from wild species (Brar and Khush 1997). However, recent trends in crop productivity indicate that traditional methods alone will not be able to keep pace with the growing demands for food, fibre and fuel. The yield increases in many food crops have hit a plateau or have fallen below the rate of population increase. Farmers in South and Southeast Asia must consistently produce an extra 30% more cereals in order to maintain current nutrition levels and food security. Biotechnology offers a challenging role to reduce the gap of yield improvement (Hossain et al. 2000; Lorz et al. 2000; Miflin 2000; Phillips 2000; Khush 2001; Datta et al. 2003a, b; Vasil 2005; Mackill 2006). This task does not become any easier with diminishing land and water resources. Plant biotechnology and, in future, nanotechnology, can bolster plant-breeding efforts to meet these new challenges in a sustainable way (Helmke and Minerick 2006).

Conventional plant breeding is often limited by reproductive barriers. The developments in the area of plant biology in the past three decades, such as plant genetic transformation, have opened up new vistas in crop improvement, thereby allowing transfer of desirable gene(s) across species and genera (overruling cross-ability barriers that limit the scope of conventional breed-

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Table 1. Some classic developments in plant biotechnology and transgenic research

Achievement in plant biotechnology and transgenics	Response/ transgene	System/method	References
Haploidy in *Datura*	Microspore development	Anther culture	Guha and Maheshwari (1964)
Cereal protoplast regeneration	Protoplast culture	Protoplast system	Vasil and Vasil (1980)
Protoplast fusion product	Protoplast fusion	Protoplast	Gleba and Hoffmann (1980)
First transformation event demonstrated in tobacco; bean phaseolin transferred to sunflower and tobacco	*gus*; phaseolin	*Agrobacterium* for both achievements	Bevan et al. (1983), Fraley et al. (1983), Herrea-Estrella et al. (1983), Murai et al. (1983)
First report of a bacterial gene expression in tobacco	*npt*II	*Agrobacterium*	Horsch et al. (1984)
Method for interspecific hybrids		Protoplasts	Sundberg and Glimelies (1986)
Biolistic transformation through particle gun bombardment established	*gus*	Biolistic	Sanford et al. (1987)
First stable soybean transgenics developed	Glyosphosphate tolearance	*Agrobacterium*	Hinchee et al. (1988)
First stable transgenic japonica rice	*Hph*	Protoplasts	Shimamoto et al. (1989)
First stable fertile homozygous transgenic indica rice	*Hph*	Protoplasts	Datta at al. (1990)
Transgenic insect-resistant cotton	*Bt*	Biolistic	Perlak et al. (1990)
Fertile transgenic indica rice	*Bar*, *gus*	Biolistic	Christou et al. (1991)
Herbicide-tolerant indica rice developed	*Bar*	Protoplasts (PEG)	Datta et al. (1992)
First successful stable herbicide-resistant wheat	*Bar*	Biolistic	Vasil et al. (1992)
Transgenic fertile japonica and indica rice	*Hph*	*Agrobacterium*	Hiei et al. (1994)
First stable independent barley transgenics developed	*Bar*, *gus*	Biolistic	Jahne et al. (1994), Wan and Lemaux (1994)
Transgenic fertile barley; transgenic red fescue	*Bar*, *gus*; *hph*	Protoplasts for both achievements	Spangenberg et al. (1994)
First detailed report on the comparative efficiency of different promoters driving agronomically important gene	*cry1A(b)*, *cry1A(c)*, *Cry1A(b)/ cry1A(c)*	Biolistic, protoplasts	Datta et al. (1998)

Table 1. (continued)

Achievement in plant biotechnology and transgenics	Response/ transgene	System/method	References
First iron-rich Japonica rice	*ferritin*	*Agrobacterium*	Goto et al. (1999)
First field testing of transgenic rice with agronomically important genes	*Bt*, *Xa21*	Biolistic	Tu et al. (2000a, b)
β-Carotene-rich (golden) rice	*Psy*, *lyc*, *crtI*	*Agrobacterium*	Ye et al. (2000)
Protein-improved potato	*Ama1*	*Agrobacterium*	Chakrovorty et al. (2000)
Nutrition improvement in commercial indica rice	*Ferritin*, *psy*, *crt1*	Molecular breeding	Datta et al. (2003a), Vasconcelos et al. (2003)
Molecular biopharming	Several genes	Chloroplast	Daniell et al. (2004)
Marker-free and enhanced carotenoids in rice	*Crt1*, *psy*	*Agrobacterium*	Paine et al. (2005), Parkhi et al. (2005)
QTL for plant regeneration; yield improvement	Rice QTL, *GN1*, *SD1*	Molecular breeding	Ashikari et al. (2005), Nishimura et al. (2005)
Environment-friendly transgenic crop	*Cry* genes	Molecular breeding	Chen et al. (2006)
Intragenic vectors (gene transfer without foreign DNA)	Intragenic vector	Molecular breeding	Conner et al. (2006)
Post-transgeneration enhanced targeted end-products	*Psy, crt1* genes	Molecular breeding	Datta et al. (2006)

ing) for developing transgenic plants with novel traits, such as built-in resistance/tolerance to several biotic and abiotic stresses, improving nutritional qualities and grain filling (Potrykus 1990; Goldberg 2001). Moreover, the advances in genetic transformation techniques provide plant breeders access to new and broader gene pools. Transgenic plants can be considered as the most recent development in our efforts to genetically improve crops.

2 Crops and Genomics

Genomics implies DNA sequencing, the routine use of DNA microarray technology to analyse the gene expression profile at the mRNA level, and improved information tools to organize and analyse such data. Genomics-based strategies for gene discovery, coupled with the high-throughput transformation process, will accelerate the identification of candidate genes. The recent reports on rice genome sequencing by Monsanto, the International Rice Genome Sequencing Project (IRGSP), the Beijing Genome sequencing (BGI) and Novartis, and completion of the genome sequencing of *Arabidopsis*, will accelerate gene discovery and further crop improvement (Datta 2004; Vasil 2005).

2.1 Addressing Issues/Concerns in the Post-Genomics Era

How do we reorganize crop breeding in the genomics era, particularly in using DNA chip/microarray? How does rice/*Arabidopsis*/tomato genome discovery help us in such an endeavour? How do we move forward with such knowledge-based intensive technology and obtain public confidence, particularly in solution to the working together of the public and private sectors? We must be sure to respect intellectual property rights (IPR) while farmers' plant varietal protection (PVP) rights also need to be respected. The awareness of mutual interest and respect will serve this purpose and will benefit all in society. How do we convince policy makers of national governments to take the advantage of the combined green-and-gene revolution to reach most farmers whose livelihood can be improved by such knowledge-based intensive technology? This task poses many challenges and will provide rewards for human welfare.

3 Genetic Transformation of Plants

Plant transformation was first demonstrated independently in 1983 by three research groups at Gent (Belgium), Monsanto (St Louis) and a collaborating group from Washington State University, St Louis and Cambridge University, UK (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983). All three groups transferred and expressed bacterial antibiotic resistance genes, using the *Agrobacterium*-mediated method. However, plant transformation became routine in the 1990s, a decade after genetically engineered human insulin went on sale. After the first report of gene transfer with the seed protein phaseolin from bean to sunflower and tobacco (Murai et al. 1983) and a bacterial gene for neomycin phosphotransferase II (*npt*II) to tobacco a year later (Horsch et al. 1984), plants have been transformed with a range of genes from other species and genera, and with those from bacteria, viruses and animals. Following dramatic progress in the improvement of transformation technology, more than 50 different species of transgenic plants have been produced, both including those of monocotyledons and dicotyledons, and some (including rice in China) are under field assessments worldwide (James 2005). A selective description of the development of biotechnological tools and product is summarized in Table 1.

Many transformation approaches have been tested in the past for their comparative efficiency and efficacy, including *Agrobacterium tumefaciens*-mediated transformation and direct gene transfer, i. e., protoplast- and biolistic-mediated procedures (Vasil and Vasil 1980; Datta et al. 1990; Potrykus 1990; Christou et al. 1991; Datta and Datta 2001; Altpeter et al. 2005; Vasil 2005).

3.1 Methods of Gene Transfer

Amongst the methods available, *Agrobacterium* and biolistic methods are the most widely explanted.

3.1.1 Agrobacterium tumefaciens

Agrobacterium tumefaciens is a soil-borne, Gram-negative bacterium which is capable of genetically colonizing susceptible host plants. It is capable of transferring any piece of DNA inserted in its T-DNA between a pair of direct repeats called border sequences, with the help of a site-specific, strand-specific endonuclease. This feature has been extensively exploited in the genetic transformation of plants. Different strains of *Agrobacterium* have different host ranges and some crop plants, particularly monocotyledons, are considered recalcitrant to *Agrobacterium* infection. Several strategies have been implemented to overcome this recalcitrance issue. Very often T-DNA integration occurs in transcriptionally active regions of the plant genome and hence the expression of the transgene becomes a routine phenomenon. A detailed insight into the *Agrobacterium*-mediated DNA transfer process into plant cells is given in the report of Zupan and Zambryski (1995). A number of variants of the *Agrobacterium*-mediated transformation protocol have been used to transform *Arabidopsis*, the model plant. Many laboratories routinely transform *Arabidopsis* using the whole plant or the floral dip method, which is efficient and easy to practice. A recent modification of the floral dip method, called the floral spray method, might help in expanding this approach to other plants (Chung et al. 2000).

3.1.2 Biolistic Transformation

Biolistic transformation (also referred to as particle gun bombardment or microprojectile bombardment) is carried out by shooting DNA-coated tungsten or gold particles into target tissue (Sanford et al. 1987). The microprojectiles can be accelerated with gun powder, helium or an electric discharge. The advantage of the method is that any tissue can be transformed, provided that tissue can be regenerated through culture into plants. Usually, transformation using this method results in complex patterns of DNA integration as compared with T-DNA transfer that usually results in precise, low-copy integrations and simple integration patterns (Tinland 1996; Parkhi et al. 2005). Co-suppression of the transgene/endogenous gene can occur due to integration of multiple copies of the transgene (Flavell 1994). Transfer of long DNA molecules can be a challenge, since the molecules can be sheared due to the forces involved in accelerating the microprojectile, unlike the case in *Agrobacterium*-mediated gene delivery (Hamilton et al. 1996). Generally, the whole plasmid representing the clone of the transgene is bombarded into the target tissue, resulting

in the integration of vector backbone into the plant genome, as is also possible in the case of *Agrobacterium*-mediated gene delivery (Ramanathan and Veluthambi 1995). Variations of the protocol in which only the transgene is introduced as a linear fragment with bombardment of a minimal expression cassette also exist with efficient transformation (Fu et al. 2000; Datta et al., unpublished data). The biolistic method is appropriate in transforming plants that are known to be recalcitrant to *Agrobacterium*-mediated transformation. Its utility in transient expression studies is also immense. Many of the commercially available transgenic plants have been developed by biolistic transformation.

3.2 Promoters Used in Transgenic Crops

The fate of the introduced gene(s) in the transgenic plant depends largely on the promoter that drives its expression as well as its position in the genome. Promoter sequences upstream to the gene(s) of interest are very important in plant transformation for determining the levels and patterns of transgene expression. Two major categories of promoters, namely constitutive and tissue-specific, are used extensively. Constitutive promoters direct the expression of a foreign gene in all plant tissues at all stages of plant development, with some variation in expression across tissues and stages of organ development. This group of promoters include the cauliflower mosaic virus 35S (CaMV 35S) promoter, rice actin I (*Act* I) promoter, maize ubiquitin (*Ubi* 1) promoter and maize alcohol dehydrogenase I (*Adh* 1, also called *Emu*) promoter; barley hordein promoter, etc. (Cho et al. 1999; Bajaj and Mohanty 2005). A hierarchy of several constitutive promoters was shown on the basis of levels of transient expression of the *gus* transgene in rice suspension cell cultures namely: *Ubi* 1 > *Act* 1 > *Adh* 1 > CaMV 35S (Li et al. 1997). However, such a comparison would be more meaningful when data become available based on the stable transformation of at least ten events for each construct (different promoters + other elements of genetic transformation remain constant). Further, comparison would also be effective when a single transgene (one copy vs. multiple copies) in an homozygous state is compared with the event of a different transgene under similar conditions (one copy vs. multiple copies). The CaMV 35S and *Act* 1 promoters have been shown to strongly drive the constitutive expression of transgenes in rice (Datta et al. 1990, 1999; Lin et al. 1995; Tu et al. 1998a).

Tissue-specific promoters drive the spatial and temporal expression of the transgene(s). Such promoters studied and used so far in rice and other monocotyledons include the maize phosphoenol pyruvate carboxylase (PEPC) promoter driving green tissue-specific expression, pith-specific, wound-inducible, root-specific, endosperm-specific, pollen-specific and stress-inducible promoters (Bajaj and Mohanty 2005). These promoters are useful for directing the expression of the transgenes in only those tissues where and when it is required.

The expression of the introduced genes also varies depending on where they are integrated in the plant genome. In order to normalize gene expression and to reduce position effects, matrix attachment regions (MARs) have been used in the gene construct for both biolistic and *Agrobacterium*-mediated transformations (Lucca et al. 2001).

3.3 Selectable Markers Used in Development of Transgenic Crops

The selection of putative transgenic tissues following transformation, irrespective of the methods of gene delivery, is the key step for the final recovery of transgenic plants. Dominant selectable markers are an integral part of plant transformation strategies. For this purpose, a selectable marker gene is used either co-integrated in the plasmid with the gene of interest or harbored on a separate plasmid for co-transformation. A number of selective agents and suitable resistance genes have been investigated concurrently with studies on gene transfer and cell culture.

The most widely used inhibitors are kanamycin, geneticin (G418) and hygromycin. All are aminoglycoside antibiotics which interfere with the translation machinery of prokaryotic and eukaryotic cells. However, they can be inactivated by phosphorylation reactions mediated by the products of either the Tn5 neomycin phosphotransferase II (*npt*II gene, also known as *aph*II or *neo*; Herrera-Estrella et al. 1983), or the hygromycin phosphotransferase gene (*hph*, also called *hpt* or *aph*-IV) originally isolated from *Eschericia coli* (Blochinger and Diddelmann 1984). Although kanamycin has been successfully used as a selective agent in plant transformation, it has some limitations, such as its low efficiency in screening transformed calli and the inability of transformed calli of some species to regenerate green plants. These problems were circumvented by the use of G418 (Peterhans et al. 1990). Currently, the hygromycin B-resistance gene is widely used as an efficient selective agent for almost all the transformation methods in several crops, including rice, without any problems relating to albino plant regeneration or plant fertility (Datta et al. 1990; Lin et al. 1995; Tu et al. 1998a, b).

The bialaphos (also called Basta) resistance gene (*bar*), encodes phosphinothricin (PPT) acetyltransferase (PAT), and acetylated phosphinothricin is no longer inhibitory to glutamine synthase. PPT or Basta has been used as a selective agent for a number of crop plants (Datta et al. 1992; Rathore et al. 1993; Ho et al. 2006). Hence, the usefulness of a particular resistance marker depends upon the characteristics of the selection agent, the resistance gene and the plant material (Angenon et al. 1994).

The use of all these genes as selectable markers poses a cautionary risk for the environmental release of the transgenic products. A recent development is based on the use of selective genes, which give the transformed cells a metabolic advantage compared with the untransformed cells, which are starved with a concomitant slow reduction in viability. Such a strategy involves

the use of mannose as the selective agent, which after uptake is phosphorylated by a hexokinase to an unmetabolized mannose-6-phosphate that accumulates in cells, resulting in severe growth inhibition. However, the phosphomannose isomerase gene (*pmi*) allows conversion of mannose-6-phosphate to fructose-6-phosphate, which is readily metabolized. The *pmi* gene as a selectable marker gene has been and is being used for plant transformation, including rice (Joersbo et al. 1998; Datta et al. 2000, 2003a, b, 2006; Lucca et al. 2001).

Similarly, in plant transformation studies, reporter (assessable marker) genes are necessary for rapid detection of DNA introduction. They are usually fused to the plant regulatory sequences in vitro and are used to determine when, where and at what level a regulatory sequence directs gene expression in vivo. Also, they can be used for protein targeting if fused to a signal peptide coding sequence. Such reporter genes of very common use include the luciferase gene (*luc*) and β-glucuronidase (*gus*) genes (Jefferson et al. 1986). The intrinsically fluorescent proteins (IFPs), such as the green, yellow and cyan fluorescent proteins, have been used as reporter genes to monitor transcriptional regulation and protein kinase activity (Dixit et al. 2006).

4 Transgenics in Stabilizing Production

A considerable proportion of the crop produce is lost due to biotic and abiotic stresses. Conventional breeding, which has often exploited the natural variablility in a species, has produced crop varieties with built-in resistance to several of these stress agents. However, in instances where the natural variability is limited or non-existent, transgenic breeding could be a viable and an alternative solution. Transgenic plants that are tolerant to biotic agents, like insect pests, and disease agents, like viruses, fungi and bacteria, have been produced, although only insect- and virus-resistant transgenic crops have been commercialized extensively. Weeds also reduce significantly crop yields. Transgenic crops with resistance to broad-action herbicides have also been commercialized in several countries. These transgenic crops allow the spraying of the herbicide in a standing crop: the weeds are killed while the crop remains unaffected, making weed control more effective and less costly. Further, it allows "no-till" cultivation aiding in soil and water conservation. Abiotic stresses have been more difficult to tackle by transgenic approaches, but some of the recent developments hold considerable promise (Shinozaki et al. 2003; Singlas-Pareek et al. 2003; Verslues et al. 2006).

4.1 Non-Segregating Homozygous Stable Lines

Isogenic lines using marker-assisted breeding or homozygous lines using anther culture may accelerate crop breeding and stabilizing the improved traits. Since the pioneering report of anther culture published by Guha and Mahesh-

wari (1964), many researchers globally have made significant contributions to crop improvement (Datta 2005). The impact of this technology has now been well appreciated and utilized in marker-assisted population studies, gene tagging and transgenic breeding.

5 Plant Protection

5.1 Insect Resistance

Transgenic crops with built-in plant protection can be cited as one of the exemplary success stories of agricultural biotechnology. The transgenic *Bacillus thuringiensis* (*Bt*) varieties are in many ways better than using *Bt* as a spray formulation. In the *Bt*-transgenics, the protein is expressed in all tissues at all times, whereas the effectiveness of the sprays is affected by lack of uniform coverage and instability of the *Bt* protein, especially on exposure to sunlight. Considerable progress has been made in developing transgenic crops with resistance to the target insect pests during the past decade. Although there have been many approaches to incorporate insect resistance in transgenic plants, transgenic plants carrying the insecticidal protein gene from *Bt* have been the most successful by far. *Bt* is a soil bacterium that makes crystalline inclusions (*cry* proteins) during sporulation. These crystals dissolve in the alkaline environment of the insect gut and release protoxin molecules that are processed by the gut proteases to give active insecticidal proteins. These proteins interfere with the ion channel pumps and ultimately lead to the death of the insect larva that ingested the crystals. Such proteins are quite specific in their host range (determined largely by ligand–receptor interaction) and this fact has been exploited in the development of transgenic plants tolerant to specific groups of insect pests. More than 50 different *cry* proteins have been characterized which have different target insect specificity.

The first transgenic tobacco plants with *Bt* were produced in 1987 (Fischoff et al. 1987; Vaeck et al. 1987). Gene truncation, use of different promoters, enhancer sequences and fusion proteins resulted in significant improvement of the amount of insecticidal proteins in the transgenic plants (Perlak et al. 1991). NewLeaf is the brand name of the first *Bt* product to be commercialized in 1995, a transgenic potato expressing *cry*3A protein to control Colorado potato beetle. The introduction of this product reportedly reduced chemical insecticide use by 40%. This was followed by the release of pest-resistant transgenic cotton and corn. Subsequently, several *Bt* crops have been released for cultivation and, in 2000, such insect-resistant crops occupied 8.2×10^6 ha globally (James 2005). It has been estimated that *Bt*-cotton alone cut the use of chemical insecticides in the United States by over 2×10^6 lb (approx. 10^6 kg) or nearly 10^6 gal (approx. 3.75×10^6 l) from 1996 to 1998. Further, the study found that *Bt*-cotton increased yields by 85×10^6 lb and farmer profits by U.S. $\$ 92\times10^6$

in 1998, whilea significant economic benefit to small-holding farmers has been reported from India and China (James 2005). This is an example of the potential of biotechnology to provide a solution for combating a problem in a manner that is more environment-friendly. Among the cereals, maize was the first one to be transformed and field-tested with a *Bt* gene exhibiting high-level resistance to European corn borer (Koziel et al. 1993). After the first transgenic crop produced with a codon-optimized and truncated *cry* gene, several reports have accumulated in the recent past for developing transgenic rice carrying single or fused *cry* genes under different constitutive or tissue-specific promoters that showed resistance to stem borers and leaf-folder insects under glasshouse as well as field conditions (Wu et al. 1997; Alam et al. 1998, 1999; Datta et al. 1998; Tu et al. 2000a; Ye et al. 2001). Two reports from the International Rice Research Institute (IRRI), Philippines, in collaboration with Wuhan University and Jhejang Agricultural University are the first reports of transgenic hybrid rice (Shan you 63) as well as an elite indica IR72 with fused *Bt*-genes that were field-tested in China (Tu et al. 2000a; Ye et al. 2001). It is reported that some farmers at Hubei province in China found it beneficial to grow and to consume this pesticide-free GM rice (Gu 2005). The reports provided quotes such as "Zhang Qifa, conducted the mainland's largest field trials on GM rice. When interviewed by Newsweek in December last year, Professor Zhang mentioned that farmers near the GM test areas in Hubei had grown and eaten such rice without any side effects". There were many challenges in the production of the first *Bt* transgenic crops, but the infrastructure for these is now well established in some countries, including the United States, Canada, China and India. An important recent finding shows that *Bt*-rice in the field does not have any significant effect on non-targeted environment-friendly insects (Chen et al. 2006).

The insecticidal protein gene, being bacterial in origin, is expressed poorly in plants. Extensive codon optimization has been carried out with many of these native bacterial genes in order to obtain useful levels of expression in plants. Low expression levels were also addressed by directly transforming the plastids of plants with transgenes (De Cosa et al. 2001). Since the plastid has a gene expression machinery similar to prokaryotes, the genes could be introduced without extensive modification and the number of plants in a given plant cell results in very high expression.

There have been some concerns regarding the use of *Bt*-transgenic crops, the two major ones being their effect on non-target organisms and the possibility of the target insects developing resistance to the *Bt* protein. A report in *Nature* (Losey et al. 1999) indicated that monarch butterfly larvae were affected when fed with pollen from *Bt*-corn; and this was widely and incorrectly interpreted to mean that *Bt*-crops were threatening non-pest insects. Several follow-up studies showed the effect of pollen from *Bt*-crops had negligible effect on non-target insects, including butterflies under field conditions (Hodgson 1999). Though *Bt*-crops have been under wide cultivation since 1995, there has not been any instance of pests developing resistance. However,

given the experience of the diamond-back moth having developed resistance to *Bt*-sprays, the development of resistance in insects cannot be discounted. As a pro-active measure, several strategies for insect resistance management have been evolved as a package for the cultivation of *Bt*-crops. These strategies include refugia (growing a small proportion of the area under a non-*Bt* crop, along with the *Bt*-transgenic crop), gene pyramiding and a high dosage of the protein in transgenic plants to prevent any insects escaping in the *Bt*-field. A recent study (Tabashnik et al. 2000) showed that the natural frequency of the recessive resistance alleles did not increase in spite of extensive cultivation of *Bt*-cotton between 1997 and 1999; and the *Bt*-cotton crop remained extremely effective against the pink boll worm.

In addition to the *Bt cry* gene, other candidate genes have been used to develop insect-resistant transgenic plants. These include protease inhibitors, α-amylase inhibitors, chitinases, lectins, vegetative insecticidal proteins from *Bt* cholesterol oxidases and toxins from predators such as mites and scorpions (Datta et al. 2003a, b).

5.2 Virus Resistance

Transgenic papaya virtually rescued the papaya industry in Hawaii from the threat of the dreaded ring spot disease (Yeh et al. 1998). The transgenic approach would be more appropriate in situations where sufficient levels of resistance to the virus are not available in the related germplasm, or resistance is difficult to transfer by normal crossing techniques, due to either reproductive isolation or linkage with other undesirable traits. The production of virus-tolerant transgenic plants has been based on several approaches. In most instances, a gene coding for the complete or part of a viral protein has been introduced into the crop by transformation.

Many different approaches have been made in an effort to build up resistance to viral pathogens in plants. The underlying mechanisms are different and complex for these approaches and not clearly understood. Genes encoding structural (coat protein) and non-structural (replicase, movement proteins) proteins have been effectively used to confer resistance to plants (Beachy et al. 1990). Antisense RNA against the coat protein gene and defective interfering transcripts were also shown to be effective in controlling viral infection.

β-1,3-Glucanases, known to be involved in plant defence against fungal infection are induced during the local lesion response to viral infection (Kauffmann et al. 1987). Their role in viral pathogenesis is not clearly established. Beffa and Meins (1996) developed transgenic tobacco plants with reduced β-1,3-glucanases by expressing antisense RNA directed against the transcript. These plants exhibited decreased susceptibility to tobacco mosaic virus and tobacco necrosis virus.

Antisense RNA molecules have been deployed in controlling viroid replication *in planta*. Yang et al. (1997) showed that ribozymes could be effectively

used to control replication of viroid RNA in the nucleus. Potato spindle tuber viroid has a small RNA genome with a nuclear replication phase. Yang et al. (1997) showed that transgenic potato plants expressing a ribozyme against the PSTVd minus strand RNA were resistant to the viroid. This is in clear contrast to the antisense RNA approach for the same viroid–host interaction. These experiences suggest that the strategy for controlling the pathogen will have to be carefully devised, taking into consideration the nature of biological interaction between the host and pathogen on a case-to-case basis.

5.3 Resistance to Fungal Diseases

Plant species deploy an assortment of defensive responses soon after infection or exposure to fungal infection. These responses involve the biosynthesis and accumulation of pathogenesis-related proteins (PR proteins;, Datta and Muthukrishnan 1999). Besides, there are resistance genes in the plant's upstream defensivee responses that recognize the avirulence genes in the pathogens, which otherwise is known as the gene-for-gene hypothesis. Considerable progress has been made in the deployment of a transgenic strategy for development of transgenic crops resistant or tolerant to fungal diseases utilizing these resistant (R) genes as well as overexpressing the PR genes. Amongst the PR genes, the most attractive initial candidates for manipulation of the single-gene defensive mechanism approach are the genes encoding chitinases and β-1,3-glucanases, because of their hydrolytic action on chitin and glucan respectively, the two major structural components of the fungal cell wall. The first report of success was the resistance of transgenic tobacco and *Brassica napus*, expressing a bean vacuolar chitinase under the CaMV 35S promoter against the necrotrophic fungus, *Rhizoctonia solani* (Broglie et al. 1991). The detail of successful reports on several crops over-expressing chitinase (PR-3 group) and glucanase (PR-2 group), cloned from different sources, singly or in combination, as well as several other PR genes of different groups is documented by Baisakh et al. (2001) and Datta et al. (2001). In rice, the first report on over-expression of a rice chitinase gene showing enhanced resistance against sheath blight fungus, *Rhizoctonia solani*, came in 1995 (Lin et al. 1995). Thereafter, regulation, expression and function of different chitinase genes have been studied in rice. A PR-5 group protein, thaumatin-like protein (TLP) from rice was introduced into rice and the high level constitutive expression of the TLP protein gave the transgenic plants enhanced resistance against *R. solani* (Datta et al. 1999).

Combinatorial expression of multiple PR genes along with the R genes could provide effective and durable resistance in crop breeding (Wenzel 1985). Transgene pyramiding has been achieved to confer resistance to bacterial blight, sheath blight and stem borer (Datta et al. 2002; Narayanan et al. 2004).

5.4 Resistance to Bacterial Infection

Considerable research effort has been directed to utilizing resistance (R) genes in crop breeding programs, including those of rice. Five different classes of R genes cloned from different plant species have been characterized and tested for their efficiency in conferring resistance against bacterial pathogens. Among these, the fifth class, representing a map-based cloned *Xa21* gene from rice conferring resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is well studied (Song et al. 1995). *Xa21* encodes a receptor-like kinase, consisting of leucine repeat regions (LRRs) in the putative extracellular domain, and a serine-threonine kinase in the putative intracellular domain, thus indicating an evolutionary linkage with the other classes of R genes at the molecular level. By virtue of its wide-spectrum resistance against bacterial blight, a serious disease of rice, *Xa21* was the first R gene to be transformed into this crop. The IRRI is focusing on the development and deployment of transgenics with *Xa21* against bacterial blight (BB), a major disease of rice. *Xa21* was transformed into rice first by Song et al. (1995), who showed resistance to a single isolate, race 6, of the BB pathogen. Subsequently, several reports showed the broad-spectrum resistance of transgenic rice with *Xa21* gene against diverse isolates from different countries, indicating the fact that a single cloned gene is sufficient to confer multi-isolate resistance (Tu et al. 1998b; Datta 2004). The transgene *Xa21* followed a Mendelian segregation (3:1) pattern in the subsequent selfing generation, indicating a single locus insertion.

The first report of transgenic rice with *Xa21* developed at IRRI was field-tested at Huazhong University, Wuhan, China (Tu et al. 2000b). Further field evaluation was carried out in the Philippines and India, which showed clearly the resistance of the transgenic homozygous lines to multiple strains and races of the BB pathogen under natural field conditions as well as after artificial inoculation (Datta 2004; Laha et al., unpublished data). This indicates the potential of transgenic breeding for broad-spectrum resistance to bacterial disease(s) with a single cloned R gene. Research is in progress in many laboratories for the deployment of novel R genes or novel members from the same family (like *Xa5* and *Xa13* in the *Xa* family) for durable, multiple and horizontal resistance (Huang et al. 1997).

5.5 Herbicide Resistance

Weeds are a major concern in several important crops, including rice, since they cause considerable yield loss. The chemical control of weeds through the application of herbicides poses a threat to human health and the environment (Pandey and Velasco 1999), apart from the heavy costs involved. Moreover, as the herbicides are usually non-selective, they do not distinguish the crop plants from the weeds. Engineering for herbicide tolerance is a new way of conferring selectivity and enhancing crop safety and production (James 2005).

Hence, herbicide technology combined with a transgenic approach may give farmers the opportunity to modernize agricultural technology with reduced labour (such as the choice of crops for rotation or double cropping) in an environmentally friendly system.

Mechanisms of herbicide resistance include: (1) a modified target site, (2) enhanced detoxification or delayed activation and (3) alterations in the uptake, translocation, or compartmentalization of a herbicide. The first two mechanisms have mainly been identified in plants. Herbicide resistance genes were isolated for several herbicides of different modes of action. Genes that coded for herbicide target or detoxification enzymes were transferred into crop plants. The transgenic plants expressing these genes were tolerant to the active ingredients of herbicides. Herbicide-tolerant transgenics are now available in several crops, including soybean, cotton, rice, rapeseed, maize, sugarbeet and alfalfa (Rai and Prasanna 2000; James 2005).

Several reports have been produced in the United States on field trials of herbicide-resistant transgenic plants in diverse crops, including rice, wheat, maize, barley, tomato, tobacco, potato, cotton, soybean, rapeseed, peanut, sugarcane, alfalfa, pea, clover, beet, cantaloupe, grape, lettuce, carnation and kiwi (Datta 2004; James 2005). The common herbicides for which herbicide tolerance has been engineered include glyphosate (Roundup), phosphinothricin (Basta, Herbiace), bromoxynil (Buctril), sulfonylurea (Glean, Oust) and 2,4-D.

5.6 Drought, Salinity and Cold Stress Tolerance

Crop productivity is affected or limited due to a variety of environmental factors, including high/low temperature stress, salinity and water supply. Modifying crops to tolerate unfavourable environmental conditions could improve their productivity under hostile conditions and also could expand the area of land under cultivation. A range of genes isolated from bacteria, animals and plants have been tested for their ability to confer stress tolerance in plants. These analyses, to a large extent, have been carried out in dicotyledonous model systems, like those of *Arabidopsis* and tobacco. However, there are a number of studies that have tested various genes in other plants, including rice and maize. Most of the abiotic stresses eventually cause water deficit. While a minor water deficit results in a reduction in photosynthesis, major deficits lead to complete inhibition of photosynthesis and the production of reactive oxygen intermediates, such as superoxides and peroxides. These intermediates affect membrane integrity and cause severe impairment of several physiological processes and biochemical reactions. Hence, a number of transgenic approaches involve the use of genes whose products can either scavenge or protect from reactive oxygen species.

The production of transgenic plants that accumulate osmoprotectants (e. g. glycine betaine) and osmolytes has been a very popular approach to study

tolerance to salinity, drought and cold stress. Osmolytes are low molecular mass molecules and may be quaternary amines, amino acids or sugar alcohols. Some plant species that have inherent tolerance to abiotic stresses have been shown to accumulate these osmolytes under stress. These molecules are known to raise the osmotic potential of cells and, as a result combat water stress. They could stabilize membranes and other macromolecular structures.

The genes that can potentially confer abiotic stress tolerance fall into three categories, since they encode: (a) enzymes that make protective metabolic products, (b) regulatory proteins and (c) protective proteins. Studies with some such genes, the expression of which could either lead to accumulation of an osmolyte or make the manufacture of a specific protein, which can scavenge radicals, are discussed below.

5.6.1 Mannitol-1-Phosphate Dehydrogenase

Targeting mannitol biosynthesis to chloroplasts in transgenic tobacco plants increased tolerance to oxidative stress (Shen et al. 1997). Transgenic plants with mannitol-1-phosphate dehydrogenase (*mtlD* gene) showed increased mannitol accumulation in seedlings with increased shoot height and greater shoot and root fresh weights under salt stress compared with non-transgenic plants.

5.6.2 γ^1-Pyrroline-5-Carboxylate Synthetase

Under water stress, many plants accumulate compatible osmolytes which could protect components of their cells from damage. Proline, a potent osmolyte, accumulates in water-stressed plants and, when the stress is removed, proline concentrations are restored and these changes in proline level are tightly regulated. The enzyme γ^1-pyrroline-5-carboxylate synthetase (P5CS) catalyses the conversion of glutamate to γ^1-pyrroline-5-carboxylate, which is then reduced to proline. Over-expression of a gene encoding for mothbean P5CS in transgenic tobacco plants resulted in accumulation of proline (Kishor et al. 1995).

5.6.3 Trehalose-6-Phosphate Synthetase Gene

The yeast trehalose-6-phosphate synthetase gene (*TPS1*) was introduced into tobacco to overproduce trehalose which is a non-reducing disaccharide that can serve as an osmolyte. Transgenic plants accumulating trehalose exhibited multiple phenotypic alterations and improved drought tolerance. Transgenic rice with over-expression of trehalose, showed abiotic stress tolerance under glasshouse conditions (Garg et al. 2003). The transgenic lines showed improved growth under drought stress in terms of their dry weight and produced larger leaves. Dry weight of leaves under stress was 85% greater in transgenic plants with the maximum trehalose accumulation in comparison with the control

plants. Leaves detected from young, well watered transgenic plants showed a better capacity to retain water when air-dried than the wild-type plants. Transgenics performed more efficient photosynthesis under stress and also accumulated non-structural carbohydrates along with glucose, fructose and sucrose. They were able to maintain a lower osmotic potential than wild-type plants, assisting them to survive better under stress. A more recent study with transgenic potato constitutively overexpressing the yeast *TPS1* gene corroborates earlier results, correlating trehalose accumulation to drought tolerance (Yeo et al. 2000).

5.6.4 Levansucrase

Fructans are polyfructose molecules that are produced by many plants and bacteria. Owing to their solubility, they facilitate plant survival under periods of osmotic stress. Their drought resistance correlates well with the amount of fructan accumulated. Transgenic plants have been developed from several other crops such as potato, tobacco, chicory and barley with levansucrase, these plants exhibiting improved growth under drought stress, compared with their wild-type counterparts (Meer et al. 1994).

5.6.5 Myo–Inositol O-Methyltransferase

Myo-inositol O-methyltransferase (*IMT1*-encoded enzyme) leads to the production of the methylated inositol D-ononitol. Expression of a cDNA encoding myo-inositol O-methyltransferase in tobacco during salt and drought stress resulted in the accumulation of methylated inositol (D-ononitol) that, in turn, conferred tolerance to both stresses. Transgenic plants had more photosynthesis and CO_2 fixation than untransformed, wild-type plants.

5.6.6 Choline Oxidase

Water stress conditions in halophytes and some bacteria result in the accumulation of glycine betaine as an adaptive response. The choline oxidase gene (*codA*) isolated from *Arthrobacter globiformis*, that converts choline to glycine betaine via betaine aldehyde, was fused with a transit peptide for plastid localization and introduced into *Arabidopsis*. The transgenic plants accumulated glycine betaine (up to 50 μM in the chloroplasts) and showed enhanced tolerance to salt and cold stress. This comprehensive study carried out by Huang et al. (2000) clearly indicates that mere installation of a betaine biosynthetic pathway was insufficient to confer cold tolerance to *Arabidopsis*, tobacco and *Brassica*. Their results indicated that the subcellular compartmentalization of the biosynthesis of glycinebetaine was a critical element in the efficient enhancement of tolerance to stress in the engineered plants.

5.6.7 Polyamines

Polyamines have been implicated in stress tolerance responses in plants.

5.6.8 Oxidative Stress–Related Genes

Stress induces several processes in plants leading to the generation of reactive oxygen species that cause oxidative stress. Plants have evolved systems to combat oxidative stress, with a battery of gene products that aid in quenching the active oxygen species that damage primarily membranes. Examples of some of the enzymes involved in such protective processes include superoxide dismutases, glutathione reductase, glutathione-*S*-transferase/peroxidase, catalases and glyoxalases.

Van Breusegem et al. (1999) developed transgenic maize plants with an *Arabidopsis* Fe-SOD expressed under the CaMV35S promoter and containing the chloroplast-targeting transit peptide signal from pea Rubisco. They observed enhanced resistance to methyl viologen (a chemical that induces oxidative stress) and increased growth rates under low temperatures in transgenic maize plants. More work is essential to understand the various oxidative stress-related genes and the interaction of their products during stress.

5.6.9 Late Embryogenesis Abundant Protein Gene hva1

A barley group 3 late embryogenesis abundant (LEA) protein HVA1 was previously characterized from barley aleurone. This gene, *hva1*, is expressed specifically in the aleurone layers and embryos during late seed development, correlating with the seed dessiccation stage. The expression of this gene is rapidly induced in young seedlings by ABA and by several stress conditions, including dehydration, salt and extreme temperatures.

5.6.10 C-Repeat Binding Factors and Dehydration Responsive Element Binding Protein

Many cold-regulated (COR) genes have a common upstream regulatory motif, called C-repeat. These are co-ordinately regulated by C-repeat binding factors (CBF). The same motif is also referred to as DRE (drought responsive element). Transcription factors bind to these motifs under stress and activate the expression of genes, resulting in tolerance to the stress. Over-expression of the *Arabidopsis* transcription factor CBF1 using the CaMV35S promoter resulted in enhanced freezing tolerance. Kasuga et al. (1999) demonstrated the utility of expressing a transcription factor in an inducible fashion to obtain stress-tolerance in plants. When CBF3 was constitutively over-expressed, the recovery of transgenic plants was affected. Also, the regenerated plants displayed stunted growth and other morphological abnormalities. However,

when the expression was driven by a cold-inducible rd29A promoter of *Arabidopsis*, the same markers obtained healthy transgenic plants that were more tolerant to drought, salt and low temperature than plants constitutively expressing CBF3. Expression of a master switch gene in an inducible manner to control stress-responsive gene expression could be a valuable approach to engineer stress tolerance in crop plants (Shinozaki-Yahguchi and Shinozaki 2000). Similar work with transgenic wheat has been reported by Pellegrineschi et al. (2004) and transgenic rice with the dehydration responsive element binding protein (DREB) gene showed significant results for drought and salt tolerance (K. Datta, personal communication, unpublished data).

6 Enhancing Shelf Life

6.1 Tomato Fruit Ripening

Tomatoes are usually harvested in the green stage to enable mechanical handling and longer shelf life. However, this prevents the development of complete flavour that is obtained with vine-ripened fruits. Expression of an antisense polygalacturonase construct resulted in delayed softening without interfering with the ripening process (Sheehy et al. 1988; Smith et al. 1988). With these transgenics, it is possible to mechanize handling after the fruits are allowed to ripen fully on the vines. Calgene marketed such transgenic tomatoes under the brand name "Flavr-Savr".

Another approach involved the inhibition of ethylene biosynthesis to delay the ripening process. This was achieved by overexpressing a 1-aminocyclopropane-1-carboxylate (ACC) deaminase isolated from a bacterial source. The enzyme degrades ACC, an intermediate in ethylene synthesis (Klee et al. 1991).

7 Improving Productivity

The primary goal of crop improvement has been to increase productivity. The first wave of transgenics has successfully addressed input traits, like insect and herbicide resistance, to sustain the maximum yield potential of the crops. The second wave of transgenic varieties is expected to enhance output traits such as yield. This could be achieved through improving the source strength by essentially increasing net photosynthesis at the leaf level. There are many steps at which photosynthetic losses can occur, including light harvesting, electron transport, carbon assimilation, partitioning of the photosynthate and respiration. Various approaches which could minimize these losses would be to alter canopy structure, improve light acclimation, improve photoprotection, incorporate features of C_4 photosynthetic pathway into C_3 crops, alter stomatal responses, modify carbohydrate metabolism, delay leaf senescence, alter

metabolite signalling, reduce respiration capacity and improve nitrogen economy. It is unlikely that achieving one of the above with a transgene would be reflected directly in yield improvement. In the source strength to sink capacity continuum, all of the above play major roles. Understanding the effect of expression of some useful genes in photosynthesis and partitioning will give clues to the useful combination of alterations that could result in discernible increases in yield. Various efforts along these lines have been recently reviewed (Horton 2000; Ashikari et al. 2005).

8 C4 Pathway in C3 Plants

The majority of plants, including important crops like rice and wheat, fix atmospheric carbon dioxide by the C_3 photosynthetic pathway. An inherent feature of the C_3 pathway that reduces the overall photosynthetic efficiency is the oxygenase reaction of Rubisco. Oxygen that is released by this activity inhibits the carbon fixation reaction and leads to loss of CO_2 through photorespiration. Environmental stresses, like high temperature and water limitation, could further decrease photosynthetic efficiency. Efforts to engineer Rubisco such that its carboxylase activity dominates over oxygenase activity may not be fruitful (Somerville 1990).

C_4 plants like maize, in contrast, have evolved mechanisms by which oxygen-mediated photosynthetic efficiency reduction could be overcome. The C_4 pathway enables increased local CO_2 concentration around Rubisco, inhibiting its oxygenase activity, which also is reflected in reduced photorespiration. Phosphoenol pyruvate carboxylase (PEPC) mediates the fixing of CO_2 into the C_4 acid, oxaloacetate. PEPC is insensitive to oxygen and has a very high affinity for bicarbonate, which is its substrate. These features of C_4 plants confer higher photosynthetic capacity and better water and nutrient use efficiency in comparison with C_3 plants, especially under low CO_2 conditions.

Efforts to constitutively over-express C_4 PEPC in C_3 plants did not yield a significant increase in photosynthetic capacity when the CaMV35S promoter was used to drive gene expression (Gehlen et al. 1996). The expression level of the transgene was found to be low in these studies. Introduction of a native maize PEPC gene into rice resulted in high expression of the gene and accumulation of high concentrations of the enzyme. Transgenic rice plants with high concentrations of PEPC were characterized by reduced quantum of photosynthesis inhibition by oxygen (Ku et al. 1999; Bandyopadhyay et al. 2006). This is a classic example that shows installing a new pathway in a plant using a single gene from a different source. Maize PEPC gene was also introduced into indica rice and the photosynthetic efficiency of the primary transgenic plants are under evaluation (Datta et al., unpublished data). Therefore, efforts are being made to install all the three key enzymes involved in the C_4 pathway [PEPC, pyruvate orthophosphate dikinase (PPDK), NADP-malic enzyme (NADP-ME)] in

rice for realizing maximum photosynthetic efficiency. Moreover, further work need to be done to study whether in the field improved photosynthesis would be seen in such transgenic rice plants under stress conditions.

It is also important to consider the impact of transgenesis in the transgenic plants. In the work carried out by Ku et al. (1999), even though the protein encoded by the transgene accounted for 12% of the total soluble protein in the leaves, phenotypic abnormalities or loss of fertility were not encountered. It is known that NADP-dependent malic enzyme (NADP-ME) is induced under low CO_2 conditions and thus might assist in improving photosynthetic capacity under stress. Tsuchida et al. (2001) over-expressed the maize NADP-dependent malic enzyme in the chloroplasts of rice.

8.1 Exploiting Heterosis

Heterosis is a proven way of increasing productivity in many crop species. However, in many grain crops the exploitation of heterosis hinges on the availability of a good male sterility and fertility restoration system. Over the past several decades, many cytoplasmic male sterility (CMS) sources have been developed and used for hybrid seed production in various crop species, including rice. In many cases, the unavailability of correct CMS sources and/or restoration systems have been a major limitation in the development of commercial F_1 hybrid varieties. Sometimes, the lack of diverse cytoplasmic sources has given rise to concerns about cytoplasmic uniformity and the consequent genetic vulnerability to pest and disease outbreaks. All these point to a need to develop alternative male sterility sources and fertility restoration systems. Transgenic plants can address this need, as has been demonstrated at least, in a few cases.

One of the earliest and successful attempts to induce male sterility by genetic engineering involved the transfer and tissue-specific expression of a "toxin" gene that disrupted normal pollen development. The toxin gene in this case was an RNase, *Barnase*, from a fungal source, which was engineered to express specifically in the tapetal tissue of developing tobacco anthers by using the tapetum-specific promoter TA29 (Mariani et al. 1990). Several similar studies have been reported in different crops, including oil seed rape (Pental 2003).

9 Nutrition-Rich Crops

Since agriculture is the primary source of nutrients and poor diets are a fundamental cause of malnutrition, it is essential to have nutrition-rich crops for solving the problems due to such deficiencies. Nutritional genomics will have a tremendous impact on the improvement of foods for human health (DellaPenna 1999). Datta and Bouis (2000) have discussed the potential of

biotechnology in improving human nutrition through the very recent breakthroughs in genetic engineering, e. g., the development of golden rice and iron-fortified crops.

Three different genes involved in the metabolic pathway, phytoene synthase, lycopene cyclase (*psy* and *lyc* cloned from *Narcissus pseudonarcissus*) and phytoene desaturase (*crtI* cloned from *Erwinia uredovora*), have been introduced into rice through *Agrobacterium*-mediated transformation that resulted in what is called as the "Golden Rice" producing beta carotene, the latter ultimately being converted to vitamin A in the human body (Ye et al. 2000). This is a remarkable achievement in transgenic research that would help eradicate blindness caused by vitamin A deficiency. Research is in progress at the IRRI, Philippines, to engineer these genes for the production of transgenic indica rice with β-carotene in different elite IRRI-bred cultivars, as well as other cultivars adapted to different developing countries (IRRI 2000; Datta et al. 2003, 2006; Paine et al. 2005; Parkhi et al. 2005).

Similarly, iron deficiency leading to anaemia, which affects about 30% of the world's population, is prevalent in the developing countries. People mainly obtain their daily iron requirement from vegetables. Consequently, increasing the iron content of plants by genetic manipulation would have a significant bearing on human health. An iron-storage protein gene, *ferritin*, driven by the constitutive CaMV 35S promoter, was transferred into tobacco where the leaves of transgenic plants had a maximum of 30% more iron than the non-transformed control plants (Goto et al. 2000b). In lettuce, the transgenic plants had 1.2–1.7 times more iron and enhanced early developmental growth and superior photosynthesis than the control plants (Goto et al. 2000a). In rice and wheat also, the use of a constitutive promoter resulted in more iron content in vegetative tissues, but not in the seed (Drakakaki et al. 2000). However, the ferritin gene placed under an endosperm-specific promoter, glutelin, was expressed in the seeds (the target tissue) in Japonica rice (Goto et al. 1999). The iron content in the endosperm of transgenic rice was significantly higher than that of non-transformed plants. All these studies used the *ferritin* gene cloned from soybean. Recently, Lucca et al. (2001) also observed increased iron content in rice seeds transformed with a *Phaseolus* ferritin gene.

Furthermore, since endogenous phytic acid inhibits iron bioavailability, a heat-stable phytase from *Aspergillus fumigatus* was introduced into rice (Lucca et al. 2001), which increased the level of phytase 130-fold, sufficient to hydrolyse the phytic acid. In addition, as cysteine peptides are considered a major enhancer of iron absorption, an endogenous cysteine-rich metallothionein-like protein was overexpressed in rice, which increased the cysteine residues 7-fold (Lucca et al. 2001). The high phytase rice, with increased iron content and rich in *cysteine* peptide, could potentially improve the iron nutrition in rice-eating populations, leaving the potential open to other crops.

Another gene, ferric chelate reductase (*FRO2*), that allows plants to uptake more iron in iron-deficient soil (Robinson et al. 1999), allows the possibility of co-integrating it with *ferritin*, *phytase* and *cysteine* peptide genes for high

iron uptake and storage in plants, together with bioavailability and absorption in the human body. At IRRI, transgenic rice has been produced with *FRO2* and ferritin genes. Interestingly, transgenic plants showed 2- to 3-fold more iron in polished seeds along with enhanced levels of zinc. Further work is in progress on the molecular breeding of transgenic rice combining both ferritin and genes for carotenoids (*psy* and *crt1*; Datta et al. 2000; Vasconcelos et al. 2003; Khalekuzzaman et al. 2006; Alamgir et al., unpublished data).

Another important essential, but limiting amino acid in rice, lysine, that promotes uptake of trace elements, is a potential candidate for the nutritional improvement of crops. Two bacterial genes, *dapA* and *lysC* from *Corynebacterium* have been shown to enhance the lysine about 5-fold in canola, corn and soybean seeds (Falco et al. 1995; Mazur et al. 1999). Keeping in view the realistic approach for the introduction of these genes into rice, IRRI is collaborating with DuPont to develop lysine-rich rice (Russel et al., unpublished data). Several food crops are now being developed with enhanced vitamin E, vitamin C, inulin, modified starch and amino acid profiles and Ama1 protein (Chakraborty et al. 2000; Galili et al. 2002; Baisakh and Datta 2004).

10 Conclusion

Biotechnology, especially that involving transgenic plants (GM crops) can improve farmer and consumer welfare in many ways, for example, through increased crop productivity by the development of built-in disease and insect resistances. Such value-added crops would have significant impact in increasing the food supply, thereby helping to reduce food prices for poor farmers. Recent significant achievements in plant genetic engineering for nutrition-rich crops would have a bearing in reducing malnutrition, while farmers will always benefit from growing such crops with a guaranteed increased financial return. However, complete realization of such biotechnological potential will take time, with a large investment in agricultural research and other public and on-farm infrastructure (Datta and Bouis 2000).

As with any other technology, controversy surrounding GM crops is no exception in a heterogeneous society. Several concerns need to be addressed on different issues like food safety, beneficiaries of the technology and conflict between the PVP and IPR of gene and technology discovery. Finally, successful product development needs extensive field trials and public understanding. Based on the experience of several field-evaluations of GM crops, it is predicted that gene technology, combined with precise plant breeding and efficient crop management, might provide the benefits that people require. Once the products of genetic engineering in agriculture reach small farms and industrial-level operations, everyone can benefit from such developments (Datta 2000). Scientists must play a sensible role in the society by convincing the policy makers to expand further the application of biotechnology in mod-

ern agriculture and human welfare. The technology must be utilized in greater co-operation with technology owners/developers, agri-industry and farming in both developing and developed countries to stay in tune with the modern era of a technology-based civil society. All people require food, and the policy makers have the responsibility to make the correct decisions for its adequate implementation and delivery.

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Section II Cereals

II.1 Rice

Y. Wang, M. Chen, and J. Li[1]

1 Introduction

Rice (*Oryza sativa* L.) is one of the most important agricultural staples that feed more than half of the world's population. The demand for rice production is likely to increase in the coming decades, especially in the major rice-consuming countries of Asia, Africa and Latin America, due to the population explosion and cropland reduction. The ideal traits for future rice varieties include high and stable production yields, good grain quality and enhanced resistance to biotic and abiotic stresses. Consequently, it is necessary to renovate current rice biotechnologies, such as hybrid rice development techniques and genetic manipulation of important agricultural traits. This chapter briefly introduces the ideas and principles of rice biotechnology and its applications in developing elite rice varieties.

2 Hybrid Rice

The application of heterosis (hybrid vigor) in the first generation (F_1) progeny has improved crop breeding universally for many decades. Davenport (1908) and East (1908, 1936) hypothesized dominance and overdominance theories in the early twentieth century to grasp the genetic basis of heterosis. However, recent studies suggest that heterosis may result from partial to complete dominance, overdominance, epistasis, or a combination of all of these effects (Comstock and Robinson 1952; Yu et al. 1997; Hua et al. 2003). Although agricultural practitioners have exploited the genetic and molecular basis to improve rice crop yields, the mechanism that underlies heterosis remains poorly understood.

Because rice is a strictly self-pollinating crop, it is not amenable to hybrid varietal production. In fact, hybrid rice emerged only 30 years ago when Chinese scientists successfully identified and transferred a cytoplasmic male sterile (CMS) wild rice trait to cultivated rice. Since then, China has pioneered multiple hybrid rice technological developments and applications. The land on which hybrid rice grows exceeds more than 50% of the total rice-growing area

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in China (Yuan 2004). Rice hybrid technology is now widely applied in most rice-producing countries, such as The Philippines, India, Indonesia, Myanmar and Vietnam.

Hybrid rice emerged in agriculture in 1973 with a three-line system. The three-line system comprises CMS, maintainer and restorer lines. Crossing CMS and fertility restorer lines produces hybrid rice seeds. Cytoplasmic male sterile lines persist by intercrossing with maintainer lines. Both maintainer and restorer lines are preserved through self-pollination. Scientists use heterosis to identify, study and apply various CMS and restorer lines to develop elite cultivars. To date, three main CMS types (wild abortion, WA; Honglian, HL; Baotai, BT) and five fertility-restoring genes (*Rf1* through *Rf5*) have been reported and the *Rf1* gene was cloned recently (Shinjyo 1984; Yao et al. 1997; Zhang et al. 1997, 2002; Huang et al. 2000; Wang et al. 2006). These are expected to facilitate future hybrid rice technological development.

Although the three-line hybrid system has gained wide recognition for increasing rice yields, hybrid seed production remains economically costly and technologically complicated. To overcome these obstacles, a two-line system was developed in 1995 and is currently being utilized. In the two-line system, the CMS line reverts under certain conditions and functions as a maintainer line. Conditions that affect sterility of CMS lines include temperature and/or photoperiod. The photoperiod-sensitive and temperature-sensitive genic male sterile (PGMS and TGMS, respectively) lines developed under long-day or high-temperature conditions, respectively, are sterile and suitable for making hybrids. Researchers can maintain these lines under short-day or low-temperature conditions by self-pollination. In China, two-line hybrid rice techniques are well developed and have been applied widely in agriculture through a super rice project initiated in 1996.

Production of hybrid seeds through three-line and two-line systems is still costly and sophisticated for most farmers, especially for those in developing countries. As a result, rice farmers must purchase new hybrid seeds each growing season from professional hybrid seed production agencies. A one-line system is ideal for producing hybrids. This is because the heterosis of the F_1 hybrid can persist by apomixis (reproduction without fertilization), thus enabling the farmers to produce hybrid seeds themselves as conventional cultivars. Despite great strides in rice apomixis technology over the past few decades, research in this area has not yet achieved a significant advance. However, studies on rice apomixis are still ongoing (Virmani et al. 2003).

3 Marker-Assisted Selection and Quantitative Trait Locus Analysis

Conventional rice breeding approaches have improved rice cultivars for thousands of years. However, progress is slow, due to the time-consuming process, the quantitative nature of most agronomic traits and difficulties in genotype se-

lection. Over the past decade, researchers have developed and applied marker-assisted selection (MAS) and quantitative trait locus (QTL) analysis techniques to rice breeding. These approaches promote new germplasm identification and new elite cultivar establishment.

A large number of molecular markers must be developed for MAS to be effective in rice breeding. They include expressed sequence tags (ESTs), restriction fragment length polymorphisms (RFLPs), cleaved amplified polymorphic sequences (CAPS), simple sequence repeats or microsatellites (SSR or MS), single nucleotide polymorphisms (SNPs), and insertions/deletions (InDels). Rice geneticists and breeders have leveraged the full-scale sequencing of rice genomes to develop molecular markers for nearly every rice gene and to select desired genotypes, even at the seedling stage. The molecular polymorphisms or markers between the *japonica* rice cv. Nipponbare and the *indica* cv. 93-11 are available to the public (Shen et al. 2004; Bertin et al. 2005; Yu et al. 2005; Zhang et al. 2005).

Most important agronomic traits are quantitative in nature, as they are controlled by multiple genes. Each single gene or QTL accounts for only a portion of the genetic variance and the final phenotype. QTL analysis dissects the genetic network regulating important agronomic traits, including grain yield, grain quality, plant architecture, flowering time, disease and pest resistance, and abiotic tolerance. By using QTL analysis, rice geneticists and breeders have identified many QTLs involved in the control of important agronomic traits. Several major QTLs were cloned or tagged. For example, flowering time or heading date of *japonica* rice is affected by 14 QTLs, eight of which have been cloned (Yano et al. 2000; Takahashi et al. 2001; Kojima et al. 2002). Therefore, the cloned QTLs or genes and their linked molecular markers will provide breeders with trait targets for effective MAS, even at the seedling stage. Furthermore, breeders can select desirable genotypes for subsequent investigation. Clearly, MAS, together with QTL analysis and gene cloning, will allow breeders to develop new rice varieties in the near future.

4 Rice Transformation and Genetic Engineering

Compared to conventional or MAS breeding, transformation of rice plants with desired genes is a time-saving, efficient and direct way to improve agronomic traits. In order to modify rice genetically, one must have a suitable gene promoter, a target gene and an efficient transformation system. Unlike dicotyledonous plants such as *Arabidopsis thaliana*, which can be transformed easily, rice transformation is a laborious process. However, in the past two decades, scientists have strived to improve rice transformation. Presently, most rice cultivars are transformation-competent.

Rice transformation can be achieved by polyethylene glycol (PEG)-mediated protoplast regeneration (Datta and Datta 1999), biolistic bombardment (Chris-

tou 1997) or *Agrobacterium*-mediated transformation (Hiei et al. 1994). Of these methods, *Agrobacterium*-mediated rice transformation displays the highest transformation efficiency, promotes minimal rearrangement of the transgene and produces a relatively high percentage of transgenic plants harboring a single transgene copy. Also, *Agrobacterium* transformation yields good transgenic plant fertility (Budar et al. 1986; Feldmann and Marks 1987; Klee et al. 1987; Zambryski 1988; Hamilton et al. 1996).

A. tumefaciens is a soil bacterium that channels a defined piece of DNA (T-DNA) from its tumor-inducing (Ti) plasmid into a receptive plant genome. The first high-efficiency *Agrobacterium*-mediated transformation producing fertile and heritable transgenic rice plants was reported in 1994 by Hiei and coworkers (Hiei et al. 1994). Currently, this method is used routinely by many laboratories worldwide working either in rice genetic engineering or on characterization of rice gene functions. A successful *Agrobacterium*-mediated rice transformation system should provide high transformation efficiency, a desirable expression profile of the transgene and a suitable vector system for delivering multiple genes.

The genetic background of rice plants has a decisive effect on the efficiency of transformation. Special attention is required for the induction of *Vir* genes, in addition to the status of calli, medium composition, the selection of bacterial strains, the plant genotype and selectable markers (Ebinuma et al. 1997; Hiei et al. 1997). In general, transformation of cultivars is much easier than that of wild rice species. In particular, transformation of *japonica* varieties is much more efficient than that of *indica* varieties. For certain rice genotypes, the medium composition must be optimized to generate competent calli for transformation. The choice of a selectable marker is also important for efficient transformation. The commonly used selectable markers for rice transformation include *hpt*, *npt*II or *bar*, which render the transformants resistant either to the antibiotics hygromycin and G418 or to the herbicide Bastar, respectively. Among them, *hpt* is the most commonly used, because it is suitable for most rice genotypes.

An appropriate promoter is always required to ensure correct spatial and temporal expression of the transgene. For stable constitutive transgene expression, the rice actin promoter (*Act1*) and two polyubiquitin promoters (*RUBQ1*, *RUBQ2*) exhibit higher activities than the relatively weak CaMV 35S promoter (McElroy et al. 1990; Wang et al. 2000; Wang and Oard 2003). GUS gene expression driven by the *RUBQ1* or *RUBQ2* promoter was 8- to 35-fold greater than that by the 35S promoter in transgenic plants. Furthermore, rice ubiquitin promoters give rise to transgenics that display less gene-silencing frequencies (Wang and Oard 2003). Thus, rice ubiquitin promoters will enjoy popularity in rice transgenic research. The CaMV 35S promoter is used successfully in dicotyledonous plants, but it does not perform as well in monocotyledonous plants, including rice (Guilley et al. 1982; Peterhans et al. 1990).

Thus far, researchers are able to introduce only one or a small number of genes at any one time per transformation. However, since multiple genes

regulate many valuable agronomic traits, transformation of a single gene or a small number of genes is insufficient to improve a target trait. Nevertheless, multiple gene transfer methods are problematic. Recently, Lin et al. (2003) developed a multigene assembly vector system for transferring different genes simultaneously by *Agrobacterium*-mediated transformation. This system combined many different genes into a TAC-based vector via *Cre/loxP* site-specific recombination system and homing endonucleases. This polygenic transformation system will contribute greatly towards generating transgenic rice varieties carrying multiple transgenes to improve yield, quality and stress resistance.

In current transformation systems, at least one selectable marker gene is co-integrated with the transferred foreign gene, allowing for the identification and separation of transformants from non-transformants. However, recent public concerns regarding transformed antibiotic and herbicide resistance genes limit their use for commercialization (Endo et al. 2002). To meet these challenges, scientists are exploring highly efficient but simple and practical strategies for eliminating selectable marker genes to generate marker-free plants. Several strategies have proven successful, including co-transformation, transposon-mediated gene repositioning, site-specific recombination, intrachromosomal homologous recombination and specialized selection (Komari et al. 1996; Ebinuma et al. 1997; Gleave et al. 1999; Zubko et al. 2000; Zuo et al. 2001).

5 Gene Isolation and Characterization

5.1 Map-Based Cloning

Map-based cloning, also termed positional cloning, is one of the most important gene cloning methods applied in rice. This procedure works by identifying a mutation locus that leads to a mutant phenotype through linkage analysis to DNA markers. Before the advent of whole-genome sequencing, map-based cloning was labor-intensive and time-consuming. High-density rice physical/genetic maps contributed to map-based cloning by providing radioactivity-labeled markers, for example RFLP markers. The availability of genome sequence data, for both *indica* and *japonica* rice, have provided PCR-based markers including CAPS, SSR, InDel and SNP markers that facilitate map-based cloning of important agronomic genes in rice.

Isolation of a rice gene by map-based cloning comprises at least three steps, namely: (1) constructing a large mapping population, (2) screening the mapping population with PCR-based markers to pinpoint the interested gene to a defined region and (3) identifying the candidate gene by sequencing and genetic complementation.

Recently, several important rice genes were identified with map-based cloning strategies. These genes involve in various developmental processes,

including plant architecture establishment (Komatsu et al. 2003a, b; Li et al. 2003b; Miyoshi et al. 2004; Ashikari et al., 2005; Luo et al., 2006; Sakamoto et al., 2006; Zhu et al., 2006), hormone signaling pathway (Ashikari et al. 1999; Ueguchi-Tanaka et al., 2005), male sterility restoration (Komori et al. 2004; Wang et al. 2006), cell wall biosynthesis (Li et al. 2003c) and stress resistance (Yamanouchi et al. 2002; Bohnert et al. 2004; Sun et al. 2004; Zeng et al. 2004; Ren et al. 2005; Chu et al. 2006).

5.2 RNA Interference

Cellular RNA interference (RNAi) is a powerful post-transcriptional gene-silencing phenomenon caused by double-stranded RNA introduction. In the past few years, RNAi has been used as an efficient and highly specific gene knockdown/knockout technology to study gene functions in a variety of organisms, including animals and plants (Kusaba 2004). In plants, RNAi is usually introduced by a transgene that produces hairpin RNA (hpRNA) with a double-stranded RNA (dsRNA) region. The hairpin structure is highly efficient for target gene silencing (Di Serio et al. 2001; Waterhouse and Helliwell 2003). Briefly, the hpRNA-producing vector is introduced into the rice genome by *Agrobacterium*-mediated transformation. The target gene is cloned into the hpRNA-producing vector as an inverted repeat driven by the maize ubiquitin promoter and the reverse repeat is spaced with an intron.

In rice, RNAi is gaining popularity among geneticists due to its advantages over other established gene-suppression (for example, RNA antisense) systems (Miyoshi et al. 2003; Jan et al. 2004; Kusaba 2004; Lee et al. 2004; Miki and Shimamoto 2004; Teerawanichpan et al. 2004; Wong et al. 2004). One important challenge for rice functional genomics by the reverse-genetic method is gene redundancy resulting from multigene families in the genome. RNAi is an effective tool for circumventing this problem (Lawrence and Pikaard 2003) and offers a promising strategy to suppress target genes, using various homologies to modulate important agronomic traits appropriately.

5.3 Gene Targeting by Homologous Recombination

Gene targeting (GT) by homologous recombination (HR) in higher plants is not as successful as it is in mice, *Escherichia coli*, yeast, or *Physcomytrella patens*. However, recent advances in *Arabidopsis* and rice stem from an HR-modified *AGL5* MAD-box regulatory gene, the first successful endogenous plant gene knockout using the GT strategy (Kempin et al. 1997). Through this revolutionary methodology, the *Arabidopsis PPO* gene involved in heme and chlorophyll biosynthesis was inhibited through HR (Hanin et al. 2001).

Integration events by HR often accompany non-homologous end-joining, giving rise to low frequency transgenic progeny (approximately 6.5×10^{-4}). To overcome this constraint, Terada et al. (2002) established a large-scale *Agrobac-*

terium-mediated transformation procedure with a strong positive–negative selection for gene targeting in rice. In this system, *hpt* is a positive selection marker for small group identification of rice calli harboring the targeted *waxy* gene from a larger number of transformed calli. Two diphtheria toxin A fragment genes provided strong negative markers to remove random integration. This study underscores the importance of GT and HR technologies to offset economic and scientific problems associated with genetic recombination studies in rice (Terada et al. 2002; Hohn and Puchta 2003).

5.4 Microarray

All cells in an organism usually contain an identical gene set, but gene activity depends on many factors, including tissue types, developmental stages and responses to environmental cues. Thus, the same genes are not active in every cell at all times. Studying which and when genes are active in different cell types under different conditions allows us to understand how these genes function normally and in concert. Before the advent of microarrays, scientists relied on rudimentary techniques to examine only a few genes at a time by traditional RNA differential expression methods, such as DDRT-PCR, RFDD-PCR and Northern blot analyses. Microarray technology and full-scale genome sequencing provide scientists with a way to examine the expression profiles of thousands of genes simultaneously under highly controlled conditions. This allows biologists to examine cell-, time- and treatment-specific regulatory pathway constituents.

There are several types of microarrays, including cDNA and oligonucleotide, and one- or two-color hybridization. Briefly, the single-stranded DNA substrate representing individual genes (cDNAs or oligonucleotides) are spotted (in the case of cDNAs) or chemically "grown" (in the case of oligonucleotides) by robot on a single square inch microscope slide termed a Gene Chip. Next, messenger (mRNA) is isolated and purified from cells or tissues of interest. The mRNA molecules are "labeled" by attaching a fluorescent dye (Cy3-dUTP or Cy5-dUTP, in the case of two-color hybridizations) and are hybridized to the Gene Chip. After hybridization, the arrays are scanned with a fluorometer. The resulting signal intensities represent the number of mRNA molecules present in the starting material and are converted into values for analysis.

Microarray technology is an invaluable tool in rice functional genomic research. Scientists are using microarrays to ask many different questions of the developing plant. For example, which genes are over- or under-expressed in mutant and wild-type plants, in response to abiotic or biotic stress. This methodology, coupled with focused and hypothesis-driven experiments, can reveal candidate genes of interest. Subsequent validation techniques are recommended, such as Northern hybridization, real-time PCR or RT-PCR. These experiments can provide a second tier of hypothesis-driven tests to define gene regulation networks and coordinated expression in rice.

Since the rice microarray project started in 1999 in Japan, various rice Gene Chips for microarray analysis have been developed, such as EST chips, full-length cDNAs and oligonucleotide-based microarray chips. Recently, Affymetrix (Santa Clara, USA) produced a high-density rice Gene Chip that contains probes to query 51,279 transcripts representing two rice cultivars, with approximately 48,564 *japonica* transcripts and 1,260 *indica* transcripts.

5.5 High-Throughput Insertion Mutagenesis

Approximately 50,000 genes are predicted in the rice genome, 50% of which share homologies with those in *Arabidopsis thaliana*, according to the comparative analysis between the two model species (Goff et al. 2002; Yu et al. 2002). In addition, most rice genes annotate with unknown or hypothetical functions. As a result, the primary challenge for future rice genomic research is to identify the functions of these genes by using high-throughput strategies.

Generally speaking, methods such as transposon (*Ac/Ds*) or retrotransposon (*TOS17*) tagging and T-DNA insertions are powerful mutant generators for gene function identification. The characteristic of each method is shown in Table 1. These *Ac*/*Ds*, *TOS17* and T-DNA insertion rice mutant databases are available online.

Table 1. Methods for high-throughput insertion mutagenesis

Method	Characteristics	References
Ac/*Ds* tagging system	Use of the maize autonomous transposable *Ac*/*Ds* element; High somatic and germinal transposition frequencies in rice; *Ds* insertions distribute randomly throughout the rice genome with bias toward chromosomes 4 and 7; *Ds* transposes preferentially into coding regions; Suitable for generating large-scale stable, unlinked and single-copy *Ds* transposon mutagenesis in rice.	Shimamoto et al. (1993), Enoki et al. (1999), Greco et al. (2001a, b), Kolesnik et al. (2004)
Tos17 retrotransposon system	Use of the endogenous retrotransposon element *Tos17*; *Tos17* is activated by tissue culture but becomes inactivated after plant regeneration; *Tos17* integration distributes throughout the rice genome; *Tos17* has a low copy number compared with other plant retrotransposons; *Tos17* prefers gene-rich regions; Suitable for large-scale gene function analysis.	Hirochika (2001), Yamazaki et al. (2001), Miyao et al. (2003)
T-DNA insertion system	T-DNA prefers gene-rich regions; Low frequency insertion in repetitive regions; Equal chance to insert in genic versus intergenic regions.	Jeong et al. (2002), An et al. (2003), Chen et al. (2003), Wu et al. (2003), Sallaud et al. (2004)

6 Application of Rice Biotechnology

The ongoing population increases in the main rice-consuming countries necessitate the rapid development of elite rice varieties. Since the Green Revolution era in the 1960s, scientists have attempted to improve rice production in these areas on the basis of advanced molecular biotechnology and conventional rice breeding, focusing on the following issues.

6.1 Modulation of Rice Plant Architecture

Among the agronomic traits affecting rice grain production, rice plant architecture is one of the most important factors that determine rice yield (Khush 2003). In the 1960s, semi-dwarf wheat and rice cultivars gave rise to a vastly improved crop production known as the Green Revolution. To further increase the production of the existing semi-dwarf rice varieties, scientists from the International Rice Research Institute (IRRI) proposed a model of the ideal rice plant architecture, which should have a low tiller number (9–10 tillers for transplanted conditions), a high number of productive tillers, 200–250 grains per panicle, dark green thick and erect leaves, and vigorous and deep root systems.

Understanding the molecular mechanisms involved in rice plant architecture is essential to rice plant modification. The green revolution rice *semi-dwarf 1* (*sd1*) cultivar was first produced in the 1960s and characterized recently as a GA biosynthesis mutant through map-based cloning (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). This revelation suggested a modulating hypothesis for plant height manipulation via biotechnology on a molecular level (Sakamoto et al. 2003).

Several other genes, such as *MOC1*, *LAX*, *FRIZZY PANICLE*, *GID1* and *CKX2*, involved in controlling rice plant architecture, were map-based cloned and characterized (Komatsu et al. 2003a, b; Li et al. 2003a, b; Ueguchi-Tanaka et al., 2005; Sakamoto et al., 2006). These genes may play crucial roles in the development of rice tillers and panicles, which are regarded as the main contributors to plant architecture. Genetic dissection and molecular manipulation of these genes have the potential to modify the rice plant architecture and thus generate new rice varieties.

6.2 Improvement of Rice Plant Resistance

Rice yield stability has been attained through the improvement of rice plant resistance, including insect and disease resistance, and drought and salinity tolerance.

6.2.1 Insect Resistance

Rice stem borers are serious rice pests. They infest plants from the seedling stage to maturity and are particularly destructive in Asia, the Middle East and the Mediterranean regions. Stem borer damage results in a severe reduction of rice grain production. In China, stem borer brings an annual 6.45×10^{12} Renminbi (RMB) loss, in addition to a 2.85×10^{12} RMB cost for their chemical control (Sheng 2003).

Bacillus thuringiensis (*Bt*) genes produce toxins in the bacterium that are detrimental to its host insects. Genes encoding various *Bt* crystal (Cry) proteins have been transferred into cotton and maize, and benefit farmers greatly. *Bt* genes, driven by an ubiquitin, inducible or tissue-specific promoter, have also been transferred into rice to produce *Bt* transgenic rice cultivars which show resistance to lepidopteran pests, including stem borer and several species of leaf folders. This resistance is achieved without causing the occurrence of other rice pests, such as the brown plant hopper (Ye et al. 2001; High et al. 2004). The *Bt* rice has been under evaluation in several countries since the beginning of its field trials in China in 1998, but not commercialized yet because of public biosafety concerns (High et al. 2004). However, the *Bt* biotechnology has already become an important component of integrated pest control methods and is compatible with the applications of pest-resistant varieties, cultural practices, insecticides and biological control. Therefore, the *Bt* rice has the potential for acceptance by several countries in Asia (Lei 2004; Huang et al. 2005).

Additionally, proteinase inhibitors (PIs) are widely used to engineer insect-resistant transgenic rice plants. These inhibitors work by disrupting the digestive systems of insects. The cowpea *CpTI* gene was the first *PI* gene to produce enhanced insect-resistant transgenic plants. Constitutive expression of the *CpTI* gene in transgenic rice plants increases the plant's resistance to two species of stem borers (Ussuf et al. 2001). Recently, a modified *CpTI* gene was introduced into Minghui 86, a rice cultivar widely used for three-line hybrid breeding in China; and the transgenic plants showed highly increased resistance to rice stem borer (Huang et al. 2005).

6.2.2 Disease Resistance

Rice bacterial blight (BB) is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). This blight is the most destructive rice disease worldwide, especially in Asian rice-growing countries. As the first step towards improving resistance to rice BB, we need to understand the corresponding regulatory genes governing signaling pathways leading to defense against pathogen invasion. At present, more than 20 BB-resistant genes have been identified, but only a few, such as *Xa21*, *Xa1*, *Xa26* and *Xa13*, have been cloned. In-depth studies revealed that *Xa21* and *Xa26* both encode a protein family that contains both leucine-

rich repeat motifs and a serine-threonine kinase-like domain, suggesting their roles in cell surface recognition of a pathogen ligand and subsequent activation of an intracellular defense response (Song et al. 1995; Sun et al. 2004). Unlike *Xa21* and *Xa26* BB-resistant genes, the deduced amino acid sequence of the *Xa1* gene product contains nucleotide binding sites (NBS) and a new type of leucine-rich repeats, suggesting that *Xa1* is a member of the NBS-LRR class of plant disease-resistant genes (Yoshimura et al. 1998). Promoter mutations in *Xa13* result in down-regulation of gene expression during host–pathogen interaction, leading to the fully recessive *xa13* that confers race-specific resistance. Among these genes, the *Xa21* gene has proved a useful tool for breeding BB-resistant rice varieties by genetic engineering due to its wide-spectrum resistance to the *Xoo* blight. It has been transferred into various rice cultivars mediated by *A. tumefaciens* to develop new varieties resistant to BB (Zhao et al. 2000; Li et al. 2001; Wang et al. 2004; Zhai et al. 2004).

However, large-scale and long-term cultivation of varieties containing a single resistance gene are most likely to make the pathogen overcome BB resistance. Singh et al. (2001) made an effort to delay this process through pyramiding three BB genes (*xa5*, *xa13*, *Xa21*) into *indica* rice by marker-assisted selection. Their work pyramided these three genes in a high-yielding but BB-susceptible cultivar, *PR106*, to generate enhanced and wide-spectrum BB resistance. Moreover, the *Xa21* gene was also pyramided with the *Bt* and chitinase genes (for tolerance of sheath blight) to produce stable elite rice lines resistant to disease and insect pests through conventional crossing of two transgenic parental lines transformed independently. The progeny carrying these three transgenes showed resistance to bacterial blight, yellow stem borer and sheath blight disease (Datta et al. 2002).

In addition to bacterial blight, blast caused by the fungus *Magnaporthe grisea* is another serious and widespread rice disease. *M. grisea* can attack the aerial parts of rice plants during all developmental stages. Infection is characterized by lesions on the leaves, nodes and panicles. *M. grisea* infects rice leaves through a series of steps. First, airborne spores attach to the rice plants and sense the waxy aerial surface. Second, the fungal conidia germinate on the leaf surface and the germ tubes differentiate into a dome-shaped penetration unit called an appressorium. Finally, the rapid reproduction of appressoria generates turgor pressure to penetrate the leaf surface and in this manner infect rice tissues (Howard and Valent 1996). The latest relevant study showed that *M. grisea* also infects plant roots (Sesma and Osbourn 2004). So far, more than 30 rice blast resistance genetic loci, denoted *Pi* genes, have been identified (Song and Goodman 2001; Talbot 2003). The availability of both host rice and pathogen *M. grisea* genome sequence data provides the opportunity to study the host–pathogen interaction and uncover effective ways to control this disease.

6.2.3 Abiotic Tolerance

As the world population continues to increase, farmers must employ inhospitable lands to grow crops. As a result, new crop varieties that can withstand long periods of drought or high-salt environments are needed.

Rice is an aquatic crop that is sensitive to water deficit (Courtois et al. 2003). Over the past decade, a severe bottleneck in rice grain production occurred in response to unusual drought circumstances. To tackle this problem, the Rockefeller Foundation is mapping and identifying traits and genes associated with rice drought tolerance in order to improve the grain yield performance of rice cultivars in the rain-fed lowland rice areas in north and northeast Thailand.

Enhancement of plant drought tolerance via transgenics is underway. Several candidate genes corresponding to abiotic stress tolerance have been incorporated into rice, leading to the accumulation of biomass under drought stress (Cheng et al. 2001, 2002). Garg et al. (2002) engineered trehalose overproduction in rice through stress-inducible or tissue-specific expression of a bifunctional trehalose-6-phosphate synthase/phosphatase (TPSP) fusion enzyme without any negative effects on rice plant growth or grain yield. During abiotic stress, transgenic plants accumulated increased amounts of trehalose and exhibited high-salt, drought and low-temperature stress tolerances. This research suggested the potential use of a transgenic approach in developing new rice cultivars with increased abiotic stress tolerance and enhanced rice productivity. In addition, cDNA microarrays were used to identify stress-inducible genes in rice (Rabbani et al. 2003). Although most of these gene functions are currently unclear, they can be utilized to investigate molecular mechanisms of drought tolerance and for applications of gene manipulation. Very recently, a novel rice QTL *SKC1* was cloned and characterized as a sodium transporter that confers salt tolerance, providing a potential tool to improve salt tolerance of rice (Ren et al. 2005).

6.3 Improvement of Rice Grain Nutrition and Quality

In many developing countries, rice is the main staple for impoverished people. However, the edible part of the rice grain lacks several essential nutrients, such as vitamins and minerals, leading to serious malnutrition. For example, rice lacks beta-carotene, which can be converted to vitamin A when digested and represents a vital dietary nutrient. As a result, in excess of 180 million children and women suffer from vitamin A deficiency in Asia and other developing countries. Furthermore, about half a million children worldwide are permanently blind due to vitamin A deficiency (Chong 2003). Fortunately, achievement of both rice biotechnology and molecular understanding of carotenoid biosynthetic pathways allows us to create elite rice varieties. Researchers at the Swiss Federal Institute of Technology created a strain of "golden" rice contain-

ing high beta-carotene concentrations (Beyer et al. 2002; Paine et al. 2005). They introduced a *de novo* biosynthetic pathway into rice endosperm with *Agrobacterium*-mediated transformation to increase the pro-vitamin A content.

A latent iron deficiency occurs in developing and some developed countries (Heath and Fairweather-Tait 2002). The ferritin genes from *Phaseolus vulgaris* and *P. limensis* have been expressed in rice, resulting in increased concentration of iron in transgenic rice grains (Lucca et al. 2002; Vasconcelos et al. 2003; Liu et al. 2004).

Amylose, one of the components of starch, determines rice eating and cooking quality. Reduction of amylose is an efficient way to improve rice starch quality (Liu et al. 2003). The *Waxy* gene encodes the granule-bound starch synthase. The *japonica* and *indica* rices differ in their abundance of mature *Waxy* RNA, as a result of a natural nucleotide substitution that confers different splicing efficiencies, leading to a varied ratio of amylose to total starch content (Isshiki et al. 1998). Liu et al. (2003) generated amylose reduction transgenic lines via *Agrobacterium*-mediated technology in both elite *japonica* and *indica* rice cultivars. In these cultivars, the starch quality was variably improved. Recently, Terada et al. (2002) also took advantage of the *Waxy* gene to carry out a GT strategy in rice (mentioned in sect. 4.3), thus providing a good example of how new biotechnology can be applied to solve economic and scientific problems.

7 Conclusions

Traditional rice breeding strives to produce elite varieties. However, this process is time-consuming and limited genetic resources leave little room for continued improvement. Rice has a small genome and was the first monocotyledonous plant to be sequenced. Through the available rice sequence data, scientists can unravel their functionality, providing researchers with abundant genetic resources for fruitful manipulation. Noticeably, increasing public concerns regarding transgenic biosafety will challenge rice-producing efforts. The goal remains to create varieties with improved production, stress resistance and grain quality and nutrition, using newly developed biotechnological approaches.

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II.2 Wheat

F.D. Meyer and M.J. Giroux[1]

1 Introduction

The era of plant transformation began in the mid-1980s with the successful uptake of foreign genes into tobacco, using *Agrobacterium tumefaciens* as a delivery system (Barton et al. 1983; De Block et al. 1984). In the following two decades, the number of transformable species diversified greatly, as well as the techniques employed for their transformation. Cereals and wheat, in particular, are important candidates for transformation because they are the primary source of calories for the world's population. The potential economic benefit arising from wheat transformation alone is considerable, and researchers are now intensely seeking transgenes that improve wheat performance. Wheat transformation has encountered many obstacles in comparison to dicotyledons and cereals such as maize and rice; the reasons for this will be discussed later. Despite these obstacles, diverse wheat cultivars are becoming increasingly transformable. This chapter discusses the two main transformation methods in use today, namely *A. tumefaciens*-mediated gene delivery and particle bombardment or biolistics. It focuses primarily on wheat transformation techniques, the strategies used to design high-expressing, stable vectors and the selectable markers used for co-transformation with the gene of interest. The molecular characteristics of transgenic wheat plants are described, including copy number, integration patterns and expression levels in transgenic populations. Finally, the chapter explores how particular traits in wheat have been modified and sometimes improved through genetic engineering. Many experiments have demonstrated improved cereal performance through the expression of specific genes. These improvements include modified bread-making qualities, grain hardness, fungal, insect and herbicide resistance, and even yield potential.

2 Particle Bombardment

It was not until 1992 that wheat was transformed with a gene encoding herbicide resistance (Vasil et al. 1992) using a biolistic delivery device, or gene

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gun. The first step in biolistic transformation is to obtain embryogenic tissue, or callus, from the desired plant species. In the case of wheat and most monocotyledons, callus is most easily initiated from the scutellum of immature embryos when immature embryos are cultured on media containing synthetic auxin (e. g. 2,4-dichlorophenoxyacetic acid). It is worthy of note that excising the scutellum from immature wheat embryos prior to bombardment does not increase transformation efficiency, as compared to using whole immature wheat embryos. In fact, efficiencies were eight times higher for whole-embryo-derived callus compared to excised scutellar callus (Huber et al. 2002). Somatic embryos require approximately one week to form, then are transferred to a medium containing an osmotic agent. Callus is bombarded with either gold or tungsten particles that are coated with precipitated DNA. These metallic "bullets" are high-density projectiles that allow the insertion of plasmid DNA into the callus tissue. Bombarded callus is placed in the dark for several weeks to allow regeneration, followed by transfer to media containing reduced auxin to permit the formation of shoots and, eventually, roots.

Regenerated plants are selected using one of several available marker genes. The marker genes include *bar* that confers resistance to bialaphos and the herbicide glufosinate (Thompson et al. 1989), *gfp* that encodes the jellyfish-derived green fluorescent protein, *hpt* (a hygromycin resistance gene) and *uidA*, which encodes a color-producing protein (β-glucuronidase or GUS). Each selectable marker requires a different procedure to screen possible transformants following bombardment. *Bar* is perhaps the most commonly used marker, as it allows screening as late as the two-leaf plant stage, well after plants have been through tissue culture and transplanted to soil. In many experiments using *bar*, plants are also screened during the tissue-culture regenerative phase, when they are placed on medium containing glufosinate, bialaphos, or phosphinothrycin. The concentration and period of screening required to optimize recovery of transgenic plants is debatable. One research group obtained a high frequency of transformation by exposing callus to glufosinate for only a short period during the regeneration phase (Sparks and Jones, unpublished data). Other experiments, noted in Table 1, rely on protocols where callus is placed onto selection medium shortly after bombardment. Wheat embryos bombarded with the hygromycin resistance gene, *hpt*, were exposed to hygromycin during the formation of callus tissue, but selection pressure after this stage was removed (Ortiz et al. 1996). The *uidA* gene encodes GUS, an enzyme capable of cleaving the substrate x-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), which in turn produces blue regions in the explant tissue. These blue regions can be visualized by applying X-gluc within a week of bombardment. However, the GUS staining procedure is cytotoxic and, although transient expression is detected, explants do not survive to maturity. The advantage of using GFP, as compared to GUS, is that it does not inhibit the production of mature plants. When excited with fluorescent light, GFP has been visualized in the leaf tissues of many species, including wheat (Pang et al. 1996). This technique is non-destructive and allows the detection of tissue-specific expression.

Table 1. Comparison of *bar* selection protocols in various wheat biolistic experiments. *bar* encodes phosphinothrycin acetyl transferase, an enzyme which confers resistance to bialaphos, glufosinate and phosphinothrycin. There are many commercial derivatives of these compounds, including Basta and Liberty. *act* Rice actin promoter, *cp* coat protein gene, *glu* glutenin promoter, *sh2r6hs* modified maize starch biosynthetic gene, *tap* tapetum-specific promoter to rice or maize, *ubi* maize ubiquitin promoter, *35S* cauliflower mosaic virus 35S promoter, *BIA* bialaphos, *GLU* glufosinate, *NS* no selection, *PPT* phosphinothrycin

Construct(s)[a]	Selective agent and selective duration[b]		Transformation efficiency[c]	Reference
	Callus induction phase	Regeneration phase		
ubi:bar, ubi:gus	BIA (1 mg l^{-1}), 5 days	BIA (1 mg l^{-1}), 100 days	0.21%	Weeks et al. (1993)
35S:bar, act:gus	NS, 14 days+; PPT (5 mg l^{-1}), 14 days	PPT (5 mg l^{-1}), 42 days	3.7%	Becker et al. (1994)
ubi:bar, ubi:gus	NS or BIA (3 mg l^{-1}), 14 days	BIA (4 mg l^{-1}), 44 days	0.1–2.0%	Altpeter et al. (1996)
35S:bar, tap:barnase	GLU (2.5 mg l^{-1}), 28 days	GLU (2.5 mg l^{-1}), 28 days	0.78%	DeBlock et al. (1997)
Act:bar, act:gus	BIA (5 mg l^{-1}), 28 days	BIA (4 mg l^{-1}), 28 days	0.16–1.71%	Takumi and Shimada (1997)
ubi:bar, ubi:cp	BIA (2 mg l^{-1}), 56 days+; BIA (5 mg l^{-1}), 28 days	BIA (2 mg l^{-1}), 28 days+; BIA (1 mg l^{-1}), 14 days	9.7%	Zhang et al. (2000)
ubi:bar; ubi:gus	NS, 35 days	NS, 28 days+; PPT (4 mg l^{-1}), 84 days	5.0%	Pastori et al. (2001)
ubi:bar, ubi:gus	NS, 35 days	BIA (3 mg l^{-1}) or GLU (2–4 mg l^{-1}), 42–63 days	1–17%	Rasco-Gaunt et al. (2001)
ubi:bar, ubi:gus, 35S:gfp	NS, 7 days+; PPT (5 mg l^{-1}), 21 days	PPT (5 mg l^{-1}), 42 days	4.9%	Huber et al. (2002)
ubi:bar	NS, 14 days	PPT (5 mg l^{-1}), 30 days	58–70%	Pellegrineschi et al. (2002)
35s:bar, glu:sh2r6hs	BIA (5 mg l^{-1}), 21 days	BIA (5 mg l^{-1}), 28 days	3.6%	Meyer et al. (2004)

[a] All references report transformation with a construct containing a promoter and *bar* (promoter:*bar*). Many citations report co-transformation with additional constructs. However, transformation efficiencies do not account for their expression. The plasmid AHC25 (Christensen and Quail 1996) was most commonly reported for transformation use carrying *bar* and *uidA* under control of the maize ubiquitin promoter. This plasmid is represented as *ubi:bar, ubi:uidA* in the table.

[b] The concentration of the respective selective agent during tissue culture is given in parentheses. Following bombardment, calli are placed on induction medium (usually in the dark) to promote somatic embryogenesis, followed by regeneration medium in the light to allow shoot formation. The time required for these phases is estimated in days. Herbicide treatments after soil transplantation may exist, but are not noted in this table.

[c] Transformation efficiency is based upon the number of calli successfully transformed with *bar*, not alternative constructs. It is calculated as: (number of resistant plants)/(number of bombarded embryos).

The concentration of fluorescing protein often depends on expression, and weak-expressing plants are often classified as non-transgenic. Most protocols call for a cauliflower mosaic virus (CaMV) 35S promoter/*bar* construct for selection purposes, since most escapes are eliminated and false negatives are reported with less frequency than GFP.

Transformation efficiency (number of independently transformed embryos per total number of bombarded embryos) varies amongst different wheat cultivars, and even between experiments using identical germplasms. One study compared the transformation efficiencies of 129 sister lines, all of which were derived from the cultivar Bob White (Pellegrineschi et al. 2002). Of the total number of sister lines bombarded with *bar*, eight lines had efficiencies above 60%. This is much greater than the average spring wheat frequency, which ranges from 1.5% (Takumi and Shimada 1997) to nearly 10% (Zhang et al. 2000). The frequency range for one experiment alone can be large, especially if small sets of embryos are bombarded and only a handful of transformants are obtained. Efficiencies varied 50-fold, ranging from 0.1% to 5.0% on a German spring wheat cultivar when bombarded with a construct containing *bar* and *gfp* (Huber et al. 2002). Based on the experimental inconsistency, it is often difficult to determine which cultivar or which selectable marker generates the optimum transformation efficiency. For example, *hpt* efficiency was more than *bar* efficiency in one comparative study, these being 5.5% and 2.6%, respectively (Ortiz et al. 1996). Yet this difference is not commonly reported, and *bar* continues to be a major selectable marker for a vast number of experiments. There is an obvious need to continually refine the biolistic technique, as it is still not possible to transform some elite wheat varieties, mostly because transformation efficiencies and regeneration rates are poor (Iser et al. 1999).

After the selection process, polymerase chain reaction (PCR) or Southern blotting is routinely performed to confirm co-integration of one or more desired genes. Some experiments rely on hybrid vectors containing two or more adjacent genes, such as *bar* and *uidA* (Pastori et al. 2001). More often, multiple constructs are precipitated on a single batch of gold particles and bombarded into wheat callus. In wheat populations bombarded with two constructs, the majority of plants expressing a selectable marker gene also usually express a second gene of interest. For instance, the co-transformation frequency of a *bar*/*uidA* construct with several genes of interest was, on average, 66% (Rasco-Gaunt et al. 2001). Another researcher found 70 *bar*-expressing plants derived from 1965 bombarded calli (Meyer et al. 2004). Amongst the 37 *bar*-positive plants analyzed by Southern blotting, 24 (65%) also expressed a starch biosynthetic gene from a separate construct, indicating co-integration and co-expression of two foreign genes (Table 2). It is interesting to note, however, the loss of *bar* expression in subsequent T1 plantlets. Eighteen of 70 T1 lines had either a reduction or complete loss of *bar* expression, evident from plant death following glufosinate treatment.

The entire tissue culture process ranges from eight to 18 weeks, depending on the transformation protocol. Protocol differences are abundant, and even

Table 2. Integration and expression of *Sh2r6hs*, a maize-derived starch biosynthetic gene, in 37 independent lines co-transformed with *bar*. Seventy plants derived from 1965 bombarded calli survived bialaphos (5 mg l^{-1}) tissue-culture treatment and glufosinate (0.1%) spraying at the two-leaf stage. Transformation efficiency was 3.6% (70 regenerants in 1965 bombarded calli). Thirty-seven *bar*-positive T0 plants were Southern and Western blotted. Twenty-four of 37 hybridized with the *Sh2r6hs* probe and 20 of 37 had a detectable signal when incubated with an anti-*Sh2r6hs* antibody. *bar* silencing occurred in 18 of 70 T1 lines. Chi-square (χ^2) values test fit of counts of resistant/sensitive progeny of T0 plants (T1 plants) to 3 : 1 ratio. *Res.* Resistant, *Sens.* sensitive

Line[a]	T0 Southern[b]	T0 Western[c]	Res./Sens. T1 plants[d]	χ^2	Line[a]	T0 Southern[b]	T0 Western[c]	Res./Sens. T1 plants[d]	χ^2
GS1	Neg	Neg	8/13	15.25	GS 28	Med	High	0/12[e]	
GS2	Low	Neg	0/12[e]		GS 32	Low	Low	11/7	1.85[f]
GS 3	Neg	Neg	13/8	1.92[f]	GS 33	Neg	Neg	0/18[e]	
GS 4	Low	High	12/8	2.4[f]	GS 34	Neg	Neg	0/19[e]	
GS 5	Med	High	21/3	2.0[f]	GS 35	Med	Neg	0/12[e]	
GS 6	High	Low	0/12[e]		GS 37	Low	Low	3/12	24.2
GS 7	Neg	Neg	0/23[e]		GS 38	High	Med	0/12[e]	
GS 8	Med	High	20/7	0.012[f]	GS 40	Neg	Neg	3/9	16.0
GS 9	High	High	19/3	1.52[f]	GS 44	Low	Low	12/8	2.4[f]
GS 10	Neg	Neg	13/4	0.02[f]	GS 45	Low	Med	14/1	2.69[f]
GS 11	Low	Low	0/12[e]		GS 47	Neg	Neg	0/7[e]	
GS 12	Low	Low	0/12[e]		GS 48	Low	Low	16/7	0.36[f]
GS 13	Neg	Neg	15/5	0[f]	GS 49	Med	Neg	0/12[e]	
GS 14	Med	High	9/3	0[f]	GS 51	Neg	Neg	0/24[e]	
GS 15	Neg	Neg	15/8	1.17[f]	GS 54	Med	High	8/4	0.44[f]
GS 16	Med	High	20/8	0.19[f]	GS 55	Neg	Neg	16/8	0.89[f]
GS 17	Med	High	22/2	3.56	GS 57	High	Low	6/6	4.0
GS 18	Med	Neg	0/12[e]		GS 66	Neg	Neg	12/12	8.0
GS 27	High	Med	0/12[e]						

[a] *Line* indicates the number designated to plants after transplantation from tissue culture to soil. *GS* refers to the pGS construct (Meyer et al. 2004) containing a *glutenin* promoter and *Sh2r6hs coding sequence*.

[b] Genomic DNA was probed with ^{32}P-labeled *Sh2r6hs* (Southern blotting). *High*, *Med*, *Low* indicate relative intensities of *Sh2r6hs* hybridization. *Neg* indicates lack of integration of *Sh2r6hs* into the wheat genome.

[c] *Low*, *Med*, *High* designate concentrations of protein detected by a SH2R6HS antibody for each line. Low, medium and high concentrations correspond to 2, 4, and 6 times the concentration of protein detected in the untransformed parental variety (HiLine), respectively (data not shown). *Neg* denotes GS lines without production of SH2R6HS.

[d] Resistant plants are those expressing *bar* from either one (heterozygous) or two (homozygous positive) chromosomes. Susceptible plants do not express *bar* (homozygous negative).

[e] Indicates silencing of *bar* in T1 seedlings.

[f] χ^2 values consistent with integration of *bar* at a single locus; fits 3:1 ratio.

the developmental stage of donor plants is not agreed universally. This factor alone appears to have a considerable impact on transformation and the frequency of the latter increased nearly 10-fold when embryos were dissected from 75-day-old plants versus 80 day-old donor plants (Pastori et al. 2001). Another controversial procedure is the excision of the root/shoot axis from scutellar tissue before bombardment. The best source of embrogenic cells is the scutellum. However, isolating the scutellum is a time-consuming process that is not proven superior to whole-embryo bombardment. The time taken for selective agents to be incorporated into the medium can also vary. One report describes a tissue culture process in which calli are formed during four weeks on induction media without selection, transferred to regeneration medium for four weeks without selection, and finally sub-cultured to regeneration medium with selection for the remaining ten weeks before transplantation to soil (Pastori et al. 2001). The concentration of the selection agent and period of selection is also highly variable. After tissue culturing, plants take six weeks to flower and reach maturity ten weeks after flowering.

Various promoters are used to biolistically transform wheat. The promoter from the maize gene ubiquitin (*ubi*) was combined with a section of the maize alcohol dehydrogenase gene, and this construct was successfully expressed in immature wheat embryos (Ortiz et al. 1997). The *ubi* promoter confers expression in many tissues, and therefore does not have the advantage of being expressed in a tissue-specific manner. The same expression pattern is seen with the CaMV 35S promoter, as viral infection in nature is not tissue-specific in most cases (Chen et al. 1998). Many promoters, including various histone and badnavirus promoters, are being developed for wheat transformation and have demonstrated transient expression of reporter genes. Histone protein promoters are good candidates for use in wheat transformation, as histone sequences are consistently expressed and are highly conserved among diverse species. Histone promoter expression has been characterized by fusing various wheat histone promoters to *uidA* (Terada et al. 1993; Yang et al. 1995). When rice and tobacco were transformed with these constructs, transient expression was detected in floral and other meristematic tissues. Some badnaviruses are an ideal source of promoters because they natively infect monocotyledons, unlike CaMV, which only infects dicotyledons (Lockhart and Olszewski 1994). Infection is limited to the host phloem with some badnavirus strains. However, the sugarcane badnavirus (ScBV) infects a greater range of tissues, and a promoter derived from ScBV confers constitutive expression of *uidA* in rice (Tzafrir et al. 1998). Similar constitutive expression from two banana streak badnavirus promoters was observed in wheat, maize and barley (Schenk et al. 2001).

Other promoters, such as the tapetum-derived promoter from maize and rice, confine expression to much more discrete locations and stages of development. A nuclear male sterility system was established by transforming wheat with the tapetum promoter and a *barnase* coding region (De Block et al. 1997). A high molecular weight (HMW) glutenin subunit promoter also conferred tissue-specific expression in wheat. GUS expression was limited to

the endosperm and was not initiated until 12 days after anthesis (Lammachia et al. 2001). Promoters like these are ideal for use in expression vectors because they restrict expression to a particular tissue and can be used to obtain a tissue-specific phenotype. These phenotypes include altering grain hardness for baking purposes and increasing seed starch biosynthesis for increased yields. Foreign gene expression with so-called tissue-specific promoters, however, does not always correlate with native gene expression. AGP2, a wheat large subunit of ADP-glucose pyrophosphorylase (AGPase), is known to be highly expressed in wheat endosperm (Thorneycroft et al. 2003). When the promoter of an AGPase gene, AGP2, was linked to GUS and introduced into wheat, expression was reported not only in endosperm, but also in carpels and pollen (Chrimes et al. 2005). In order to advance biolistic gene expression, it is necessary to discover a greater number and diversity of tissue-specific promoters. These promoter discoveries will allow more precise control of the timing and location of transgene expression and will expand the potential of biolistic modification.

3 Integration and Expression of Biolistic Transgenes

The nature of biolistic gene integration into the wheat genome has been extensively studied but is not fully understood. Because potential transgenic cells are literally bombarded with plasmid DNA, the chromosomal location, number of copies and intactness of the transgenic sequence all vary amongst different transformation events. Researchers cannot determine whether a true correlation exists between copy number and gene stability, since a large amount of data recognizes multiple factors that affect transgene stability. These factors include the sequence homology of the transgene to endogenous genes (Muller et al. 1996), the region disrupted by the transgenic insertions and even environmental factors. Although multi-copy insertions do not always lead to gene instability, it is generally believed that single-copy insertions, when available, provide the best means of obtaining reliable gene expression and stability.

When physically mapped with fluorescence in situ hybridization (FISH), wheat transgene integration varied among 13 transgenic lines (Jackson et al. 2001). Integration sites contained tandem repeats with and without interspersed segments of unknown DNA, ranging in copy number from six to 17. There is some evidence that integration occurs more frequently at the distal region of chromosomes (Pedersen et al. 1997). While it is believed that chromosomal insertion site and copy number has some effect on transgene expression, the latter is more affected by other factors, such as promoter efficacy.

Transgene integration may interrupt native genes or hinder homologous pairing during meiotic and mitotic phases. In these cases, the potential benefit of the transgene would be inconsequential, as native gene interruption would result in plant death or reduced vigor. In addition, transgenes are of-

ten silenced when loci contain inverted or direct sequence repeats. Silencing can also stem from the interaction between transgenic loci or the interaction between transgenic and native loci (Matzke and Matzke 1995). Large repeats lend to a greater frequency of homologous recombination and instability of the transgenic locus.

The biolistic uptake of transgenes into the wheat genome is a complex event. Because of the stresses induced from tissue culture and the variation in transgene integration, the frequency of producing viable plants that integrate transgenes is low (< 5%) for essentially all wheat transformation research thus far. The ultimate objective of wheat transformation is to produce multiple transformant lines per bombardment. These lines must each have independent, relatively simple transgenic integrations, good transgene expression and stability, stable inheritance of the transgene in subsequent generations, a detectable phenotypic change conferred by the gene of interest, and field performance comparable to the wild-type cultivar. Selecting such lines is a formidable challenge, and can be accomplished only when a large number of transformant lines are available to choose from which a choice can be made.

4 *Agrobacterium*-Mediated Transformation

The soil bacterium *A. tumefaciens* causes crown gall disease in many dicotyledonous species by infecting host cells with DNA carried on tumor-inducing (Ti) plasmids. The Ti plasmid contains transfer DNA (T-DNA) that is copied to form a T-strand. The latter is moved into the host's genome with the aid of virulence (*vir*) genes (Hiei et al. 1997). In engineering theT-DNA, a segment of T-DNA is often excised from the plasmid in vitro, then replaced with a selectable marker gene and another gene of interest. An effective transformation system utilizes two plasmids, and is called a binary vector system. One Ti plasmid provides *vir* expression and function, while another, smaller plasmid, contains an artificial T-DNA with a selectable marker gene and a gene of interest (An et al. 1988).

Many dicotyledonous plants, including tobacco, peanut, pea and cotton (Hinchee et al. 1988; Umbeck et al. 1989; Cheng et al. 1996) are readily transformed with *A. tumefaciens*. However, gene insertion with this technique proved more difficult with monocotyledons. Rice (Hiei et al. 1994), maize (Ishida et al. 1996), barley (Tingay et al. 1997) and wheat (Cheng et al. 1997) transformants were eventually generated, after the problems of poor T-DNA expression and inheritance in the early 1980s had been encountered and overcome (Hess et al. 1990). Current maize transformation efficiencies are as high as 30% using *Agrobacterium* vectors (Komari et al. 1998), but barley and wheat efficiencies are generally less than 5%. This is attributed partially to the weak wound response of wheat and barley to infection, which is vital in signaling *vir* gene expression. Wheat transformation was initially limited to a few varieties,

but more recent improvements have increased the list of cultivars. These improvements include the expression of highly effective *vir* genes, or superbinary approach (Khanna and Daggard 2003), and optimizing embryo size, length of preculture and the timing and strength of selection (Wu et al. 2003).

5 Biolistics Versus *Agrobacterium*: Which Method is Preferred?

As previously mentioned, several problems were encountered with the original biolistic wheat transformation experiments, including a low frequency of transformant recovery, a high frequency of escapes, instability or silencing of transgenes, and lack of transgene transmission from parents to progeny (Demeke et al. 1999). Despite these early biolistic attempts, both *Agrobacterium*-mediated and biolistic transformation methods have improved in the past 15 years, and one method is not currently viewed as superior to the other. It has been suggested that *Agrobacterium* vectors provide greater transformation efficiencies and transgene expression than biolistic vectors. A comparison of the two transformation systems, however, concluded that efficiency and expression levels were similar between methods for wheat, barley, maize and rice (Stoger et al. 1998). However, the precise nature of *Agrobacterium*-inserted transgenes may be advantageous over biolistic transgene insertion. Copy number was reportedly less for *Agrobacterium*-transformed compared to biolistically-transformed plants (Hansen and Wright 1999). Biolistics often integrates several rearranged copies into the host genome, sometimes with accompanying plasmid sequence, that are tightly linked and therefore are unlikely to segregate in further generations. The nature of these insertions increases the probability of gene silencing and rearrangement. In an attempt to improve transformation efficiency, decrease copy number and reduce plasmid sequence integration, one report describes the integration of *Agrobacterium* and biolistic techniques ("agrolistics"). Two constructs were co-bombarded into maize and tobacco, one encoding the gene of interest flanked by T-DNA inserts, the other encoding virulence genes necessary for *Agrobacterium*-mediated T-DNA transfer (Hansen and Chilton 1996). However, only 20% of the transgenic population contained true agrolistic sequences, that is, genes inserted with the aid of virulence proteins. The decrease in efficiency outweighs the benefit of *Agrobacterium*-like insertion; therefore, agrolistics has not become a routinely used transformation technique. Another attempt to improve biolistic integration involved a recombinase gene within the vector (Srivastava et al. 1999). Site-specific recombination reportedly resolved multi-copy insertions into single-copy insertions and also removed *bar*. Overall, biolistic transformation may be slightly favored over *Agrobacterium* transformation in wheat, because *Agrobacterium* does not naturally infect wheat, and because studies suggest no difference in transgene rearrangement or integration efficiencies between either technique, at least in rice (Kohli et al. 1999).

Alternative transformation methods involve isolating protoplasts from cell suspension cultures, then transferring DNA with the protoplasts with either electroporation (Sorokin et al. 2000) or polyethylene glycol (PEG) treatment (Zhang and Wu 1988). Although these methods were introduced along with biolistics and *Agrobacterium* transformation, they are not widely used for wheat transformation. Culturing protoplasts is more time-intensive and tedious than obtaining embryogenic callus necessary for direct transformation (i. e. *Agrobacterium* and biolistic) procedures. Refining protoplast methods could potentially benefit the field of wheat transformation, especially if some cultivars continue to produce extremely poor efficiencies when transformed with *Agrobacterium* or biolistic devices.

6 Improving Wheat Field Performance

The process of transforming wheat can have very negative effects on agronomic traits. The insertion of a foreign gene into the wheat genome can often disrupt flanking gene expression, and many tissue-culture-induced mutations may occur at sites essential for plant survival. These mutations are often referred to collectively as somaclonal variation. Reduction in transgenic plant vigor is commonly reflected in the literature. A 2004 field trial concluded that nearly all yields were reduced in a population expressing the grain hardness genes *puroindoline A* and *puroindoline B* (Fig. 1). Another experiment was conducted with plants expressing a gene for wheat streak mosaic virus (WSMV) resistance. While the WSMV resistance gene may have conferred viral resistance, and therefore a yield advantage over controls, concurrent yield losses associated with transformation made this yield advantage undetectable, and a significant yield drop was noted versus the wild-type variety, HiLine (Sharp et al. 2002). Other growth parameters can be affected by somaclonal variation and foreign gene insertion. Several populations carrying multiple marker genes (*bar*, *uidA*) and/or a seed protein storage gene exhibited later heading dates than controls (Barro et al. 2002).

Breeders often backcross transgenic plants with parents to reduce somaclonal variation and to restore parental vigor. However, multiple backcrosses are time-consuming and only improve disruptions not associated with the transgenic locus. Disrupted regions that are tightly linked to the selectable marker insert and/or the gene of interest are not easily improved with backcrossing because recombination between these loci is rare. Biolistic delivery can disperse DNA along a large chromosomal region, sometimes over 100 kb (Jackson et al. 2001). In these cases, it is extremely difficult to backcross transgenic lines and remove deleterious mutations without also removing the gene of interest.

Despite the loss of vigor associated with transformation, many genes are now implemented to boost wheat field performance in specific environments.

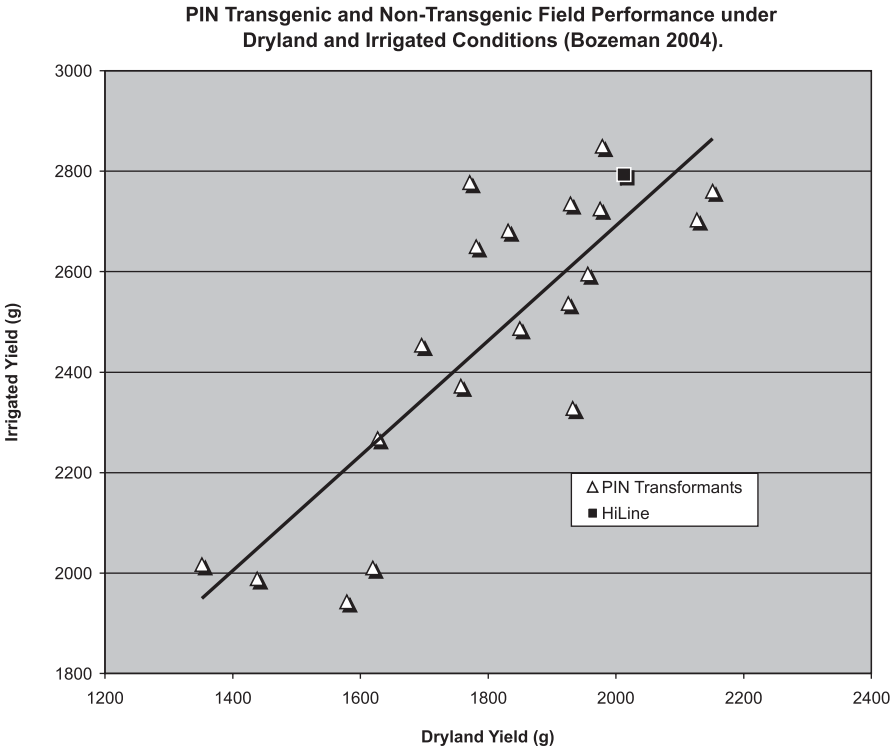

Fig. 1. Comparison of puroindoline (*PIN*) yields to control yields obtained from four-row, replicated plots. Yields were derived from homozygous independent T_4 lines transformed with one or two grain hardness gene constructs, puroindoline A (*pinA*) and/or puroindoline B (*pinB*). The mean control yield was obtained from the parental cultivar HiLine

These environments include areas where fungi are threats to wheat productivity. One fungal pathogen, the powdery mildew caused by *Erysiphe graminis*, ranks as a leading cause of European cereal crop damage (Oerke et al. 1994). Yield loss can be quite severe, especially in warm and humid climates where fungal pathogenesis is favorable. Fusarium head blight, caused by *Fusarium graminearum*, can infect 50–100% of wheat and barley crops during epidemics in China, and accompanying yield losses may reach 40% (Chen et al. 1999). Traditional fungal control methods include fungicide application and so-called "integrated pest management", which relies on the use of fungus-resistant wheat cultivars. The downfall for fungicides is that it is expensive and environmentally toxic, and specific fungal strains may eventually acquire fungicide resistance over time. The downfall for breeding and growing fungus-resistant varieties is that it is difficult to incorporate the large number of genes needed to confer broad-spectrum resistance, rather than resistance to specific fungal pathogens. Single genes or gene combinations conferring fungal resistance in

barley and rice have been transformed into wheat to reduce fungal infection. Chitinases are enzymes secreted from plants that breakdown fungal cell wall components and are a subclass of defense proteins known as pathogenesis-related (PR) proteins. A barley seed-derived chitinase gene, under control of the maize ubiquitin promoter, was stably expressed in wheat. These wheat transformants exhibited increased resistance to *E. graminis* infection (Bliffeld et al. 1999). Similarly, head blight development was delayed on transgenic wheat plants expressing a rice thaumatin-like protein and a rice chitinase gene (Chen et al. 1999).

7 Improving the End-Use Quality of Wheat

Many cereals have also been modified with genes that enhance grain quality. The major constituents of wheat endosperm are starch (approx. 70%) and protein (approx. 15%). The viscoeleastic property of dough is attributable to the largest protein polymer in the endosperm, gluten (Wrigley 1996). Gluten composes the polymeric glutenins and the monomeric gliadins. Blechl and Anderson (1996) transformed wheat with a high-molecular-weight glutenin subunit (HMW-GS) hybrid gene, whose expression could be monitored independent of native HMW-GS gene expression. High levels of the hybrid gene transcript were measured, resulting in increased total glutenin content and different viscoelastic properties in dough-making experiments. A similar HMW-GS gene, *1Ax1*, was cloned and expressed in wheat. Additional copies of *1Ax1* also boosted gluten content relative to controls, and increased dough elasticity (Vasil et al. 2001). Similarly, wheat puroindolines have been used in transformation experiments to modify wheat grain hardness (Beecher et al. 2002) and milling and baking quality (Hogg et al. 2004, 2005). Over-expression of puroindoline a and b under control of the wheat glutenin promoter decreased grain hardness and starch damage after milling, and created a chalky opaque endosperm from a hard vitreous parent variety (for a review, see Giroux et al. 2003).

8 Conclusions

Wheat is a dietary staple in over 60 countries, and worldwide annual wheat yields frequently gross over 500×10^6 t (FAO 2000). Efficient transformation systems have been established for several agronomically important species, resulting in the production of crops with desirable traits (e. g. insect tolerance, herbicide resistance). Unfortunately, a reliable transformation system has not been developed for wheat, and transformation efficiencies remain low and inconsistent. Developing stable transgenic wheat lines is essential to meet

the world's increasing demand for carbohydrate. To consistently obtain stable wheat transformants, it is necessary to refine many, if not all, of the steps in the biolistic process. These steps include obtaining a reliable source of totipotent cells from callus tissue, using constructs which contain high-expressing, tissue-specific promoters, delivering these constructs into concise chromosomal regions, and providing the correct level of selection, so that escape frequency is low and transgenic resistance is definitive during tissue culture.

The literature describes several ways of obtaining callus and regenerating plants following bombardment. Most protocols generate callus by placing dissected embryos on media containing a synthetic auxin. The concentration of auxin in the callus-induction medium may determine whether or not transformable cells are obtained, since the absence of auxin rapidly leads to organ formation. The scutellum is believed to be the best source of totipotent cells. However, excising the scutellum before placement onto medium does not increase transformation efficiency relative to non-excised scutellums. Embryonic maturity plays a large role in transformational success, as past experiments show that efficiencies vary significantly when embryos differ in age by as little as four days. The period of callus formation before and after bombardment may also affect the success of transformation. Embryos placed on callus induction medium any time from three days to several weeks produce transgenic plants. Therefore, it is difficult to determine the optimal callus induction period. Basta and hygromycin resistance are both good screening tools, but resistance may not always be complete and subsequent generations may no longer be resistant.

Modifying the bombardment procedure and the composition of constructs could potentially advance wheat transformation. Chromosomal insertion during bombardment is a random event, evident from the variable copy number and sometimes dispersed nature of transgenes and vector sequences. While *Agrobacterium*-mediated transformation inserts DNA into a discrete location, efficiencies are usually lower than biolistic efficiencies, mostly because *A. tumefaciens* does not naturally infect wheat. All biolistic devices have an inherent "shot-gun" quality that disperses DNA throughout the cell's genome. While it is doubtful this quality will be radically improved, smaller measures should be researched that will optimize gene uptake. More research is needed to test the effect of gold particle size, delivery pressure and delivery distance on callus tissue. Once these parameters are optimized, the next objective is in developing promoters that control high expression and are compatible in wheat. It is sufficient to use ubiquitous, constitutive promoters when efficiency experiments are conducted (e. g. transforming with *bar* or *uidA*), but as more non-wheat genes are characterized, the need to find promoters that control both the timing and the location of gene expression will become even greater.

A handful of biolistically modified wheat lines, although not yet commercially available, are superior to wild-type cultivars in a number of ways. We have addressed fungal resistance and modified grain hardness in wheat, but there are also reports of insect-tolerant (Altpeter et al. 1999) and Roundup-resistant wheat (Zhou et al. 1995). Wheat breeders are only beginning to in-

corporate genetically modified traits into current well-adapted varieties. As biolistic methods are refined and consistent transformation becomes possible, commercial cultivars will become available, and the quality and productivity of wheat crops worldwide will increase.

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II.3 Maize

F. Torney, B. Frame, and K. Wang[1]

1 Introduction

Maize (*Zea mays* L. ssp. *mays*) is one of the three most cultivated crops in the world, along with wheat and rice. Modern maize is quite different from its proposed ancestor, the teosintes (*Zea mays* L. ssp. *parviglumis* or *mexicana*), grown by the pre-Columbian and Mesoamerican civilizations. Centuries of selection and genetic improvement of domesticated maize have influenced its evolution into the hundreds of landraces now grown worldwide. Genetic improvement of maize has been sustained by an increase in the variety of uses of this crop (Johnson 2000). In addition to genetic improvement through breeding, recent developments in plant genetic transformation have introduced new possibilities for trait improvement in maize.

1.1 Old and New Uses of Maize

Maize does not exist in the wild. It was created through the domestication of teosinte by the Mesoamerican civilizations for which it was the major staple food. Today, maize is the most important food and feed crop worldwide. In the past 20 years, it has also been increasingly used in industrial processing; and in 2004, close to one-third of United States maize production was for food, feed or industrial use (Fig. 1).

New industrial uses of maize include plastic, sweeteners and ethanol. Zein, a major protein component of the maize kernel, is a good example of this diversification of use. Its thermoplastic and film-forming properties are used by industry to produce paper and paperboard adhesive, additives in oil cloth and linoleum, moisture and oxygen barriers and varnish substitutes (Johnson 2000). In the United States, maize seed is currently refined to produce six major outputs: high fructose corn syrup (HFCS), fuel alcohol, beverage alcohol, glucose and dextrose, starch and food (Fig. 2). In the past 25 years, production of sweeteners (HFCS), and in particular fuel alcohol, has increased dramatically. Production of ethanol from maize is a rapidly developing area of industrial crop utilization, due in part to the search for alternative energy sources. Although any starch-containing grain is theoretically suitable for ethanol production,

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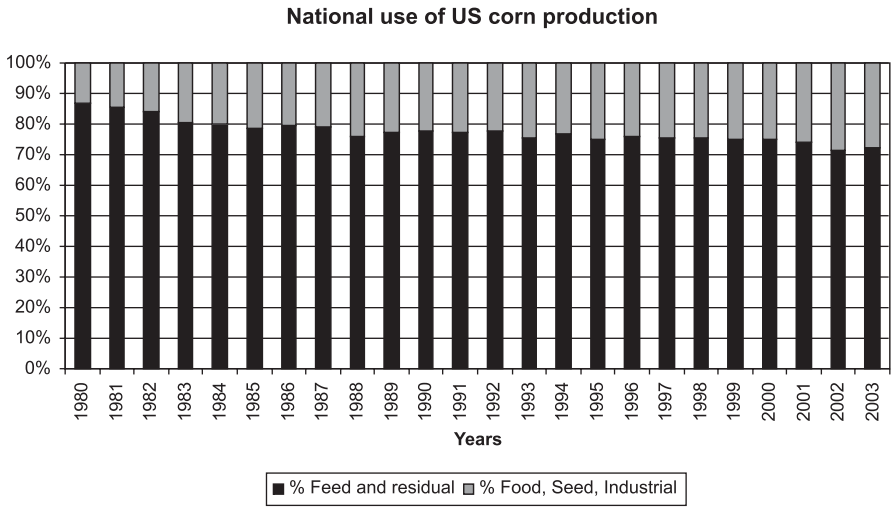

Fig. 1. United States national use of maize from 1980 to 2003. The proportion of industrial use (gray) has steadily increased in 23 years. Data compiled and published in the Corn Refiners Association Annual Report (http://www.corn.org/)

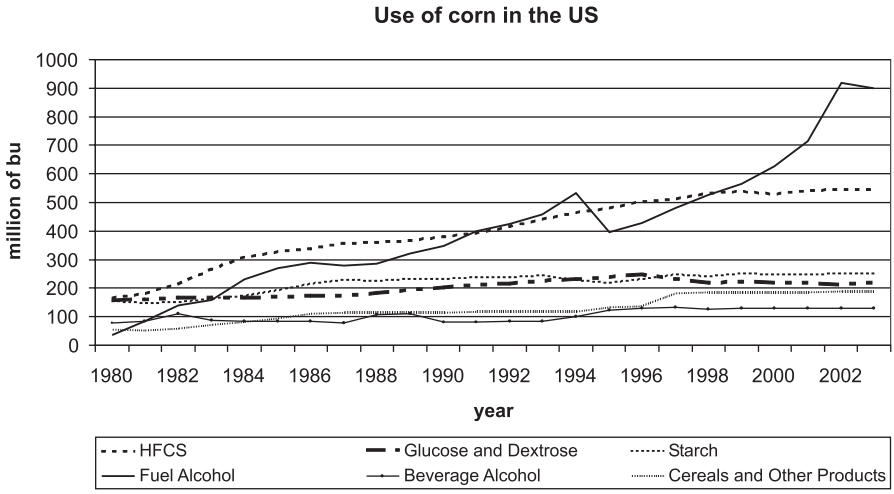

Fig. 2. Diversity and evolution of maize use in the United States from 1980 to 2003 (USDA Economic Research Service, Feed Outlook)

maize represents 95% of the current starting material for this process. For sweeteners, maize competes with sugar beet and sugar cane, the two major sweetener-producing crops in the world.

All continents (except Antarctica) produce maize, which ranks second to wheat in metric tonnes (t) of crop production world-wide. The United States produces and consumes more maize than any other country, with an annual

Table 1. Production and consumption of maize in the world ($\times 10^3$ t). Source: USDA, Foreign and Agricultural Services

Country	2002/2003		2003/2004		2004/2005	
	Production	Consumption	Production	Consumption	Production	Consumption
Brazil	44,500	37,500	42,000	38,600	35,500	38,900
Canada	8,999	12,576	9,600	11,238	8,836	10,905
China	121,300	125,900	115,830	128,400	128,000	131,500
Egypt	6,000	10,900	5,740	9,500	5,780	10,600
India	11,100	12,000	14,720	13,200	13,600	13,600
Indonesia	6,100	7,500	6,350	7,350	6,500	7,200
Mexico	19,280	24,700	21,800	26,400	22,000	27,900
Nigeria	5,200	5,200	5,500	5,500	6,500	5,900
Romania	7,300	7,200	7,020	7,200	12,000	9,400
Serbia Montenegro	5,585	4,850	3,800	4,450	6,274	5,300
South Africa	9,675	8,520	9,700	8,677	12,000	8,950
European Union	49,360	49,526	39,861	46,814	53,350	52,500
United States	227,767	200,748	256,278	211,723	299,917	224,420
World total	601,714	627,224	623,711	647,185	706,263	680,472

production nearing 257×10^6 t and an annual consumption of about 210×10^6 t (Table 1). Second is China, producing and consuming half that of the United States. Most of the United States production and, therefore, the world maize production, is localized in the "corn belt" of Illinois, Iowa, Indiana, Ohio, Minnesota, Missouri and Nebraska.

One of the major turning points in maize production occurred in the 1940s during the green revolution. Maize hybrids, a concept first outlined by Shull (1908) reached 2 t ha^{-1} during this period due to continued germplasm improvement and increased use of agronomic inputs, such as fertilizer and herbicides. Since 1962, maize yields have doubled in the United States and the world average maize yield almost tripled (Fig. 3). In recent years, novel tools such as plant genetic engineering have again revolutionized our approach to crop improvement. Tremendous resources have been directed towards using these technologies to complement traditional breeding strategies in order to improve the productivity of maize. If crop improvement in the green revolution was defined by the synergistic use of plant breeding and increased agronomic inputs, today's gene revolution (http://www.fao.org/newsroom/en/news) is catalyzed by the use of genetic engineering as a complementary tool for improving crop productivity.

1.2 Maize Improvement Through Genetic Engineering

For a ninth consecutive year, the increase in agricultural land cultivated to genetically engineered crops has reached double digits. Globally, land cultivated

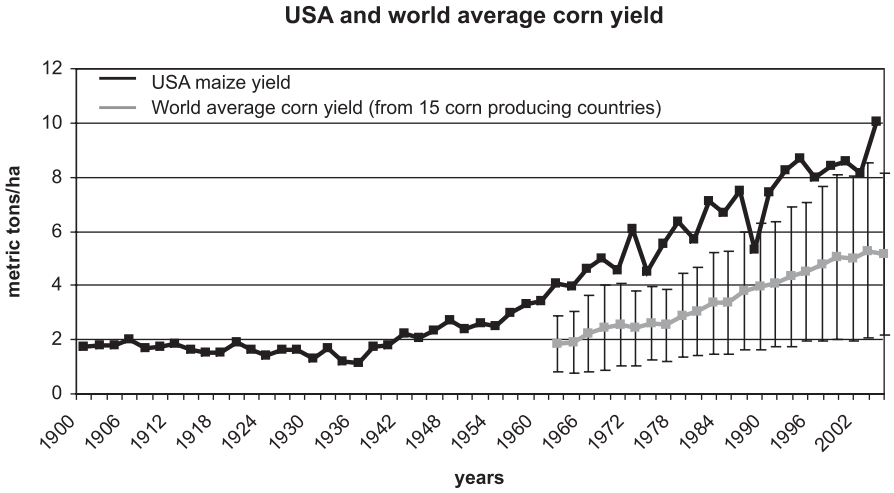

Fig. 3. Progression of maize yield in the United States from 1900 to 2004 (USDA-NASS Statistical Database) and average world maize yield increase from 1962 to 2004 (FAOSTAT 2004). The world data includes Argentina, Australia, Brazil, Canada, Chile, China, France, Germany, India, Kenya, Mexico, Nigeria, South Africa, Spain and Turkey

to biotech crops increased by 20% between 2003 and 2004; and it has increased 47-fold since 1996 (James 2004). Biotech maize (19.3×10^6 ha) is second to soybean (48.4×10^6 ha) in cultivated area devoted to biotech crops world-wide. In 2004, this represented 23% of the total land area planted to biotech crops and 14% of the total land for maize cultivation.

When these numbers are broken down into genetically engineered traits across all biotech crops, herbicide tolerance represents by far the most widely cultivated trait. For maize specifically, it is not herbicide tolerance, but Lepidoptera resistance (*Bt* technology) which represents 14% (11.2×10^6 ha) of the total biotech crop worldwide and 12.9% of the total maize cultivated area (FAOSTAT 2004). In addition, a new tendency is emerging for that of "double trait" biotech crops. The stacking of both herbicide resistance and *Bt* traits in maize, as well as in cotton, represents a major improvement in the agronomic usefulness of these genetically modified crops. More recent developments include the novel use of plant biotechnology for producing pharmaceuticals and industrial products. This remains a controversial development due to concerns about the potential for contamination of food supplies (http://www.plantpharma.org/forum/index.php).

Now, 2005 is the closing year of the first decade of biotech crop commercialization. The fact that the rate of increase in land area committed to these crops continues to increase, even though the purchase price of the seed is generally greater than non-biotech seed (Moschini et al. 2000), is testimony to the success of biotech crops worldwide.

1.3 Maize Genetic Transformation: The Critical Points

Genetic modification of plants through hybridization and selection is the basis of successful crop improvement in agriculture. However, genetic modification of maize using recombinant DNA technology became possible only in the final decade of the past century. The first fertile transgenic maize plants were reported in 1990 (Gordon-Kamm et al. 1990), while the first attempt at inserting a foreign gene into maize was reported as early as 1966 (Coe and Sarkar 1966). In this earliest experiment, DNA from one maize variety was injected into apical meristems of developing seedlings of another maize variety. The donor seedlings from which the DNA was extracted were homozygous for various dominant traits such as red anthers or purple sheaths. The targeted recipient seedlings were homozygous recessive for the same traits. If successful, red pigment was expected to appear in the 242 recipient plants. However, phenotypic evidence of genetic transformation was not observed. Although unsuccessful, this precedent experiment outlined two critical points for achieving maize transformation, namely: penetration and competence.

- Penetration. How effectively can DNA be delivered into plant cells? Coe and Sarkar (1966) emphasized that the cell wall may be a major barrier to DNA delivery. Since then, several DNA delivery techniques have been developed, amongst which the two most efficient techniques for maize are biolistic (Fromm et al. 1990; Gordon-Kamm et al. 1990) and *Agrobacterium*-mediated (Ishida et al. 1996) DNA delivery. The development of both methods required, of course, the correct target tissue.
- Competence. This involves a cell's capacity to first be genetically transformed and then to regenerate into a fully fertile plant. These attributes jointly address the issue of target tissue. For maize transformation, several possibilities have been tested with varying degrees of success. In vitro techniques and development of the appropriate combination of media and selection agent has often been the key for achieving genetic transformation of maize. In turn, the transformation competency of a given target tissue is tightly linked to that of the delivery method.

As techniques for maize genetic transformation improve and the number of transgenic events produced is no longer a limiting factor, considerable focus can now instead be directed towards transgene integration and the stability of transgene expression. This emphasis on the quality of transgenic events underlines the dual nature of efforts to improve maize genetic transformation technology.

This review outlines how maize genetic transformation has improved from the pioneer work of Coe and Sarkar (1966) to the present and emphasizes particular challenges overcome to transform maize. In addition, it presents the application of some new technologies to maize genetic transformation.

2 Penetration – Delivery Methods

The challenge of introducing DNA coding for a gene of interest into a cell is tightly linked to two factors, namely the delivery method and target tissue. Many combinations have been tested over the years and few were successful.

The uptake of DNA by plant cells was demonstrated beginning in the 1960s (Stroun et al. 1966; 1967a, b; Ledoux and Huart 1968; Kleinhofs and Behki 1977). However, these early studies disagreed on the fate of the DNA following uptake. Some groups reported that the DNA was integrated into the plant genome and replicated, while others suggested that the DNA was taken up by cells but then degraded. Even when needles were used to directly inject the DNA solution into maize apical meristems, the cell wall was described as a potential barrier to DNA penetration and the authors suggested that the cell wall may have to "be disrupted mechanically or chemically or otherwise circumvented" (Coe and Sarkar 1966).

2.1 Some Unsuccessful Attempts to Genetically Transform Maize

Numerous additional efforts to achieve maize transformation were based on very interesting concepts, but were unsuccessful and therefore abandoned. Attempts were made to transplant petunia nuclei into maize protoplasts (Potrykus and Hoffmann 1973), but the experiments were carried out under non-sterile conditions and no further analysis was done. Uptake of the blue-green alga *Gloeocapsa* into maize protoplasts was demonstrated, but no long-term persistence of the introduced alga was observed (Burgoon and Bottino 1976). Most of this early work in plant transformation using non-conventional methods was discussed by Kleinhofs and Behki (1977). Two less controversial attempts at introducing exogenous DNA into maize cells were reported 20 years after the work of Coe and Sarkar (De Wet et al. 1985; Ohta 1986). In these experiments, DNA was mixed with pollen and applied to the silks. Some seeds displayed predicted phenotypic changes, but molecular analysis was not provided by the authors (Ohta 1986). Later, Bennetzen and Lin (1988) attempted to rescue alcohol dehydrogenase (adh) defective maize pollen by injecting DNA carrying the cloned *adh-1* gene into spikelets. After staining the pollen for *adh-1* activity, the authors calculated delivery frequency to be 0.005% and concluded that further use of this technique was limited, since it was time-consuming, inefficient and required a large amount of glasshouse space for the donor plants.

More sophisticated strategies were undertaken. Incubating protoplasts in a polyethylene glycol (PEG) solution along with DNA proved to be a successful approach to achieving DNA uptake by maize cells (Armstrong et al. 1990; Golovkin et al. 1993; Omirulleh et al. 1993). Since protoplasts were used, the cell wall barrier was not an issue. The frequency of transformation under optimal conditions using Black Mexican Sweet (BMS) maize cell suspension cultures

was 0.3% (Armstrong et al. 1990). Although this frequency may seem low, it was calculated as 300 independent stable transformation events out of a million cells, and isolating a million protoplasts was feasible by a routine protocol. The DNA was efficiently delivered into maize protoplasts, but plants could not be regenerated from transgenic callus events. This inability to recover plants was a common problem encountered when starting with protoplasts from suspension cultures (Phillips et al. 1988) and likely one of the reasons that PEG-mediated maize transformation was not adopted extensively by the community. Only one laboratory reported successful PEG-protoplast transformation and regeneration of fertile transgenic maize (Golovkin et al. 1993; Omirulleh et al. 1993). In this study, a maize embryogenic suspension culture of genotype HE/89, a line developed by selection for its favorable tissue culture response (Mórocz et al. 1990), was used for transformation. The authors were able to obtain a large number of fertile transgenic maize plants from transformation and regeneration of protoplasts using this line. In addition to these intrinsic tissue culture difficulties, the PEG-protoplast technique itself was more difficult to perform than electroporation, another technique originally developed for protoplasts (Fromm et al. 1985).

2.2 Electroporation

Because the cell wall appeared to be a major barrier to DNA penetration, the use of protoplasts as a starting material for maize transformation was a natural choice. DNA penetration and expression of the introduced transgene was demonstrated in electroporated maize cells (Fromm et al. 1985). BMS microcalli were incubated with a mixture of cellulase, hemi-cellulase and pectinase to digest their cell walls. Purified protoplasts were electroporated in a solution containing plasmid DNA bearing the chloramphenicol acetyl transferase (CAT) gene, which was subsequently detected in the electroporated cells. Stable transformation of protoplasts derived from maize callus was subsequently achieved (Fromm et al. 1986). Eventually, transgenic maize plants were produced using embryogenic cell suspension cultures of the maize line A188 (Rhodes et al. 1988), but these plants were not fertile. Conversely, non-trangenic plants that were fertile were recovered (Prioli and Sondahl 1989; Shillito et al. 1989). Thus, although electroporation enabled penetration of DNA into maize cells, it proved to be an unsatisfactory combination of delivery method and target tissue for obtaining fertile transgenic maize plants.

Several teams developed tissue, as opposed to protoplast, electroporation techniques, including immature zygotic embryos or type I callus (D'Halluin et al. 1992), A188×B73-derived suspension cultures (Laursen et al. 1994) and type II callus (Pescitelli and Sukhapinda 1995). In all cases, successful transformation of maize cells occurred only when the target tissue was wounded either by partially digesting the cell wall (D'Halluin et al. 1992; Laursen et al. 1994), or by mechanical wounding and plasmolysis of cells (D'Halluin et al.

1992; Pescitelli and Sukhapinda 1995). Stable transgenic maize plants were recovered in all cases.

Although electroporation of either protoplasts or entire tissue has been documented, the low efficiency of this approach is unlikely to provide a workable system for the emerging area of functional genomics. Nevertheless, protoplast electroporation has proven to be a valuable tool for analysis of gene function through transient expression assays (Sheen 2001).

2.3 Silicon Carbide Whiskers

While electroporation was being developed, other research teams were following different paths for genetically transforming maize cells. Physical delivery of exogenous DNA into target cells was demonstrated using two major "brute force" methods that circumvented the cell wall barrier, namely biolistic and whiskers. Both techniques rely on the penetration of exogenous particles, and DNA with them, into plant cells.

Silicon carbide whiskers (SiC), needle-shaped crystals used by industry in the production of composite materials (Braun 2004), are one of the simplest ways to introduce DNA into maize cells. In the first report of DNA penetrating a maize cell using SiC, BMS suspension culture cells were mixed with DNA and whiskers, vortexed for one minute, and *gus* gene expression was monitored (Kaeppler et al. 1990). In all samples tested, transient β-glucuronidase activity was detected and, two years later, stable transformation of BMS cells was obtained using this method (Kaeppler et al. 1992). The first fertile transgenic maize plants generated using this method were produced from A188×B73-derived suspension cells (Frame et al. 1994).

Using whiskers to transform maize is an attractive method because it is simple and inexpensive. However, a major drawback of this delivery method is that it has been successful with only a limited number of regeneration proficient tissues, such as well dispersed maize suspension cultures (Frame et al. 1994) or callus cultures (Petolino et al. 2000), for which the latter authors concluded that the system was "not particularly efficient for large-scale transgenic production".

2.4 Biolistics

Development of a high-velocity micro-particle transformation apparatus in 1987 opened new possibilities for cereal transformation (Klein et al. 1987). At that time, *Agrobacterium tumefaciens*-mediated transformation was in its infancy and, in theory, was restricted to dicotyledonous plants. This new idea was to force the penetration of DNA into the cell by accelerating microparticles coated with DNA into the target tissue, thereby overcoming the dilemma of DNA penetration through the cell wall. Because the biolistic delivery system circumvented the need for cell wall removal, more organized tissues could be

targeted for transformation. This invention marked a turning point in the history of maize transformation. *Bt* maize, a transgenic product of this transformation methodology, was commercialized in 1996, only six years after the first reports of fertile transgenic maize production using this technique (Fromm et al. 1990; Gordon-Kamm et al. 1990). Recent research has outlined advantages to targeting maize immature embryos using *A. tumefaciens*-mediated methods instead of the biolistic gun (Zhao et al. 1998; Shou et al. 2004). Nevertheless, biolistic transformation of maize immature embryos remains a widely used technology in many laboratories (Songstad et al. 1996; Brettschneider et al. 1997; Frame et al. 2000).

2.4.1 The Biolistic Armory

"Biolistic" is a generic term for micro-particle bombardment and derives from *biological ballistic* (Armstrong 1999). The original biolistic gun was reported by Klein et al. (1987) and, a year later, its efficacy for delivering DNA to intact BMS maize suspension cells was demonstrated (Klein et al. 1988). By 1990, the production of fertile transgenic maize using this device was reported (Gordon-Kamm et al. 1990). Its original design used a gunpowder charge to propel the DNA-coated microparticles through a vacuum chamber into target cells from the surface of a plastic disk referred to as the macrocarrier (Klein et al. 1987). The macrocarrier, which is also propelled downward by the burst, is intercepted by a metal stopping screen, while the micro-particles continue through the screen to penetrate the target tissue placed towards the bottom of the chamber. Since the late 1980s, the system has been improved and helium gas has replaced gunpowder to provide the kinetic energy that propels the particles (Sanford et al. 1991).

Other particle acceleration methods based on similar concepts have also been developed. Like the biolistic gun, the air gun (Oard et al. 1990) uses a macro-carrier and tungsten micro-projectiles, but it has the advantage of being less costly (Songstad et al. 1995). Transient *gus* gene expression has been observed in bombarded maize suspension cells. In the ACCELL system, DNA-coated gold particles are loaded onto a metal sheet, also called a macro-carrier. An electrical impulse accelerates the particles at a precise velocity controlled by the voltage. The greater control of particle penetration by this device makes it a versatile tool for delivering DNA to a variety of crops, genotypes and tissues (McCabe and Christou 1993).

The Particle Inflow Gun (PIG) was first described by Takeuchi et al. (1992) and later improved by Finer et al. (1992). It directly accelerates DNA-coated particles into plant cells with a gentle burst of gas without the use of a macro-carrier. Minimal damage to target tissue transformed using this gun is attributed to the low-pressure helium stream required to accelerate only micro- and not macro-projectiles. Stable transformation of embryogenic maize suspensions has been achieved (Vain et al. 1993a).

2.4.2 Optimization of Biolistic Parameters

The biolistic gun is a relatively simple, robust and reproducible method for targeting a variety of maize explants for transformation. Stable maize transformation has been achieved using BMS cell suspensions (Klein et al. 1989), embryogenic maize suspension cultures (Fromm et al. 1990; Gordon-Kamm et al. 1990; Register et al. 1994), type II callus (Fromm et al. 1990; Walters et al. 1992; Armstrong et al. 1995; Pareddy and Petolino 1997; Frame et al. 2000), type I callus (Wan et al. 1995) and immature zygotic embryos (Koziel et al. 1993; Songstad et al. 1996; Brettschneider et al. 1997; Frame et al. 2000). The biolistic gun has also been used to reproducibly (although inefficiently) recover stable transgenic maize from shoot apical meristem-derived cultures (Lowe et al. 1995; Zhong et al. 1996; O'Connor-Sanchez et al. 2002; Zhang et al. 2002).

Following original optimization experiments using non-regenerable BMS maize cells (Klein et al. 1988), several reports undertook to optimize biolistic transformation parameters for regenerable maize tissues. A four-fold reduction in the amount of gold used at bombardment increased stable transformation efficiency of pre-cultured H99 immature embryos (Brettschneider et al. 1997). Similarly, a reduction in gold particle size from 1.0 μm to 0.6 μm (Frame et al. 2000) increased stable transformation efficiency in Hi II (Armstrong et al. 1991) type II callus and transient expression of an anthocyanin marker gene in type I callus (Randolph-Anderson et al. 1997). These authors attributed improved transformation rates to a reduction in damage to targeted cells at bombardment, as proposed by Kausch et al. (1995).

Increasing particle velocity by increasing rupture disk pressure from 900 psi to 1,300 psi (1,000 psi = ca. 6.89 MPa) increased stable transformation efficiency for H99 immature zygotic embryos, which produced a type I callus response in culture (Brettschneider et al. 1997). Interestingly, neither transient *gus* gene expression nor the frequency of post-bombardment somatic embryogenesis differed at these two helium pressures; only the rate of stable clone recovery was affected. The authors concluded that the greater rupture disk pressure facilitated DNA delivery to cell layers in which cells competent for transformation and regeneration in these target embryos were located. Similar observations by Dunder et al. (1995) led the authors to recommend greater rupture disk pressures when targeting type I maize cultures and reduced pressures for type II callus cultures. In our laboratory, rupture disk pressures as low as 650 psi are routinely used for targeting both immature embryos and type II callus (Frame et al. 2000).

The type of particle used in biolistic delivery also plays an important role in stable transformation efficiency. Gold particles cost more per unit weight than tungsten particles but have a more homogeneous size and smoother surfaces. These attributes are believed to minimize cell damage at bombardment (Russell et al. 1992). Moreover, tungsten is reported to have a toxic effect on some cells and may impair regeneration (Russell et al. 1992). Comparisons of transient

expression following bombardment of BMS maize cells with gold or tungsten particles coated with the CaMV35S-driven *gus* gene or the rice actin-driven *gus* gene showed more expressing cell clusters when gold was used (Hunold et al. 1994).

In addition to physical parameters, improvements in culture methods have contributed to increased transformation efficiencies using biolistic delivery methods. Four hours prior to bombardment, embryogenic cell suspensions were placed on a medium containing 0.2 M sorbitol and 0.2 M mannitol (Vain et al. 1993b). Osmotic treatment prior to bombardment was hypothesized to minimize cell damage at particle impact by reducing turgor pressure in the cell. The same treatment was applied after bombardment to help cell recovery. The transformation efficiency was significantly greater when both pre- and post-osmotic treatments were applied to the target tissue than when one or the other or neither was done (Vain et al. 1993b). Similar results were achieved using 12% sucrose to increase the osmotic potential of the medium (Dunder et al. 1995). In addition, pre-culturing explanted immature embryos for a period of days prior to bombardment was shown to increase biolistic transformation efficiency (Songstad et al. 1996).

2.5 *Agrobacterium tumefaciens*

While using "brute force" to facilitate penetration of exogenous DNA into plant cells was proven to be very effective, an alternative approach was also being developed.

The role of the soil-borne bacterium *A. tumefaciens* in tumor development in plants was described as early as 1907 (Smith and Townsend 1907). It was confirmed subsequently that this tumor-inducing ability could be attributed to the transfer of a bacterial DNA sequence into the plant host genome (Chilton et al. 1977). Finally, evidence that a non-oncogenic (disarmed) strain of *A. tumefaciens* could be used to introduce a T-DNA sequence modified with exogenous DNA into a plant cell, and that this foreign DNA sequence could be expressed, was reported independently in 1983 by three laboratories (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983). Since *A. tumefaciens* was known to be a pathogen of dicotyledonous and not monocotyledonous plants, its use as a vector for stable plant transformation was initially limited to dicotyledons. Maize infection by *A. tumefaciens* was demonstrated as early as 1986 (Grimsley et al. 1986) and transgene expression in maize cells after *A. tumefaciens* infection was reported by several teams (Schlappi and Hohn 1992; Ritchie et al. 1993; Shen et al. 1993). Recovery of maize plants containing the *gus* and *npt*II genes after inoculation of cut shoot apices with an *Agrobacterium* suspension was reported as early as 1991 (Gould et al. 1991), but not until 1996 was a robust protocol reported for production of stable transgenic maize using an *Agrobacterium*-mediated gene-delivery method (Ishida et al. 1996).

2.5.1 Penetration of the T-DNA into Plant Cells

Penetration of T-DNA from bacterial cells into plant cells is efficient enough to produce transgenic plants, although the mechanism is not completely understood. Two 25-bp border sequences bracket and define the T-DNA region, which is the only part of *Agrobacterium* Ti (tumor-inducing) plasmid DNA transferred into the plant cell. Another critical component on the Ti plasmid is the virulence (*vir*) region located outside of the T-DNA. The *vir* genes produce a number of enzymes and proteins that facilitate the T-DNA transfer. The T-DNA is believed to be cleaved from the Ti plasmid as a single-strand molecule. This single-stranded T-DNA, the T-strand, is coated by *vir*E1 and E2 proteins and capped by *vir*D2 protein in the bacteria. In a process similar to conjugation, *A. tumefaciens* uses a type IV secretion system to transfer this nucleoprotein complex into the plant cell (Gelvin 2000).

Until recently, most efforts to enhance transformation efficiency using *A. tumefaciens* have focused on modifying bacterial components. Using strain LBA4404 to harbor a binary vector containing extra copies of certain *vir* genes (called the super binary vector) resulted in high transformation efficiencies in rice (Hiei et al. 1994) and maize (Ishida et al. 1996; Zhao et al. 1998).

Using forward and reverse genetics, plant mutants have been identified that react differently to the same *A. tumefaciens* strain (Gelvin 2000, 2003; Hwang and Gelvin 2004). This indicates that plant factors are also involved in mediating the transformation process. Modifying the target plant so that it becomes the "ideal" recipient for *A. tumefaciens* may also be one way to enhance transformation efficiency. Gelvin's group identified *Arabidopsis thaliana* "rat" mutants (resistant to *Agrobacterium* transformation; Zhu et al. 2003). One of them was deficient for the *HTA-1* gene coding for H2A-1 histone. Transient expression was observed in this mutant, but stable expression was not obtained (Mysore et al. 2000). Penetration of the T-DNA was observed in the rat-5 mutant by transient *gus* expression, although the plant cells were not competent for stable transformation. Interestingly, *Agrobacterium* transformation by the flower dip procedure (Clough and Bent 1998) was still possible for this mutant. Expression of the wild-type *HTA-1* gene in the rat-5 mutant of *Arabidopsis* restored the competency for stable transformation via tissue culture and enhanced efficiency for transformation using the floral dip method (Mysore et al. 2000).

2.5.2 The Super Binary Vector

The super binary transformation system for maize was the second major turning point in maize genetic transformation after the 1990 reports using the biolistic gun. Ishida et al. (1996) used immature zygotic embryos from the maize line A188 as a target tissue for their seminal work in stably transforming maize using *Agrobacterium tumefaciens*. One of the key points in their success was the use of a Ti plasmid "super binary" system, in which a DNA segment containing *vir*B, *vir*C and *vir*G genes were cloned into the binary vector that

normally carries only genes of interest (Komari and Kubo 1999). The presence of extra copies of these *vir* genes, in addition to the endogenous *vir* genes on the disarmed Ti plasmid, allowed the authors to reach transformation efficiencies as high as 30%. The transformation system used the *bar* gene as a selectable marker and the intron-containing *gus* marker gene, both driven by the CaMV35S promoter. Molecular analysis by Southern blot showed that *A. tumefaciens*-mediated transformation produced large numbers of single- and low copy number transformation events. This high transformation efficiency and high proportion of single-copy events using the super binary vector system was later confirmed using the maize Hi II genotype (Zhao et al. 1998).

2.5.3 The Standard Binary Vector

A. tumefaciens-mediated transformation of maize proved to be a low-cost technique generating transgenic events with simple transgene integration patterns (Ishida et al. 1996) and improved transgene expression stability (Shou et al. 2004) and reports of using standard binary vector system for DNA delivery have also been forthcoming (Frame et at. 2002; Zhang et al. 2003). One advantage of the standard binary vector is that no homologous recombination step is required for introducing the gene of interest into the *Agrobacterium* strain. In our laboratory, *A. tumefaciens* strain EHA101 harboring the standard binary vector pTF102 (Frame et al. 2002) is used to infect 10- to 13-day-old Hi II immature zygotic embryos. Co-cultivation is carried out for three days on medium supplemented with the anti-oxidant L-cysteine, following earlier work in soybean (Olhoft and Somers 2001). Bialaphos-resistant type II callus events are recovered eight weeks after infection at 5% frequency and regenerated to produce stably transgenic R_0 plants and stably transformed R_1 progeny.

3 Competence and Regenerability – Target Tissue

Discussion of maize genetic transformation in this chapter focuses on systems that lead to stable transformation. In order to achieve this end, a transformation-competent targeted cell must first be able to receive DNA fragments and stably integrate them into the nuclear genome. The transformed cell must then, in most cases, proliferate under a suitable selection scheme to distinguish it from non-transformed cells. Finally, a fertile maize plant must be recovered that is germ-line transformed and thus transmits the introduced trait to its progeny.

3.1 Selectable Markers

As aptly emphasized by Vain et al. (1993a), the effectiveness of any target tissue and gene-delivery method is highly dependent on a selectable marker system

and the promoters for recovering transformed cells, although promoters will not be a subject of this chapter. Recent successes in efficient maize transformation have, in part, been attributed to the move away from the aminoglycoside selection agents that had proven so effective for the rapid advancement of dicotyledon transformation in the1980s to the use of the *bar* (bialaphos-resistant) selectable marker gene which confers resistance to phosphinothrycin, the active ingredient in several commercial herbicides (Gordon-Kamm et al. 1991). The use of this selectable marker gene for recovering stable transformants of bombarded maize cells was described by Spencer et al. (1990) using non-regenerable BMS cultures and, shortly thereafter, by Gordon-Kamm et al. (1990) using embryogenic suspension cells in the first report describing the recovery of fertile transgenic maize. More recently, transgenic maize events produced using *Agrobacterium*-mediated methods have been recovered at high frequency, also using this selection system (Ishida et al. 1996, 2003; Zhao et al. 2001). Other herbicide-resistant genes that have been used effectively as selectable marker genes to recover fertile transgenic maize in combination with their complementary herbicides are ALS (acetolactate synthase and chlorsulfuron; Fromm et al. 1990) and EPSPS (5-enolpyruvylshikimate-3-phosphate synthase and glyphosate; Howe et al. 1992).

An effective selectable marker gene for maize that is neither antibiotic nor herbicide-based is the *pmi* (phosphomannose isomerase) gene. A cell expressing this gene can metabolize mannose as a carbon source, thereby conferring a positive advantage to transformed cells under selection on mannose-containing medium (Evans et al. 1996). This selectable marker system has also been reported to work very effectively in *Agrobacterium*-mediated maize transformation systems (Negrotto et al. 2000). The search for so-called "safe" selectable marker systems (Reed et al. 2001), such as *pmi*/mannose, is likely to maintain its momentum and interest as one approach to producing transgenic events that do not carry antibiotic- or herbicide-resistant genes. Recently, the use of D-amino acid oxidase (DAAO) in *Arabidopsis* has shown that, depending on the D-amino acid used, negative selection or a positive selection for transformed cells can be achieved (Erikson et al. 2004). D-Alanine, for example, was an effective negative selectable marker agent for *Arabidopsis* and these authors also described its toxicity in maize. This preliminary evidence suggests the possibility of using D-alanine and the DAAO gene as a new selection system for maize.

3.2 Stable Integration of Transgenes

The first requirement for competency of a targeted cell is that it be capable of stably integrating exogenous DNA. Depending on the transfer method, exogenous DNA penetrates the cell in different forms. Physical delivery methods such as electroporation, whiskers and the biolistic gun use naked DNA, whereas *Agrobacterium* transformation transfers a nucleoprotein complex.

These differences lead to downstream consequences regarding exogenous DNA integration. Both processes remain largely unknown, but recent progress has shed light on various aspects of transgene integration into the plant genome. Most of the information available on the integration mechanism comes from sequencing and analysis of transgene integration sites.

3.2.1 Transgene Integration via Physical Delivery Methods

Current understanding of the process of exogenous DNA integration is quite poor. The transgene is understood to integrate into the genome by illegitimate recombination (IR) or non-homologous end-joining (NHEJ; Kohli et al. 2003). One of the hypotheses is that DNA integration is a two-phase process (Kohli et al. 1998).

The first phase is a pre-integration one in which the exogenous DNA is processed by the cell before being integrated into the genome. After bombardment, the cells enter a stress-response phase as demonstrated by callose formation (Hunold et al. 1994). During this phase, multiple enzymes are activated, including those for DNA repair and DNA degradation. It is believed that a combined action of those two types of enzymes on the freshly introduced DNA may result in multimerization of complete and incomplete exogenous DNA molecules (Kohli et al. 1998), which may explain the complex integration patterns found when sequencing transgene integration sites (Makarevitch et al. 2003).

The second phase occurs at integration. Double-stranded break-points in the host genome may serve as landing sites for the intact or rearranged exogenous DNA by stimulating the cell's recombination machinery (Kohli et al. 1998). Such temporary "hot spots" may become the site for IR or NHEJ, since separately introduced transgenes frequently integrate at the same site in the genome (Register et al. 1994; Kohli et al. 2003). These sites vary in targeted cells, resulting in the multitude of integration patterns observed when a number of independent transgenic events are compared. Interestingly, transgene copy numbers assessed by detailed Southern blot analysis could not reveal the real complexity of an insertion site as effectively as could the use of sequencing (Kohli et al. 2003).

3.2.2 T-DNA Insertion

How the T-DNA is inserted into the host genome remains somewhat of a mystery. Some pieces of the puzzle have, however, been elucidated. T-DNA enters the cell as a nucleoprotein complex. The VirD2 protein that caps the T-DNA and the VirE2 protein both contain nuclear localizing signals (NLS). The VirD2 NLS signal interacts with a host importin-α (Ballas and Citovsky 1997). Modification of the VirD2 NLS alters the nuclear localization of the T-DNA nucleoprotein complex and affects transformation efficiency (Shurvinton et al. 1992; Mysore

et al. 2000). Interestingly, the VirE2 NLS does not interact with the same host protein, but rather with the VirE2 interacting protein 1 (VIP-1) to allow nuclear import (Tzfira et al. 2001). *A. tumefaciens* seems to have developed two parallel systems to insure the nuclear import of its transferred T-DNA. The molecular process of the insertion itself is not yet fully understood, although some groups suggest that micro-homologies between the T-DNA and the insertion site aid in integration (Mayerhofer et al. 1991; Kohli et al. 2003).

3.3 From Targeted Cell to Fertile Plant

Several plants, like tobacco, can be regenerated from leaf explants (Vasil et al. 1964; Vasil and Hildebrandt 1965). However, early work with maize tissue cultures demonstrated totipotency only from shoot meristem or some embryo-derived cells (Green and Phillips 1975). Although immature embryos from six inbred lines were cultured in this study, embryogenic scutellar callus capable of regenerating plants was produced from only inbred line A188, or its reciprocal crosses with inbred line R-navajo. These results demonstrated the totipotency of immature embryo-derived callus but also emphasized that its production was genotype dependent, a characteristic that still presents problems for maize genetic transformation 30 years later.

The ideal maize explant to target for transformation would be one for which germ-line transformation is ensured with as short a period of in vitro culture as possible. Potential maize transformation target explants, ranked from high to low for morphological complexity, are presented in Fig. 4. In this simplistic depiction, the less complex the targeted explant, the greater the probability of germ-line transformation. In turn, if prolonged culture is re-

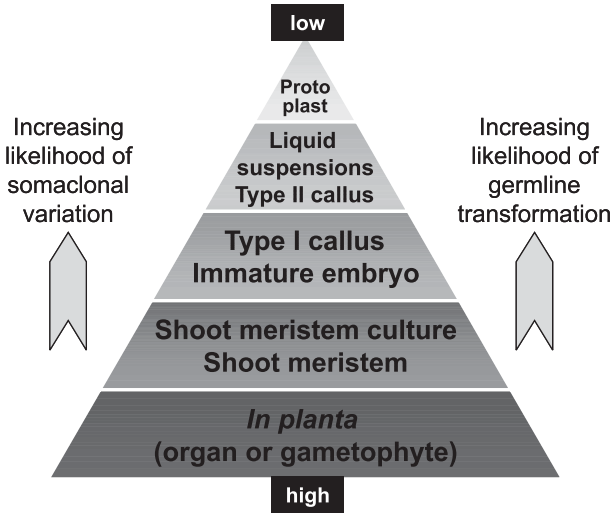

Fig. 4. Morphological complexity of potential maize target explants

quired to achieve this undifferentiated or dissociated state, the probability of somaclonal variation increases (Lee and Phillips 1987). Unicellular protoplasts were considered an ideal target to ensure germ-line transformation and recovery of non-chimeric maize plants, but failure to recover fertile transgenic plants from transformed protoplasts was attributed to loss of totipotency after prolonged culture (Rhodes et al. 1988). Tissue culture-induced variation would be all but eliminated were *in planta* transformation achieved, but targeting a plant organ would also reduce the likelihood of germ-line transformation unless it was a gametophyte. Although the latter scenario has been achieved in *Arabidopsis thaliana* using the floral dip method (Bechtold et al. 1993), no conclusive evidence for maize gametophyte or *in planta* transformation has yet been reported. Interestingly, this *in planta* approach to transformation was that taken in some of the earliest attempts to transform maize and remains an intriguing prospect today.

Since the introduction of the biolistic gun as a method for transforming maize, three maize target tissues have been commonly used, namely the immature zygotic embryo, immature embryo-derived scutellar callus and callus-derived liquid suspension cultures. Although the immature embryo is the starting ex-plant for all these target tissues, it was the last amongst them to be targeted for stable transformation, in part because of the trade-offs illustrated in Fig. 4. Instead, early biolistic experiments were carried out using liquid suspension cultures or friable type II callus, based on the premise that targeted explants had to be reduced to their most dissociated state (least morphologically complex) prior to DNA delivery to ensure that non-chimeric transgenic plants would be regenerated from single transformed cells. The first report demonstrating stable maize transformation (using electroporation) from more organized explants such as immature embryos and type I callus (D'Halluin et al. 1992) stated that "embryogenic, friable, type II cell cultures were no longer a prerequisite for genetic transformation of maize". Furthermore, the authors argued that Mendelian inheritance of their transgene demonstrated the non-chimeric nature of their primary transformants and, therefore, the single cell origin of their transgenic events. By challenging the premise that morphologically complex explants were less competent transformation targets, these results helped expand the range of potential target tissues in maize.

3.3.1 Callus or Suspension Cultures

Embryogenic callus can be produced from scutellum cells of maize immature zygotic embryo explants dissected 10–14 days after pollination and placed with their adaxial side in contact with auxin containing medium. Beginning 2–3 days later, embryogenic callus forms from rapidly dividing cells in the abaxial, basal region of the scutellum (Fransz and Schel 1990).

Maize callus phenotypes are characterized as type I or type II. Using maize genotype A188, high concentrations of proline and N6 salts (Chu et al. 1975),

friable, rapidly growing, highly embryogenic callus was developed (Armstrong and Green 1985) and called type II to differentiate it from that which had been observed previously (designated type I). In contrast to type II callus, in which embryogenic structures are independently suspended in the friable callus matrix, the latter is compact and embryogenic lobes show a high degree of association.

Type II embryogenic callus, like non-regenerable BMS callus (Sheridan 1982), could be used to produce finely aggregated liquid suspension cultures. While BMS cell cultures were used for preliminary optimization experiments with the biolistic gun (Klein et al. 1988) and whiskers (Kaeppler et al. 1990, 1992), it was suspension cultures produced from highly embryogenic type II (A188×B73) callus that facilitated production of the first fertile transgenic plants using the gene gun (Gordon-Kamm et al. 1990) and whiskers (Frame et al. 1994). Recovery of some somaclonal variant transgenic lines using these target cells (Gordon-Kamm et al. 1990) was attributed to prolonged time in culture and cryo-preservation was used by these and other authors (Register et al. 1994) to extend the "shelf-life" of particularly desirable cell lines. As early as 1990, stable biolistic transformation of type II callus was also reported (Fromm et al. 1990) and was rapidly followed by several reports of targeting type II callus using the gun (Walters et al. 1992; Pareddy and Petolino 1997; Frame et al. 2000) and whiskers (Petolino et al. 2000).

Stable transgenic maize plants have been regenerated from type I callus transformed using electroporation (D'Halluin et al. 1992) and the biolistic gun (Wan et al. 1995; Wang et al. 2003). Because many inbred or elite maize genotypes often produce type I callus instead of type II callus from cultured immature embryo scutella (Duncan et al. 1985), establishing the transformation competence of this more structurally complex tissue was a significant development for expanding the inventory of maize genotypes accessible to transformation. However, when compared with friable type II callus, the compact, differentiated and slow growing characteristics of type I callus require that increased selection pressure and labor be used for effective recovery of transgenic events after transformation (Pareddy and Petolino 1997).

Finally, targeting callus cultures for transformation provides access to maize genotypes that display notoriously low embryogenic callus induction frequency from immature zygotic embryos. Callus cultures (type I or type II) can be bulked up from the few responding embryos of a recalcitrant genotype, providing access via biolistic transformation (Wan et al. 1995; Wang et al. 2003) to a callus phenotype and genotype of choice.

3.3.2 Immature Zygotic Embryos

Seventeen years ago, Klein et al. (1988) observed transient *gus* gene expression in scutellar cells of maize immature zygotic embryos following particle bombardment. Armed with the additional knowledge that totipotent callus could

be produced from this explant (Green and Phillips 1975; Armstrong and Green 1985), the authors stated that the immature embryo would be a prime target for transformation. Today, where dedicated glasshouse or growth chamber space is available for embryo donor plant production, this has become the explant of choice for biolistic or *Agrobacterium*-mediated transformation.

While the target explant is the immature embryo scutellum, it acts as a repository for abaxial somatic cells located from one (Fransz and Schel 1990) to five cell layers deep (Pareddy and Petolino 1997) which, when precultured in the presence of auxin, undergo rapid division and differentiation into embryogenic cell clusters to form embryogenic callus. It is from these proliferating, totipotent cells that transgenic events are recovered after selection. Importantly, prolonged tissue culture steps are not required to produce this population of transformation competent cells, thereby minimizing loss of totipotency due to somaclonal variation (Songstad et al. 1996). Immature embryos have been directly targeted for routine stable transformation using both the biolistic gun (Koziel et al. 1993; Songstad et al. 1996; Frame et al. 2000) and *Agrobacterium*-mediated methods (Ishida et al. 1996, 2003; Zhao et al. 1998, 2001; Negrotto et al. 2000; Frame et al. 2002).

Development of the hybrid Hi II line (Armstrong et al. 1991), in conjunction with a medium regime on which 100% of F_1 or F_2 immature embryos produced type II embryogenic callus (Armstrong and Green 1985; Songstad et al. 1996), has enabled widespread use of the immature embryo explant for transgenic research and production using either biolistic or *Agrobacterium*-mediated transformation methods. Our laboratory, for example, routinely transforms Hi II immature zygotic embryos using the PSD-1000/He delivery system with an average transformation frequency of 15 bialaphos resistant events per 100 bombarded embryos.

Recently, this "transformability" of Hi II germplasm was transferred to an elite stiff stalk line using marker assisted breeding; and an efficient *Agrobacterium*-mediated protocol for transforming this type II elite line callus was developed (Lowe et al. 2004).

3.3.3 Maize Inbred Line Transformation

In spite of the outlined advantages to targeting immature embryos, transformation of maize inbred or elite lines for crop improvement using this explant is limited by the quality and frequency of embryogenic callus induction from immature embryos of a given genotype on a given medium. Except for some inbred lines such as A188 (Armstrong and Green 1985), the response frequency is generally low, despite attempts to identify a genotype-independent culture system for this explant (Duncan et al. 1985; Carvalho et al. 1997). Use of breeding to improve the tissue culture response is one approach to targeting elite lines (Armstrong et al. 1992; Lowe et al. 2004). Some success has also been reported for directly targeting immature embryos of inbred or elite lines (other

than A188) using the biolistic gun (Koziel et al. 1993; Brettschneider et al. 1997; Wang et al. 2003) or *A. tumefaciens* (Ishida et al. 2003; Frame et al. 2005; Huang and Wei 2005).

3.3.4 Shoot Meristems

Poor induction of embryogenic callus from the immature embryo explants of many maize inbred lines led researchers to investigate alternative explants from which to develop transformation competent cells. If, in addition, mature seeds could be used as starting material for producing target cells, one would not have to rely on greenhouse space to grow maize plants for weekly production of immature embryos. Targeting shoot apical meristems of germinated seeds was the subject of the first attempt at maize genetic transformation 40 years ago (Coe and Sarkar 1966) and recently has been one of renewed interest.

Because the fate of cells in the apical meristem is predetermined by their location in that meristem (Bowman and Eshed 2000), the challenge to using these cells as transformation targets is either to deliver DNA into a germ-line progenitor cell, or to reprogram the cell by manipulating culture conditions before or after transformation, to ensure stable transformation. If not, meristem transformation and regeneration produces a chimeric plant which does not transmit the transgene to its progeny (Cao et al. 1990; Gould et al. 1991).

Two approaches have been used to produce stable transgenic progeny from maize shoot apical meristem explants. In the first system, the apical domes of coleoptile-stage immature embryos were bombarded with an antibiotic selectable marker and a *gus* reporter gene, and plants germinated. Sectors in the apical meristems of these regenerated plants that were chimeric for the introduced transgene were proliferated on medium containing a cytokinin and the antibiotic selection agent that inhibited chloroplast development. In this way, chimeric plantlets could be regenerated and, through sequential vegetative propagation of de novo meristems, transgenic plants and progeny were eventually obtained (Lowe et al. 1995).

In the second system, the authors used medium supplemented with both cytokinin and auxin to induce heterogenous multiple shoot meristem cultures from the shoot apical meristem (SAM) of germinated mature seeds (Zhong et al. 1996). In spite of concerns that biolistic targeting of morphologically complex shoot tip cultures would lead to chimeric plant production, stable transformation and transmission of the transgene to progeny was demonstrated, although at low frequency, and chimeric plants were not reported. This was attributed to the morphologically elastic phenotype of the bombarded SAM cultures used (Zhong et al. 1992). For example, by varying relative concentrations of cytokinin and auxin, these cultures could also be induced to produce somatic embryos directly from the SAM, or from callus derived from the SAM. Inter-conversion between embryogenic and organogenic callus in SAM-derived cultures of subtropical and tropical maize was observed

using a similar medium supplemented with adenine (O'Connor-Sanchez et al. 2002). Using the biolistic gun, stably tranformed plants and progeny were also produced in this latter study.

One important advantage to targeting SAM-derived cultures for transformation is that their production is reported to be somewhat genotype-independent. SAM cultures were produced in 70% of 45 temperate zone inbred and hybrid lines tested (Li et al. 2002a) and all nine tropical and subtropical lines reported (O'Connor-Sanchez et al. 2002). In addition, SAM cultures of the well known but notoriously recalcitrant inbred line B73 have been transformed using the biolistic gun, although at low frequency (Zhang et al. 2002).

Progress towards stably transforming these heterogeneous, shoot meristem-derived tissues using *Agrobacterium* has been reported (Li et al. 2002b). Alternatively, shoot apical meristems were first incubated with *Agrobacterium* cells and then induced to form either somatic embryogenic callus or organogenic shoot meristem cultures from which R_0 transgenic plants, but no progeny, were recovered (Sairam et al. 2003). When embryogenic callus was first induced (directly or indirectly) from mature seed derived nodal sections and then co-cultivated with *A. tumefaciens* (Sidorov et al. 2006), stable transformation was achieved and transgenic progeny plants were recovered.

In a recent study, potentially novel, embryogenic tissues derived from the embryo explant of mature seeds of seven maize inbred lines, including Mo17, were described (Huang and Wei 2004) but not targeted for transformation. While implementation of this methodology has yet to be reported by other laboratories, it is indicative of the widespread interest in developing readily available, genotype-independent, competent cells for maize crop improvement using genetic transformation technology.

4 Future Prospects

As already discussed, targeting a wider range of maize genotypes, and some inbred lines in particular, for research or trait improvement purposes will continue not least of all in an effort to marry transformation technology with current genomic characterization of specific inbred lines. Maize transformation (both biolistic and *Agrobacterium*-mediated methods) is now a routine but not a trivial task. The production and maintenance of large numbers of transgenic maize plants requires committed resources and efficient organization. As functional genomics demands more transgenic plants for gene analysis, the importance of improving transgenic plant quality becomes imminent.

Currently, the quality of transgenic events is addressed by using the "numbers game" strategy. Hundreds of independent events are produced routinely to select for a few "successful" events (Z.Y. Zhao, personal communication). For most academic laboratories, this production is an unattainable task. In the next stage of improving maize transformation, the quality issue will likely be addressed using different strategies.

For research programs, it may be sufficient to simply choose transgenic events that strongly and stably express the transgene under study. However, for a commercial program, the quality of a transgenic event also pertains to how "clean" it is; i. e., does it (1) only contain one copy of the gene of interest, (2) only contain the desired and essential sequences and (3) not contain the selectable marker. Furthermore, control of pollen dissemination to avoid cross-pollination with non-transgenic maize may be of particular interest especially if maize producing pharmaceutical and industrial products is commercialized.

4.1 Clean Insertion Strategies

4.1.1 Isolated Cassette Bombardment

Biolistic transformation is known to generate complex integration patterns, which is believed to cause transgene silencing (Fagard and Vaucheret 2000) and may also be a legal concern for biotech crop commercialization. In order to overcome these drawbacks, several strategies have been tested.

In a standard biolistic transformation experiment, the entire vector sequence is delivered together with the transgene into a targeted cell, such that they cannot be separated as Mendelian traits. One solution to avoid their insertion is to use transgene expression cassettes only instead of complete plasmids carrying the vector backbone and the antibiotic resistance gene for bacterial selection. In rice, using a supercoiled plasmid, or a double-stranded isolated gene expression cassette for transformation, resulted in no loss of transformation frequency using the latter (Breitler et al. 2002). Furthermore, the occurrence of silencing decreased drastically in T_0 plants. However, this approach did not seem to decrease the complexity of the integration site based on the Southern blots shown in the work (Breitler et al. 2002).

4.1.2 Site-Directed Recombination

Site-specific DNA integration, as well as specific excision of a DNA fragment, are attractive ways of solving many issues inherent to genetic engineering (randomness of the transgene insertion or presence of undesired sequences in the transgene integration site, for example). Both phenomena can be achieved through site-specific recombination: the recombination of two precisely oriented sequences (target sites), either in two separate DNA molecules or in the same DNA molecule, catalyzed by a specific enzyme, the recombinase (Ow 2002). For instance, if the target sites bracket the selectable marker gene (e. g., *bar* or *npt*II) in the same orientation, it becomes possible to remove the undesirable gene by expressing a recombinase (Lyznik et al. 1996; Ow 2001; Zhang et al. 2003). Other applications can be envisaged for this sequence removal strategy. Simplification of complex integration sites by removing extra sequences is possible. This was demonstrated using wheat as a model and a modified

Cre/lox system (Srivastava et al. 1999). In all four independent transgenic events produced with lox target site-containing plasmids, and crossed with a Cre recombinase expression line, simplification of transgene integration pattern was observed as well as selectable marker sequence removal (*bar* gene). In similar experiments with maize, simple insertion events were also obtained (Srivastava and Ow 2001).

Another application for site-directed recombination is site-directed insertion, the most desired feature for transgenic transformation. However, to achieve this illusive goal, an integration site (a sequence to be targeted) must first be established, either through genomic characterization or genetic transformation to allow insertion of the gene of interest by the second round of transformation (Albert et al. 1995; Srivastava and Ow 2002). The concepts and possible applications are reviewed by Ow (2006).

Site-directed recombination appears to be operable, but more data is required to substantiate its effectiveness. Thanks to the numerous site-specific recombination systems in existence, several variations to this approach are available (Lyznik et al. 1996; Sugita et al. 2000; Ow 2001). However, some issues may be associated with future adoption of the method. For example, the lox sites are undesirable for validation of commercial products because of their bacterial origin. The presence of the recombinase gene in the final product is also not desirable and requires an extra breeding step for removing it through segregation.

4.1.3 A. tumefaciens Vector/Strain Improvement

One of the important features in *Agrobacterium*-mediated transformation is that the bacterium transfers only its T-DNA region to the recipient plant genome, such that transgenic events generated by this method should contain only the desired T-DNA segment. In fact, it appears that the *Agrobacterium* method is not as "clean" as had been hoped. Using this method, a high percentage of vector backbone elements were carried into transgenic plants (Kononov et al. 1997; Meza et al. 2002; Shou et al. 2004). Even if these elements do not influence transgene expression (Meza et al. 2002), undesirable backbone sequences can hinder legal validation of a transgenic event. This problem is far from negligible, since the presence of the vector backbone has been detected in 50–75% of independent events investigated in two separate studies (Kononov et al. 1997; Shou et al. 2004). Strategies, such as using double T-DNA border sequences (Kuraya et al. 2001) and negative selection markers inserted into the backbone, are currently being tested to reduce the occurrence of vector backbone integration.

Another feature of the *Agrobacterium*-mediated method has been to allow removal, by segregation, of the selectable marker gene. Two separate T-DNAs are used instead of one. One carries the gene of interest and the other carries the selection marker. This approach has been tested in rice and tobacco (Depicker

et al. 1985; Komari et al. 1996; McCormac et al. 2001; Breitler et al. 2004). Depending on the strategy used to deliver both T-DNAs, the co-transformation efficiency varies from study to study. However, in all of them, some transgenic events, both in rice and tobacco, show segregation of the two T-DNA in the progeny. A two T-DNA binary system was used in maize to achieve high-efficiency transgene segregation in co-transformed maize plants (Miller et al. 2002).

Recently, the ability has been demonstrated of other bacteria (*Sinorhizobium meliloti*, *Rhizobium* sp. NG234, *Mesorhizobium loti*) to transfer DNA into plant cells (Broothaerts et al. 2005). Although these transformation efficiencies are low compared to *Agrobacterium*-mediated transformation, using other bacteria to mediate exogenous DNA delivery to plant cells may open new possibilities for crop biotechnology (Gelvin 2005).

4.2 Control Pollen

Pollen dissemination, leading to unwanted out-crossing of transgenic pollen with non-transgenic plants, is a major issue for maize transgenic crop production. Several approaches can be envisaged to address this issue. One strategy is simply to physically and temporally isolate a transgenic maize crop from other non-transgenic maize fields. In 2003, a one mile (1.6 km) distance was defined as the minimal isolation radius for open pollination tests for pharmaceutical-producing maize plants in addition to delayed planting for 28 days (USDA-APHIS 2003). An extensive study on transgenic maize pollen flow showed that very little cross-pollination could be found at 300 m away from the transgenic pollen source (Stevens et al. 2004).

Although physical and temporal isolation minimizes any possible pollen contamination, biological containment measures are being developed. Two other possibilities can be investigated for pollen control, namely male-sterile lines (natural or induced) and organelle transformation.

4.2.1 Cytoplasmic Male Sterility

One way to implement transgenic pollen containment is to prevent the production of pollen. Cytoplasmic male sterility (CMS) has been studied in maize in the context of hybrid seed production because it prevents labor costs associated with manual de-tasseling (Levings 1993). CMS is a maternally inherited trait preventing formation of viable pollen. It is generally associated with mitochondrial defects (Mackenzie et al. 1994). The first CMS to be described in maize was the Texas cytoplasm (cms-T; Rogers and Edwardson 1952). It was used for hybrid seed in the United States until the Southern corn leaf blight epidemic in the 1970s (Ullstrup 1972) demonstrated a high susceptibility of this male sterile germplasm to the disease (Levings 1993). Despite this drawback, it is

possible to envisage the use of CMS as one approach to pollen containment when transgenic maize is produced on a small scale.

Another possibility is to use genic male sterility (GMS), which is triggered by a defect in a nuclear gene and therefore segregates as a normal Mendelian trait. Recent work described a system in which transcription of an inverted repeat of the Ms45 gene promoter led to transcriptional gene silencing of the Ms45 gene. This induced a high frequency of male-sterile plants lacking the Ms45 transcript (Cigan et al. 2005). Such a system, if added to a traditional gene of interest expression system, would bring about the desired biological containment of the transgene by producing male sterile plants.

4.2.2 Plastid Transformation

Another biological containment approach for transgenic pollen drift is to place the transgene in the plastid genome. Because inheritance of chloroplast traits is maternal in maize (Daniell and Varma 1998), Mendelian transmission of a transgenic locus associated with the nuclear genome would be circumvented and pollen grains would not carry the transgene of interest. The first successful plastid transformation was achieved using the biolistic gun to transform *Chlamydomonas reindhartii* (Boynton et al. 1988) and this delivery method is still the tool of choice for plastid transformation. Plastid transformation offers many advantages over nuclear transformation, but is lagging behind nuclear transformation because of its current low efficiency and the issue of heteroplasty.

Two major plastid transformation strategies have been used so far, namely (1) transformation of the chloroplast genome by homologous recombination (Maliga 2004) and (2) use of an autonomous replicating vector in the plastid (Staub and Maliga 1994). Using the first approach, the gene of interest is bracketed by two regions highly homologous to a precise part of the chloroplast genome and the construct is introduced by biolistic transformation allowing a targeted insertion within the chloroplast genome (Lutz et al. 2001; Maliga 2004). A derivative of a homologous recombination strategy using a phiC-31 phage site-specific recombination system has been described (Lutz et al. 2004), in which a target site was first inserted using homologous recombination. In a second round of transformation, a simpler vector was used in combination with either a nuclear integrated or a transiently expressed recombinase gene. Targeted insertion was observed as frequently as 17 independent transformation events per bombardment.

The use of plastids as targets for transformation offers the added advantage that recombination protein production yields are higher than in nuclear transformants. Such an approach was recently described by Tregoning et al. (2004) for the production of vaccine in plants.

5 Concluding Remarks

Maize is an economically important crop with rich genetic information that, like wheat, has benefited from human intervention for agronomic trait improvement. Because of its importance, the need for improving production levels and broadening the uses of maize has warranted development of genetic engineering technologies to meet this goal. Although maize genetic transformation is now a routine application in some laboratories, it is far from being a trivial technique. Since the first attempts of genetic transformation, many approaches have been explored by the research community to achieve stable and quality maize transformation events. Through the aim of achieving reproducible inbred line transformation, it is feasible that completely new approaches may evolve from those presently described. The issue of what approach is taken towards crop genetic engineering remains a society debate that scientists, politicians and the public will undertake for maize and all other biotech crops.

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II.4 Rye

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1 Introduction

Cultivated rye (*Secale cereale* L.) is assumed to have originated from either *S. montanum* Guess subsp. *montanum*, a wild species found in southern Europe and nearby parts of Asia, or from *S. montanum* Guess subsp. *anatolicum*, a wild rye found in Syria, Armenia, Iran and Turkistan. Rye was found as a weed widely distributed in wheat and barley fields in southern Asia. It apparently had co-evolved with wheat and barley for more than 2000 years until its value as a crop was recognized. Although rye first grew wild in southwest Asia, it was cultivated initially in north and northeastern Europe, where it remains a staple food and the most common flour used for baking breads (Bushuk 2001).

2 Economic Importance of Rye

Winter rye is the most productive cereal grain crop under conditions of low temperature, acidic pH, low fertility and drought (Madej 1996). World rye grain production during the past decade is estimated at 20×10^6 t year^{-1}. About 94% of world rye grain is produced in Europe, about 2% in North America and the rest in other continents. In Europe, the greatest rye producers are Russia, Poland, Germany, Belarus and Ukraine (FAO 2005).

The total rye production and cultivation area in Europe decreased during the past 50 years to about 20×10^6 t from 9.4×10^6 ha in 2004. During the same period, the average grain yield increased from 11 dt ha^{-1} to 20 dt ha^{-1}. It is remarkable that, after introduction of the first rye hybrids in 1984, average yields increased considerably in Germany (Miedaner 1997). In 2004, about 60% of rye seed production in Germany was from hybrids.

Rye has a higher nutritional value compared to other cereals. Soluble fiber, which may help reduce the risk of heart disease by helping to lower blood low-density lipoprotein (LDL) cholesterol levels, makes up about 17% of the dietary fiber found in whole grain rye foods. Whole grain rye also contains many important vitamins and minerals, such as B vitamins, vitamin E, calcium,

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magnesium, phosphorus, potassium, iron, zinc and folate (Rakowska 1996). Although the total production of rye has diminished, its use as food for humans increased slightly during the 1990s and its demand will likely increase as research indicates a positive role of rye dietary fiber for human health (Lange et al. 1994). Rye is used to make whiskeys and vodkas, crisp flat breads, crackers, porridges, puddings, pumpernickel and sourdough breads, and many other foods. However, more than 60% of rye is used as animal feeds. The majority of this is used as a component of mixed feed (Bushuk 2001). Rye has enough gluten to be made into a rising, yeasted bread, but it has less than wheat, making it denser, darker and heavier than wheat breads. Rye could be used to produce starch as a raw material for biopolymers, straw as a fiber base for paper or insulation materials, and biomass as an energy source. Recent economical and political trends in European agriculture towards the reduction of fertilizer, pesticides and growth regulators may lead to the increasing interest in rye cultivation.

3 Molecular Markers, Genetic Mapping and Marker-Assisted Selection

Conventional breeding of a new rye variety takes between eight and 12 years. Hence, breeders are extremely interested in molecular marker technology, which is expected to accelerate selection in rye breeding. In recent years, different marker systems such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), sequence tagged sites (STS), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) or microsatellites, single nucleotide polymorphisms (SNPs) and others have been developed and applied to a range of crop species including cereals. The relative advantages and disadvantages of these markers are summarized in Table 1.

Table 1. Comparison of the most commonly used marker systems in rye

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10.0	0.02	0.5–1.0	0.05	0.05
Required DNA quality	High	High	Moderate	Moderate	High
PCR-based	No	Yes	Yes	Yes	Yes
Number of polymorph loci analyzed	1.0–3.0	1.5–50.0	20–100	1.0–3.0	1.0
Amenability to automation	Low	Moderate	Moderate	High	High
Ease of use	Not easy	Easy	Easy	Easy	Easy
Reproducibility	High	Unreliable	High	High	High
Development cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

Table 2. Genetic maps of rye

Cross between rye cvs./inbred lines	Population structure	Marker type(s)	Reference
DS2 × RXL10	99 F_2s	RFLP	Devos et al. (1993)
		RFLP	Philipp et al. (1994)
Two inbred lines	137 F_2s	RFLP, RAPD	Senft and Wricke (1996)
Two inbred lines	54 F_2s	RFLP, RAPD	Loarce et al. (1996)
P87 × P105	375 F_2s	RFLP	Korzun et al. (1998)
Consensus map	F_2s	RFLP, SSR	Börner and Korzun (1998)
UC-90 × E-line, King II × Imperial	184 F_2s	RFLP, SSR	Ma et al. (2001)
P87 × P105	375 F_2s	RFLP, SSR	Korzun et al. (2001)
DS2 × RXL10	282 F_2s	RFLP, RAPD	Masojć et al. (2001)
		AFLP, SSR	Saal and Wricke (2002)
Composite map	F_2s	SSR	Khlestkina et al. (2004)

RFLP, SSR and AFLP markers are most effective in detecting polymorphism. However, given the large amount of DNA required for RFLP detection and the difficulties in automating RFLP analysis, AFLPs and SSRs are currently the most popular markers for cereals. An increasing amount of sequence information and the determination of the gene function in cereals will lead to the preferred use of new marker types, such as SNPs. Analyzing the co-segregation of markers and traits in defined populations allows the construction of genetic maps (Table 2).

F_2 populations resulting from narrow or wide crosses, single seeds descent-derived recombinant inbred (RI) populations, or doubled haploids were used to construct genetic maps of rye. The first linkage maps of rye were based on RFLP markers (Devos et al. 1993; Philipp et al. 1994; Senft and Wricke 1996). Using selected anchor probes of already published maps of rye (Devos et al. 1993; Philipp et al. 1994; Senft and Wricke 1996), wheat (Gale et al. 1995), barley (Graner et al. 1991; Heun et al. 1991) and oats (O'Donoughue et al. 1995) and combining them, Korzun et al. (2001) published a linkage of rye consisting of 139 RFLPs, 19 isozyme and protein markers, 13 microsatellites, ten known function sequences and two morphological genes. This map allowed users to choose additional probes of wheat, rye or barley for saturating rye genome regions of their interest. Recently, another extended linkage map of rye was published, containing a total of 91 microsatellite markers mapped into the RFLP frameworks of four rye mapping populations, consisting of 139, 64, 58 and 60 RFLPs, respectively (Khlestkina et al. 2004). The availability of a "grass genome" composite map detailing the similarities in gene order between many species of the Gramineae will enable genetic studies in relatively small genomes, such as rice, to be applied to large genome species, such as wheat, barley and rye (Gale and Devos 1998). Maps of the related grass species may be constructed rapidly by using a set of anchor probes derived from the composite map.

One of the main objectives of plant breeding is the introgression of favorable genes from a donor parent into the background of an elite variety. Knowing the location of specific genes/traits and their alleles offers the possibility to apply marker-assisted selection (MAS) in rye (Table 3). In rye more than 200 genes were identified and localized in certain chromosomes, using special cytogenetics tester systems and several linkage maps (compilation of Schlegel et al. 1998). MAS allows plant selection at the juvenile stage from an early

Table 3. Tagging and molecular mapping of traits in rye using molecular markers

Trait	Gene/QTL	Marker type(s)	Chromosome	Reference
Morphological/physiological traits				
Reduced plant height	*ct1*	RFLP	7R	Plaschke et al. (1995)
(Compactum)	*ct2*	RFLP	5R	Plaschke et al. (1993)
Reduced plant height	*Ddw1*	RFLP	5R	Korzun et al. (1996)
Vernalization response	*Sp1*	RFLP	5R	Plaschke et al. (1993)
Flowering time	*QTL*	RFLP	2R,5R,7R	Börner et al. (2000)
Florets per spike	*QTL*	RFLP	6R	Börner et al. (2000)
Self-fertility	*S*	RFLP RAPD	1R	Senft and Wricke (1996) Voylokov et al. (1998)
	Z	RFLP RAPD SSR, STS	2R	Senft and Wricke (1996) Voylokov et al. (1998) Hackauf and Wehling (2005)
	S5	RFLP	5R	Voylokov et al. (1998)
Biotic/abiotic stress response				
Leaf rust	*Lr-a*	RFLP	6R	Ruge et al. (1999)
	Lr-c	RFLP, SSR	1R	Ruge et al. (1999)
	Lr-g	RFLP	1R	Ruge et al. (1999)
	Pr3	SSR	1R	Roux et al. (2004)
	Pr4	SSR	1R	Roux et al. (2004)
	Pr5	SSR	1R	Roux et al. (2004)
Powdery mildew	*Pm*	RFLP	1R	Wricke et al. (1996)
Cereal cyst nematode	*CreR*	RFLP, RAPD	6R	Taylor et al. (1998)
Aluminium tolerance	*Alt1*	RAPD, SCAR	6R	Gallego et al. (1998)
	Alt3	AFLP	4R	Miftahudin and Gustafson (2001)
Restoration of fertility				
	Rfg1	RFLP RAPD RAPD, CAPS, AFLP, SCAR	4R 4R 4R	Börner et al. (1998) Miedaner et al. (2000) Stracke et al. (2003)
Quality				
Secalins	*Sec2*	RFLP	2R	Malyshev et al. (1998)
	Sec5	RFLP	2R	Malyshev et al. (1998)
Waxy endosperm	*Wx*	RFLP	4R	Korzun et al. (1997)

generation. Unfavorable alleles can be eliminated or greatly reduced during the early stages of plant development. For simply inherited traits, PCR-based markers are frequently used and allow efficient screening of large populations of segregating progenies.

4 Androgenesis for the Production of Double Haploids

Selection of parental lines for rye breeding is more effective at the level of homozygous inbred lines than at the heterozygous level, especially with respect to quantitative characters. Homozygous inbred lines are also very useful in test crosses, particularly in hybrid breeding. Conventional production of inbred lines in a heterozygous crop, such as rye, is frequently complicated by self-incompatibility and requires at least five generations of controlled self-fertilization. Several methods of haploid production have been investigated in cereals, including microspore and/or anther culture (androgenesis), ovule culture (gynogenesis), *Hordeum bulbosum* L. or maize (*Zea mays* L.) pollination methods (alien species chromosome elimination) and an alien cytoplasm system (Dunwell 1985; Kasha 1989). In contrast to other methods, androgenesis has been adapted for haploid formation in rye. Following the culture of anthers or isolated microspores, embryo formation from immature pollen and subsequent regeneration of embryos into plants can result in the formation of haploid plants. The formation of double haploids occurs either spontaneously, or is induced by colchicine treatment.

Early studies on the production of double haploids in rye, begun in the 1970s, concentrated on anther culture (Wenzel and Thomas 1974; Thomas et al. 1975; Wenzel et al. 1977; Flehinghaus et al. 1991; Dainel 1993; Flehinghaus-Roux et al. 1995). The best results were obtained using progenies of *Secale cereale* × *vavilovii* (Flehinghaus-Roux et al. 1995), a genotype which lacks agronomic importance. Immonen and Anttila (1996) and Rakoczy-Trojanowska et al. (1997) identified responsive true rye lines. Deimling et al. (1994) reported the successful regeneration of plants from isolated cultured microspores of semi-wild rye SC35. The culture of isolated microspores eliminates the risk of plants arising from diploid tissue (septum, anther wall or tapetum). Guo and Pulli (2000) demonstrated relatively high callus induction and green plant regeneration from isolated cultured microspores of true rye (*Secale cereale* L.). Compared to anther culture, cultured isolated microspores resulted in more green double haploid plants per anther in four rye genotypes (Ma et al. 2004). Most advances toward improving anther or microspore culture methods used specific genotypes and "stress" treatments, like cold or osmotic pretreatment, to induce androgenesis from the preprogrammed gametophytic to the sporophytic pathway. Compared to barley and wheat, the development of an efficient androgenic cell culture system for rye is less advanced. Problems associated with rye anther and microspore culture include poor embryogenic callus in-

duction, limited green plant regeneration, a high proportion of albinos and severe genotype dependency. More effective methods are needed for producing double haploids from a wide range of genotypes.

5 Genetic Transformation

Genetic transformation is a powerful tool for crop improvement. Injection of plasmid DNA directly into floral tillers represented the first attempt to genetically transform rye (De la Pena et al. 1987). Since conclusive molecular evidence could not be generated to demonstrate the success of this approach, attention moved to tissue culture-based genetic transformation approaches. In comparison to other cereals, rye is very recalcitrant in tissue culture. Despite reports of successful regeneration of plants from embryogenic cultures derived from leaves (Linacero and Vázquez 1986), immature embryos (Rybczynski and Zdunczyk 1986; Zimny and Lörz 1989; Rakoczy-Trojanowska and Malepszy 1995) or immature inflorescences (Linacero and Vázquez 1990; Rakoczy-Trojanowska and Malepszy 1993), the reproducibility and efficiency of these protocols was the limiting factor for the development of an efficient genetic transformation protocol for rye. Rye is a cross-pollinated species. The resulting genotypic variability within a cultivar also reduces the reproducibility of the culture response from rye cultivars (Popelka and Altpeter 2001). A gametophytic self-incompatibility mechanism prevents selfing in rye (Geiger and Schnell 1970). Self-fertile forms have also been identified in several rye populations and are used for developing inbred lines in rye-hybrid breeding programs (Miedaner 1997). In the early transformation experiments, cross-pollinating rye populations were used to initiate embryogenic tissues, resulting in the first few transgenic rye plants (Castillo et al. 1994). The identification of inbred lines displaying a good regeneration response from cultured tissues (Popelka and Altpeter 2001) and the optimization of gene transfer, selection and culture parameters allowed the development of a reproducible and efficient biolistic transformation protocol for rye (Popelka and Altpeter 2003a). The selection of marker-free transgenic plants, or plants with a simple transgene integration pattern, were also reported following biolistic gene transfer to rye (Popelka et al. 2003). Compared to *Agrobacterium*-mediated gene delivery, biolistic gene transfer is usually successful in a wider range of genotypes, and co-transfer of multiple genes is facilitated (Altpeter et al. 2005). *Agrobacterium*-mediated gene transfer also offers potential advantages, including preferential integration of T-DNAs into transcriptionally active regions (Czernilofsky et al. 1986; Koncz et al. 1989). The elimination of selectable marker genes is also facilitated by the typical integration of co-transformed T-DNAs into separate chromosomes (McKnight et al. 1987; Komari et al. 1996). The transfer of T-DNA and its integration into the plant genome is influenced by the bacterial strain (McKnight et al. 1987), vector-plasmid (Tingay et al. 1997; Klee 2000), the

addition of *vir*-gene inducing compounds (Hoekema et al. 1983), culture conditions, media composition and osmotic stress treatments (Usami et al. 1988) during and before *Agrobacterium* infection, the plant genotype, explant and tissue culture protocol, as well as the suppression of *Agrobacterium* after co-cultivation (Nauerby et al. 1997). In the past decade, convincing molecular evidence of stable *A. tumefaciens*-mediated gene transfer was also presented for several cereals, including rice (Hiei et al. 1994), wheat (Cheng et al. 1997), barley (Tingay et al. 1997) and, more recently, rye (Popelka and Altpeter 2003b). The development of efficient genetic transformation protocols for rye (Popelka and Altpeter 2003a, b) has enabled, to date, the engineering of rye seed protein composition. Expression of the high molecular weight glutenin subunits *1Dy10* and/or *1Dx5* from wheat in transgenic rye resulted in a drastic increase of the polymeric glutelin fraction. Expression of *1Dy10* significantly enhanced the bread-making quality of rye (Altpeter et al. 2004; Wieser et al. 2005).

6 Conclusions

Efficient genetic transformation protocols and molecular markers have been recently developed and currently support the molecular improvement of rye.

The future direction of marker development and marker-assisted breeding in rye will continue to target improvement for disease resistance (especially leaf rust and virus resistance), factors determining bread-making quality of harvested grain like pre-harvesting sprouting and dissection of heterosis mechanisms to support higher grain yields. One of the major factors for the stable yield of hybrid rye and reduction in infection by the ergot fungus is the efficiency of the fertility restoration system. Partial restoration of male fertility causes a reduction in the amount of viable pollen, which encourages infection by the ergot fungus (*Claviceps purpurea*). Ergot infection contaminates rye grains with toxic alkaloids. To reduce or avoid this risk, rye hybrids need effective restorer genes. Recently, a new restorer source was found in an Iranian primitive rye population "IRAN IX". This exotic material displays a significantly higher level of restoration than the currently used European lines. However, despite the excellent restoration ability, this primitive rye population contains many undesirable agronomic characters. The development of new rye cultivars which combine an excellent pollen restoration with a high agronomic performance can be accelerated by MAS (for more details see http://www.pollen-plus.de).

The increasing amount of sequence information and number of cloned genes from cereal crops might support the development of a SNP-based marker system in rye. This will reduce costs and increase the efficiency of marker-assisted breeding.

Transgenic approaches are likely to focus on disease resistance (e. g. rust and ergot resistance), grain composition and quality, for example reduction

of pentosans to improve feed quality, co-expression of low and high molecular weight glutenin subunits from wheat to improve bread making quality or improvement of starch content for bioethanol production. Additional targets are improvement of drought tolerance and nutrient uptake efficiency to further enhance the comparatively high stress tolerance of rye. Containment of transgenes is desirable to enhance the biosafety of transgenic rye and might be achieved by using natural or transgenic male sterility systems or by developing a chloroplast transformation protocol for rye.

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II.5 Pearl Millet

M. Girgi[1] and M.M. O'Kennedy[2]

1 Introduction

"We are talking about a crop that is virtually unimprovable – a crop that grows where not even weeds can survive; a crop that has been improved by farmers and through natural selection for thousands of years; a crop that produces nourishment from the poorest soils in the driest regions in the hottest climates; a crop that grows straight out of sand dunes; a crop that survives sand storms and flash floods" (ICRISAT 1996).

2 Species Origin and Economic Importance

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) is the sixth most important cereal world-wide and is the main food source in the poorest regions of India and the African continent. It is a high-yielding, diploid C4 summer grass with $2n = 14$ chromosomes. Amongst the major cereals, pearl millet is highly tolerant to heat and drought, to saline and acid soils and is easy to grow in arid regions where rainfall is not sufficient for maize or even sorghum (FAO 2004). Pearl millet is descended from wild grasses native to the central Saharan plateau region of Niger, from where it spread to East Africa and India. Developing countries, mainly in Asia and Africa, account for about 94% of the global output of millet, where annual production exceeds 10×10^6 t, to which India contributes nearly half. In 2003, world-wide millet production was estimated at 29.8×10^6 t harvested on 36.3×10^6 ha, an area larger than that used for wheat production in the USA (FAOSTAT 2004). Five countries in West Africa (Nigeria, Niger, Mali, Burkina Faso, Senegal) produce 85% of the continent's total pearl millet crop. Almost all millet is produced by small-scale farmers for household consumption and localised trade.

Pearl millet is used for food, feed, brewing and as a building material. It is consumed primarily as a thick porridge ("toh"), but it is also milled into flour to prepare unfermented breads and cakes ("roti"), steam-cooked dishes

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("couscous"), fermented foods ("kisra and gallettes"), non-alcoholic beverages and snacks. Roasted young ears are a popular food for children. Furthermore, it is the most preferred cereal grain grown in the Sahelian countries, Senegal, Mali, Niger and Burkina Faso and is consumed in preference to sorghum. In northern Nigeria, pearl millet is used in making a popular fried cake known as *masa*.

Feeding trials conducted in India have shown that millet is nutritionally superior to maize and rice. It is a "high-energy" cereal with starch amounts of 70% in the dry grain. Its protein content of 16% is higher than in maize with a good balance of amino acids. Further, it contains 5–7% fat, which is greater than the values in most maize varieties; and it is particularly high in calcium and iron. It has low contents of fibre and most vitamins, whereas it is rich in vitamin A (NRC 1996; DeVries and Toenniessen 2001).

Although resistant to many diseases, pearl millet is susceptible to several pathogens, amongst which *Sclerospora graminicola*, the causal agent of downy mildew, is economically the most important, causing high annual yield losses. Smut, caused by *Moesziomyces penicillariae*, and top rot, caused by *Fusarium moniliforme*, present further fungal pathogens of pearl millet. The root parasite *Striga hermonthica* and the stem borer *Coniesta ignefusalis* also belong to its important pests (Wilson 2000; FAO 2004).

3 Pearl Millet Biotechnology

The African continent is dominated by agriculture and about 70% of its population live off farming. Africa has the highest percentage of agriculturally working population and the second highest cultivated area world-wide. Nevertheless, yields are the world's lowest (DeVries and Toenniessen 2001). In the past decades, impressive advances have been achieved in the productivity of the major three cereal crops, wheat, rice and maize, which helped to mitigate the disasters of population explosion. In the years to come, considering global warming and overpopulation, Africa's native grains will dominate, having the greatest untapped potential, since they still retain many of the robust properties of their wild ancestors.

Pearl millet is considered a "lost" crop of Africa (NRC 1996). Its available gene pool with traditional breeding methods is restricted by sexual incompatibility in many interspecific and intergeneric crosses. Most pearl millet cultivars are grown in Africa using minimal levels of purchased input. Breeding is aimed towards the capacity of surviving harsh conditions rather than increasing yield. Like other native African crops, pearl millet is still poorly supported by both science and politics; and biotechnology research in this field remains an underdeveloped resource for improved crop production in African agriculture. Also, the practical utilisation of biotechnological advances has been limited. A large number of African scientists have acquired skills and knowledge in

biotechnology but they are often unable to apply the techniques on local crops due to a lack of facilities and research funding. Furthermore, most African countries do not possess legal requirements and need consumption regulations for genetically modified organisms (GMOs). Biotechnological products aimed at Africa and improved in foreign laboratories await only the appropriate regulatory licences for importation (DeVries and Toenniessen 2001).

To date, the improvement of plants by means of genetic transformation and in vitro culture has been successfully implemented. Most activities on pearl millet, such as molecular breeding for downy mildew resistance, stover quality, increased beta-carotene content, drought and salinity tolerance (ICRISAT 2004), are localised at the International Crops Research Institute for the Semi–Arid Tropics (ICRISAT) in India. Applications of biotechnology to pearl millet are being investigated regarding methods of in vitro culture, such as micropropagation through tissue culture, genetic engineering and marker-assisted selection (DeVries and Toenniessen 2001).

There is an urgent need for an increased focus on crops relevant to the small farm holders and poor consumers in the developing countries of the humid and semi-arid tropics (Sharma et al. 2002). Pearl millet is the only major staple cereal that reliably produces both grain and forage on poor, sandy soils under the hot, dry conditions of Africa and Asia (Goldman et al. 2003), yet fungal phytopathogens such as *Sclerospora graminicola* have devastating effects on grain production. Genetic manipulation and in vitro culture provide a means for the plant gene pool to be broadened. Besides eliminating time-consuming back-crossing procedures in the field, gene technology offers transfer methods for specific genes controlling well-defined traits which are not available by classic breeding. Thus, the improvement of the native crops of Africa could directly benefit the people in greatest need. Transgenic pearl millet, conferring increased resistance to fungal invasion and developed through biotechnological methods, could complement current breeding programmes. Nevertheless, transgenic pearl millet will have to be tested as stringently as any other cultivars released. The World Health Organisation/Food and Agricultural Organisation (WHO/FAO) have protocols for a rigorous assessment and testing of genetically modified (GM) foods (Halsberger 2003) which would be applicable to transgenic pearl millet expressing selected valuable transgenes. Furthermore, it is essential to exclude gene flow from GM pearl millet to non-GM pearl millet. The pollen-mediated flow of transgenes can be controlled by cytoplasmic or nuclear male sterility. As cytoplasmic male sterility is a maternally inherited trait (Budar and Pelletier 2001; Feil and Stamp 2002), it can minimise the possibility of gene flow to non-GM pearl millet, which might otherwise present the threat of contaminating the gene pool of pearl millet. Furthermore, cytoplasmic male sterility systems already contribute significantly to increasing the productivity in pearl millet breeding (Thakur et al. 2001).

Finally, the gains in food production provided by the "Green Revolution" have reached their climax, while the world population continues to grow (Wisniewski et al. 2002). A new "Green Revolution" will necessitate the application

of recent advances in plant breeding, including new tissue culture techniques, marker-assisted selection and genetic modification (Wisniewski et al. 2002), in order to aid mankind's increasing food requirements, with cereal grains playing a pivotal role (Hoisington et al. 1999). The affluent nations can afford to adopt elitist positions and pay more for food produced by the so-called natural methods; the one billion chronically poor and hungry people of this world cannot (Wisniewski et al. 2002). Therefore, despite the diverse and widespread beneficial applications of biotechnology products, there remains a critical need to present these benefits to the general public in an understandable way that stimulates an unbiased and responsible public debate (Sharma et al. 2002) and pro-GMO government policies.

3.1 Somatic Embryogenesis and Plant Regeneration

An efficient transformation system has to be available in order to enhance the genetic pool of pearl millet and to apply recombinant DNA technology. Furthermore, high frequency plant regeneration from cultured explant material is a prerequisite for the successful transformation of this crop, as the limiting step in the development of genetic engineering technologies for the improvement of selected cereal genotypes lies in the in vitro culture step. Cultures of cereals, in general, show strong genotypic dependency and development of appropriate cultures is generally restricted to certain genotypes (Lambé et al. 1999). The improvement of efficient in vitro regeneration systems for pearl millet is therefore a major precondition to achieve applicable plant transformation.

Different in vitro regeneration systems for pearl millet have been reported within the past 20 years, such as regeneration of plants from protoplasts (Vasil and Vasil 1980), immature embryos and inflorescences (Vasil and Vasil 1981a; Swedlund and Vasil 1985) and from suspension cultures derived from immature zygotic embryos (Vasil and Vasil 1981b) or shoot apical meristems (Devi et al. 2000). Recently, efficient regeneration systems targeting selected pearl millet genotypes, based on shoot apices and immature zygotic embryo-derived embryogenic tissue, were developed by Oldach et al. (2001) and O'Kennedy et al. (2004a). These studies tasked the optimisation of explant source, culture media including carbon sources, and phytohormone concentrations and ratios (Fig. 1).

The duration needed to obtain mature pearl millet regenerants averages up to six months and is long compared with that of other cereals. Furthermore, the culture of certain pearl millet genotypes is characterised by an extensive production of phenolic substances, that oxidise and provoke browning of the culture medium and the plant tissue. Their accumulation slows down plant growth and increases mortality, leading to retarded plant regeneration. The addition of particular compounds to the culture medium, such as silver nitrate, can antagonise and thus mitigate this effect. Furthermore, callus induction medium supplemented with L-proline improved regeneration efficiencies to

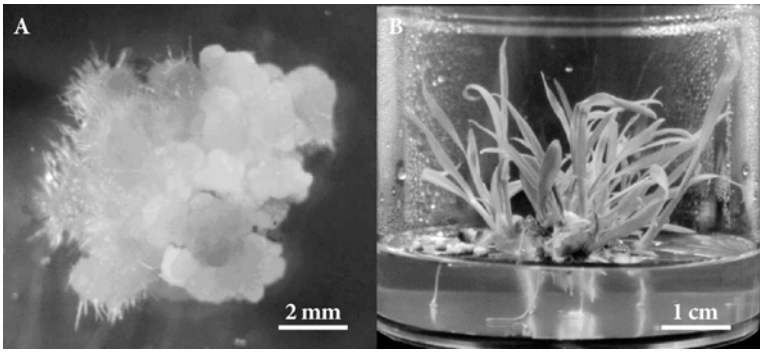

Fig. 1. Regeneration of pearl millet from immature zygotic embryos, according to Oldach et al. (2001). **A** Callus induction and early regeneration. **B** Root induction on regenerating plants

nine plants per immature zygotic embryo, six plants per shoot apex (Oldach et al. 2001) and 80 plants per immature zygotic embryo (O'Kennedy et al. 2004a).

3.2 Genetic Transformation

Although successful in vitro regeneration and transformation systems for pearl millet have been published (Lambé et al. 2000; Goldman et al. 2003), transformation of this crop is still limited by relatively low and erratic stable transformation efficiencies. Until recently, reports were restricted to transient reporter gene expression (Taylor and Vasil 1991) and expression of selectable marker genes in long-term callus cultures which often could not be regenerated to transgenic plants (Lambé et al. 1995, 2000). Establishing an efficient routine transformation protocol for pearl millet would therefore form the technological basis for the genetic enhancement of this crop and provide the means of introducing commercially important genes. Lately, transgenic, fertile pearl millet plants were regenerated from scutellum cells of cultured immature zygotic embryos, the cells being transformed with either the PDS 1000/He gun (BioRad) or the particle inflow gun (PIG) devices, using the herbicide selectable marker gene, phosphinotricin acetyltransferase from *Streptomyces hygroscopicus* ("*bar*") or *Streptomyces viridochromogenes* ("*pat*"; Girgi et al. 2002). Transformation rates ranged between 0.04% and 0.7%, requiring a regeneration period of 10–12 months for the various genotypes used.

It is well documented that only a minor fraction of the treated cells will integrate the foreign DNA during the transformation process, while the majority of the untransformed cells need to be eliminated by selection. During negative selection by herbicides or antibiotics, most of the cells in the cultured tissue die. These dying cells release toxic substances, which in turn affect the regeneration of transgenic tissue and may form a barrier between the medium and the transgenic cells, thereby preventing or slowing the uptake of nutrients. Although the stable integration of such selection genes makes it possible

to identify and select transgenic plant cells, their lingering presence in crops complicates the regulatory process and negatively affects public acceptance of the final product. Thus, positive selection systems, based on enzymes from sugar metabolism, favour the regeneration and growth of the transgenic cells, while the non-transgenic cells are starved but not killed. Finally, transgenic plants expressing these enzymes have no potential risk to animals, humans or environmental safety, which is essential since pearl millet is indigenous to Africa. Recent investigation resulted in significantly higher transformation frequencies in other cereal crops when the phosphomannose isomerase gene *pmi* from *E. coli* was used for selection, in maize (Negrotto et al. 2000) and rice (Lucca et al. 2001). Similarly, fertile, transgenic pearl millet plants were produced using *manA*, also encoding a phosphomannose isomerase, resulting in a higher transformation efficiency of 0.7–3.0% (O'Kennedy et al. 2004b).

Beyond the establishment of pearl millet transformation, first approaches concerning genetic enhancement of pearl millet towards fungal resistance were published recently. Selected genes, like the chemically synthesised *pin* gene (Latha et al. 2006), and the *afp* gene encoding the anti fungal protein from the mould *Aspergillus giganteus* (Girgi et al. 2006) were successfully transformed into different pearl millet cultivars and showed significant enhancement in fungal resistance against downy mildew and rust infection.

3.3 Genomics in Pearl Millet Breeding

During the past ten years, resources have been established for the genetic analysis of pearl millet (Qi et al. 2004). Among these are detailed genetic maps containing both homologous and heterologous restriction fragment length polymorphism (RFLP) markers, and simple sequence repeats (SSRs). In 1994, the first genetic map of pearl millet was generated by Liu et al. (1994). It contained 181 RFLP markers covering the seven pearl millet chromosomes and spanning a genetic distance of 303 cM. Subsequently, a subset of these markers was transferred to a series of different crosses that segregate for agronomically important traits. Quantitative trait loci (QTL) for downy mildew resistance (Jones et al. 1995, 2002), drought tolerance (Yadav et al. 2002, 2003, 2004) and characteristics involved in domestication (Poncet et al. 2000, 2002) have been mapped. The integration of markers, previously mapped in other grass species, enables the alignment of the pearl millet linkage groups to other cereal genetic maps, including the model species rice (Qi et al. 2004). The pearl millet genome appears to be highly rearranged compared to rice. Nevertheless, regions of colinearity between the two species can be clearly identified (Devos et al. 2000). These regions present a framework for utilising rice genomic sequences as a source of new markers and candidate genes underlying traits in pearl millet. The maps and markers provide a base for future genomic and comparative analysis of pearl millet and for the application of marker-assisted selection in breeding programmes (Qi et al. 2004).

4 Conclusions

There is an urgent need to increase nutritional supplies to help attain food security, especially in developing countries. Due to their high adaptation to environmental conditions, the improvement of local crops presents a capable strategy for small farm holders and poor consumers. Pearl millet is the only major staple cereal that survives under the hot and dry climate in Asia and Africa (Sharma et al. 2002). Nevertheless, its gene pool can be broadened towards economically important traits such as resistance to fungal pathogens, higher yields and nutritional properties. Besides classic breeding, gene- and biotechnology offer promising strategies for the selective genetic enhancement of crops. Until recently, plant breeding relied solely on the sexual transfer of genes between plant species. Today, advances in plant molecular biology and genomics now give access to the knowledge and understanding of plant genomes and genetic engineering (Job 2002). Research on in vitro technologies, genetic transformation and molecular techniques have to be intensified for pearl millet. In order to achieve this purpose, more scientific and political investigations are required. Supporting technology transfer and the development of the legal requirements to handle GMOs will help to establish the needed prerequisite for an autonomous research base to match the demands in those countries where pearl millet is cultivated as a major crop.

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II.6 Barley

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1 Introduction

Barley (*Hordeum vulgare* L.), one of the earliest crops to be domesticated, is genetically suited to improvement through the use of biotechnology since it is a self-pollinated diploid ($2n = 2x = 14$) that does not cross-hybridize with species outside of its primary gene pool. Barley has been cultivated since 8000 BC and possibly as far back as 17,000 BC. Germplasm collections are numerous, as barley has been adapted to a wide variety of environments around the world.

The book "*Diversity in Barley*" edited by von Bothmer et al. (2003) is an excellent source on the history of the crop. It covers the distribution of barley from its origins in the Fertile Crescent of Asia to various parts of the world. This book also details the wide diversity of mutants in barley (Lundqvist and Frankowiak 2003), as this species was a major crop in the nineteenth century for both mutation and genetic research. "*Diversity in Barley*" also contains details of various other types of diversity, such as good cytogenetic and molecular tools, biotic and abiotic stress resistance or tolerance, as well as details of the "Barley Core Collection" germplasm.

Cultivated barley is considered to have evolved from two ancestral subspecies, *Hordeum vulgare* ssp. *vulgare* and ssp. *spontaneum*, that today also serve as the primary gene pool for germplasm diversity. Of the other 30 *Hordeum* species (von Bothmer et al. 2003), the only one with which barley might hybridize in nature is *H. bulbosum*; and it serves as the secondary genepool. *H. bulbosum* exists in both diploid and autotetraploid forms in nature and is out-crossing, enhanced by a self-incompatability system. With the aid of embryo culture, this interspecific hybrid of *H. bulbosum* and barley was shown to produce haploids of barley through preferential *H. bulbosum* chromosome elimination after hybridization (Kasha and Kao 1970; Subrahmanyam and Kasha 1973). The genomes of the other *Hordeum* species are different from those of *H. vulgare and H. bulbosum*, although some polyploids may contain the I genome of barley.

The karyotype and chromosome numbering for barley has changed over the years and this knowledge is important for biotechnological research, par-

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ticularly for molecular mapping and transformation. The original karyotype numbering followed the traditional system so that chromosome 1 was the longest, chromosome 2 the second longest and this pattern continued except for the satellited chromosomes which were placed last (6 and 7 in barley). However, using the C-banding technology on trisomic lines for each chromosome, Noda and Kasha (1978) demonstrated that the linkage groups assigned to each chromosome, as originally numbered, were not correct for the three longest chromosomes. Thus, the karyotype was rearranged to fit the linkage groups; and chromosome 3 with the most median centromere became 1, the longest chromosome (1) became chromosome 2 and the original chromosome 2 became chromosome 3. Linde-Laursen et al. (1997) proposed that the barley genome chromosomes be numbered with the symbol H to be consistent with the homoeologous chromosomes within the Triticeae genomes. Thus, in the literature published most recently, the symbol H is used with the number and chromosomes 1, 5 and 7 are changed in order, becoming 7H, 1H and 5H, respectively. The numbers of the other four chromosomes remain unchanged and are referred to as 2H, 3H, 4H and 6H. The Seventh International Barley Genetics Symposium adopted this system. Often the relationship between the two systems is pointed out when gene linkage or molecular markers are assigned to chromosomes.

Barley has one large genome with about 5.6×10^9 bp of DNA. About 80% of the genome consists of highly repetitive DNA which is mostly retrotransposons (Schulman et al. 2004).

In this review, papers from the Proceedings of the Ninth International Barley Genetics Symposium held in Brno, Czech Republic, in 2004 and from other recent reviews are cited for information in order to reduce the large numbers of references that could be cited for this broad topic. This, unfortunately, means that many of the original papers on topics are not cited, but may be traced through the citations used. The paper on transformation in barley by Lemaux et al. (1999) is an extensive and excellent review of the early literature on this topic, particularly when only transient gene expression occurred. This area will not be discussed here. The papers of Varshney et al. (2005a, b; 2006) should also be consulted for a review of molecular markers and maps of barley, although it will be briefly reviewed here.

Within the topic of biotechnology are included both tissue culture and molecular marker developments, as they are basic to the bioengineering of barley. Tissue culture procedures are essential, as totipotent cells are the targets for gene delivery and for the regeneration of transgenic plants. Low regeneration from tissue culture of most genotypes has been a problem for barley transformation using *Agrobacterium tumefaciens*. Molecular markers have revolutionized gene identification, tagging and isolation and are used for mapping and as tags for marking and selecting genes of importance in the improvement of barley.

2 Economic Importance of Barley

Barley ranks fourth in the world in cereal production, behind wheat, rice and maize. In 2004/2005, it was grown on approximately 56×10^6 ha (USDA-FAS 2005). About 85% of the barley grown around the world is used for animal feed and most of the remainder is used as malt for brewing and distilling, or as foods. While barley has decreased in world acreage in recent years, it appears to have levelled out in 2004/2005. The advent of molecular tools for biotechnology may stop this decline, as barley becomes improved and more acceptable as livestock feed and human health food. Genes that can improve barley for poultry feed and for human consumption have been added and are being tested, as are genes for disease resistance (von Wettstein 2004). Recently, barley was designated as a "health food" in North America similar to oats for human consumption. This should lead to further markets and increased production of barley.

3 Current Research and Development

This topic is discussed in three sections in the order of tissue culture, molecular markers and genetic transformation. As the transformation techniques rely to a large extent on the first two topics, they are summarized in that order.

3.1 Cell and Tissue Culture

Barley transformation has been lagging behind some of the other cereals because of difficulties in the regeneration of plants of most genotypes using cell and tissue culture. However, recent studies would indicate that this situation may be improving (for reviews, see Cheng et al. 2004; Murray et al. 2004; Tiidema and Truve 2004). The tissues or cells used for barley transformation are embryos (immature or mature), shoot meristems, microspores and protoplasts. Initially, immature embryos were the most suitable targets.

To date, the systems for the culture of anthers and isolated microspores for haploid production in barley are more advanced than in other cereals. These systems are also effective in breeding new improved cultivars of barley (Thomas et al. 2003; Devaux and Pickering 2005). Transformation of single-cell microspores has been successful in barley only through particle bombardment (Jähne et al. 1994; Yao et al. 1997). *Agrobacterium* does not appear to be able to penetrate the microspore cell wall and has not been compatible with uninucleate microspore survival. However, recent reports indicate that, once the microspore wall is broken, *Agrobacterium* can effectively produce transgenics from these multicellular structures, which may be haploid or doubled haploids (DHs; Kumlehn et al. 2006). Regeneration from callus, or through secondary embryogenesis produced from embryos, has also been difficult, with only a few

genotypes responding (Lemaux et al. 1999). However, as mentioned earlier, recent studies suggest that this genotype problem may be overcome through the use of mature embryo or shoot meristem explants. The culture of protoplasts has also been extensively studied for purposes of gene insertion or cytoplasmic organelle transfer between species or lines (Shillito 1999). With isolated protoplasts, electroporation or polyethylene glycol (PEG) systems are suitable for transformation. However, the predominant methods of transformation in barley today are the bombardment of microspores and embryos or co-culture of embryos or 7- to 8-day-old cultured microspores with *Agrobacterium*.

Embryo culture has been a tool used for many years in cereals following wide hybridization where small embryos have been rescued by culturing them in nutrient media. The use of this approach in barley, hybridization with *H. bulbosum*, led to a system of haploid production in barley (Kasha and Kao 1970) that worked across genotypes with sufficient frequencies to make it efficient for barley breeding programs. Thus far, about 60 cultivars of barley have been produced around the world by this wide hybridization method (Thomas et al. 2003; Devaux and Pickering 2005). More recently, most haploid-derived barley cultivars have been produced by anther or isolated microspore culture (IMC). In total, more than 100 barley cultivars have been produced through time-saving haploid systems, more than in any other crop species. In addition, the DH lines have been used extensively in mutation and cell culture selection and are basic for collaborative molecular marker mapping and map construction.

Anther and IMC culture protocols have also been developed that work well for haploid production in barley, although there are slightly more problems with genotype response limitations, albinism and linkage disequilibrium. However, genotype differences and albinism are not much of a problem today because of the large numbers of haploids produced through IMC. It is recommended that donor plants of the anthers or microspores be grown under a controlled environment, such as in growth rooms or glasshouses in order to obtain consistent results. Any stress to the donor plants through nutrition problems, pests or temperatures can reduce the response in culture (Devaux and Pickering 2005). Various pretreatments of the anthers or microspores at the uninucleate stage are required to induce a high frequency of microspores to enter the embryogenic pathway. In barley, this is usually a mannitol and/or cold pretreatment, although other stresses can also be successful. Various protocols for the induction, culture and regeneration of plants from anthers or IMC are presented in the book edited by Maluszynski et al. (2003). One of the advantages of IMC in barley is that about 80% of the progeny become DHs from processes that occur during the first (PMI) or second (PMII) mitotic division in the microspores. Kasha et al. (2001) demonstrated that failure of cell wall formation occurs due to the induction pretreatment and can lead to nuclear fusion, resulting in a plant that is completely DH and fertile. Nuclear fusion as the main pathway to doubling the chromosomes has been verified cytologically (Gonzalez-Melendi et al. 2005; Shim et al. 2006). The use of anti-microtubule agents during the initial stage of anther or microspore culture can also produce

high frequencies of chromosome doubling (see Kasha 2005). Giménez-Albián et al. (2004) described how the microtubule cycle during mitosis in binucleate mitotic cells could be responsible for nuclear ploidy; and this may be one way that anti-microtubule agents increase ploidy as well as induce embryogenesis.

Regeneration from barley cell cultures for purposes of obtaining transgenic plants has been a problem and has limited the genotypes that can be used in transformation procedures. The main problems are maintaining the regeneration potential of the culture over a three to four month period and preventing the increase of albino plant formation during this same culture period. Efforts to overcome these problems have centered on the evaluation of culture media components (Bregitzer et al. 1998) and the source of somatic cells. Immature embryos, and their scutellum in particular, have been the main target for transformation and regeneration. Most barley cultivars now can be regenerated from these tissues (Rikiishi et al. 2003). Ganeshan et al. (2003) used four commercial barley cultivars and compared plant regeneration from immature embryos, mature embryos and leaf base apical meristems. It was observed that, using the plant growth regulator thidiazuron, direct shoot regeneration from mature embryos and leaf base apical meristems was feasible, with the best being the mature embryos. In the search for systems to obtain better shoot regeneration and overcome genotype effects, Zhang et al. (1999) were successful in barley transformation using in vitro shoot meristematic cultures from germinated seedlings. The use of shoot apical meristem cultures for cereal transformation has been reviewed by Sticklen and Oraby (2005). Both mature embryos from seeds and shoot apical meristems should widen the range of genotypes that can be used for barley transformation. Direct embryogenesis can avoid somaclonal variation that can be induced in callus cultures (Karp and Lazzeri 1992). Using the correct balance of nutrients and hormones in the media (Ziauddin and Kasha 1990; Ziauddin et al. 1992) can also reduce such variation.

A number of improvements in procedures have also led to a wider range of genotypes that can be used in transformation (Cho et al. 1998; Nuutila et al. 2000; Roussy et al. 2001; Manoharan and Dahleen 2002; Chernobrovkina et al. 2004; Murray et al. 2004; Sharma et al. 2004; Tiidema and Truve 2004). However, the first cultivar Golden Promise to respond to *Agrobacterium*-mediated transformation methods still tends to provide higher transgenic frequencies than other cultivars.

3.2 Development of Molecular Markers

Molecular markers are now used routinely in many barley breeding programs (Varshney et al. 2005b). They are used to track important agronomic loci through the selection of parents for crossing and through segregating progeny from crosses. They are also helpful in locating genes on the chromosome regions, thus reducing the need to isolate genes or to use transformation.

Molecular markers have also been used to develop maps for the chromosomes and to identify quantitative trait loci (QTLs) for many agronomic traits, pest or stress resistance and quality traits. The web site http://barleyworld.org will lead readers to most of the molecular maps and mapping projects around the world. Within that site, GrainGenes will provide the molecular mapping of various cereal species including barley. Lörz and Wenzel (2005) have edited review papers on all aspects of molecular markers in many crops. The introductory chapter by Langridge and Chalmers (2005) in that book provides an overview of the identification and application of molecular markers.

The types of molecular markers developed have evolved rapidly over the past 20 years. They can be divided into two classes: those molecular markers closely linked to important genes and those that result from expressed gene sequences. Over time, the molecular markers have been refined to enable more rapid handling and have become smaller in size. The first molecular markers mapped in barley were restriction fragment length polymorphism (RFLPs; Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993). Next to be developed in barley were the randomly amplified polymorphic DNA (RAPDs); and these were quickly followed by simple sequence repeats (SSRs) or microsatellites. La Rota et al. (2005) observed the non-random distribution and genome frequencies of microsatellite markers derived from expressed sequence tags (ESTs) in rice, wheat and barley. Varshney et al. (2005a) showed the interspecific transferability and comparative mapping of barley EST-SSR markers with those in wheat, rice and rye. Varshney et al. (2006) assigned EST-derived SSR markers to BACs and among the most abundant recent markers are single-nucleotide polymorphisms (SNPs) that are developed from expressed barley sequences. Bundock et al. (2006) demonstrated an allele-specific PCR procedure with three primers to produce robust SNP markers with a system that does not require sophisticated equipment. However, ESTs that usually are the partial cDNA sequences produced by reverse transcriptase PCR of mRNA have become the most valuable tools for gene identification, isolation and mapping.

The original linkage maps in barley were based upon genes for morphological traits and biochemical markers such as isozymes. It had taken many years of work to develop these maps. However, with the advent of molecular markers, new molecular marker maps quickly surpassed the old gene maps in both density and coverage of the chromosomes. The integration of the many molecular maps that developed with various types of molecular markers has been difficult but achievable (Varshney et al. 2005a). The first RFLP molecular marker maps were made using populations of DHs produced from the F_1 of crosses between good cultivars. Since each DH produced is genetically homozygous for a different combination of alleles of genes, the DHs could be maintained eternally and the DNA extracted from them could be sent to many collaborators working together on developing molecular maps. These same DH lines could be grown in replicated field trials at various locations to identify and locate QTLs for various agronomic traits such as yield, pest and

stress resistance, as well as for quality traits. One of many such collaborative groups was the North American Barley Genome Mapping Project (NABGMP), consisting of 49 researchers from the United States and Canada, with Kleinhofs et al. (1993) providing the first of many map publications.

For use in breeding, the mapping of QTLs also meant that they could be tagged with molecular markers located close to each QTL. The molecular markers could be assigned to a physical location on the chromosomes by in situ hybridization (ISH) and, subsequently, by their relationship to chromosomal interchange breakpoints (Sorokin et al. 1994; Kunzel et al. 2000). Such studies have confirmed the uneven distribution of recombination frequency and more precisely located the molecular markers or genes along the chromosomes, with the majority being located in the distal regions of the barley chromosomes. Varshney et al. (2005b) concluded that 4.9% of the physical map developed from the Igri × Franka cross contained high recombination rates and 47.3% of 429 markers assigned to the map.

The eventual goal of mapping is to identify the genes and to sequence them. For this purpose, large insert DNA libraries have been constructed, both as bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) contigs (Varshney et al. 2005b). A number of additional BAC libraries are under construction or completed (Kleinhofs 2004). In addition, a collaborative approach has produced a large number of ESTs that have been placed in a common database (http://www.ucbi.nih.gov/UniGene/). It lists 344,352 ESTs, as of March 2005. These ESTs are thought to include about 40,000 distinct genes or about 85% of the gene compliment of barley (Varshney et al. 2005b). The aim is to develop a high-density transcript map using about 1000 ESTs that would complement the existing molecular marker consensus map. The latter consists of 510 RFLP, 234 SNP and 185 SSR markers, with at least 100 markers in total on each of the seven chromosomes. Another valuable crop EST data base (CR-EST) has been established online with public access by Kunne et al. (2005) and it contains over 200,000 ESTs. It provides online access to sequence, classification, clustering and annotation data derived from cDNA libraries of four species: barley, wheat, pea and potato. About one-third of these ESTs are derived from barley. Sequences are clustered in species-specific projects and have generated a non-redundant set of about 22,600 consensus sequences and about 17,200 singleton sequences.

Comparative mapping of marker and gene maps is extremely useful in determining synteny between different cereal and grass species and thus, in the study of their evolution. Moore (1995) proposed that the grass genomes are made up of conserved segments and all are derived from a common ancestor. The early molecular markers such as RFLPs permitted the rough assignment of homoeologous chromosome regions between species with various different basic numbers (x) of chromosomes (Moore 1995).

The number of mapped genes in barley exceeds 1100 and these markers are preferred over random DNA markers such as RFLP and SSR, as genes are more conserved in evolution and useful in crop improvement. The ESTs are also being

developed in other related species and this helps in identifying more genes in all species (Waugh et al. 2004). In rice, for example, there are 6591 ESTs placed on a physical contig map (Wu et al. 2002). When comparing mapped barley ESTs against this rice database, sequence identity was more than 80% (Varshney et al. 2005b) and there were more than 30 syntenic genes on each barley chromosome. Thus, ESTs represent a means for rapidly identifying genes in all cereal and grass species. La Rota et al. (2005) found a non-random distribution of EST-derived microsatellite markers in cereals. This might be expected since the genes appear to be in small clusters and located more distally than centrally on the chromosomes. More recently, Varshney et al. (2006) attempted to map EST-derived SSR markers on a BAC library in barley. They found a non-uniform distribution of genes in the barley genome, concluding that the barley genome contains gene-rich and gene-poor regions.

An important collaborative project (Close et al. 2004) has been the development of a micro-array chip (Barley 1 GeneChip) fabricated by Affeymetrix, which contains about 22 000 unique 25-mer 3′ end ESTs screened from over 400 000 ESTs in barley. This chip will greatly facilitate the identification and location of various genes by their expression on the matrix. It, as well as the database of Kunne et al. (2005) will be extremely useful for comparing identified ESTs from other cereal species and we can expect a very rapid advance in numbers of known genes in barley.

A number of other tools are being used to assist in assigning genes and gene function in barley. Koprek et al. (2000) introduced Ds transposable elements into barley to help detect genes and gene function. Doležel et al. (2004) used flow cytometry chromosome sorting to develop libraries for individual chromosomes or parts of chromosomes. In barley, only chromosome 1H could be distinguished by sorting, but through the sorting of unequal chromosomal interchange stocks, it was possible to sort segments of the chromosomes ranging from 5% to 9% of the total genome. Because of the numerous markers mapped and those yet to be mapped in barley, Kleinhofs and Graner (2001) proposed that the chromosome regions be divided into 10 cM (centi-Morgan) "bins", with a distinct marker at each end of a "bin". Newbigins et al. (2004) discussed functional genomics that requires the technology to identify large sets of genes that influence a particular biological process. Such research includes genomics, proteomics and metabolomics and would require high-throughput data collection and analysis of gene function and structure. Barley DNA micro-arrays, high-density genetic maps and barley EST and mutant libraries provide critical support for this research. In the future, the development of procedures to measure gene activity by monitoring and assessing the amount of mRNA produced from such genes in a related group will provide insight into the expression of quantitative traits of agronomic importance and biochemical processes (Waugh et al. 2004). Recent reports on using proteomics to analyze traits in barley include those of Corrado et al. (2005), working on the photosynthetic apparatus, Maeda et al. (2005) and Wong et al. (2002), working on thioredoxin h relative to seed proteins, and

Finnie et al. (2004) working on stress and fungal resistance as well as malting quality.

3.3 Transformation in Barley

Genetic transformation of barley, like many other grass or cereal species, has been hindered by the lack of a system that is easy to handle and that will produce sufficient numbers of transgenic plants. Many systems for transformation have been tested in barley (Mannonen et al. 1994; Lemaux et al. 1999; Murray et al. 2004). However, only three systems have produced transgenic plants. The first reports with these systems are the electroporation of protoplasts (Mannonen et al. 1994), particle bombardment of immature embryos (Wan and Lemaux 1994; Ritala et al. 1994), microspores (Jähne et al. 1994) or shoot meristems (Zhang et al. 1999) and the inoculation of immature embryos with *Agrobacterium* (Tingay et al. 1997).

There are many reasons why barley transformation has lagged behind other cereals. The slow development of efficient tissue culture systems for plant regeneration after three to four months in culture is one; and this is associated with increased albino plant production with length of time in culture. High frequencies of somaclonal variation were indicative that improved embryogenic response from cultures was required. The first report with *A. tumefaciens* (Tingay et al. 1997) used the cv. Golden Promise and this cultivar remains the best for regeneration of transgenic plants. Recently, Wang et al. (2001) and Murray et al. (2004) were able to obtain very low frequencies of transgenic plants with three Australian cultivars using the *Agrobacterium* system. Tiidema and Truve (2004) reported improved regeneration with some Nordic cultivars, while Roussy et al. (2001) evaluated the transformation and regeneration capacities of five Nordic barley elite cultivars. The limitations of using electroporation of protoplasts are similar to those of using *Agrobacterium*, namely the difficulty in regeneration of plants from protoplasts and the frequencies of the production of albino plants.

The majority of barley transformation successes, to date, have been from particle bombardment of immature embryos, or the scutellum from such embryos, because success with *Agrobacterium* has been limited by strong genotype dependency. Southgate et al. (1995) and Klein and Jones (1999) reviewed the factors affecting transformation of plants by microprojectiles. Microprojectile bombardment has a few desirable attributes, such as being highly versatile and adaptable to a wide range of tissues and cells. It is simple to utilize and is widely used for the study of gene expression and to test the efficiency of different gene promoters.

Although the *Agrobacterium* system has had limited success, it has some advantages. One is that the majority of transgenic plants have a single intact gene copy inserted at one site. In contrast, particle bombardment tends to introduce more than one copy at a site and some of the copies may not be com-

plete and transgene instability is higher (Harwood et al. 2004; Travella et al. 2005). Svitashev and Somers (2001) showed through fiber-FISH (fluorescence in situ hybridization) studies that the multiple inserts are found at one site and are interspersed with host genomic fragments of various sizes following bombardment. However, methods to select plants with a single transgene copy and greater stability following bombardment are available and such lines are currently under field evaluation (von Wettstein 2004). Fang et al. (2002) studied the sequences of the T-DNA inserted by *Agrobacterium* and found the right T-DNA ends were highly conserved, while the left ends were more variable. They showed the lack of transmission of the vector sequences, suggesting a mode of molecular T-DNA transfer similar to that in dicotyledonous plants. Another advantage of *Agrobacterium* transformation is that, with double-cassette vectors, it is possible to remove the selectable marker cassette by selection in segregating generations following transformation (Matthews et al. 2001; Stahl et al. 2002).

Koprek et al. (2001) demonstrated a method to produce single transgene copies using transposons delivered via particle bombardment. Taking the Ac/Ds system from maize, the gene of interest was placed between inverted-repeat Ds ends and transformed into the host cells. Regenerated plants were then crossed with a plant containing the Ac element that activated the movement of Ds. Thus, many plants that had a single copy of the gene of interest were selected among the F_2 progeny. Such plants were shown to be highly stable in the expression of the inserted gene, allowing one to obtain stable transgenic plants following transformation by particle bombardment. Cooper et al. (2004) were able to map the Ds insertions in barley using a sequence-based approach. von Wettstein (2004) and colleagues successfully used this system in transforming barley with genes valuable for malting, non-ruminant feed and disease resistance.

The bombardment of isolated immature microspores offers the potential to obtain DH plants homozygous for the transgene. Jähne et al. (1994) obtained homozygous transgenic DH plants when using a long 28-day cold (4 °C) pretreatment of spikes with immature microspores before bombardment, whereas Yao et al. (1997) obtained only hemizygous transgenic plants when they pretreated by incubating in 0.3 M mannitol at room temperature for four days. Lemaux et al. (1999) suggested that the difference between the results of Jähne et al. (1994) and Yao et al. (1997) was that the microspores treated only in mannitol at room temperature would be at an older stage when bombarded and, therefore, would be hemizygous for the transgene. Shim and Kasha (2003) demonstrated that, during the pretreatment in mannitol for only four days at room temperature, most of the microspores would have completed PMI (first post-meiotic mitosis) without cell wall formation. This permitted nuclear fusion (Kasha et al. 2001) and a few nuclei had entered PMII before bombardment. However, when using a cold plus mannitol pretreatment, the microspores were held at the uninucleate microspore stage, whereas after a 21-day cold pretreatment most microspores had completed the S phase of the cell cycle and many

had gone through PMI. Thus, it was not clear why Jähne et al. (1994) had obtained homozygous transgenic DH plants. However, González-Melendi et al. (2005) cytologically showed that chromosome doubling in barley microspores can occur after the subsequent mitotic divisions by nuclear fusion of multinucleate microspores. Therefore, the transgene could be incorporated during the S phase in these subsequent nuclear divisions prior to nuclear fusion. Other advantages of barley transformation using the culture of isolated microspores are that success can be obtained across genotypes and a high proportion of regenerates are derived by direct embryogenesis, reducing the potential for somaclonal variation. While the frequency of transgene-expressing microspores is very low, large numbers of microspores are available from which can be obtained adequate numbers of transgenic plants.

The treatment of uninucleate barley microspores with *A. tumefaciens* has not been successful to date because the microspores do not survived the 2-day *Agrobacterium* treatment (Kasha and Gu, unpublished data). When the barley microspores become multicellular after 6–8 days in culture, they can survive *Agrobacterium* treatment, but the goals of obtaining completely fertile DHs that are homozygous for the transgene and free from chimeric sectors are lost. However, Kumlehn et al. (2006) showed that, once the microspore wall has been disrupted by the multicellular structures within, the *Agrobacterium* can induce transformation and large numbers of transgenic plants can be selected and regenerated. The transgenic haploid barley plants can then be treated with anti-microtubule agents to induce chromosome doubling and to produce plants homozygous for the transgene. Such plants will only be partially doubled but will produce sufficient seed to obtain the homozygous transgenic line.

Trifonova et al. (2001) studied a number of factors involved in *Agrobacterium*-mediated transformation using immature embryos of the barley cv. Golden Promise. Using 12 different procedures, they obtained transgenic plants with five of these procedures; and the frequency of success ranged from 1.7% to 6.3%. Southern analysis proved transgene integration with a copy number from one to six, but most had a single integration with the expected Mendelian inheritance patterns.

Travella et al. (2005) compared the production of transgenic barley lines by particle bombardment with *Agrobacterium*-mediated techniques on immature embryos. They compared transformation efficiency, transgene copy number, expression, inheritance and location of the transgenes. The efficiency of *Agrobacterium* transformation was 2%, whereas that following bombardment was 1%. The *Agrobacterium*-mediated transgenic plants had from one to three copies of the gene, while 60% of the plants produced following bombardment had more than eight copies. Relative to inheritance pattern, all six lines tested from the *Agrobacterium* system showed the expected inheritance patterns, while only three of nine lines from bombardment showed the expected inheritance pattern. A high frequency of gene silencing was observed after DNA delivery by bombardment, which is consistent with the earlier study of Bregitzer and Tonks (2003). This study strongly suggests that *Agrobacterium*

is more effective for transformation using immature embryos as targets in barley. However, the limitation of only a few responding genotypes needs to be resolved and this might be achieved by using microspore culture and transformation, as demonstrated by Kumlehn et al. (2006).

Through the use of in situ hybridization, a number of studies examined the sites on the barley chromosomes where the transgenes were inserted (Pedersen et al. 1997; Salvo-Garrido et al. 2001, 2004; Choi et al. 2002). Some inserts appeared to be at random sites amongst the seven chromosomes, while other studies showed a more limited distribution. Large numbers were not examined in any study, but the general conclusion is that inserts tend to be towards the ends of the chromosomes and, more often than expected, in regions of active genes. For example, about 75–80% of the barley genome is made up of highly repeated retrotransposons; and von Wettstein (2004) mentioned that, when chromosome sites were determined, only 12 of 46 transgenic sites were located in the retrotransposons. There is a trend in the findings of all studies showing that the locations of transgenes are distributed more in the telomere or subtelomere regions of the chromosomes (Choi et al. 2002; Salvo-Garrido et al. 2004). This is consistent with the reports of von Wettstein (2004) and Varshney et al. (2005a), showing that transgenes and molecular markers, respectively, are located more often in small gene clusters.

In attempts to improve the efficiency of transformation in barley, many factors have been examined. Cheng et al. (2004) reviewed the factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. These factors include plant genotype, explant type, *Agrobacterium* strain, plasmid construction and the use of binary vectors. For the *Agrobacterium* infection stage, the recovery of plants is influenced by osmotic pretreatment, antioxidants and bactericides, desiccation of the target explants and the inoculation and co-culture media composition. For example, Y.S. Shim (unpublished data) found improved transgenic plant recovery when adding arabinogalactan protein (AGP) to the culture media following bombardment. AGP has been shown to improve the viability of microspores in culture and improve the frequencies of recovered plants from isolated microspore culture of wheat (Letarte et al. 2006).

Many types of selectable markers have been used and various promoters attached to them have been studied. Antibiotics and herbicide resistance have been used extensively as selectable markers in cell cultures, while green fluorescent protein (GFP; Sheen et al. 1995; Pang et al. 1996) and the firefly luciferase gene (Harwood et al. 2002) are the main markers used to follow the stability of transgene expression. Because of the undesirability of having selectable marker genes and other foreign DNA in food products, transformation systems have been developed for producing marker-free transgenic barley (Xue et al. 2003; von Wettstein 2004). Xue et al. (2003) used binary vectors with the marker gene on a separate vector from the transgene of interest, so that the marker could be removed in subsequent segregating generations. Hensel and Kumlehn (2004)

reported a protocol for *Agrobacterium* transformation on immature barley embryos.

The stability of transgene expression in subsequent generations is a concern and must be examined for a number of generations following the selection of the transgenic plant. The copy number of insert is a major factor, as is the construction of the plasmid/gene constructs used, relative to stability. *Agrobacterium* is favored over other methods used for introducing genes as it tends to have fewer copy numbers inserted into the plant host. This would reduce the number of transgenic plants required in order to obtain stable transgenic plants. The promoters used for the gene to be inserted have also been found to influence the stability of the transgene in the host plant; and tissue-specific promoters are also valuable in some instances (Furtado and Henry 2005). Cho et al. (2002) and Choi et al. (2003) observed that the barley endosperm-specific hordein promoters driving *uidA* or *sgfp* marker genes were much more stable through the T4 and later generations than the maize ubiquitin promoter. Schunmann et al. (2004) studied the promoters from the phosphate transporter genes (*Pht1*) in barley and found a 20-fold increase in marker gene expression when an intron was utilized, supporting the concept that introns incorporated into gene constructs are important for expression. They concluded that the *Pht1* promoters were ideally suited for driving the expression of foreign genes associated with nutrient uptake. Petersen et al. (2002) demonstrated that including matrix attachment regions (MARs) in the plasmid/gene construct both enhanced transformation frequencies and improved transgene expression in barley.

4 Practical Applications of Biotechnology in Barley

The applications of transformation in barley have, to this point in time, concentrated on inserting disease, stress and pest resistance genes. There is also interest in using transformation to improve the quality and malting characteristics of barley and, in the future, in developing new foods from barley with improved or new quality traits. Because barley is grown around the world for feeds and food, there has been little or no interest in using barley as a host species for the production of pharmaceuticals.

While regeneration from somatic cell and tissue culture has been problematic in barley, the production of haploids from wide crosses or microspore culture has been exemplary (Thomas et al. 2003; Devaux and Pickering 2005), with well over 100 DH-derived cultivars being released world-wide. The extensive development of molecular marker maps in barley (Varshney et al. 2005a) has been accompanied by the tagging of genes and QTLs. That has then led to the quite extensive use of molecular marker-assisted selection (MMAS, or more simply MMS). Ullrich et al. (2004) reviewed some of the North American

research on MMAS and listed web sites for different areas of the world where the studies are posted. Results with MMAS are encouraging and many breeders now have the facilities to exploit this approach. The markers are shifting from random DNA molecular markers to genes, facilitated by ESTs that also simplify locating and sequencing genes (Sato et al. 2004). The ESTs are also used to select markers like SNPs and SSRs (Kota et al. 2003) that can then be used to mark the genes themselves. The Barley 1 GeneChip disc will also greatly facilitate gene identification and sequencing (Close et al. 2004), as will the CR-EST resource (Kunne et al. 2005).

Transformation of barley is expected to progress more rapidly now, with the identification in many cereals of genes through the available ESTs. At present, a number of genes for disease resistance have been isolated and transformed into susceptible barley cultivars (von Wettstein 2004). These include the *Rpg1* stem rust resistance (Horvath et al. 2003; Rostoks et al. 2004), the endochitenase gene (*ThEn42*) from the fungus *Trichoderma harziamun* that can confer resistance to one or more *Rhizoctonia* root rot organisms (von Wettstein 2004), the *mlo* gene for powdery mildew resistance (Bieri et al. 2004) and virus resistance (Wang et al. 2001; Stein et al. 2005). Genes for stress tolerance and malting quality are also of interest, the latter more specifically for food processing. To convert barley from a low nutritional value to a high nutritional value for poultry, a gene (1,3-1,4)-β-glucanase from *Bacillus* was transformed into barley (von Wettstein 2004) which improved the nutritive value to that of maize. In tests with chickens, the addition of 0.02% transgenic grain to barley feed achieved as high a nutritive value as adding commercial enzymes to barley feed for non-ruminant animals. Xue et al. (2003) increased cellulose production in barley by inserting a hybrid cellulose gene. Matthews et al. (2002) studied both α-amylase production and the transport of gibberellin in malting grain by inserting a marker tagged α-amylase gene into barley. Kim et al. (2003) found that thioredoxin h over-expression enhanced selenite resistance and uptake during germination, while Maeda et al. (2005) found it was involved in the regulation of protein in barley seeds. Wong et al. (2002) observed that thioredoxin h influenced communication between the embryo and aleurone. Antisense constructs of limit dextrinase inhibitor (LDI) protein in barley modulates quality factors such as starch granule size, amylopectin structure and starch composition (Stahl et al. 2004). The evaluation of transgene stability under field conditions and in breeding programs is also necessary (Horvath et al. 2001). However, an expressed inserted gene may not always improve the trait desired, as Rae et al. (2004) observed with a high-affinity phosphate transporter gene in barley.

Delhaize et al. (2004) engineered high aluminum tolerance in barley by inserting a gene (ALMT1) from wheat that is associated with malate efflux and aluminum tolerance. The barley *Lem 1* gene promoter drives expression in outer floret organs in wheat and may be useful in engineering organ-specific *Fusarium* resistance (Somleva and Blechl 2005).

5 Conclusions and Future Challenges

The production of haploids in barley through wide hybridization and microspore culture for the development of new improved cultivars has been very successful, with more cultivars produced than in any other crops. Haploid-produced populations have also been essential for much of the molecular marker mapping in barley, as the DHs are eternal populations of homozygous genetic lines. Such lines are also valuable for mutation and selection research (Szarejko 2003). Further exploration of the potential to obtain fertile DHs that are homozygous for transgenes is warranted, although Kumlehn et al. (2006) provided evidence for a method using 7- to 8-day-old microspore cultures.

The development of molecular marker maps and, more recently, gene maps through the use of ESTs, has accelerated the identification of genes in barley. This will be further enhanced by similar work in related species because of the synteny of genes and chromosomes. Gene isolation in barley through DNA sequencing aided by BAC and YAC libraries and the use of transposable elements will also continue to be valuable. In the near future, we can expect the identification of genes involved in many traits. For example, through the combination of QTL and EST studies, we can expect many of the genes involved in grain quality to be identified and examined for their roles. The ability to identify the genes involved in a process (functional genomics) is feasible and this will open up a much more precise monitoring of plant physiology and gene expression in the development of traits (Newbigins et al. 2004). This will enable biotechnology to change genes for the improvement of feed and food quality. The study of proteomics (proteins produced by known genes and their interactions) will become a major area of research.

For transformation, research on identifying the genes and their locations is important for the incorporation of transgenes by homologous recombination, leading to more stable transgenics (Monostori et al. (2003). Much more research on the methods of producing transgenics in barley and all cereals is required. Gene constructs with tissue-specific promoters and their delivery into and selection among totipotent cells are also in need of improvement.

Barley has the potential to be classed as a health food like oats and this will open new markets for barley producers.

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II.7 Oat

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1 Introduction

Cultivated oat (*Avena sativa* L.) is an important agronomic cereal crop both locally and globally. The oat crop is primarily produced for animal feed and human food, but recent research has elevated its potential dietary value for human consumption. There are several coordinated public breeding programs focusing on the genetic enhancement of oat. Despite these efforts, a yield and quality losses due to stress, pathogens, and insects continue to occur. The continued development of biotechnological approaches to genetically study and manipulate oat would aid significantly in oat enhancement efforts.

Oat biotechnology has been advancing at a similar pace as that for the rest of the cereals. There are several published protocols for initiating embryogenic callus from various explants and for recovering transgenic oat plants with different selectable markers. Many molecular markers are available to detect polymorphisms in oat. Despite the challenge of creating a map of a large hexaploid genome, there are a number of recombinant inbred (RI) populations for which recombinant maps have been created. Many complex quantitative traits have been investigated to create quantitative trait loci (QTL) predictions using these populations. Knowledge from defined biochemical pathways has also allowed matching map positions of candidate genes to defined QTLs. The expansion of both the availability and diversity of cereal gene sequence and bioinformatic tools is increasing the potential for oat biotechnology to further define the oat genome and aid in crop improvement.

2 Economic Importance

The global oat crop has retained its place as an important cereal, despite a decline in hectarage. Oat was produced for animal feed and for human food in over 70 countries during the past decade (FAOSTAT 2006). Recent recognition of health components within the grain, such as β-glucan and antioxidants, has bolstered consumer interest in oat. Cereal β-glucan has been shown to be beneficial in both helping reduce cholesterol and stabilize blood serum in

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type II diabetes. Research has also shown that oat produces unique antioxidants which, when fully characterized and capitalized on through breeding and biotechnology, may further increase the dietary value of the crop.

2.1 Oat Production and Value

The genus *Avena* contains many species of oat, but *A. sativa* has been used in the majority of oat cultivation. Warmer climate-adapted, hexaploid, red oat (*A. byzantina* C. Koch) and diploid, hull-less oat (*A. nuda* L.) are also produced commercially. Some species of hexaploid wild oat, such as *A. fatua*, are considered noxious weeds (Youngs et al. 1982).

Global oat production averaged 27×10^6 t and was grown on over 13×10^6 ha of land in the past decade (FAOSTAT 2006). However, national and world oat production has declined in favor of other crops during the past century. Before the development of mechanized farms, oats were an important crop. In 1917, oat accounted for almost 20% of the cereal hectarage planted in the United States (Wilson and Warburton 1918), compared to <2% now. Despite this decline, oat has held its position amongst the cereals. In the past four decades, oat has ranked seventh in area harvested in world cereal production, exceeded by the other cereals discussed in this book, namely wheat, rice, maize, barley, sorghum and millet. During this time, the area of oat harvested has declined by 70%, but an increased yield of the crop has limited the decline in global production to about 1% per year. Recent research discovering health components in oat promises to stabilize the declining value of the crop.

2.2 Emerging Importance of Healthy Grain Quality Traits

Oat and barley have a high concentration of β-glucan, a unique water-soluble fiber, in the cell walls of the endosperm. The β-glucan from oat has been shown to reduce cholesterol in humans (Davidson et al. 1991). The first study to document this was published in 1963, when a daily diet of 140 g of rolled oat reduced human cholesterol levels by 11% in three weeks (De Groot et al. 1963). In 1997, the United States Food and Drug Administration allowed oat products to use the term "may reduce heart disease risk". It was concluded that the β-glucan soluble fiber of whole oat is the primary component responsible for reducing total and low-density lipoprotein (LDL), or "bad" blood cholesterol in diets by including oat fiber at appropriate levels. However, to qualify for the claim, whole-oat foods must provide at least 0.75 g of soluble fiber per serving. About 3 g of soluble fiber is needed daily for an effect on cholesterol concentration (FDA 1997). In addition to this claim, β-glucan has been shown to have an impact on stabilizing the rise in blood sugar concentration in type II diabetes patients (Reyna et al. 2003).

Food products are available that use β-glucan. Oatrim is oat flour that can be used as a fat replacement in cooking and was developed by the United

States Department of Agriculture. Recently, Finland's Finn Cereal Company has started to market Natureal, an oat flour that acts as a blood sugar stabilizer.

Oat is also a source of antioxidants (Adom and Liu 2002) that can be used by the body to fight oxidative stresses believed to be a source of chronic diseases. While oat cereal and oatmeal are high in antioxidants (Yu et al. 2002), the whole grain is the optimal antioxidant source as milling reduces the antioxidants available (Decker et al. 2002). The individual antioxidant components are part of the vitamin E family (Emmons et al. 1999) and include tocopherols and avenanthramides. The tocopherols are common to most cereals, with oat producing α-tocotrienol (Panfili et al. 2003). Avenanthramides are only produced in oat and have been shown to be beneficial antioxidants in human tissue tests (Nie et al. 2005).

In addition to the β-glucan and antioxidant grain components, oat also has the highest relative protein quality and level of the amino acid lysine, the first limiting amino acid, compared to any of the routinely consumed cereal grains (Ranhotra and Gelroth 1995). While the current level of production is declining, the continued demand for oat as a feed and the growing awareness of oat as a healthy component of the human diet should ensure that oat remains an economically important crop long into the future.

3 Current Research and Development

The current research on oat biotechnology can be divided into tissue culture, genetic transformation and molecular genetics. Much of the development of oat tissue culture came from general advances in cereal tissue culture protocols. The first report of oat transformation occurred over a decade ago. Since then, protocol and media improvements have led to successful transformation of oat by numerous groups. The size and hexaploid nature of the oat genome has hindered the creation of a complete recombination map. However, several mapping populations have been developed and many simple and quantitative plant traits have been studied.

3.1 Tissue Culture

Oat tissue culture and plant regeneration was first reported over 30 years ago. Initial conditions produced callus from immature and mature embryos, which could be regenerated into plants (Cummings et al. 1976). Like most cereal tissue culture, early callus regeneration protocols used callus that had been in culture for relatively long periods (several months to a year). The level of aneuploidy in the regenerants ranged over 12–50% for those in culture for 4 months and 48–88% for those in culture for 20 months (McCoy and Phillips 1982). Regeneration protocols continued to advance utilizing additional tissue as explants, including leaf base (Chen et al. 1995), the leaf within the coleoptile

(Torbert et al. 1998c), and tissue from the mature leaf (Gless et al. 1998a). A protocol for oat anther culture was developed (Kiviharju et al. 2000), but the non-synchronous developmental architecture of the oat panicle prevented success with isolated microspore culture. All of these protocols can produce regenerable target tissue for genetic transformation.

3.2 Transformation

Genetic transformation of oat callus and regeneration of fertile, transgenic plants was first reported by Somers et al. (1992). Selection of transgenic oat was achieved later using an antibiotic marker (Torbert et al. 1995) and direct selection for expression of green fluorescent protein (GFP) with an epifluorescent microscope and no herbicide or antibiotic selection (Kaeppler et al. 2000). The ability to monitor the fluorescent phenotype of GFP during each stage of regeneration permitted the calculation of regeneration efficiencies and revealed the limiting steps in the protocol (Kaeppler et al. 2001). Transgenics have been generated from both the leaf within the coleoptile (Torbert et al. 1998c) and from the mature leaf (Gless et al. 1998b). Originally, this technique was limited to tissue culture amenable lines. However, it since has been expanded to include elite cultivar transformation (Torbert et al. 1998b; Zhang et al. 1999).

Early successes with *Agrobacterium tumefaciens*-based transformation were limited to dicotyledonous plants. Eventually, this barrier was overcome and most of the major cereals have been transformed using *Agrobacterium*, including rice (Chan et al. 1993), maize (Ishida et al. 1996), wheat (Cheng et al. 1997), and barley (Tingay et al. 1997). To date, however, there are no published reports of *Agrobacterium*-mediated transformation of oat.

3.3 Molecular Genetics

The production of oat genomic maps using data from populations of recombinant inbred lines has allowed researchers to locate, identify, and verify genes for simple and quantitative traits. Recombinant mapping in oat was first conducted using a diploid population derived from the cross of *A. atlantica* with *A. hirtula*, using restriction fragment length polymorphism (RFLP) markers (O'Donoughue et al. 1992). O'Donoughue et al. (1995) also produced the first hexaploid oat map using a recombinant inbred population derived from *A. byzantina* C. Koch cv. Kanota × *A. sativa* L. cv. Ogle. This map was updated in 2003 to include new markers (607) and a larger population (133 vs. 71) of RI lines, resulting in the reduction of the number of linkage groups to 29 (Wight et al. 2003).

There have been many successful mapping studies in oat, which have analyzed a number of traits that are segregating within their population. There is an inherent weakness in these studies, however, as the oat parents chosen

to create the mapping population may not contain segregating genes for all the major loci that contribute to the trait. For brevity, this chapter will review examples of genetic mapping in oat for grain quality traits (oil content and β-glucan) and disease resistance (crown rust: *Puccinia coronata* f. sp. *avenae*).

3.3.1 Molecular Genetics of Oil Content

The oil content of the oat seed generally ranges from 7% to 10%, but is a flexible trait that can be altered through recurrent selection. For example, recurrent selection for high oil content resulted in an increase in oil to 16% of the groat (Frey and Holland 1999). High oil content provides a more dense energy source for animal feed, but also increases the susceptibility for turning rancid in the preparation for human food.

The genetic factors underlying grain oil percentage were investigated using four mapping populations (Kianian et al. 1999; Groh et al. 2001; DeKoeyer et al. 2004; Zhu et al. 2004) and each revealed three to four QTLs. The first QTL study on oil revealed the major QTL on linkage group 11, which was linked to a candidate gene, acetyl-CoA carboxylase (ACCase), previously characterized as the enzyme for the first step in de novo fatty acid synthesis. This QTL was also identified in two of the three other mapping studies (Groh et al. 2001; Zhu et al. 2004). Knowledge of the genomic regions affecting grain oil percentage provides the basis for future marker assisted breeding for altering oil content.

3.3.2 Molecular Genetic Analysis of β-Glucan Content

Oat breeders have begun to select for increased oat grain β-glucan concentrations to enhance the positive effects on human health and for decreased β-glucan to reduce negative effects of β-glucan on animal feed quality. Understanding of the genetic basis of β-glucan content will allow marker-assisted breeding and aid in gene discovery and transgenic manipulation.

QTLs for β-glucan grain content were first identified in barley (Han et al. 1995) and studied for their effects on malt quality. Three oat QTL studies later included β-glucan in their analysis (Kianian et al. 2000; Groh et al. 2001; DeKoeyer et al. 2004). Different numbers of QTLs were identified depending on the parentage. In the study using the Kanota × Ogle (KxO) population, three significant QTLs were identified, while four significant QTLs were reported using the Kanota × Marion (K × M) population. However, only regions on linkage groups 11 and 14 influenced β-glucan concentrations in both populations over environments. Barley QTL regions were compared to oat and mapped to oat linkage groups 3 and 11. The candidate gene for involvement in β-glucan production is (1-3,1-4)-β-glucanase. This gene was mapped to linkage group KO24_26_34 (Wight et al. 2003). A QTL for β-glucan was later identified on TM_18, which is homoelogous to KO24_26_34 (DeKoeyer et al. 2004). These

studies may provide oat breeders with genetic markers to aid in the selection of different β-glucan profiles.

3.3.3 Molecular Genetics of Crown Rust Resistance

Crown rust is the most damaging and widespread disease of oat (Harder and Haber 1992). Classic breeding approaches have identified sources of resistance identified as *Pc* genes, mainly introgressed from wild oat (*A. sterilis* L.). The competing evolution of host defense and pathogen virulence has revealed that specific *Pc* genes can recognize specific rust races. The *Pc* genes of oat have not been cloned, but are most likely resistance R genes (as defined by Staskawicz et al. 1995; reviewed by Dangl and Jones 2001). This assumption is based on evidence of sequenced resistance genes from cereals that are classified as R genes, for example the L gene family in flax (Ellis et al. 1999) and the rp1 gene family in maize (Collins et al. 1998).

The goal of genetically mapping crown rust resistance genes is to develop markers to assist breeding attempts to pyramid the genes into a single line, to clone and study *Pc* genes, and to remove defeated resistance genes and suppressors. Plant breeders have assigned individual *Pc* labels to rust genes that do not complement each other and many of these genes have been placed on the KxO map (reviewed by Cheng et al. 2002) by direct or comparative mapping. Zhu and Kaeppler (2003) used molecular mapping to locate two resistance QTLs that were introgressed from *A. sterilis* L. and were previously uncharacterized. The development of molecular markers linked to known *Pc* genes has also been accomplished (Wight et al. 2004).

There is also an interest in utilizing "slow rusting" as a resistance scheme where there is no single dominant R gene resistance, but the disease does not progress to the point where it harms the plant. Molecular mapping of genes underlying the slow rusting trait are being conducted to better understand and manipulate the genes involved (Portyanko et al. 2005). Seven total (four major) QTLs were found to affect slow rusting and a negative correlation with flowering time was observed. The basis of this relationship was hypothesized to be that late-flowering plants allowed the rust longer to harm the plant, and thus the plants showed reduced fitness. Significant QTLs affecting slow rusting may be used in marker-assisted selection by oat breeders to select for slow rusting-based resistance while breaking their correlation with maturity.

4 Practical Applications of Transgenic Plants

There have been numerous reports of stable transgenic oat production in the literature over the past 15 years (Table 1). Several of the initial publications established the tissue culture explant systems or selectable markers and promoters. Only a few groups have published practical applications of transgenic

Table 1. A list of published transgenic oat plants. Markers comprise: *Bar* phosphinothricin phosphotransferase, *Npt* neomycin phosphotransferase, *Hyg* hygromycin phosphotransferase, *GFP* green fluorescent protein. *Purpose* sorts each publication into a category for the advancement of oat tissue culture and transformation: *A* proof of concept for an explant tissue, *B* proof of concept for a selectable marker, *C* promoter test experiment, *D* involves elite cultivar transformation, *E* involves abiotic stress resistance transgenes, *F* involves biotic stress resistance transgenes

Marker	Purpose	Cultivar(s)	Reference
Bar	A, B	Gaf/Park	Somers et al. (1992)
Npt	B	Gaf/Park	Torbert et al. (1995)
Bar	F	Gaf/Park	McGrath et al. (1997)
Bar	A, B	Jumbo, Fuchs	Gless et al. (1998b)
Bar	D	Belle	Torbert et al. (1998b)
Npt	A	Gaf/Park	Torbert et al.(1998c)
Npt	C	Gaf/Park	Torbert et al. (1998a)
Npt	F	Gaf/Park	Koev et al. (1998)
Bar, Npt	A, D	Garry	Zhang et al. (1999)
GFP	B	Gaf/Park	Kaeppler et al. (2000)
Bar	D	Melys	Kuai et al. (2001)
Bar	E	Ogle, Pacer, Prairie	Maqbool et al. (2002)
Bar	C, D	Melys, Bullion, Bulwark, Lexicon	Perret et al. (2003)
Bar, Npt, Hyg	B, C	Gary	Cho et al. (2003)

oat plants, focusing on elite cultivar transformation and disease or abiotic stress resistance. Currently, there are no transgenic oat plants released as publicly available cultivars.

5 Conclusion and Future Challenges

The framework for further successes in oat biotechnology is in place. The global demand for the oat crop helps support several conventional breeding programs and molecular genetics research efforts. Breeding programs continually release new cultivars with increased yield and improved important agronomic traits, and are able to rapidly address evolving abiotic and biotic stresses. Molecular maps and mapping studies have supplied the infrastructure for gene isolation and tagging for molecular breeding. Efficient tissue culture and transformation protocols have been established and utilized in the transformation of several oat cultivars. Together, molecular and breeding laboratories can use oat biotechnology to address the future challenges that face the crop.

Advances in oat biotechnology are limited by a number of factors. The large size of the oat hexaploid genome limits the ability to positionally clone oat genes. The hexaploid nature of cultivated oat has complicated the goal of a complete map with 21 linkage groups. The lack of a functional *Agrobacterium* transformation protocol in oat means that researchers have to rely on biolistic

transformation. The decline in global oat hectarage is decreasing the crop's competitiveness with other cereals for research funding.

Progress in other cereal biotechnology research, however, may alleviate some of the barriers to oat biotechnology advancement. Utilization of novel biotechniques in gene discovery, positional cloning, genomic sequencing, and functional genomics are already common among rice, wheat, and maize. Extrapolation of these advances and their application to oat is feasible and promises to continue to expand oat biotechnology research and accelerate oat genome investigations.

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Section III Vegetables

III.1 Tomato

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1 Origin of *Solanum lycopersicum*

The wild ancestral tomato species originated in western South America, from Chile through the mountainous Andean belt to lowland coastal reaches in Peru and to Ecuador in the North. Indeed today, the greatest diversity of tomato wild species resides along the Peruvian coastal strip up to the Andean foothills. The small, yellow ancestral tomato species migrated in a northerly direction towards Mexico and was used as a cultivated crop by the Aztecs. From South America the tomato was transported across the Atlantic Ocean to Europe, probably by Spanish conquistadors some time in the early 1500s. The first known recorded account of tomatoes appearing in Europe was made in 1554 by the Italian botanist Pietro Andrea Matthiolus, who described the fruit as *pomi d'oro* (golden apple; Gould 1983). This name was later translated by the French to *pomme d'amour* (love apple). The fruit was not initially widely accepted as a food source due to its morphological similarity with fruits of the toxic nightshade family. Instead, the plants were grown as exotic ornamentals, with some believing the fruit to have an aphrodisiac effect. By 1578, the tomato, as an ornamental, had arrived in England (Gould 1983). In the mid 1700s, English colonists transported the ornamental tomato from England to North America. Italian and French immigrants settled in and around the New Orleans area and used the fruit in new recipes. By the late 1700s, the tomato as a food source had grown in popularity. As an attractive ornamental and developing food source the rough skinned yellow tomato was hybridised to produce orange and red varieties. These were taken back to Italy. By the mid 1800s, plants with the more attractive red fruit were cultivated in North America and to a greater extent in the Mediterranean. Cultivars that produced larger fruit with increased sugar content were selected and the fruit became widely accepted, rapidly increasing the economic importance of the tomato as a crop plant. In 1863, an American seed catalogue listed the 'heirloom' cultivar 'Trophy', believed to be the first large, red, smooth-skinned variety to be marketed. Access to the new cultivars gave breeders the opportunity to develop traits that were more attractive to the developing market.

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2 Current Uses of Tomato Fruit

2.1 Fresh Market Tomatoes

Tomato fruit forms an important constituent of the human diet, providing essential vitamins and antioxidants. The organoleptic quality of a tomato involves attributes such as taste, aroma, colour and texture. Most consumers have a preference for fresh tomatoes with a firm and crunchy texture and a strong disliking for mealy fruit. Mealiness within tomatoes is a direct result of the separation of cells, without rupture, thereby reducing the juiciness of the fruit. This trait is found in the majority of fresh round tomato varieties. In season, tomatoes are often picked just as they start to ripen, ensuring that an attractive crop arrives at the market. Some growers harvest green fruit, with forced ripening via the introduction of ethylene gas.

Fresh tomatoes are produced and sold on the open market. Superior fruit quality drives the import/export trade. A world-wide goal for tomato breeders has been the development of excellent quality coupled with a long shelf-life. Once harvested (hand-picked, sometimes mechanised), fruit are graded on size and maturity then packed. Uniformity allows for efficient packing and increases the buyer's confidence in the product. Higher production costs and market uncertainty generate increased and more variable price ranges for the fresh market tomato compared with the processing tomato.

2.2 Tomatoes for Processing

The tomato processing industry requires varieties that contain a higher proportion of soluble solids to efficiently make tomato paste and other concentrates. Tomato paste is manufactured and packed in large containers that can be stored for up to 18 months before being used. The paste is sold in bulk to the food industry as a raw ingredient for the production of soups, sauces and other products. Tomatoes grown for processing are usually produced under contract between growers and processing companies. Processing tomatoes are machine-harvested to cope with the large volume of fruit required. Considerable time and money has been invested into the development of processing machinery and the breeding of tomato varieties that are better suited for the processing industry. The fruit should be large, smooth (peels more easily), ripened evenly and of a clear, red colour. Varieties that have many seeds are not acceptable. Tomatoes are produced in every state in the United States, with more than 20 states supporting large commercial-scale units. The largest producer of processed tomatoes is the state of California, which by itself leads all the nations of the world in the production of processing tomatoes (ERS 2006).

2.3 Importance of Tomato in the Human Diet

Modern trends in healthy eating, coupled with growing consumer awareness, have increased the popularity of the tomato. Tomatoes are rich in antioxidants,

such as lycopene, vitamins E and C, β-carotene which serves as a provitamin A, and phenolics, such as ρ-coumaric and chlorogenic acids. Lycopene, in particular, has received much attention in recent years, owing to its ability to help protect the human body against mutagenic free radicals. This property of lycopene has triggered intensive research into developing varieties with increased lycopene content and interspecific crosses between *S. lycopersicum* L. and *S. pimpinellifolium* L. (naturally high lycopene) have been in progress for some time (Chen et al. 1999). Tomatoes grown in glasshouses contain less vitamin C compared with those grown on the vine in natural sunlight. Tomatoes that are cooked also lose vitamin C content compared with fresh fruit. Tomatoes also contain several trace elements, including iron, phosphorus, magnesium, niacin, potassium, thiamine, riboflavin and folate. The pericarp tissue provides a source of fibre (cellulose and lignin). The tomato only contains about 0.3% fat and as such is a cheap ideal food supplement.

3 Tomato Biodiversity

The family Solanaceae contains approximately 90 genera and 3,000–4,000 very diverse species, many of which are *Solanum* spp. The genus *Solanum* is placed within the subfamily Solanoideae, characterized by flattened seeds with curved embryos (Hunziker 1979). Within the Solanoideae, *Solanum* belongs to the large and complex tribe Solaneae, which encompasses about 50 genera in the scheme of Hunziker (2001; available at Solanaceae Source, http://www.nhm.ac.uk/botany/databases/solanum/). Recently, the genus *Lycopersicon* has been (re-)integrated into *Solanum* and the naming conventions given in a short guide to the revised nomenclature, courtesy of Sandra Knapp (Natural History Museum, London, UK; available at http://www.sgn.cornell.edu), have been adopted.

The tomato, *S. lycopersicum,* belongs to a large clade with a chromosome base number of $x = 12$. Muller (1940) originally subdivided the genus into two groups: Eulycopersicon (coloured) and Eriopersicon (green, fruited species). Rick (1976) differentiated between the species, based not on morphological characteristics but on the species ability to be crossed with the cultivated tomato. Species that could be crossed to *S. lycopersicum* L. formed the 'esculentum-complex', namely: *S. lycopersicum* L., *S. pimpinellifolium* L., *S. cheesmaniae* (L. Riley) Fosberg, *S. galapagense* (S Darwin, IE Peralta; endemic to the Galápagos Islands; Rick 1956), *S. neorickii* (DM Spooner, GL Anderson, RK Jansen), *S. chmielewskii* (CM Rick, Kesicki, Forbes, M Holle) DM Spooner GJ Anderson, RK Jansen, *S. habrochaites* S Knapp, DM Spooner, *S. pennellii* Correll. The species that could not be crossed to *S. lycopersicum* formed the 'peruvianum-complex', namely: *S. chilense* (Dunal) Reiche, '*S. N. peruvianum*', L., '*S. N. peruvianum*' var. *humifusum* CH Mull, '*S. N. peruvianum*' var. Marañon race (Atherton and Rudich 1986).

The modern cultivated tomato species *S. lycopersicum* L. (formerly *Lycopersicon esculentum* L. Miller) is believed to be a descendant of the 'cherry-like' cultivar *S. lycopersicum* var. *cerasiforme*. The 'cherry' tomato is widely grown in Mexico and can still be found growing in a semi-wild state across Central America. Cluster analysis based on inter-sample sequence repeat (ISSR) polymorphisms (Kochieva et al. 2002) and phylogenetic data based upon mitochondrial DNA suggest that the 'cherry tomato' is a descendent of the red currant-like wild species *S. pimpinellifolium* (Nesbit and Tanksley 2002). Both species can be freely intercrossed with *S. lycopersicum*. As a result of its popularity in the human diet, *S. lycopersicum* is distributed world-wide, with many domesticated escapees surviving in a wide range of wild habitats. Today, the modern cultivated tomato has a closed anther cone that effectively conceals the stigma, resulting in the species being exclusively inbreeding. This feature has enabled breeders to maintain specific phenotypes within populations, allowing for predictable fruit and limiting potential losses.

4 Molecular Biology Tools for Tomato Improvement

4.1 Genetic and Genomics Resources

The tomato community has an excellent set of genetic resources and information about these collections can be found at the Tomato Genetics Resource Centre (TGRC; http://tgrc.ucdavis.edu/) and on the Solanaceae Genomics Network (SGN; http://www.sgn.cornell.edu).

4.2 Monogenic Mutants and Mutant Libraries

Two large collections of mutants exist. The TGRC collection was founded by the late Dr Charles M. Rick in the Department of Vegetable Crops at the University of California–Davis. The site currently lists 994 monogenic stocks (at 614 loci). Another equally impressive collection that was generated more recently is the SGN Mutation Library in the M82 background (Menda et al. 2004). Thirteen thousand M2 families derived from EMS and fast neutron mutagenesis have been extensively catalogued. Data, including images, for more than 3400 of them so far, with many that are not in the collection of the TGRC, can be accessed via the SGN Mutation Library (http://zamir.sgn.cornell.edu/mutants/).

Many of these mutants have been instrumental in revealing the biological basis of important developmental events. For example, there are a small number of pleiotropic mutants that almost completely inhibit the ripening process. These include *rin* (*ripening-inhibitor*; Robinson and Tomes 1968), *nor* (*non-ripening*; Tigchelaar et al. 1973), *Nr* (*Never-ripe*; Rick 1956), and *Cnr* (*Colourless non-ripening*; Thompson et al. 1999). The genes at these loci have all been

identified using positional cloning strategies, providing important insights into the control of the ripening process.

The size of normal tomato cultivars has often proved a constraint on developing large mutagenised populations, especially when the plants need to be grown under glass. The miniature cultivar, Micro–Tom, offers a solution to these problems and can be grown at very high density (Meissner et al. 1997). Additionally, a new activation tagged resource was recently described for Micro–Tom by Mathews et al. (2003).

4.3 Interspecific Introgression Lines

Genetic variation for specific traits is usually quantitative and a range of populations is now available to study quantitative traits. Of these, some of the most important resources are permanent mapping populations where a portion of a wild tomato species has been introgressed into the genome of a cultivated variety. These can be evaluated for a wide range of traits thereby creating a comprehensive phenotypic database (Paran and Zamir 2003). Several sets of interspecific introgression lines (ILs) are available. The best characterised are those developed by Eshed and Zamir (1994) represented by 76 lines, each containing a small introgression of the wild green fruited *S. pennellii* in a *S. lycopersicum* (cv. M82) background. The introgression lines overlap to provide genome wide coverage with a single introgression per line and the ILs represent the entire genome and partition the genetic map into 107 bins (Liu et al. 2003). Other introgression resources include those with portions of the *S. habrochaites* genome in the *S. lycopersicum* background (Monforte and Tanksley 2000a, b). These types of introgression resources have proved useful in identifying quantitative trait loci (QTL) and in cloning the genes underlying these loci. Based on screening the *S. pennellii* ILs,Holtan and Hake (2003) reported 30 QTL affecting morphological diversity in tomato leaves. Genes underlying QTL for tomato fruit quality have been cloned using a map-based approach. Frary et al. (2000) reported the identification of a gene underlying a QTL on chromosome 2 that changed fruit weight by up to 30%. A major QTL controlling pear-shaped fruit in tomato was cloned by Liu et al. (2003). Most recently, an invertase gene underlying a QTL for enhance sugar yield in tomato fruits has been isolated. Here, a single nucleotide change resulted in a 25% increase in fruit sugar content depending on genetic background and environmental conditions (Fridman et al. 2004). Seeds for both of these resources can be readily obtained from the TGRC.

4.4 The Tomato Genetic Map and Genome Sequencing Programme

There is an excellent tomato genetic map (Tanksley et al. 1992; also see Tomato Genetic Maps, available at http://www.sgn.cornell.edu/cgibin/mapviewer/mapviewerHome.pl) which makes it possible to undertake map-based cloning in

tomato; and several important genes have been isolated via this route. In many cases, the task of identifying these genes was long and arduous mainly because of the absence of tomato genomic resources, including a genome sequence.

As part of the prelude to sequencing the tomato genome, a large programme was set up to isolate and characterise expressed sequence tags (ESTs) and this can be accessed online through the Tomato Digital Expression Database (http://ted.bti.cornell.edu). A recent paper from the Giovannoni laboratory at Cornell describes the analysis of the database in relation to fruit ripening (Fei et al. 2004).

The tomato genome is comprised of approximately 950 Mb of DNA, much of which is repetitive and largely devoid of genes and will be sequenced as the cornerstone of an International Solanaceae Genome Project (ISGP 2006). This programme involves at least ten countries and began in late 2004. The plan for sequencing the genome is to use available tomato bacterial artificial chromosome (BAC) libraries. Each sequenced anchor BAC will serve as a seed BAC from which to radiate out in a minimum tiling path in either direction along the chromosome of interest. A draft sequence for the tomato genome could be available within four years. This project will provide the road map to pinpointing the genes responsible for quality and disease resistance traits in tomato. Although the genome sequence will come from tomato, it will be applicable to numerous other members of the Solanaceae. The members of this plant family include other important crops such as potato, pepper and ornamentals such as petunia. There have been no large-scale gene duplication events in the Solanaceae and the genome of the different family members show large degrees of synteny (Ku et al. 2000). The family evolved largely in the equatorial regions of South America and members have adapted to a very wide range of climatic conditions. This means that comparative genomics will provide the means to link genotype and phenotype differences. For more information on the International Tomato Genome Initiative the reader should refer to the SOL 'White Paper' published on the SGN website (http://www.sgn.cornell.edu).

5 Applications of Biotechnology to Tomato

Tomato is often chosen as a model to study fruit ripening because the tomato plant is readily and easily transformed, has a short life-span and has a good genetic map. The fruit are also easy to work with at a biochemical level as they contain little protease activity. However, the tomato is itself a very important crop and, as such, the tools described above (mutants, QTL, map-based cloning, genetic modification) have or are being employed to alter several quality traits within the tomato plant.

Agronomic traits such as yield, fruit size and growth pattern are likely to be controlled by a complex array of genes and as such lend themselves to analysis by the determination of QTL; and indeed loci related to these traits

have been identified by several groups (Eshed and Zamir 1995; Frary et al. 2000; Causse et al. 2002, 2004). This information can then be used to identify candidate genes within these loci likely to control these traits. However, to date the identity of such candidate genes has only been achieved in a limited number of cases – as exemplified for increased sugar yield and a fruit-specific invertase LIN5 (Fridman et al. 2004). An alternative approach may be to carry out genetic modification of transcription factors involved in the regulation of the trait. This approach can be exemplified by the constitutive expression of the *Arabidopsis* APETAL1 gene in tomato plants (Ellul et al. 2004). This is a MADS box gene involved in the regulation of vegetative development. Its over-expression in transgenic tomato plants results in major changes to the growth pattern. These changes include flowering being initiated earlier and producing plants with determinate growth characteristics with reduced pruning requirements.

Other important complex traits for which QTL in tomato have been identified include salt (Foolad and Jones 1993) and drought tolerance (Foolad et al. 2003). There are several ways to enhance salt tolerance (Flowers 2004), although attempts to achieve this in tomato have been limited to date. Those applied to tomato were reviewed recently (Foolad 2004). One attempt to modify salt tolerance in tomato was by the expression of the yeast HAL1 gene (Gisbert et al. 2000). The product of this gene is involved in the regulation of K^+ transport and expression of this gene in transgenic plants resulted in an increase in the K^+ content, relative to controls, of the cytoplasm when plants were grown under salt-stress conditions. More recently, tomato plants were transformed with the *AtNHX1* gene from *Arabidopsis* (Zhang and Blumwald 2001) which encodes for a vacuolar Na^+/H^+ antiport protein. Plants expressing this gene were able to withstand 200 mM NaCl, a level of salt that was lethal to non-transgenic controls. These plants also flowered and produced a good yield of fruit under these saline conditions.

Disease resistance is another important trait for which QTL have been identified in tomato plants. These include QTL for resistance to *Alternana solani* (early blight; Zhang et al. 2003) and *Phytophthora infestans* (late blight; Brouwer et al. 2004). Several QTL were identified in each case and, in the case of resistance to late blight, several of these were confirmed by analysis of the corresponding near isogenic line (NIL; Brouwer and St Clair 2004). Disease resistance can also be addressed through genetic modification. One approach to exemplify this is the expression of pear polygalacturonase inhibitor protein (PGIP) in tomato plants (Powell et al. 2000). This protein inhibits fungal polygalacturonase and its expression in transgenic tomato plants results in a reduction in lesions and a slowed expansion of the disease.

5.1 Modification of Fruit Ripening

The applications described above have been largely aimed at modifying whole plant traits. However, the major attention has been on the manipulation of traits

within the fruit itself and as such a modification of ripening. Ripening can be delayed by holding the fruit at reduced temperatures. An alternative approach is to retain the fruit under modified or controlled atmospheres (Goodenough et al. 1982) in which the concentration of oxygen has been reduced and that of carbon dioxide increased. However, more recently genetic modification has been employed in an attempt to manipulate ripening. There are several mutants available in which one or more aspects of ripening have been influenced. Of particular note are those mutants that exhibit pleotrophic effects such as *rin* (Robinson and Tomes 1968) and *Nr* (Rick 1956). The genetic basis for several of these mutants has been elucidated; thus, the lesion associated with the *rin* mutation has been shown to reside in a gene encoding a MADS box transcription factor (Vrebalov et al. 2002) and that for *Nr* in a gene encoding a receptor for the 'ripening' hormone ethylene (Wilkinson et al. 1995). Since ethylene is essential for both the initiation and continuation of ripening, its synthesis and perception are an obvious target for the manipulation of ripening.

The ethylene biosynthetic pathway was described by Adams and Yang in 1979. They showed that a key intermediate was 1-aminocyclopropane-1-carboxylic acid (ACC). There are two key enzymes that are specific to this pathway: namely, ACC synthase and ACC oxidase. Genes encoding these two enzymes have been described in many plants and represent an obvious target for any genetic manipulation of ripening. Indeed, the first fruit to have its ethylene synthesis manipulated in this way was the tomato (Howie et al. 1996). Silencing of ACC oxidase resulted in fruit that produced about 5% normal concentrations of ethylene during ripening and, as such, ripened more slowly than normal (Hamilton et al. 1990). Silencing of ACC synthase resulted in a much more dramatic reduction in ethylene production (Oeller et al. 1991) and, in the absence of any exogenously added ethylene, fruit did not ripen. Whilst this approach can be used to modify ripening in general, it is often more useful to change specific aspects. The most important are probably the softening of the fruit and its flavour and nutritional quality.

5.2 Fruit Texture

Softening is a major factor determining the shelf-life of tomato fruit. Softening may be brought about by a combination of factors, but the most important is likely to be the degradation of the cell wall that accompanies ripening. The structure of the tomato cell wall is beyond the scope of this review, but it is similar to that of most dicotyledons and has been reviewed (McCann and Roberts 1991; Carpita and Gibeaut 1993; Carpita et al. 2001). The changes in the wall accompanying ripening of tomato are also beyond this review, but again are those common to many fruit and have also been extensively reviewed (Fischer and Bennett 1991; Tucker 1993; Brummell and Harpster 2001; Giovannoni 2001).

A number of enzymes have been found in tomato fruit that are likely to be able to degrade the cell wall in vivo and those targeted at pectin include: (1) polygalacturonase (PG), a hydrolytic enzyme that results in pectin depolymerisation, (2) pectinesterase (PE) that can reduce the degree of esterification and (3) β-galactosidase (β-gal) which can remove galactosyl residues. All have been targets for genetic manipulation, in particular by the application of gene-silencing technology (Brummell and Harpster 2001; Tucker 2004).

Two groups have independently down-regulated PG activity in tomato fruit (Sheehy et al. 1988; Smith et al. 1988). This resulted in a reduction in PG activity to below 1% of that normally detected and the depolymerisation of the pectin occurring during normal ripening was markedly inhibited (Smith et al. 1990). Transgenic fruit handled better with less cracking (Schuch et al. 1991) and this enabled fruit to be harvested from the vine later in ripening and as such with an improved flavour. A commercial version of this fruit was marketed in the United States under the trade name 'Flavr Savr'. Another commercial advantage was that puree made from the transformed fruit had improved quality (Schuch et al. 1991; Kramer et al. 1992; Brummell and Labovitch 1997) and this was again commercialised. Despite these commercial advantages, the transgenic fruit were only slightly firmer than controls (Kramer et al. 1992; Langley et al. 1994; Errington et al. 1998). The lack of effect on texture could have been explained by the fact that, whilst PG activity had been reduced to around 1% of normal, this still represented a significant level of enzyme activity, given the unusually high level of this enzyme found in tomato fruit. More recently, Cooley and Yoder (1998) produced a line in which the PG gene was disrupted by a transposon insertion. This resulted in almost complete elimination of PG activity but did not appear to result in any further reduction in firmness.

Independently, two groups have also down-regulated the expression of PE activity (Tieman et al. 1992; Hall et al. 1993). In both cases, expression of PE activity was reduced to around 10% of that in normal fruit. In tomato fruit, PE activity can be separated into at least three isoforms, the major isoform, PE2, accounting for about 80–90% of the activity in ripe fruit. It was this isoform that was targeted for gene-silencing. The expression of PE2 in the transgenics was found to be almost completely blocked, whilst expression of the other two isoforms, PE1 and PE3, continued as normal and accounted for the residual 10% of activity (Tucker and Zhang 1996). The degree of esterification was between 15% and 40% higher in transgenic fruit compared with controls (Tieman et al. 1992; Hall et al. 1993) and this was accompanied by a reduced depolymerisation of the polyuronides in ripe fruit and a decrease in the amount of chelator-soluble pectin of around 20–30% (Tieman et al. 1992). These changes in pectin structure had little or no effect on fruit firmness during normal ripening (Tieman and Handa 1994). However, they were associated with a complete loss of tissue integrity in over-ripe fruit (Tieman and Handa 1994) and with an increase in quality of processed juice made from the transgenic fruit, these having higher soluble solids and viscosity (Thakur et al. 1996).

β-Galactosidase (β-gal) has been shown to occur in tomato fruit in at least three isoforms but only one of these, β-gal II, is thought to be capable of degrading cell wall pectin (Pressey 1983; Carey et al. 1995). Thus, β-gal II has been targeted for gene silencing. At least seven putative β-gal genes (*TBG1–7*) have been identified in tomato fruit (Smith et al. 1998; Smith and Gross 2000) and, of these, *TBG4* may be the most significant gene for the expression of cell wall-directed galactanase activity associated with tomato fruit ripening. Antisense suppression of this gene resulted in a 90% reduction in exo-galactanase activity in transgenic fruit (Smith and Gross 2000) and, if this occurs very early in ripening, then there is a 40% increase in the firmness of the ripe fruit (Smith et al. 2002).

Pectins form only one domain of the plant cell wall, another being the cellulose/hemicellulose framework. The glycans associated with this domain have been shown to undergo depolymerisation during the ripening of tomato (Huber 1983; Brummell et al. 1999a) and, as such enzymes that might be implicated in this, have also been targets for genetic manipulation. Endo-glucanase (EGase) may act to cleave bonds within the hemicelluloses (Brummell et al. 1994). The EGase activity in tomato plants is thought to arise from at least eight separate genes; and the two which show ripening-related accumulation have both been silenced: namely *LeCel1* (Lashbrook et al. 1998) and *LeCel2* (Brummell et al. 1999a). In neither case was there any detectable effect on fruit texture. Xyloglucan endotransglycosylase (XET) is another enzyme that might be involved in the modification of hemicelluloses. In tomato fruit, there are at least two genes involved in the production of XET activity and both of these have been silenced by genetic modification – *LeEXGT1* (Asada et al. 1999) and *LeXETB1* (de Silva et al. 1994). In neither case was an effect on the texture of the ripe fruit reported. Another protein which has been shown to influence the structure of the cellulose/hemicellulose framework is expansin (McQueen-Mason and Cosgrove 1995). In tomato fruit, at least six expansin genes have been identified; the most highly expressed being *LeExp1* whose suppression resulted in a 15–20% increase in firmness (Brummell et al. 2002), whilst a three-fold increase in expression resulted in fruit which softened more extensively (Brummell et al. 1999b).

In none of the cases described above was a major alteration to the texture of the tomato fruit reported. Given the complex nature of the cell wall, it is likely that softening involves several hydrolytic activities. Thus, simultaneously targeting several enzymes involved in the degradation of the wall may be more effective. This can be achieved either by crossing plants in which individual enzymes have been down-regulated or by using chimeric constructs targeted at two or more endogenous genes, as described by Seymour et al. (1993) for PG and PE2. Combined silencing of PG and PE2 did not effect texture, but more recently silencing PG and expansin resulted in a significant alteration to the texture of tomato fruit (Powell et al. 2003). Another method to 'silence' several enzymes at once would be to target the general secretion of proteins into the cell wall. A protein thought to be implicated in this trafficking, rab11,

was silenced in tomato and resulted in fruit which retained their firmness for nearly twice as long as controls (Lu et al. 2001).

5.3 Fruit Flavour

Sugars and acids are major determinants of flavour and, as such, targets for manipulation (Kader et al. 1978). As a sink tissue, tomato fruit obtain their carbohydrate in the form of sucrose but the predominant sugars in ripe fruit are glucose and fructose. Metabolism of sucrose is associated with the activities of sucrose synthase and invertase, the activity of the latter increasing during the later stages of ripening (Wang et al. 1993; Schaffer and Petriekov 1997). One target would be to inhibit this metabolism and accumulate sucrose directly within the fruit. Reduction of sucrose synthase activity to around 1% of normal has been shown to have no effect on the accumulation of either starch or sugar in transgenic fruit (Chengappa et al. 1999). Inhibition of a soluble acid invertase (T1V1), however, resulted in an increased accumulation of sucrose (Klann et al. 1996) and also in smaller fruit. These findings are consistent with QTL data from introgressed lines. Yelle et al. (1988) demonstrated that acid invertase levels correlated with sucrose concentrations in the wild-type tomato *L. chmielewskii* and that the *sucr* loci associated with this trait appeared to be linked to the acid invertase gene *T1V1* (Yelle et al. 1991). Invertase activity is also associated with the cell wall and one of these enzymes, LIN5, has been shown to be linked to a QTL (Brix-9-2-5) associated with increased sugar yield in *S. pennellii* (Fridman et al. 2004). Sucrose phosphate synthase (SuPSy) is thought to be the major enzyme implicated in the synthesis of sucrose. Overexpression of SuPSy in transgenic tomato plants has been shown to increase sucrose unloading and hence increase the strength of the fruit as a potential sink (Nguyen-Quoc et al. 1999).

There are about 400 flavour volatiles produced by ripening fruit, but it is thought that a much smaller number may be responsible for the characteristic aroma of the fruit (Teranishi et al. 1990). The major volatiles appear to be those related to amino acid, carotenoid or lipid breakdown. In the latter case, these include C6 compounds that are produced by a 'cascade' of enzymes including lipases, lipoxygenases, hydroperoxide lyases, isomerases and alcohol dehydrogenase. Such compounds include hexanal and (Z)-3-hexenal, which are formed from the 13-hydroxyperoxides of linoleic and linolenic acids, respectively. These aldehydes are then transformed to their corresponding alcohols by alcohol dehydrogenase. There are at least five genes associated with lipoygenases in tomato, Tomlox A and B (Ferrie et al. 1994), Tomlox C and D (Heitz et al. 1997) and Tomlox E (Chen et al. 2004). Attempts to manipulate these flavour volatiles have included the production of transgenic fruit with modified lipoxygenase (Griffiths et al. 1999; Chen et al. 2004) and alcohol dehydrogenase activities (Speirs et al. 1998; Prestage et al. 1999). Silencing of Tomlox A and B, whilst having a very significant effect on the total lipoxyge-

nase activity in transgenic fruit, had no effect on volatile production (Griffiths et al. 1999). Silencing of Tomlox C, however, did result in a marked alteration in flavour volatile production (Chen et al. 2004). The final approach to the manipulation of flavour has been to express genes for sweet-tasting proteins such as monellin (Penarrubia et al. 1992) or more recently thaumatin (Bartoszewski et al. 2003) in transgenic tomato fruit.

5.4 Nutritional Quality of Tomato Fruit

Tomato is a widely consumed fruit and, as such, is a major contributor of phytonutrients to the diet. The fruit is particularly rich in the antioxidants lycopene, β-carotene (which also acts as provitamin A) and vitamin C; but it also provides other antioxidants such as vitamin E and phenolics, as well as other vitamins such as folate.

The major carotenoid pigment found in tomato, lycopene, has aroused considerable interest. Intake has been particularly implicated in protection from prostate cancer (Kucuk et al. 2002). Several approaches have been used to enhance lycopene levels. Natural mutants of tomato are available, such as high pigment (*hp1*, *hp2*), and these may be employed in breeding strategies. These mutations result in the general elevation of all carotenoids within the fruit (Cookson et al. 2003) and, in the case of *hp1,* has been shown to represent a mutation in the tomato *UV-DAMAGED DNA-BINDING PROTEIN 1* homologue (Liu et al. 2004). This same research group have also identified two light signal transduction genes that act as either negative or positive regulators of fruit pigmentation. The repression of one of these genes, *LeCOP1LIKE*, results in elevated fruit carotenoids (Liu et al. 2004). There is a high level of natural genetic diversity in nutrient levels between tomato fruit. Thus, Hanson et al. (2004) have shown that *L. pimpinellifolium* tends to have much higher levels of lycopene, as well as vitamin C, phenolics and general antioxidant activity than *L. esculentum*; and a study using recombinant inbred lines of tomato has identified QTL for lycopene and carotene levels (Causse et al. 2002).

Carotenoid concentrations in tomato fruit have also been enhanced through genetic modification. Over-expression of phytoene synthase activity, a key enzyme in the carotenoid pathway, resulted in a three-fold increase in β-carotene concentration (Romer et al. 2000) and an enrichment of 1.8- and 2.2-fold for lycopene and β-carotene, respectively, in the fruit (Fraser et al. 2002). The expression of a yeast *S*-adenosyl methionine decarboxylase in tomato fruit also resulted in an enhancement of lycopene concentration (Mehta et al. 2002). However, this effect on lycopene concentration was unexpected and difficult to explain.

Other important antioxidants in fruit include polyphenolics such as flavonols. In the normal tomato fruit, flavonoids are at a low level and localised in the skin. Induction of flavonoid synthesis in the flesh was achieved by expression of two transcription factors LC and C1 from maize (Bovy et al. 2002) and resulted

in the production of high levels of the flavonol kaempherol. More recently, Niggeweg et al. (2004) increased the concentration of another nutritionally important phenolic, chlorogenic acid, in tomato fruit by over-expression of a key enzyme in its biosynthesis, namely hydroxycinnamoyl transferase.

6 Conclusions

There has been a significant amount of effort put into the improvement of tomato fruit quality. Currently, the application of genetic modification is meeting with considerable public resistance and the exploitation of natural genetic variation, in terms of QTL, is likely to be the way forward in the foreseeable future. The availability of a genome sequence, as is currently being generated, is essential and will greatly facilitate the cloning of genes underlying QTL. It will also assist in any map-based cloning targeted at identifying the genetic basis of any tomato mutants. This information can then be exploited in marker-assisted breeding programmes.

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III.2 Cucumber

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1 Introduction

Cucumber is an edible fruit and the plant belongs to the family Cucurbitaceae. The genus *Cucumis* contains more than 30 species, of which two are economically important crops, namely cucumber (*C. sativus* L., $2n = 14$) and melon (*C. melo* L., $2n = 24$). As a vegetable crop, the economic importance of cucumber varies with the region (Table 1). As a fresh market vegetable in the culinary sense, it is very popular in Europe and the United States. Most cucumbers are grown worldwide for the fresh market, with China as the leading producer. Indeed, cucumber is among the top ten vegetables produced globally. The plant is believed to have been domesticated in India, then spread to Greece and Italy and was introduced into China around 100 BC. It has been cultivated for food for at least 3000 years; it appeared in France in the ninth century, England in the fourteenth century and North America by the middle of the sixteenth century.

Table 1. Data concerning world cucumber and gherkin production (source: FAO 2004)

	Production (t)	Area of harvest (ha)	Yield (kg ha^{-1})
Africa	1,037,727	145,670	737.1
Asia	33,037,786	1,915,554	1,724.71
Europe	3,923,230	200,839	1,953.42
North and Central America	2,059,441	126,540	1,627.5
South America	76,430	5,237	1,459.42
Oceania	19,490	1,285	1,516.73
World total	40,190,104	2,395,125	1,678.0

Cucumber chromosomes are small in size, with a basic number of $x = 7$, and a relatively small haploid genome of 367 Mbp/C (Arumuganathan and Earle 1991). The plant is unique as the three genomes show different transmissions, namely maternal for chloroplast, paternal for mitochondrial and biparental for the nuclear DNA (Havey 1997; Havey et al. 1998). The mitochondrial genome belongs to the largest amongst all eukaryotes. However,

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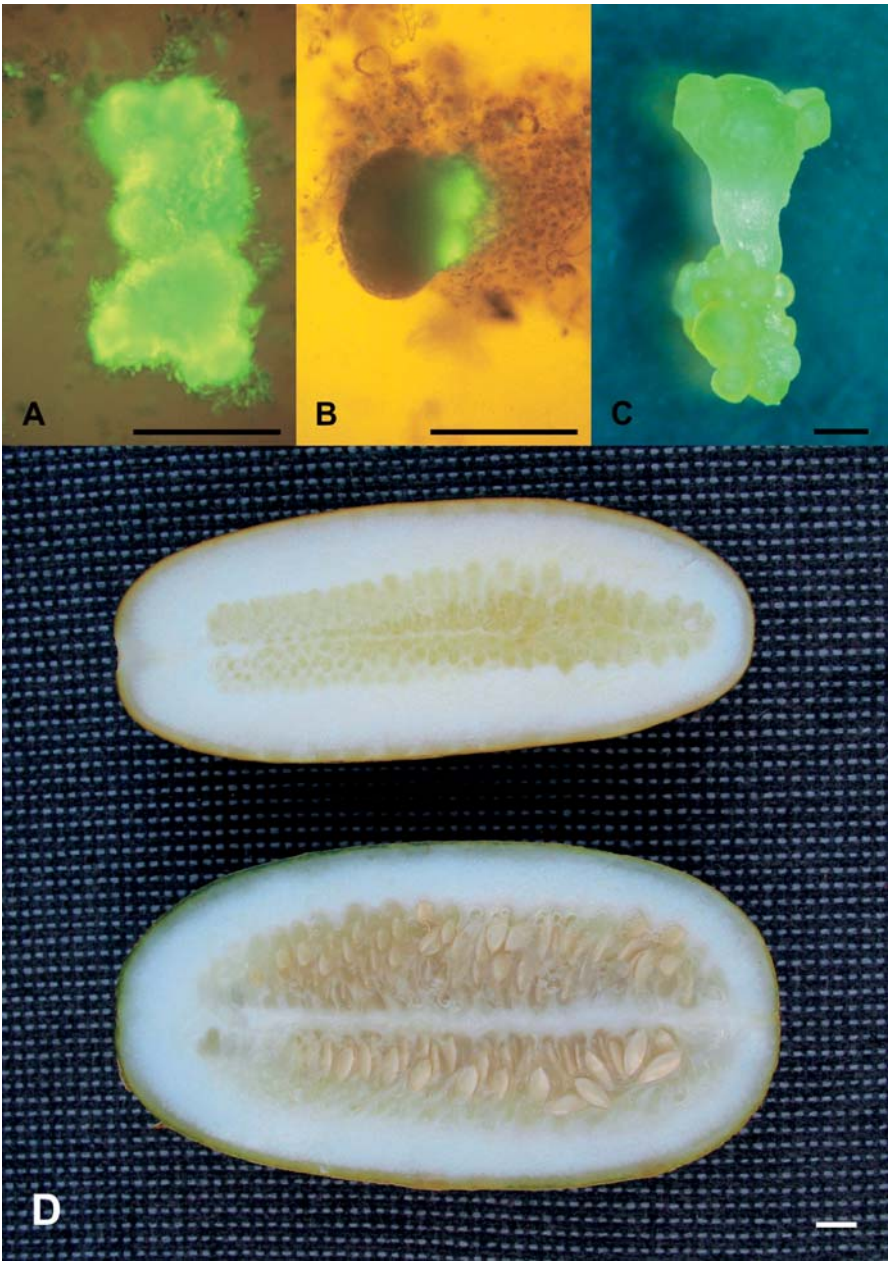

cucumber has a narrow genetic base, with a genetic variability of only 3–8%. This lack of variability greatly hinders breeding programs. Although cucumber is a diploid cross-pollinated species, it is nearly insensitive to inbreeding. Different types of sex expression occur in this species, such as monoecious,

◀ **Fig. 1.** Cucumber biotechnology – the somatic embryo formation from green autofluorescence (GAF)-emitting tissue derived from a cytokinin-dependent embryogenic suspension (CDS) of the cucumber line Borszczagowski (**A**, **B**, **C**) and the effect of transgene introduction (**D**). **A** Strong expression of GAF in cell aggregates proliferating in modified MS medium supplemented with 2 mg l^{-1} BA (after excitation with light at 450–490 nm). **B** Globular-stage embryo developed from GAF-emitting cell aggregate 1 week after transplanting to growth regulator-free medium. **C** Cluster consisting of globular to early cotyledon-stage embryos 3 weeks after transplantation (bright field micrograph). **D** The introduction of the *DefH9*:*iaaM* construct into a monoecious variety Borszczagowski results in the production of parthenocarpic seedless fruit: *below* fruit from a non-transgenic recipient with full seed set, *above* its transgenic (seedless) version. *Bars* 0.5 mm in **A**, **B**, **C**, 1 cm in **D**

dioecious, hermaphroditic and male or female. For example, many of the older cultivars are monoecious, while many of the newer ones are gynoecious. A detailed description of *Cucumis* species and their systematics, origins and chromosome numbers has been reviewed by Kirkbride (1993) and Jeffrey (2001). The papers cited in an earlier article (Malepszy 1988) are not discussed in this chapter.

2 Current Research and Developments

2.1 Tissue Culture

2.1.1 Micropropagation

An efficient procedure for cucumber micropropagation was reported by Hisajima et al. (1989). It consists of a multi-stepped protocol for shoot induction from seeds, followed successively by shoot multiplication, rooting (with 100% efficiency) of single shoots and the successful semi-aquaculture of regenerated plants. It has been estimated that 1,600,000 cucumber shoots can be obtained from a single shoot within one year using micropropagation. This system appears to be promising for vegetative propagation as no deviants were observed amongst the regenerated plants. However, the procedure requires further investigation aimed at the yield potential and quality of the plants produced. In another micropropagation system, Burza and Malepszy (1995a) reported regeneration of two to five shoots from leaf microexplants (2–3 mm^2) of *C. sativus* and *C. anguria* during a 5-week culture period. This system may be adapted to other genotypes differing in auxin sensitivity and/or nitrogen (ammonia/nitrate) source requirements. In a similar procedure, Colijn-Hooymans et al. (1994) concluded that cotyledonary explants from younger seedlings (3- to 5-day-old) should be used, as the regeneration frequency of cotyledons from 7-day-old and older seedlings decreased dramatically. In addition, most of the plants regenerated from the older explants were polyploid/mixoploid with an abnormal morphology.

An effective embryo rescue method is necessary to extend genetic variation. The ability of embryos at different developmental stages of *C. sativus* and in the wild species *C. zeyheri* and *C. metuliferus* to regenerate plants *in vitro* was studied by Custers and Bergervoet (1990). When proembryos (0.03–0.05 mm) and embryos at the early-globular stage (0.05–0.08 mm; surrounded by the embryo sac) were cultured on MS medium (Murashige and Skoog 1962) supplemented with 3.5% sucrose, 0.1 mg l^{-1} kinetin and 0.01 mg l^{-1} indole-3-acetic acid, they developed into plants at a frequency of up to 21%. However, embryos at the late-globular (0.08–0.1 mm) and heart stage (0.1–0.3 mm) cultured under the same conditions developed into plants at a much higher frequency of up to 48% and 83%, respectively. An unexpected observation was that, from the early cotyledon stage onwards, the frequency of plant formation gradually decreased, reaching a minimum at the late cotyledon stage. Relative to the wild species, embryos of *C. sativus* showed lower percentages of plant formation. In the case of triploids, the good embryo formation is in the 4× by 2× cross direction and, depending on the parental lines, 11.7–45.8% of embryos developed into plants (Malepszy et al. 1998).

The carbohydrate source may be a critical factor in cucurbit morphogenesis (Kim and Janick 1989). Ladyman and Girard (1992) investigated the possibility of using cucumber somatic embryos as artificial seeds by testing the number of regenerable embryos. They found that, although embryogenic tissue was most successfully induced on a medium semi-solidified with agar (0.7%), there was no effect of gelling agent (0.15%, 0.3%, 0.6% Gelrite; 0.35%, 0.7% agar) on tissue proliferation. Moreover, when sucrose, glucose, or fructose were used in the proliferation medium, there was no effect on tissue growth. However, when fructose was compared with sucrose as a carbon source, the highest proportion of somatic embryos (about 50%) germinated following proliferation on a medium supplemented with 3% sucrose and gelled with Gelrite (0.15%). Maltose (3%) was observed to inhibit severely embryogenic tissue proliferation.

2.1.2 Suspension Cultures

Liquid media are preferred over their solidified counterparts for the large-scale production of somatic embryos. An improved method for obtaining a stabilized embryogenic cucumber suspension culture, in which specific type-1 cells and proembryogenic masses (PEMs) are present, was reported by Wróblewski et al. (1995). This system resembles the carrot model. The correct successive development of somatic embryos was achieved by choosing the appropriate nitrogen ratio and content. From 1 ml of suspension, 21 embryos were obtained on a hormone-free medium 3 weeks after inoculation; and 3.2% of the embryos ultimately regenerated into plantlets.

An alternative method for the initiation and maintenance of long-term embryogenic suspensions was described by Kreuger et al. (1996). In this method,

the application of high concentrations (45 μM) of 2,4-dichlorophenoxyacetic acid (2,4-D), which are toxic in the initiation phase, was necessary during the proliferation phase to increase the embryogenic potential. It is possible to obtain genetically stable diploid cell lines if fully diploid explants are used for culture initiation, or exclusively diploid PEMs are selected for subsequent proliferation. The established cell lines could be maintained for over 3 years without loss of embryogenic capacity. Approximately 20% of the PEMs formed embryos; and up to 60% of the embryos ultimately regenerated into plantlets. Burza and Malepszy (1998) observed similar results with respect to genotype, long-term embryogenicity and genotype independency, but used a different mechanism by cytokinin-dependent cell suspension (CDS). Their medium was supplemented with a cytokinin as the sole growth regulator and they used a suspension that was easily dispersed, diploid over a 2-year period and produced a substance that emitted a green autofluorescence (Fig. 1A for details, see Burza et al. 2006). The intensity of this fluorescence correlated with the capacity of the suspension to produce somatic embryos following removal of the cytokinin from the medium (Fig. 1B,C; Burza et al., unpublished data). Both the embryo yield and their conversion into the plants was high and the plants obtained were diploid if the appropriate procedure was used (Table 2).

2.1.3 Protoplast Culture

Punja et al. (1990) described a procedure in which cotyledon and mesophyll protoplasts were cultured and plant regenerated. They obtained 18 plants, but there is a paucity of information regarding the development of these plants and seed-setting proficiency. Efficient plant regeneration via direct somatic embryogenesis (5000 embryos per gram tissue) from cell suspension-derived protoplasts was described by Burza and Malepszy (1995b). The procedure is simple and differs from those already known in some major respects, including: (1) high parameters of both protoplast yield (maximal 1×10^7 protoplasts per gram tissue) and plating efficiency (approximately 25%) and (2) a short time (6–8 weeks) for obtaining of plantlets and a high uniformity and quality of R_0 plants. This procedure, if incorporating the green autofluorescence emitting suspension, produced the labelling protoplasts for the fusion system (Burza et al., unpublished data).

2.1.4 Haploid Production and Chromosome Doubling

Przyborowski and Niemirowicz-Szczytt (1994) obtained a number of cucumber haploids by stimulating parthenogenetic embryo development through pollination with 300-Gy-treated pollen grains and subsequently applying embryo rescue to regenerate plants. Embryo yield was dependent on the variety used. In the best case, e. g. variety Polan F1, 1.2–3.1 embryos were obtained per 100 seeds; and ca. 50% of these developed into plants. All of the plants

Table 2. Somaclonal variation by different type of culture from cucumber B line (inbred line from cv. Borszczagowski) measured as the frequency of plants with altered ploidy and R_1 families segregating new traits (according to Plader et al. 1998b; Ladyzynski et al. 2002). *ADS* Auxin-dependent suspension (1 mg l^{-1} 2,4-D); *ADS1.7* is the same medium with 1.7× ammonia content and without nitrate. *CDS* Cytokinin-dependent suspension (2 mg l^{-1} BA); *CDS1.7* contains increased ammonia without nitrate. *LMC* Liquid meristematic clumps (shoot primordia); LMC_{18} and LMC_{12} indicates the duration of the culture (in months). These R_0 plants result from regeneration and R_1 are their progeny after self-pollination. The effect of several types of *in vitro* culture differed in tetraploid presence/absence and the segregation of new phenotypes in R_1 progeny. Some types of culture produced diploids only, others produced diploids or tetraploids depending on the experiment; and the ADS produced the tetraploids only. Leaf direct regeneration was only one type of culture, free from new phenotype segregation in R_1

Culture type	Ploidy of R_0 plants		% of R_1 families segregating new traits[b]	Culture age (months)
	Number of plants examined	% of tetraploid plants		
Seed control	120	0.0	0.0	–
CDS	28	0.0	9.7	10
CDS1.7	9	33.3	15.5	18
LMC_{18}	21	0.0	10.9	18
LMC_{12}	18	0.0	5.1	12
ADS	40	100	–	36
ADS1.7	30	100	–	24 and 12
Leaf callus				
I[a]	60	0	8.5	4–5
II[a]	25	12	–	4–5
Protoplasts from embryogenic suspension				
I[a]	20	0	45.0	12
II[a]	15	26.6	–	12
Leaf direct regeneration	39	0	0	1.5

[a] I and II indicate independent experiments.

[b] These data do not consider the mitochondrial MSC mutants (against the original papers).

obtained, with one exception, were haploids or aneuhaploids. Chromosome doubling involving callus induction from haploid leaf explants and plant regeneration from long-term callus culture was reported by Faris et al. (1996). The plants regenerated from callus were predominantly diploid (70–100%); and the number of polyploids obtained was lower than for methods employing colchicine. The main limitations of this procedure are that it is influenced by genotype and is time-consuming (several months to more than a year). The method using gynoecious haploids (Faris et al. 2000) has been incorporated into the breeding program of "Spojnia", a Polish plant breeding company (Hodowla i Nasiennictwo Ogrodnicze, Srem, Nochowo). The search for a long-term storage protocol for haploids was undertaken by Niemirowicz-Szczytt

et al. (2000). They observed that, when haploid plants were stored for longer than 2 years, the result was physiological ageing, manifested by a continuous flowering stage. Vegetative reproduction could be prolonged only after rejuvenation through direct shoot regeneration from the first young leaf of the haploid parent plants. Gémes-Juhász et al. (2002) carried out various trials to develop a protocol for regenerating gynogenically derived haploid plants *in vitro*. Most embryos were obtained from unpollinated cucumber ovaries isolated at the best developmental stage (cellularization stage during embryo-sac formation) following 2–4 days of heat (35 °C) induction treatment on medium containing thidiazuron. The highest frequency of gynogenesis and plant regeneration was 18.4% and 7.1%, respectively; and nearly 90% of the plants were haploid.

Haploid production by two cultivars from anther culture was recently described by Ashok Kumar et al. (2003). The optimal rate of embryogenic calli/embryos production was induced on B5 medium supplemented with 2.0 mM 2,4-D and 1.0 mM benzylaminopurine following a 2-day cold shock (4 °C). Each embryo was shown to produce 1.02 and 1.36 embryos; and 87% and 70% haploid plants were obtained in the cultivars Calypso and Green Long, respectively.

2.1.5 Quality of Somatic Embryos and Plant Regeneration

A good knowledge of the physiology of somatic embryo (SE) development is important for the successful application of artificial seed technology in cucumber. Suspension culture-derived (diploid and triploid) SEs have been found to differ substantially from their zygotic counterparts in two vital respects. One is the concentration of abscisic acid (ABA), which is extremely low (0.005–0.011 mg l^{-1}) and the other is that no significant changes in ABA concentration occur during embryo development (Gawronska et al. 2000). These physiological differences are probably responsible for the non-dessication and precocious germination of SEs observed in our *in vitro* system described previously. Consequently, the addition of exogenous ABA to the maturation medium seems to be necessary.

Various factors can affect *in vitro* systems, such as a relatively high concentration of growth regulators (Burza et al. 1994), or inappropriate regeneration systems (Murkowski et al. 1999). These may result in the regenerated cucumber plants displaying a poor physiological state and having a high susceptibility to suboptimal environmental conditions. The applications of adequate monitoring methods of various parameters of chlorophyll fluorescence induction and delayed luminescence decay kinetics allow the estimation of the actual physiological state of the regenerants, as well as their potential productivity. This state depends on the regeneration procedure used, as shown by Malepszy et al. (1990), who investigated the yield from embryogenic callus-derived plants, and by Burza et al. (1996), who examined direct regeneration from leaf

microexplants. In the first case, the reduction in yield was as high as 70%, while in the second, the reduction was much lower (15%). However, seed-derived plants also showed a 10% reduction in yield following germination on agar. Consequently, it may be concluded that the leaf microexplant regeneration protocol itself only resulted in a 5% decrease in yield. An improvement in the adaptation procedure may remove this difference.

2.1.6 Genetic Variation

The various sources of genetic variation for cucumber have been described. These include somaclonal mutants, mutants arising from the application of a selective agent during the *in vitro* step and wide hybridization. New artificial amphidiploids were obtained in the last case. Interspecific hybridization is an efficient method to transfer useful characters from wild relatives to the cultivated species. In the past, considerable efforts have been expended to apply this method to cucumber. Chen et al. (1997) described successful interspecific hybridization between *C. sativus* and *C. hystrix*. A new species, *Cucumis* × *hytivus*, was obtained by applying the somaclonal approach. This amphidiploid had double the normal chromosome number ($2n = 38$) and the genome status HHCC (Chen and Kirkbride 2000). When *C. hystrix* is used as a female parent, the diploid hybrid ($2n$=19) can set fruit without seeds, whereas the amphidiploids ($2n = 38$) can produce fruit with viable seeds. The hybrid has a higher protein (0.78%) and mineral (0.35%) content than the normal pickling cucumber (0.62% and 0.27%, respectively) and a higher root-knot nematode resistance (partially transmitted from the wild species).

Most of the culture procedures produce somaclonal mutants, which then segregate following the selfing of the regenerants. Only some of these somaclonal mutants give rise to tetraploids directly in R_0 (Table 2); and these may be employed for tetraploid production. The variations are nuclear-dependent as are the mitochondrial mutants with the mosaic (MSC) phenotype that occur. The leaf direct regeneration procedure is the only protocol free from nuclear somaclonal variation. All of the culture procedures listed in Table 2 induce changes in the mitochondrial genome. MSC mutants can be stabilized following self-pollination and selection (Malepszy et al. 1996); and they possess various rearrangements in the mitochondrial genome (Lilly et al. 2001; Bartoszewski et al. 2004). One of the established MSC mutants has somatic embryogenesis impairment, but shows no differences with respect to growth intensity and regeneration via liquid meristematic clumps when compared with the wild type (Ziolkowska et al. 2005). *In vitro* plant regeneration influences the metabolite profiles of plants in the first generative progeny; and these changes are different in the particular types of culture (Filipecki et al. 2005). This aberrant variation does not disturb vegetative propagation in any obvious fashion, because it appears almost exclusively in the first sexual generation. However, it does disturb some of the procedures with the elite material.

The variation occurred in culture was used to select for *Fusarium oxysporum* f.sp. *cucumerinum*-resistant plants (Malepszy and El-Kazzaz 1990) and for cadmium resistance (Gzyl and Gwozdz 2005). The selection agent used was the *F. oxysporum* culture filtrate (5.0%, 7.5%) and the R_1 progeny showed resistance to both the pathogen inoculation and the filtrate. However, seeds were produced after the crossing of mutants (as female) with the control, due to unsuccessful self-pollination.

The effect of genotype on the results of culture is a very well known phenomenon and is a serious limitation of many applications. In a plant regeneration procedure from leaf callus, the effect of genotype had a high heritability (0.77–0.43); and two complementary genes with additive interaction were responsible for the differences observed (Nadolska-Orczyk and Malepszy 1989). The difference between a frequently regenerating line (B) and an intermediately regenerating line (Gy-3) depended on one gene, while the difference between the former and an occasionally regenerating line (Gy-3) depended on three genes. It was subsequently shown that the B and Gy-3 lines and their F_1 hybrids have a different relationship in a cell suspension (Gy-3 produced more embryos than B) and differ in several parameters, such as rate of ammonium and nitrate uptake, kinetics of 2,4-D utilization and dynamics of pH changes (Wróblewski et al. 1998). Thus, genotypes can specifically change some of the medium parameters which may support or inhibit the somatic embryogenesis. In short-term cultures, the relationship has been documented between morphogenetic response *in vitro* and the intensity of nitrogen uptake by seedlings from the culture medium. Several genes expressed during somatic embryogenesis were isolated, sequenced and functionally characterized (Filipecki et al. 1997; Linkiewicz et al. 2004; Malinowski et al. 2004) and two of which, *CsXTH1* (xyloglucan endotransglucosylase/hydrolase) and *CsXTH3*, were localized on chromosomes 2 and 5, respectively.

2.2 Transgenic Technology

2.2.1 Genetic Transformation

Reviews covering the methodology of transgenic cucumber technology and the agronomic value of transgenic cucumber plants were recently published by Yin et al. (2005a, b). For cucumber, all of the current transformation procedures use tissue culture; to date, no floral dip method has been developed for transformation. A wide variety of explants have been used, of which the most common are young leaf microexplants, young cotyledons, leaf petioles and hypocotyls, all of which have been transformed after a short preculture period. The most frequently used explants are young leaf microexplants and young cotyledons. All of the constructs used for transformation contain a marker gene, usually *neomycin* phosphotransferase II (*npt*II) or hygromycin phosphotransferase (*hpt*), but occasionally *bar* (conferring tolerance to phosphinothricin). Trans-

formation has been mostly *Agrobacterium tumefaciens*-mediated, although in a few cases biolistics has been used. It takes from 10 weeks to 6 months from the inoculation of the explants to the time when the plants are ready to be transferred into greenhouse. Transformation efficiency (the percentage of explants regenerating shoots or transgenic plants) ranges from 1.5% to 6.3% for regenerated shoots and from 1.4% to 10% for the ratio between the number of transgenic plants obtained and the total number of explants inoculated.

Two transformation procedures, namely leaf microexplant direct regeneration and long-term established embryogenic suspension culture, were described recently (Burza et al. 2006). These authors reported a transformation efficiency of 0.8–6.5% for primary explants and 6.4–17.9% for embryogenic aggregates (see Sect. 2.1.2), depending on the construct and/or other experimental conditions. In the case of embryogenic aggregates, the treatment with antibiotics for bacteria elimination is not necessary if a milder bacteria strain, e. g. LB 4404, is used. Recently, a selectable marker system was described (He et al. 2006) which used the *pmi* gene encoding the phosphomannose isomerase, enabling positive selection utilizing mannose as a carbon source. The highest transformation rate was 23% and the PMI activity considerably varied between the nine transformants analysed.

2.2.2 Transgenic Plant Evaluation

About 30 different constructs have been introduced into cucumber and, with 20 of these, plants were regenerated and transgene integration and/or expression occurred. Inbred lines (six), hybrid (three) and non-hybrid (nine) varieties have been used as recipients. Fourteen different constructs have been introduced into inbred line B (derived from the Borszczagowski line) and almost 100 homozygous transgenic lines obtained (Yin et al. 2005a, b, unpublished data). In the following examples, the expected feature was observed at the plant level. An enhanced biotic resistance was observed following the introduction of the cucumber mosaic virus coat protein (CMV-cp) gene (Chee and Slightom 1991; Nishibayashi et al. 1996), the zucchini green mottle mosaic virus coat protein (ZGMMV-cp) gene and chitinase genes (Punja and Raharjo 1996; Raharjo et al. 1996; Tabei et al. 1998). A CR32 transgenic line (obtained by Tabei et al. 1998) exhibited resistance to *Phytophthora* rot (*Phytophthora nicotianae* var. *parasitica*), but not to *Fusarium* wilt (*Fusarium oxysporum* f. sp. *cucumerinum*; Kishimoto et al. 2003). The introduction of the *Solanum sogarandinum*-derived dehydrin gene *pGt:Dhn 10* increased the chilling tolerance of the transgenic plants in physiological tests (Yin et al. 2004b). The *pGT:Dhn24* construct expressing another *S. sogarandinum* dehydrin was recently produced, three lines subsequently showing chilling tolerance, while one line showed chilling and freezing tolerance (Yin et al. 2006a).

A new characteristic was obtained following the introduction of the sweet protein-thaumatin II cDNA from *Thaumatococcus danielli*, which enhances

a sweet taste in fruits (Szwacka et al. 2002). The *mSOD1* gene caused a higher level of superoxide dismutase (SOD) and, as such, the transgenic plant may be more useful as a functional cosmetic material as no transgenics until now have been used for this purpose (Lee et al. 2003). The introduction of the *UGT* and *ACB* genes resulted in an increased yield in transgenic cucumber plants (Salyaev et al. 2002), while the insertion of the *DefH9:iaaM* construct containing a *Pseudomonas syringae* tryptophan monooxygenase gene led to facultative parthenocarpy (Fig. 1D Yin; et al. 2006b). The transgenic line I44, which is homozygous for the putative 54-kDa replicase gene, was immune to cucumber frit mottle mosaic virus (CFMMV) infection by mechanical and graft inoculation and to root infection following planting in CFMMV-infested soil (Gal-On et al. 2005). A substantial delay of symptom appearance was observed following infection by three additional cucurbit-infecting tobamoviruses. When used as a rootstock, line I44 protected susceptible cucumber scions from soil infection by CFMMV. The tobacco *PR 2d* promoter is inducible in cucumber transgenics following exposure of the plant to various biotic and abiotic stresses, with the highest induction rate occurring after a cold stress (Yin et al. 2004a).

Tabei et al. (1999) carried out an environmental risk assessment of transgenic cucumber lines containing the *RCC2* gene (rice chitinase cDNA clone) in a closed and a semi-closed glasshouse. Field trials with transgenics were also conducted. In one field trial, Gonsalves et al. (1992) investigated the effectiveness of coat protein-mediated protection. Gajc-Wolska et al. (2001, 2003) compared several lines harboring the *p35S::thaumatinIIcDNA-pnos*::*npt*II construct and found that the yield, physical parameters and nutritional value of the transgenic fruits were similar to those of the control (Gajc-Wolska et al. 2001). High scores in the sensory assessment of both the fresh and processed transgenic fruits verified their high taste and consumption values. The fruits of the transgenic and non-transgenic plants were similar with respect to taste quality and chemical composition, dry matter content (average 4.3%) and vitamin C content (6.4 mg g^{-1} fresh weight). The highest potassium content was found in the non-transgenic control lines and the highest calcium concentration in the transgenic lines. Twardowska (2003) described vegetable dishes using thaumatin-containing cucumbers that were highly prized by the consumers. The high scores in the sensory assessment of both fresh and processed transgenic fruits prove their high taste and consumption values.

The relevance of genetic modification is reflected by several parameters. The most crucial one is the number of the independent transgenic lines showing the improved phenotype (which allows the selection of optimal transgenes and host plant interaction). Equally important is obviously the practical value of transgenic phenotype. In some of the examples already described, these data were provided. Eight out of 14 transgenic lines exhibited a high degree of immunity to CFMMV, one out of 16 lines had a remarkably sweet thaumatin-derived fruit and four of the eight lines produced 74–87% of parthenocarpic fruits, while one of the three lines tested possessed an improved chilling and freezing tolerance.

2.3 Molecular Genetics

2.3.1 Genetic Mapping

Cucumber breeders and geneticists are faced with a paucity of genetic markers and close linkages with important economic traits. Although the length of the cucumber genetic map is relatively small as compared to other crop species (estimated at 750−1,000 cM; Staub and Meglic 1993), the genetic maps constructed for cucumber are relatively unsaturated (mean marker interval 8−10 cM). Additionally, cucumber linkage maps have been produced using different types of molecular markers and different mapping. The published maps spanned regions from 95 cM (Vakalounakis 1992) to 766 cM (Kennard et al. 1994). The construction of a consensus linkage map was completed for cucumber using six previously published maps, along with AFLP data (Bradeen et al. 2001). As a result, narrow- (within *C. sativus*) and wide-based (*C. sativus* × *C. sativus* var. *hardwickii*) maps were created with 255 (ten linkage groups) and 197 (15 linkage groups) markers, respectively. These maps covered 538.6 cM for the narrow-based and 450.1 cM for wide-based maps and were smaller than that reported previously (Kennard et al. 1994), despite an increase in the number of markers mapped. This difference may be due to the different software used. Kennard et al. (1994) used MAPMAKER and Bradeen et al. (2001) used JOINMAP, the latter utilizing more precise algorithms for linkage estimation. In the next step, 14 polymorphic single sequence repeats (SSRs) were added to the cucumber map (Danin-Poleg et al. 2000). These SSRs were rather randomly distributed throughout the cucumber genome, providing effective anchor points between maps. This research was also the first report of synteny between two *Cucumis* species, namely cucumber and melon.

Finally, 28 new markers were added to a previously published cucumber map, resulting in a genetic map with seven linkage groups, which is equal to the haploid chromosome number in cucumber (Fazio et al. 2003). This map spanned 706 cM and consisted of 131 markers, with a mean interval of 5.6 cM between markers. Also, *F* (gynoecy) and *de* (determinate habit) were associated with two SSR loci at 5.0 cM and 0.8 cM, respectively. The addition of molecular and morphological markers to the cucumber map will allow for cultivar identification and plant variety protection.

2.3.2 Ribosomal DNA

An extensive research program conducted over a 20-year period by Hemleben et al. (1992) has resulted in precise and detailed information on cucumber ribosomal DNA (rDNA). rDNA represents approximately 2–10% of the total cucumber nuclear DNA, which is equivalent to 2,000–10,000 copies per haploid genome (Ganal et al. 1986). The rRNA genes in cucumber are arranged as tandemly repeated sequences and each unit is composed of the coding regions for 18S, 5.8S and 25S rRNA, separated from each other by an intergenic spacer

(IGS). The coding regions are highly conserved amongst all eukaryotes, while non-coding spacers are very variable. These variations found among different species (King et al. 1993) and even between individuals belonging to the same species, e. g. *Cucumis sativus* (Plader et al. 1998), are the focal point of evolutionary studies. Transcription initiation sites (TIS) and several transcription termination sites (TTS), due to their presence within the duplication region, as well as transcription regulation, have been extensively described (Zentgraf et al. 1990; Zentgraf and Hemleben 1992). Cytological studies of cucumber chromosomes by C-banding (Hoshi et al. 1998) and staining using the fluorochromes chromomycin A (CMA) and 4′,6-diamidino-2-phenylindole (DAPI; Plader et al. 1998a) enabled physical mapping of the cucumber genome. Fluorescent in situ hybridization (FISH) with rDNA as a probe indicated that the gene clusters are located at the pericentromeric regions of chromosomes 1, 2 and 5. Silver staining for nucleolar organizing regions (NOR) located the transcriptional activity of the largest ribosomal cistron to the pericentromeric region of chromosome 2 (Hoshi et al. 1999). These results were confirmed independently by Jin-Feng et al. (1999) and Dal-Hoe et al. (2002). Small variations in the results reported by these three groups may be due to the variability in rDNA loci which is found in the genus *Oryza* (Fukui et al. 1994). Transgene integration sites in four independent lines of transgenic cucumber were shown by FISH to be preferentially located in the euchromatic regions of chromosomes 1, 2, 3 and 4 (Tagashira et al. 2005).

Palmer (1982) described a physical map of chloroplast DNA (cpDNA) based on the analysis of fragments obtained from restriction enzyme digestion. Since that time, only 11 accessions have appeared in the computer database EMBL, mostly describing a single gene as a direct submission. The entire sequence of cucumber cpDNA is now known and characterized (Plader 2005; NCBI database, accession number Aj970307), which opens new possibilities for basic and applied research (Maliga 2004).

A complete list of genetically characterized cucumber loci was updated together with cloned genes and presented in the Cucurbit Genetics Cooperative for the first time (http://cucurbitsvr.hort.ncsu.edu). This list consisted of 95 genes divided into ten groups, namely genes involved in seed germination or seedling development (16 genes), photosynthesis and photorespiration activity (18 genes), expressed mainly in the root (four genes), controlling flowering (12 genes), fruit development and maturation (ten genes), cell wall loosening and cell enlargement (ten genes), induced or repressed by plant hormones (15 genes), resistance genes (three genes), somatic embryo genes (one gene) and repeated DNA sequences (six genes; Xie and Wehner 2001). Only those sequences appearing in both reports and in the GeneBank database were included in the list. The newest update of the list was published in July 2005. To the best our knowledge, several accessions have been added to the database since the list was published and not more than 50 entries has been deposited, excluding expressed sequence tag (EST), tRNA, rRNA, organellar, unknown and uncompleted sequences.

Sex determination in cucumber is controlled by simple system of three main genes (*a*, *gy*, *F*), which is different from the MADS-box genes as previously expected. Only one of these genes, the *F* gene, which determines gynoecism, has been characterized molecularly. It was shown to be an additional copy of the main gene involved in ethylene biosynthesis, namely ACC synthase (*CS-ACS1G*; Trebish et al. 1997); and the full sequence has been described (Mibus and Tatlioglu 2004).

We are aware of three public cucumber bacterial artifical chromosome (BAC) libraries in existence. In fact, there are two prepared libraries, respectively using *Bam*HI and *Eco*RI as a cloning site (Nam et al. 2005). The first one was constructed by the Laboratory for Plant Genomics & GENEfinder Genomic Resources (public) at Texas A&M University; and the source of the high-molecular-weight (HMW) DNA was an inbred F_6 line derived from the cucumber cultivar Suiseifushinari 2-go (http://hbz7.tamu.edu/index.htm). Another, commercial cucumber BAC library was constructed by KeyGene Genomics (Wageningen, The Netherlands) using the TMG-1 genotype as the HMW DNA template (unpublished data). A more recent BAC library was constructed with the *Hin*dIII restriction enzyme; and it contains 25 500 clones with average insert size of 130 kb and derived from the cv. Borszczagowski inbred line (Przybecki, personal communication; zbigniew_przybecki@ sggw.pl).

3 Conclusion and Future Challenges

Extension of our knowledge is essential about cucumber genetics, functional genomics and molecular biology. Cucumber biotechnology has been applied in several different ways. One is the development of new varieties through the use of double haploids. This approach would be even more efficient through enhanced frequencies of parthenogenetic haploids. Anther derived haploids were produced recently for the first time in cucumber. These androgenic haploids may complement well with gynoecious haploids and offer the promise of further development of new breeding methods. Disease- and pest-resistant varieties have been obtained by mutation and the development of transgenic plants. An artificial amphidiploid from cucumber and *C. hytivus* may be a novel source of resistance genes. The development of a dense genetic map for cucumber will stimulate marker-assisted selection.

Genetically manipulated (GM) cucumbers have not yet been introduced into production. However, promising new varieties have been described and evaluated in field or glasshouse experiments. The success of GM cucumber will require transformation procedures that are free of marker genes, the development of plastid transformation and identification of new genes and regulatory sequences. An important goal would be the development of in vivo transformation procedures, like the floral dip of *A. thaliana.* The reproduction biology of cucumber seems very suitable for this procedure.

One of the main limitations for the development of cucumber biotechnology is genotypic differences for fundamental processes, like plant regeneration ability and transformation. Recently, this limitation was circumvented by the development of suspension cultures, especially those emitting green autofluorescence. However, further progress and a better understanding of these phenomena are required.

One of the most important biotechnological approaches is the application of non-pathogenic microorganisms like fungi and yeast to combat diseases (Punja and Utkede 2003). Fungal and yeast biological control products are commercially available to manage cucumber diseases. A better understanding is necessary about the mechanism of protection and genetic manipulation of the microorganisms to make them more efficient control measures.

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III.3 Eggplant

M.V. Rajam[1] and S.V. Kumar

1 Introduction

Eggplant (*Solanum melongena* L.) ($2n = 24$), commonly known as aubergine, guinea squash or brinjal, is an important vegetable crop of tropical and temperate regions of the world. It is a good source of vitamins and minerals, especially iron, making its total nutritional value comparable with that of tomato (Kalloo 1993). Besides being used as an important vegetable, eggplant has been exploited extensively in traditional medicines (Khan 1979). For example, tissue extracts have been used for the treatment of asthma, bronchitis, cholera and dysuria; fruits and leaves are beneficial in lowering blood cholesterol. Recent studies have shown that eggplants also possess antimutagenic properties. The medicinal and economic value of eggplant can be found in the Sanskrit literature (Khan 1979; Hinata 1986; Kalloo 1993).

Eggplant probably originated from India, with Indo–Burma, China and Japan as the secondary centres of origin (Gleddie et al. 1986a). The plant has been cultivated in Asia for over 1500 years. Arabs introduced this crop to the west during the fifteenth century (Hinata 1986). Germplasm resources and collections have been well documented, evaluated and conserved throughout the world (Sarathbabu et al. 1999). Eggplant has been divided into three main types based on the fruit shape. These include egg-shaped (*S. melongena* var. *esculentum*), long and slender in shape (*S. melongena* var. *serpentium*) and dwarf types (*S. melongena* var. *depressum*; Kalloo 1993).

Various cultivars of eggplant are susceptible to a variety of stress conditions, which limits crop productivity significantly. Plant improvement using conventional breeding methods has been directed towards fruit size, weight and shape, and resistance to diseases and pests (Kalloo 1993). This has led to the development of numerous varieties of eggplant with improved characteristics and yield. There are also many wild species of eggplant that are resistant to pests and pathogens and are the source of agronomically important genes that can be exploited for eggplant improvement (Collonier et al. 2001a; Kashyap et al. 2003). Efforts to impart disease and pest resistance to cultivated varieties have been achieved with only limited success due to sexual incompatibilities with the source species or wild relatives (Daunay and Lester 1988; Kashyap et al. 2003). In addition, introgression of desired traits such as parthenocarpy,

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improved nutritional value and post-harvest qualities into the cultivated varieties is difficult to achieve due to the lack of appropriate sexually compatible varieties or species (Collonier et al. 2001a).

Recent advances in biotechnology and genetic engineering have provided a new impetus to eggplant improvement programs. The development of efficient tissue culture systems for plant regeneration has facilitated rapid multiplication of desired genotypes, while protoplast culture and fusion methods are important for the production of novel somatic hybrids and cybrids, thereby surpassing sexual incompatibility. Genetic engineering enables trans-Kingdom gene transfer, permitting gene introgression and crop improvement at a faster pace through precision breeding. Excellent progress in eggplant research has been made over the past two decades and many transgenics with improved traits have been developed. This chapter reviews the progress in eggplant biotechnology.

2 Tissue Culture

An ability to regenerate plants from cultured cells and tissues at high frequency has been considered as the corner stone of plant biotechnology, as it serves as an alternative means of vegetative propagation and is a pre-requisite for the production of genetically modified crops. In eggplant, plants have been regenerated from somatic cells and tissues, or from anthers and microspores via organogenesis and/or embryogenesis.

2.1 Organogenesis

Cultivated and wild species of eggplant have been regenerated through organogenesis (Fig. 1). Organogenesis has been achieved using various explants (Rao and Narayanaswami 1968; Kowlozyk et al. 1983; Gleddie et al. 1985; Kashyap 2002), including those of hypocotyl (Kamat and Rao 1978; Matsuoka and Hinata 1979; Alicchio et al. 1982; Matsuoka 1983; Sharma and Rajam 1995a), leaf (Alicchio et al. 1982; Magioli et al. 1998; Sharma and Rajam 1995a), cotyledon (Alicchio et al. 1982; Sharma and Rajam 1995a) and root (Franklin et al. 2004). Regeneration has also been reported in cell suspensions (Fassuliotis 1975; Fassuliotis et al. 1981) and protoplast cultures (Guri et al. 1987; Sihachakr and Ducreux 1987). However, hypocotyl explants have been most commonly used for organogenesis, compared to cotyledon explants (Sharma and Rajam 1995a). Organogenic response varies considerably with genotypes and explants (Kamat and Rao 1978; Alicchio et al. 1982; Sharma 1994). In addition, the variation has also been detected within a single explant that follows a basipetal pattern. This variability in morphogenetic response correlates to changes in the spatial distribution of polyamines (Sharma and Rajam 1995a, b). This finding is supported by the results of a study showing that organogenesis in cotyledon

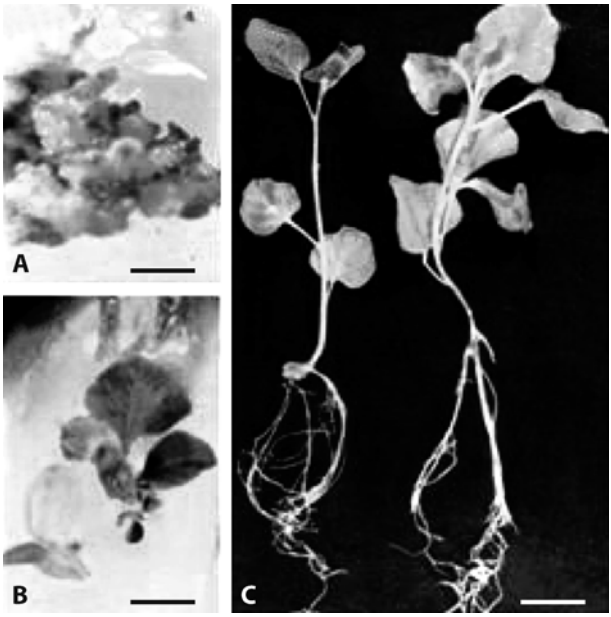

Fig. 1. Organogenesis in eggplant from leaf explant. **A** Multiple shoot bud formation from callus. **B** Rooting of shoots in vitro. **C** Regenerated plants. *Bar* 1 cm

explant correlates with the hormone-mediated enhancement of biosynthesis and conjugation of polyamines (Scoccianti et al. 2000).

The formation of shoot buds can be induced from cultured explants grown in medium containing cytokinins (Gleddie et al. 1983). Direct organogenesis in eggplant has been reported in the presence of a combination of benzylaminopurine (BAP) and indole-3-acetic acid (IAA; Sharma and Rajam 1995a), kinetin (Mukherjee et al. 1991) or thidiazuron (TDZ; Magioli et al. 1998). Indirect shoot regeneration, via the intervening callus stage, was obtained using combinations of IAA with BAP, kinetin or zeatin (Kamat and Rao 1978; Sharma and Rajam 1995a), α-naphthaleneacetic acid (NAA) and BAP (Matsuoka and Hinata 1979), or combinations of IAA, 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, followed by IAA and 2-isopentyladenine (2iP; Fassuliotis et al. 1981). In contrast, cell suspension cultures grown in the presence of IAA and 2iP gave rise to a nodule-like structure that, however, failed to develop into shoots (Fassuliotis et al. 1981). Shoot formulation occurred after the culture was transferred to medium containing ascorbic acid or the antiauxin, *p*-chlorophenoxyisobutyric acid.

In order to generate lines resistant or tolerant to diseases or abiotic stress, tissue and cell cultures of eggplant have been used to regenerate plants on media supplemented with pathogen toxins or abiotic stress agents (Mitra et al. 1981; Mitra and Gupta 1989; Sadanandam and Farooqui 1991; Asao et al. 1992; Ashfaq Farooqui et al. 1997). A similar approach has also been employed in an attempt to understand gene function and activity under stress conditions (Alicchio et al. 1984). Electrolyte release from regenerating eggplant

calli has been used in screening for resistance and susceptibility to *Verticillium dahliae* (Cristinzio et al. 1994). The induction of laterals in eggplant root cultures in hormone-free liquid medium was reported by Sharma and Rajam (1997) and this system was used to demonstrate the role of polyamines in root growth and differentiation of lateral roots. These workers showed that polyamines, particularly spermidine, were intimately involved in root growth and differentiation of lateral roots.

2.2 Somatic Embryogenesis

Somatic embryogenesis (SE) in eggplant has been studied extensively (Fig. 2) and a significant advancement has been made with respect to the genetic and physiological factors associated with the process (Collonnier et al. 2001a); Kashyap et al. 2003; Kantarajah and Golegaonkar 2004). Synthetic seeds have also been developed by encapsulating somatic embryos in sodium alginate and calcium chloride (Rao and Singh 1991; Mariani 1992).

Auxins alone can induce SE in eggplant and cytokinins have been shown to be inhibitory (Matsuoka and Hinata 1979; Gleddie et al. 1983). The first

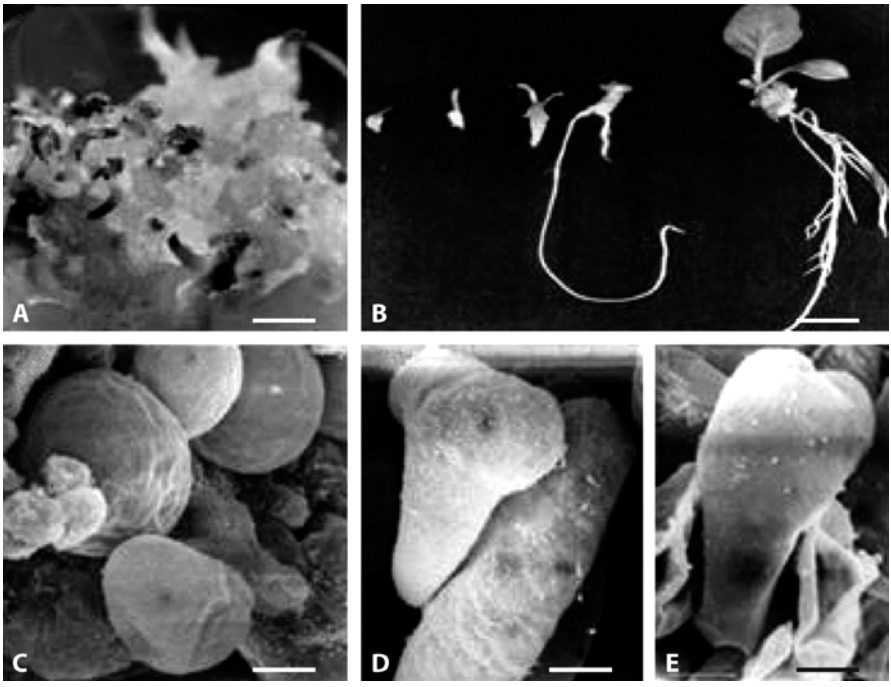

Fig. 2. Somatic embryogenesis in eggplant from a leaf explant. **A** Somatic embryo formation from callus (*bar* 1 cm). **B** Various stages of plant regeneration via somatic embryogenesis (*bar* 1 cm). **C–E** Scanning electron micrographs of various stages of somatic embryogenesis: globular (**C**, *bar* 30 μm), heart-shaped (**D**, *bar* 8 μm) and torpedo (**E**, *bar* 8 μm) somatic embryos

induction of SE in eggplant using IAA was reported by Yamada et al. (1967). Other auxins such as NAA (Matsuoka and Hinata 1979; Sharma and Rajam 1995a, b; Hitomi et al. 1998) and 2,4-D (Hitomi et al. 1998) were also used, with the former enhancing somatic embryo differentiation. However, the optimal auxin concentration for SE varied with the type of explant and plant genotype. Leaf explants required 2–6 mg l^{-1} NAA for SE, with 6–10 mg l^{-1} for hypocotyls (Matsuoka and Hinata 1979; Gleddie et al. 1983; Sharma and Rajam 1995a). SE was also affected by the nitrogen concentration and sucrose in the medium, with the optimal NO_3^-/NH_4^+ ratio of 2 : 1 and a sucrose concentration of 0.06 M (Gleddie et al. 1983). Seedling explants prepared from seeds germinated in the dark was found to be beneficial for SE induction (Ali et al. 1991). The use of an aseptic ventilative filter in the lid of culture vessels was also helpful for SE induction and plant regeneration (Saito and Nishimura 1994).

Sharma and Rajam (1995a, b) reported that the most important factor influencing SE in eggplant was the plant genotype. Genotype-dependant variation in physiology and metabolism in the explants was thought to determine the capacity of somatic embryos induction. It was shown that varietal differences were also related to growth regulator requirements for SE (Rao 1992). The results from several studies indicate that somatic embryogenic potential is controlled genetically (Ali et al. 1991; Afele et al. 1996).

Explant type is also an important factor affecting SE (Ali et al. 1991; Sharma and Rajam 1995a; Kantarajah and Golegaonkar 2004). Various explants used to induce SE induction include leaves (Gleddie et al. 1983; Rotino et al. 1987a; Rao and Singh 1991; Sharma and Rajam 1995a), cotyledons (Fobert and Webb 1988; Saito and Nishimura 1994; Sharma and Rajam 1995a) and hypocotyls (Matsuoka 1983; Ali et al. 1991; Sharma and Rajam 1995a). However, leaves and cotyledons were found to be better than hypocotyls (Sharma and Rajam 1995a). Significant differences in morphogenic potential within the different regions of a single explant were also observed, which was thought to be attributed to the variation in spatial distribution of polyamines (Sharma and Rajam 1995a, b; Yadav and Rajam 1997).

Polyamines are a new class of endogenous growth regulators (Galston and Kaur-Shawney 1990). They have been shown to play a regulatory role in several plant developmental processes, including SE (Robie and Minocha 1989; Minocha et al. 1991; Koetje et al. 1993; Walden et al. 1997; Rajam et al. 1998; Pua 1999). The results of our previous study showed that variation in the embryogenic potential within the single explant was correlated with temporal changes in polyamines (Sharma and Rajam 1995a). We further demonstrated that SE was associated with the spatial distribution (Yadav and Rajam 1997) and temporal regulation (Yadav and Rajam 1998) of polyamines in explants. Results also showed that explants from different regions of the leaf showed differential SE potential, which was correlated with the concentration of polyamines, especially putrescine. Modulation of polyamine titres in the explants by exogenous application of polyamines resulted in improved SE. These results clearly suggest a regulatory role of polyamines in SE. In view of the pivotal role of

polyamines in SE in these and other studies (Pua 1999), it was suggested that polyamines might be used as biomarkers for plant regeneration (Shoeb et al. 2001).

Attempts have also been made to elucidate the molecular mechanisms underlining SE of eggplant. Momiyama et al. (1995) reported the identification of a group of genes expressed differentially during SE, using a differential display technique. In a comparative study of embryogenic and non-embryogenic eggplant tissues, several unique transcripts were detected in the former, but not in the latter (Afele et al. 1996). The results of a recent study on the transgenic eggplant that expressed a chimeric gene consisting of a β-glucouronidase (GUS) coding sequence under the control of *Atgrp*-5 promoter from *Arabidopsis thaliana* indicated that early stages of somatic embryo development in eggplant is associated with *Atgrp*-5 expression (Magioli et al. 2001).

2.3 Protoplast Culture and Somatic Hybidisation

Successful isolation of protoplasts and plant regeneration can be achieved in both cultivated and wild species of eggplant (Guri et al. 1987; Sihachakr and Ducreux 1987). Mesophyll tissues of eggplant have been most commonly used as a source of protoplasts due to their high yield (Bhatt and Fassuliotis 1981; Jia and Potrykus 1981). Other sources of protoplasts with high regeneration potential include in vitro-grown shoots (Sihachakr et al. 1993) and stems and petioles (Sihachakr and Ducreux 1987; Sihachakr et al. 1993). In general, plant regeneration from protoplasts is highly genotype-specific (Gleddie et al. 1983, 1985).

Somatic hybridisation through protoplast fusion has been reported to transfer novel cytoplasmic and nuclear traits to cultivated eggplant from wild relatives that are sexually incompatible (Table 1). The first successful somatic hybrid was developed between cultivars of *S. melongena* and *S. sisymbriifolium* through polyethylene glycol (PEG)-mediated protoplast fusion (Gleddie et al. 1986b). Since then, several somatic hybrids have been reported by fusing protoplasts of cultivated varieties with various wild species. Interestingly, some somatic hybrids derived from *S. melongena* and *S. sisymbrifolium* were shown to possess only the *S. sisymbrifolium* chloroplast genome and were highly resistant to root knot nematodes and potentially resistant to spider mites (Gleddie et al. 1986b). Similar somatic hybrids were also generated recently that displayed resistance to wilt caused by *Verticillium dahliae* as well as *Ralstonia solanacearum* (Collonnier et al. 2003a). However, further exploitation of these hybrids through breeding has been limited because of hybrid sterility. In somatic hybrids of eggplant and *S. torvum*, plants were resistant to *Verticillium* wilt and nematodes and partially resistant to spider mites (Guri and Sink 1988a; Sihachakr et al. 1989, 1994) and *Ralstonia* (Collonnier et al. 2003b). Sihachakr et al. (1988) reported that hybrids of eggplant with *S. khasianum* were resistant to shoot and fruit borer. It was demonstrated that herbicide (atrazine) resistance could be transferred from the resistant biotype *S. nigrum* to somatic

Table 1. Somatic hybridisation in eggplant

Parents	Remarks	Reference
Inter-specific		
S. melongena × *S. sissymbrifolium*	Nematode and mite resistance	Gleddie et al. (1986b)
S. melongena × *S. sissymbrifolium*	Resistance to *Verticillium* and *Ralstonia*	Collonnier et al. (2003a)
S. melongena × *S. khassianum*	Resistance to *Leucinodes orbonalis*	Sihachakr et al. (1988)
S. melongena × *S. torvum*	Resistance to *Verticillium* wilt and mites	Guri and Sink (1988a)
S. melongena × *S. torvum*	Resistance to *V. dahliae* and nematodes	Sihachakr et al. (1989, 1994)
S. melongena × *S. torvum*	Asymmetric hybrids resistant to *V. dahliae*	Jarl et al. (1999)
S. melongena × *S. torvum*	Resistance to *Verticillium* and *Ralstonia*	Collonnier et al. (2003b)
S. melongena × *S. nigrum*	Resistance to atrazine	Guri and Sink (1988b)
S. melongena × *S. macrocarpum*	Inter-specific hybrids	Gowda et al. (1990)
S. melongena × *S. aethopicum* (*Aculiantum* group)	Resistance to *R. solanacearum* wilt	Daunay et al. (1993), Collonnier et al. (2001b)
S. melongena × *S. aethopicum* (*Aculiantum* group)	Resistance to *Fusarium oxysporum* wilt	Rotino et al. (1995)
S. melongena × *S. aethopicum* (*Aculiantum* and *Gilo* groups)	Resistance to *Ralstonia* wilt	Collonnier et al. (2001b)
S. melongena × *S. aethopicum* (*Gilo* group)	Resistance to *Fusarium* wilt	Rizza et al. (2002)
S. integrifolium × *S. violaceum*	Resistance to *Ralstonia* wilt	Tamura et al. (2002)
Inter-generic		
S. melongena × *N. tabaccum*	Production of inter-generic hybrids	Toki et al. (1990)
S. lycopersicoides × (*L. esculentum* × *L. pimpinellifolium*)	Production of inter-generic hybrids	Guri et al. (1991)
S. melongena × (*L. esculentum* × *L. pimpinelli*)	Asymmetric kanamycin-resistant inter-generic hybrids	Liu et al. (1995), Samoylov and Sink (1996)

hybrids (Guri and Sink 1988b). All hybrids resistant to 0.1 M atrazine were shown to possess *S. nigrum* chloroplast DNA. Daunay et al. (1993) also generated fertile somatic hybrids of eggplant with *S. aethiopicum*, which exhibited improved pollen fertility (30–85%) compared with their sexual counterparts

(20–50%). Moreover, some hybrids showed fruit production increased by three to four times compared with the parental lines. Fertile somatic hybrids of eggplant and *S. integrifolium* (*S. aethiopicum*) were also reported and characterised (Rotino et al. 1995, 1998).

Inter-generic asymmetric somatic hybrids have also been developed between the inter-specific tomato sexual hybrid (*Lycopersicon esculentum* × *L. pennellii*) and eggplant (Liu et al. 1995). In a similar study, Samoylov and Sink (1996) reported that the regeneration ability of asymmetric somatic hybrids depended on the irradiation dose used for elimination of chromosomes from one parent. The usefulness of tetraploid symmetric somatic hybrids in breeding programmes depends on their ability to be backcrossed to the recurrent genotype of eggplant. It is important to reduce the ploidy level to the dihaploid status by haploidisation methods before backcrossing.

2.4 Androgenic Haploids

Haplodisation of diploid plants is an efficient strategy to obtain homozygosity by chromosome doubling of haploid plants. These homozygous plants can be used as inbred lines in breeding programs. In *S. melongena*, haploid plants can be obtained either from cultured anthers (Raina and Iyer 1973; Isouard et al. 1979) or isolated microspores (Gu 1979; Miyoshi 1996). Haploid plant regeneration in eggplant is influenced by genotype, temperature, culture conditions, growth regulators and anther stage (Rotino et al. 1987b; Rotino 1996). High temperature appears to be essential for inducing morphogenic response in cultured anthers (Miyoshi 1996). Other treatments such as sucrose starvation, together with a short period of high temperature during initial culture, have been shown to suppress gametophytic development and DNA synthesis of cultured microspores and stimulated androgenesis (Miyoshi 1996).

Anther culture has been employed to reduce the ploidy of somatic hybrids to the diploid status (Rotino et al. 2001; Kashyap 2002; Rizza et al. 2002), which is necessary for the hybrids to cross more easily with cultivated eggplant. In vitro androgenesis has been used increasingly by eggplant breeders to rapidly obtain fixed lines from heterozygous material and to produce commercial F_1 hybrids (Collonnier et al. 2001a; Kashyap et al. 2003). Apart from their usefulness as potential breeding material, dihaploids may be utilised to determine the inheritance of morphological and agronomic traits.

3 Genetic Engineering

3.1 Genetic Transformation Mediated by *Agrobacterium Tumefaciens*

Genetic transformation is an important method for crop improvement by insertion of genes encoding useful agronomic traits into the plant, which is

also valuable material for breeding programmes. The method also facilitates insertional mutagenesis and gene tagging that help to identify and to isolate genes of interest. Eggplant transformation by *Agrobacterium tumefaciens* was achieved as early as 1988, using a co-integrate vector pMON 200 harbouring the *npt*II gene (Guri and Sink 1988c). Since then, several studies on eggplant transformation have been reported (Komari 1989; Rotino and Gleddie 1990; Fari et al. 1995; Iannacone et al. 1995; Billings et al. 1997; Franklin and Sita 2003). Although the protocol for eggplant transformation has been relatively well developed, production of transgenic plants expressing gene for agronomically important traits has been limited.

In eggplant transformation, only the *npt*II gene (encoding neomycin phosphotransferase II) has been used as a selection marker (Table 2). This is considered as a major hurdle for eggplant improvement by gene pyramiding through co-transformation (Kashyap et al. 2003). Several reporter genes such as *gus* (encoding β-glucuronidase; Rotino et al. 1992; Sunseri et al. 1993; Chen et al. 1995; Fari et al. 1995; Billings et al. 1997; Jelenkovic et al. 1998), *cat* (encoding chloramphenicol acetyl transferase; Rotino and Gleddie 1990) and *luc* (encoding luciferase; Komari 1989) have also been used for eggplant transformation (Table 2). Recently, we used a chimeric *gfp*:*gus* reporter gene (Fig. 3) to monitor transgene expression in an attempt to develop an efficient transformation system.

Table 2. Genetic transformation protocols for eggplant using reporter genes

Marker gene	Reporter gene	Remarks	Reference
*npt*II	–	First successful *Agrobacterium*-mediated genetic transformation	Guri and Sink (1988c)
*npt*II	*luc*	*Agrobacterium*-mediated transformation using callus derived from leaf explants	Komari (1989)
*npt*II	–	Transgenic plants obtained from cotyledon and leaf explants	Fillippone and Lurquin (1989)
*npt*II	*cat*	Protocol for transformation of leaf explants using binary vector	Rotino and Gleddie (1990)
*npt*II	*gus*	Transformation in wild species *S. integrifolium* using leaf explants	Rotino et al. (1992)
*npt*II	*gus*	Transformation, segregation of *npt*II gene in transgenics	Sunseri et al. (1993)
*npt*II	*gus*	Transformation of leaf and cotyledon explants; plant regeneration through somatic embryogenesis	Fári et al. (1995)
*npt*II	–	Transformed in *S. gilo*, a wild species; influence of explant type	Blay and Oakes (1996)
*npt*II, *hpt*	*gfp:gus*	Efficient transformation protocol by modulating *vir* gene induction	Kumar (2003)

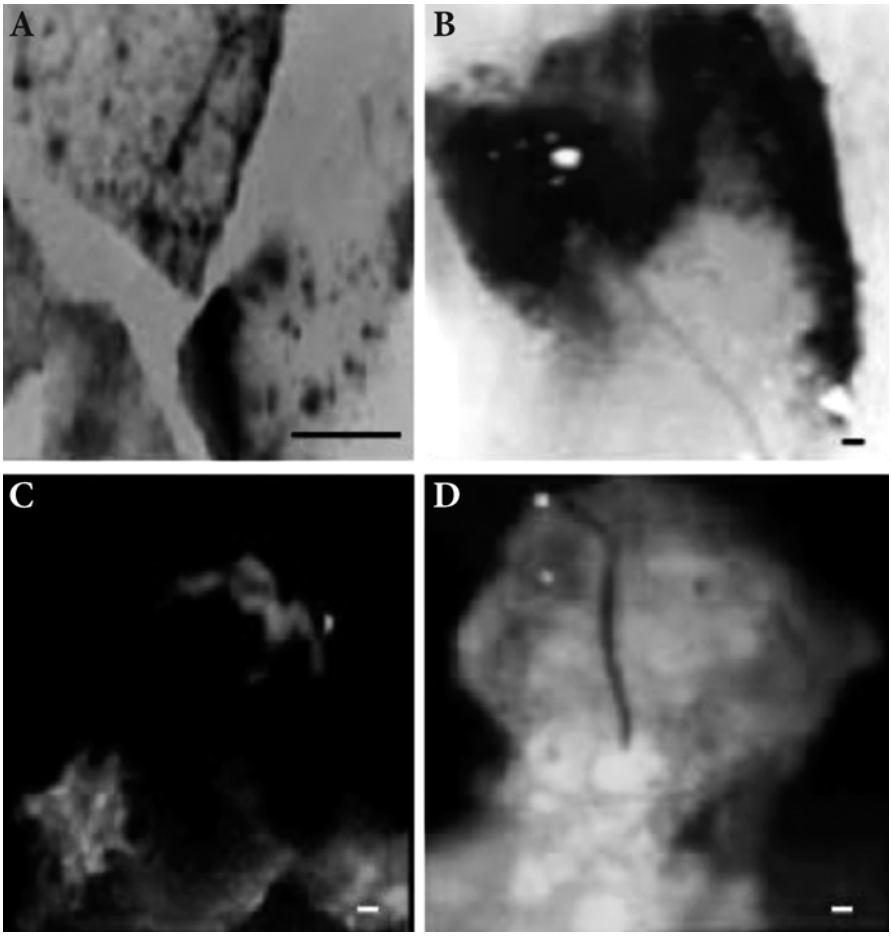

Fig. 3. Eggplant genetic transformation via *Agrobacterium tumefaciens*. **A** GUS reporter gene expression in transformed leaf explant (*bar* 1.5 cm) **B** GUS expression in a transformed shoot bud (*bar* 1.75 mm). **C, D** GFP expression in transformed shoot bud (*bar* 2 mm)

The influence of antibiotics and growth regulators on transformation efficiency has been investigated (Billings et al. 1997) and the protocols for eggplant transformation reported. However, comprehensive studies on the optimisation factors affecting eggplant transformation are limited. In view of this, we recently conducted a study showing that cotyledon explants were superior to leaf and hypocotyls for eggplant transformation (Kumar and Rajam, unpublished data). Transformation was also associated with other factors, such as bacterial density and selection procedure. The ability of eggplant explants to induce *Agrobacterium vir* genes was also studied and the result showed that eggplant was a poor inducer of these genes (Kumar 2003). The variability in transformation efficiency amongst the explants was correlated with their differential

vir gene induction potential. It was speculated that enhancement of *vir* gene induction during transformation could improve transformation efficiency.

3.2 Transgenic Eggplants with Improved Traits

Although genetic transformation of eggplant was demonstrated some time ago, the application of this technology for genetic improvement is still in its infancy. To date, only a few traits of agronomical importance have been introduced into cultivated eggplants. These traits include insect resistance, parthenocarpic fruit production and tolerance to salinity and drought stress (Table 3).

The major achievement in eggplant biotechnology is the development of insect-resistant transgenic plants by over-expressing the *Bacillus thuringiensis* (*Bt*) genes that encode the crystal protein endotoxin (Table 2). *Bt CryIIIb* was used to develop plants resistant to Colorado potato beetle (CPB; Rotino et al. 1992). Field trials showed that insect-resistant transgenic lines had a significantly higher yield (Arpaia et al. 1997; Acciarri et al. 2000). Introduction of a synthetic *Bt CryIAb* gene into eggplant was shown to confer protection against fruit borer, *Leucinodes orbonalis* (Kumar et al. 1998).

Another significant achievement is the development of transgenic eggplants with parthenocarpic fruits by manipulating the concentrations of endogenous auxin during fruit development. This was demonstrated by the production of transgenic eggplants expressing a chimeric *iaaM* gene from *Pseudomonas*

Table 3. Transgenic eggplants with improved agronomic traits

Transgene	Remarks	Reference(s)
Bt (Cry IIIB)	Resistance against insects	Chen et al. (1995)
Bt (Cry IIIB)	Modified *Bt* gene confers resistance to Colorado potato beetle (CPB)	Arpaia et al. (1997)
Bt (Cry IIIB	Insect resistance	Billings et al. (1997)
Bt (Cry IIIA)	Resistance to fruit borer	Hamilton et al. (1997)
DefH9-iaaM	Parthenocarpy in transgenic plants	Rotino et al. (1997), Donzella et al. (2000)
Bt (Cry III)	Resistance against insect pests in *S. integrifolium* and *S. melongena*	Iannacone et al.. (1997)
syn.Bt (Cry IAb)	Resistance against fruit borer *(Leucinodes orbonalis*)	Kumar et al. (1998)
syn.Bt (Cry IIIA)	Resistance to neonate larvae and adult CPB in T_0 and F1 population	Jelenkovic et al. (1998)
Bt (Cry IIIB)	Insect resistance; studied ecological impact assessment of transgenics	Acciarri et al.(2000)
Δ-9 desaturase	Increased 16:1, 18:1, and 16:3 fatty acids, resistance to *Verticillium* wilt	Xing and Chin (2000)
*mtl*D	Salinity, drought and chill tolerance	Prabhavathi et al. (2002)

syringae, which encodes tryptophan mono-oxygenase, under the control of an ovule-specific *DefH9* promoter from *Antirrhinum majus* (Rotino et al. 1997). In the absence of pollination, transgenic eggplants developed seedless parthenocarpic fruits in the absence of exogenous phytohormones, even at low temperatures which normally prohibit fruit production in untransformed lines (Rotino et al. 1997). When pollinated, the parthenocarpic plants produced seeded fruits. Trials in an unheated glasshouse showed significantly higher yields in transgenic plants than in untransformed controls and a commercial hybrid (Donzella et al. 2000).

Over-expression of a yeast Δ-9 desaturase gene in eggplant has been attempted with the objective of developing disease resistant plants. Transgenic plants were shown to contain higher concentrations of 16 : 1, 18 : 1 and 16 : 3 fatty acids and exhibited increased resistance to *Verticillium* wilt (Xing and Chin 2000). Transgenic plants challenged by *Verticillium* could also result in a marked increase in the content of 16 : 1 and 16 : 3 fatty acids. Results also showed that *cis-*Δ9 16 : 1 fatty acid was inhibitory to *Verticillium* growth. In our laboratory, we also generated transgenic eggplants resistant to fungal diseases by over-expression of pathogenesis-related genes, such as those for glucanase, chitinase and thaumatin (Rajam et al., unpublished data).

More recently, transgenic eggplant showing increased tolerance to osmotic stress induced by salinity, drought and chilling have been obtained by expression of a bacterial mannitol-l-phosphodehydrogenase (*mtlD*) gene (Prabhavathi et al. 2002), which is involved in the synthesis of the sugar alcohol (polyol) mannitol. Analysis of these transgenic plants under in vitro and in vivo growth conditions revealed increased tolerance to salt (200 mM NaCl),

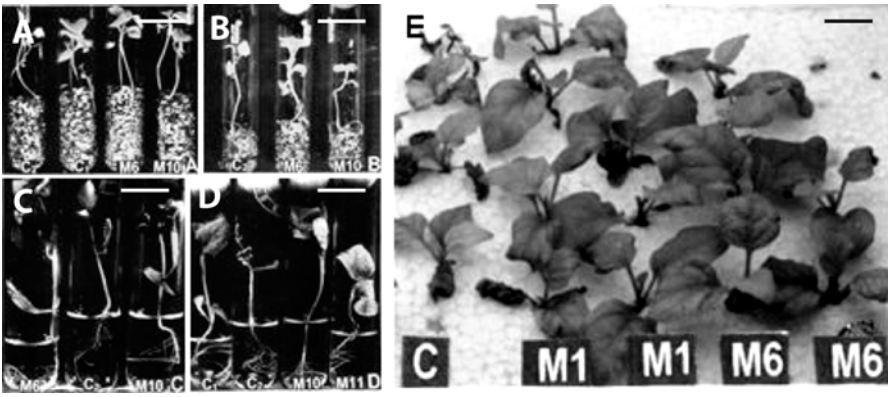

Fig. 4. Enhanced tolerance to salinity and drought by transgenic seedlings of eggplant expressing mannitol synthesis gene *mtl* D. Salt and drought stress induced by adding 200 mM NaCl and 10% polyethylene glycol solution to the test tubes containing the seedlings which are grown in 1 : 1 soil:vermiculite (**A**, **B**, *bar* 2.5 cm), or by including the stress agents in 10% MS liquid medium (**C**, **D**, *bar* = 2.0 cm) and a hydroponics system (salt stress; **E**, *bar* 2.0 cm). C, C_1 and C_2 represent the seedlings of untransformed control and M_1, M_6, M_{10} and M_{11} the seedlings of transgenic lines

drought (mediated by PEG 10%) and chilling (4–6 °C) stress (Fig. 4). Interestingly, *mtlD* transgenic eggplant was also resistant to wilt caused by *Verticillium* and *Fusarium* (Prabhavathi and Rajam, unpublished data). Furthermore, various transgenic eggplants over-expressing different genes encoding enzymes in the polyamine metabolic pathway have also been generated in our laboratory. These transgenic plants show increased tolerant to both biotic (fungal pathogens) and abiotic stress (salinity, drought, extreme temperature, heavy metals). A gene pyramiding approach has been added to our programs, in which the genes responsible for various agronomical important traits, including abiotic stress tolerance and fungal disease resistance, are being introduced in combination into eggplant through co-transformation.

4 Future Prospects

Eggplant has been proven to be amenable to in vitro manipulation for achieving plant regeneration and also for the development of transgenic plants with improved characteristics. Plant regeneration via SE and organogenesis has been reported in different genotypes. Protoplast culture and development of somatic hybrids and cybrids through protoplast fusion have generated lines resistant to several fungal and bacterial pathogens. Efficient protocols have been developed for haplodisation. This opens the possibility of exploitation of the dihaploid population to identify molecular markers linked to disease resistance, thereby facilitating the introgression of these traits into eggplant via marker-assisted selection. Androgenesis has also been helpful in the development of dihaploid plants from tetraploid somatic hybrids. The usefulness of the somatic hybrids in breeding programmes depends on their ability to be backcrossed to the recurrent genotype of eggplant. A major constraint in this area is the relatively low fertility of the somatic hybrids, as well as their dihaploids. *Agrobacterium*-mediated transformation of eggplant has been achieved and transgenic plants have been generated for insect resistance, production of parthenocarpic fruits and tolerance to abiotic stresses (Collonnier et al. 2001a; Kashyap et al. 2003). However, transgenic technology has yet to be used for the improvement of other agronomic traits, including disease and pest resistance, abiotic stress tolerance and the quality and shelf life of fruits. Gene pyramiding would be helpful in harnessing the benefits of agronomically important genes in an additive manner. Further efforts are to be taken to develop transgenic technology for quality improvement of eggplant by maximally utilising the capabilities of biotechnological tools, especially genetic engineering, coupled with conventional breeding programmes.

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III.4 Lettuce

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1 Morphology, Origin and Production of Cultivated Lettuce

Lettuce (*Lactuca sativa* L.) is an annual, self-fertile species in the family Asteraceae, with a chromosome complement of $2n = 2x = 18$. The plant is classified on the morphology of its fleshy leaves, with seven types being recognised by the International Code of Nomenclature for cultivated plants, namely: (1) Crisphead, Iceberg or Cabbage, (2) Butterhead, (3) Cos, (4) Leaf or Cutting, (5) Latin, (6) Stem or Asparagus and (7) the Oilseed Group (de Vries 1997; Ryder 1999). Crisphead varieties have compact, large heads of tightly folded leaves, while Butterhead types have crumpled, soft-textured leaves. Cos types are characterised by long, oval, dark upright green leaves, forming oblong heads or hearts. Leaf types, as the name implies, produce a rosette of loose leaves, while Latin cultivars (cvs.) are intermediate between the Butterhead and Cos types in forming loose heads with oval leaves. The leaves of stem-type cvs. are coarse and unpalatable, but the young fleshy stems are eaten after being cooked. Oilseed type lettuces progress rapidly through the rosette stage, bolt early and are probably primitive forms of *L. sativa*. Classification of lettuce is based on differences in leaf shape and size and less on pigmentation (Ryder 1999).

Whilst the centre of origin of lettuce is uncertain, the areas proposed include Egypt, the Mediterranean region, the Middle East and south-west Asia. Cultivated lettuce may have originated in south-west Asia from the area of the Euphrates and Tigris rivers, since most related *Lactuca* species are endemic to this region. Lettuce probably spread from south-west Asia to Egypt and then to Greece and Rome, with early lettuces being Cos and Leaf types. Evidence of lettuce cultivation in north-west Europe dates from the late 1400s, with its introduction to America a few years later, ultimately leading to the selection of Crisphead cvs. (de Vries 1997). In attempting to advance understanding of the lettuce genome, Frijters et al. (1997) constructed a bacterial artificial chromosome library containing large *Eco*R1 and *Hin*dIII genomic fragments, while Waycott et al. (1999) and Ryder et al. (1999) mapped genes for morphology to molecular markers. A molecular study that assessed variation at restriction fragment length polymorphism (RFLP) loci in 67 accessions of *L. sativa* and five related *Lactuca* species led Kesseli et al. (1991) to suggest

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a polyphyletic origin for *L. sativa*. Subsequently, Johnson et al. (2000) reported a molecular comparison of cultivated lettuce and its wild progenitor, *L. serriola*. Dziechciarková et al. (2004) reviewed aspects of protein and molecular marker technologies that have contributed to elucidating various aspects of the taxonomy, biodiversity, genetics and breeding in the genus *Lactuca*.

The main areas of production and consumption of lettuce are the United States and Europe. In the United States, more than 3.18×10^9 t are harvested each year, with excess of 70% of the United States production as an outdoor crop located in California (Ryder 1999). The amount harvested in the United States has an estimated value of U.S. $\$ 9.8\times10^8$ per annum (USDA 2006). Italy, Spain, France, The Netherlands and the United Kingdom are the main producers in Europe. Outdoor production of lettuce in Northern Europe is confined to the summer months, with the crop being cultivated under glass at other times. Additional regions of cultivation include Canada, Northern Mexico, South America, South Africa, the Middle East, Japan, China and south-east Australia. Globally, 19×10^6 t were produced in 2002 (FAOSTAT 2006). The market for lettuce has increased in the past two decades, accompanied in some cases by a change in the range of cvs. being grown, especially for processed mixed salad. Nutritionally, lettuce contains only moderate concentrations of phosphorus, iron, sodium, copper, ascorbic acid and vitamin A, ranking it about 26th in comparison to other fruits and vegetables in terms of its contribution to human diet. In the United States, it follows tomato and orange with respect to bulk consumption.

2 Genetic Improvement of Lettuce: Conventional Breeding and Biotechnological Approaches

The selection and domestication of wild *Lactuca* species has resulted in a decrease in latex content and bitter taste, the acquisition of a rosette habit with loss of prickles, an extended vegetative phase with head formation accompanied by increased succulence of leaves, and an increase in seed size with non-shattering properties. Breeding objectives for cultivated lettuce include modifying leaf shape and colour, manipulating head formation and delaying bolting. Other targets are the introduction of male sterility and resistance to insects, herbicides and diseases, in particular, downy mildew and lettuce mosaic virus (LMV; Ryder 2002), *Fusarium* wilt (Garibaldi et al. 2004), root rot (Tsuchiya et al. 2004) incited by *F. oxysporum* f. sp. *lactucae* and corky root disease (Dufresne et al. 2004; Mou and Bull 2004). The generation of plant material with resistance to dieback (Grube and Ryder 2003; Grube et al. 2005), drop caused by *Sclerotinia minor* (Grube and Ryder 2004) and downy mildew resulting from infection by *Bremia lactucae* (Jeuken and Lindhout 2002; Lebeda and Petrželová 2004) have also been identified as targets of breeding programmes. Improved leaf quality with reduced nitrate accumulation during winter culti-

vation under glass and extended shelf-life following harvest (de Vries 1997) are also relevant to this crop.

Conventional lettuce breeding through sexual hybridisation is hindered by floral structure and events during anthesis, which normally result in self-fertilisation. The stamens are fused to form an anther sheath onto the inside of which pollen collects as the flower opens. Pollen is shed onto the stigma as the style elongates and emerges from the anther sheath. However, cross-hybridisation may be achieved, for example, by removing anther sheaths prior to style emergence. The most practical method is to wash pollen from the stigma with a fine stream of water immediately after the style emerges from the anther sheath. Foreign donor pollen is then applied to the style (Ryder 1986). The gene pool from which desirable traits may be acquired is restricted, since cultivated lettuce is sexually compatible with only a limited number of the 100 or so species within the genus *Lactuca*. The three wild relatives, *L. saligna*, *L. serriola* and *L. virosa*, have been exploited most extensively in breeding programmes. *L. serriola* crosses freely with *L. sativa*. Some Asian and South African *Lactuca* species are potential gene donors, but studies on these wild relatives remain limited (Zohary 1991; de Vries 1997).

Important traits that have been introgressed from *L. virosa* into *L. sativa* include resistance to LMV, decreased susceptibility to tipburn, reduced bitterness and a more extensive root system. Other useful traits that have been identified include resistances to downy mildew and LMV from *L. serriola*, leaf aphid resistance (*L. virosa*) and cabbage lopper resistance (*L. saligna*; de Vries 1990). An emphasis of breeding programmes has been to identify and to introduce novel genes for resistance to viral and fungal pathogens (Witsenboer et al. 1995). The fungal pathogen *Bremia lactucae*, which thrives in the cool wet climate of Northern Europe, is the cause of downy mildew, the most destructive fungal disease of lettuce. Most lettuce breeding programmes also incorporate mildew resistance. LMV, transmitted by aphids, is the most destructive viral disease of lettuce (Revers et al. 1997). Aphids and whitefly cause significant damage to lettuce, in addition to acting as viral vectors. Jeuken and Lindhout (2004) developed backcross inbred lines (BILs) in which chromosome segments from the wild species, *L. saligna*, were introgressed into *L. sativa*, through four to five backcrosses and one generation of selfing, with marker-assisted selection commencing in the backcross fourth generation. BIL association mapping enabled the location to be determined of 12 morphological traits and amplified fragment length polymorphic (AFLP) markers. Such BILs will be essential in current and future genetic studies in *Lactuca*.

Weeds reduce crop production and quality in field-grown lettuce, with an estimated loss of U.S.$ 2.4×10^6 in Eastern Canada in 1992 (Anderson 1996). Undoubtedly, the use of herbicides has increased the efficiency of modern crop production and compounds are now available to control weeds in most crops. However, environmental and health issues, with the appearance of herbicide-resistant biotypes in weed species related to the sustained use of herbicides (Tonks and Westra 1997), have contributed to developing alterna-

tives to chemical herbicides, or at least to a reduction in their application. The generation of herbicide-resistant lettuce could reduce dependence on the chemical control of weeds. In this respect, conventional breeding has been used to transfer sulfonylurea resistance into cultivated lettuce from *L. serriola* (Mallory-Smith et al. 1990). However, exploitation of sexual hybridisation to transfer herbicide resistance is restricted because of the lack of resistance gene(s) in the gene pool, and the incompatibility of herbicide-resistant *Lactuca* species with cultivated lettuce. Protoplast fusion, which is discussed later, offers an alternative approach, the limiting factor being the availability of *Lactuca* species carrying suitable genes that are available for somatic hybridisation with *L. sativa*.

Product quality at harvest is important, particularly in relation to uniformity of size, yield, post-harvest shelf-life and nitrate content. Accumulation of nitrate in leafy vegetables is undesirable, since it produces nitrite in humans. Nitrite combines with haemoglobin to form methaemoglobin, the latter being incapable of binding oxygen. Nitrite may also react with secondary amines in the stomach, to generate carcinogenic nitrosamines which may induce gastric cancers (Gunes et al. 1995). Since the consumption of uncooked lettuce is considerably more than that of other leafy vegetables, efforts are being made to limit nitrate in this crop (Gaudreau et al. 1995; Santamaria 1997). Year-round cropping, uniformity of size and time to maturity are essential commercial targets. Quality and appearance govern saleability; immature heads lack firmness, while over-mature heads often have cracked, discoloured ribs and a bitter flavour. Prolonged post-harvest shelf-life is important because of the perishable nature of the crop, while salt tolerance is another character for introduction into lettuce to permit cropping on marginal lands. The generation of high-yielding varieties for glasshouse cultivation is relevant to breeders in Europe, especially where there may be a restricted amount of land for lettuce cultivation. In relation to maximising crop yield, Arkhipova et al. (2005) reported that inoculation of plants of the lettuce cv. Lollo Rosso with suspensions of the bacterium, *Bacillus subtilis*, increased plant shoot and root weight by approximately 30% over an 8-day period, with 10-fold increases in zeatin and its riboside in leaf tissues within 2 days of inoculation with the bacterium. Changes in abscisic acid and indole-3-acetic acid (IAA) also accounted for this increased growth.

Tissue culture-based technologies provide an adjunct to conventional breeding for the genetic improvement of lettuce. Importantly, *L. sativa* and its wild relatives are amenable to culture in the laboratory, with efficient shoot regeneration systems existing for cultured organs, tissues, cells and isolated protoplasts of several species. An important target has been to circumvent sexual incompatibility of cultivated lettuce with other *Lactuca* species, by somatic hybridisation involving the fusion of isolated protoplasts. Tissue culture has been exploited to expose somaclonal variation in lettuce, while the development of reliable transformation systems has permitted the introduction of potentially useful genes into this crop. Biochemical and molecular techniques

are being used to characterise the lettuce genome and to identify potentially useful genes, such as those for disease resistance.

3 Culture of Tissues and Isolated Protoplasts of Lettuce

The response of lettuce in culture enabled investigation of the effects of combinations of several growth regulators, media components and environmental parameters, such as light and temperature, on adventitious bud initiation from cultured seedling hypocotyls of the lettuce cv. Wayahead (Sasaki 1975, 1979a, b, c, 1982). The general responses of explants, as reported in the early studies, were reviewed by Michelmore and Eash (1988). Such investigations optimised shoot regeneration from different explants in several cvs., including Greenfields and Summer Gem (Crisphead types), Bronze Mignonette and Green Mignonette (Butterhead types), Salad Bowl (Leaf type) and Cos and Green Cos (Romaine types; Xinrun and Conner 1992). Ampomah-Dwamena et al. (1997) screened 22 lettuce genotypes belonging to different morphological groups for their shoot regeneration response on Schenk and Hildebrandt (SH) medium containing 3% (w/v) sucrose, 0.1 mg l^{-1} IAA, 0.5 mg l^{-1} kinetin and 0.05 mg l^{-1} zeatin (Schenk and Hildebrandt 1972). Reproducible shoot regeneration was achieved with Bambino and Iceberg (Crisphead types), Cobham Green and Sweet Butter (Butterhead types), Simpson Elite (Leaf type) and Rosalita and Paris White (Cos types). Although the composition of shoot regeneration media differs between studies, satisfactory regeneration can be achieved using a medium based on the formulation of Murashige and Skoog (MS) with 3% (w/v) sucrose, 0.04 mg l^{-1} α-naphthalene acetic acid (NAA), 0.5 mg l^{-1} benzylamino purine (BAP) and semi-solidified with 0.8% (w/v) agar at pH 5.8 (Murashige and Skoog 1962). An incubation temperature of $23 \pm 2\,^{\circ}C$ with a 16-h photoperiod (18 µ mol m^{-2} s^{-1}, daylight fluorescent tubes) is satisfactory.

In several studies, seedling cotyledons have been the source of explants because of their uniformity and their rapid shoot regeneration response. For example, cotyledons from 4-day-old seedlings were reported as being the preferred starting material for root and shoot initiation (Webb et al. 1984). Normally, shoot regeneration in cultured tissues of lettuce proceeds by organogenesis, although Zhou et al. (1992) induced somatic embryos on cotyledons cultured on MS-based medium with either 2.0 mg l^{-1} benzyladenine (BA; synonym BAP) and 0.2 mg l^{-1} NAA, or 0.2 mg l^{-1} BA and 2.0 mg l^{-1} NAA. Callus from an embryogenic line exhibited more peroxidase activity than a non-embryogenic culture.

Light is a parameter that is often not given adequate consideration in studies of shoot regeneration. However, Hunter and Burritt (2004) germinated seeds of the four lettuce cvs. Bambino, Greenway, Red Coral and Red Oak Leaf in the dark or under white, red or blue light, followed by culture of cotyledon explants

for 28 days on a shoot-inducing medium under white light. Germination of seeds in the light reduced the number of shoots regenerated, suggesting that light stimulates the competence for organogenesis from cotyledon explants. Shoot regeneration from explants taken from seedlings initially grown under white light was inhibited by blue light, while red light stimulated regeneration or had no effect on shoot production compared to controls. The authors suggested that phytochrome and cryptochrome can influence shoot regeneration from lettuce cotyledons and that blue light inhibits organogenesis, but only during a short developmental period.

Marked differences may occur in callus initiation and shoot regeneration from different lettuce cvs. (Xinrun and Conner 1992), while hyperhydricity may impair shoot regeneration in some cvs., such as Flora. A potential beneficial "nurse" effect was indicated of cell suspensions of *Nicotiana plumbaginifolia* in stimulating shoot regeneration from cultured cotyledons of the cv. Cobham Green, but these results were inconsistent (Michelmore et al. 1987). In general, investigations of plant regeneration from cultured cells have exploited batch cultures. However, Teng et al. (1993) studied the regeneration of plants from cells cultured in liquid medium in a 2-l bioreactor, focusing on parameters that influence cell growth and differentiation. Such parameters included foaming, the wall effect of the culture vessel, aeration and dissolved oxygen. Adhesion of cells of the inoculum to the walls of the bioreactor above the liquid meniscus accounted for a 10% loss of inoculum within 24 h of culture initiation. The wall effect resulted from foaming caused by the interaction of bubbles used to aerate the culture and the inoculum. Sieving the inoculum through a screen of pore size 400 μm, thorough washing of the inoculum with distilled water to remove single cells and debris and admitting air through a screen column with 150-μm pores prevented foaming and removed the wall effect. This resulted in shoot regeneration comparable to that from cells in batch culture in 125-ml flasks.

Takano et al. (1988) cultured shoot tips for the rapid multiplication of F_1 hybrid lettuce plants, while embryo rescue permitted the recovery of sexual hybrids between *L. sativa* and *L. virosa* (Maisonneuve et al. 1995). Protoplast-to-plant systems, as a basis for gene transfer by somatic hybridisation, have been developed for *L. sativa* and some wild *Lactuca* species, including *L. serriola*, *L. saligna* and *L. virosa* (Berry et al. 1982; Engler and Grogan 1984; Brown et al. 1986; Enomoto and Ohyama 1989; Tanaka et al. 1991; Webb et al. 1994). Details of the source material, the enzyme mixtures and the culture conditions employed in these investigations are given in the relevant publications.

4 Somaclonal Variant Plants Regenerated from Cultured Tissues and Protoplasts of Lettuce

Somaclonal variation, generally observed as morphological differences Lettuce Between individuals, is relatively common in plants regenerated from cultured

cells and tissues. In some cases, 30–40% of regenerated plants may exhibit at least one variant character, with up to 3% of the plants showing variation in a specific trait (Daub 1986). The phenomenon has been reviewed in detail (Karp 1995; Kaeppler et al. 2000), although the precise mechanisms involved are still not clear. Changes in chromosome complement, structure and DNA sequence have been observed (Choi et al. 2000). However, this phenomenon is probably related to several factors, including an increase in DNA methylation following the "shock" of introducing tissues into culture, chromosome breakage and rearrangement (Jain 2001), interference with the normal cell cycle and amplification of repetitive DNA sequences (Gyulai et al. 2003). Transposable elements may also be activated (Jain 2001) and changes induced by the action of growth regulators, especially 2,4-dichlorophenoxyacetic acid (2,4-D; Arun et al. 2003). Tissues from highly differentiated organs, such as stems, leaves and roots, often give plants with more somaclonal variation than explants with pre-existing meristems, such as shoot tips and axillary buds (Sahijram et al. 2003). In addition to simple phenotypic analyses, molecular techniques using RFLP markers, polymerase chain reaction (PCR)-based random amplification of polymorphic DNA (RAPD) and AFLP DNA technologies (Polanco and Ruiz 2002) have been developed to study somaclonal variation in tissue culture-derived plants (Rahman and Rajora 2001; Sahijram et al. 2003).

In lettuce, somaclonal variation has been reported as a result of the culture of tissues (Sibi 1976; Koevary et al. 1978; Berry et al. 1982; Alconero 1983; Brown et al. 1986) and isolated protoplasts (Engler and Grogan 1984). Brown et al. (1986) described a somaclone of the cv. Salad Bowl that was more vigorous with an early flowering habit and increased chlorophyll concentration compared to its parent. In addition, somaclonal variants were observed with reduced susceptibility to both LMV and *B. lactucae*. Engler and Grogan (1984) regenerated protoclones from mesophyll protoplasts of the cv. Climax. Mutant phenotypes exhibited variation in pigmentation, ranging from light green to yellow, with elongated and crinkled leaves, dwarfism and increased vigour. To date, most of the somaclonal variants reported in lettuce have exhibited some deleterious characteristics, such as reduction in vigour, pigmentation and fertility. However, this approach remains worthy of evaluation, since there remains the possibility of exposing genetically stable, agronomically useful characteristics.

5 Somatic Hybridisation of Lettuce

Protoplast fusion circumvents the pre- and post-zygotic barriers often associated with conventional sexual hybridisation, enabling the introgression of useful genetic traits into *L. sativa* from sexually incompatible *Lactuca* species. The precise details of protoplast isolation (Nishio et al. 1988), chemical and/or electrical fusion procedures and subsequent culture conditions employed to

generate somatic hybrid plants in the genus *Lactuca*, can be obtained by consulting the limited number of primary publications relating to this topic. More general background information, discussion of the relevance of protoplast fusion to generate novel somatic hybrid and cybrid plants, together with details of the culture conditions, are presented in recent reviews (Davey et al. 2000a, b, 2005a, b, c). Examples of somatic hybrids in lettuce include those between *L. sativa* and *L. perennis* or *L. tartarica* (Chupeau et al. 1994; Maisonneuve et al. 1995), *L. sativa* with *L. virosa* (Matsumoto 1991), *L. sativa* with *L. deblis* or *L. indica* (Mizutani et al. 1989) and *L. sativa* with *L. serriola* (Matsumoto 1987). Somatic hybrids of *L. sativa* (+) *L. tartarica* were found to be sensitive to climatic conditions (Maisonneuve et al. 1995). Although the hybrids grew vigorously to form a rosette, leaf development ceased, roots became stunted and leaves necrotic. However, modification of the environmental conditions permitted growth in some of these plants with limited seed production following pollination by donor cultivars. Resistance to downy mildew was shown to be transferred from *L. tartarica* into *L. sativa*, but fertility was low. Somatic hybrids of *L. sativa* (+) *L. virosa* were generated to transfer resistance to *Nasonovia ribis niger* (leaf aphid), *Erysiphe cichoraceanum* (powdery mildew) and *Pseudomonas cichorii* (bacterial rot) from the wild species into the cultivated crop. In an extension of their earlier investigations, Mizutani and Tanaka (2003) performed genetic analyses of isozymes in lettuce and the wild species *L. serriola*, *L. saligna*, *L. virosa* and *L. indica*. Enzyme polymorphism revealed that the somatic hybrid between lettuce and *L. indica* was composed of four genomes of *L. sativa* and two genomes of *L. indica*. These authors concluded that such polymorphisms are useful genetic markers in lettuce breeding. Unfortunately, the full potential application of somatic hybridisation technology to commercial breeding programmes has not been realised for lettuce.

6 Transformation for the Genetic Manipulation of Lettuce: Direct DNA Uptake into Protoplasts and *Agrobacterium*-mediated Gene Delivery

6.1 Introduction of Reporter and Selectable Marker Genes into Lettuce

Early investigations of transformation in lettuce focused upon the introduction of reporter and marker genes for selection of transformed cells in order to establish reliable transformation protocols for this crop. DNA uptake into isolated protoplasts was the procedure initially adopted. Protoplasts isolated from expanded leaves of lettuce were electroporated in the presence of either the plasmid CAMV CAT, carrying the chloramphenicol acetyltransferase (*cat*) gene driven by the cauliflower mosaic virus (CaMV) 35S RNA promoter, or pABD1 with the neomycin phosphotransferase (*npt*II) gene (Chupeau et al. 1989). The *cat* gene was employed to demonstrate transient gene expression in

this experimental system. The selection of kanamycin-resistant plants following transformation with pABD1 and subsequent analysis of their seed progeny showed that resistance to kanamycin was inherited as a dominant Mendelian trait. Integration of foreign DNA into the genomic DNA of antibiotic-resistant plants was confirmed by DNA–DNA hybridisation. In general, few lettuce genotypes have been transformed using this procedure and the use of direct DNA uptake into lettuce protoplasts has been superceded by less labour-intensive and more reliable *Agrobacterium*-based transformation systems.

Exploitation of the natural ability of the Gram-negative soil bacteria, *A. tumefaciens* and *A. rhizogenes*, to genetically engineer plant cells provided the major approach for gene transfer into a range of crop plants of both dicotyledons and, more recently, monocotyledons including cereals. Background information relating to the molecular basis of *Agrobacterium*-mediated gene transfer into plants, the construction of chimaeric genes and the development of vectors for gene introduction into target plant species is summarised in excellent detailed reviews (Zupan and Zambryski 1995; Tinland 1996; Newell 2000; Cheng et al. 2004).

Agrobacterium-mediated gene transfer into lettuce was achieved initially by Michelmore et al. (1987) using wild-type octopine (ACH5) and nopaline (C58) strains of *A. tumefaciens*. Galls were induced by inoculating with agrobacteria explants of cotyledons from 4-day-old seedlings of the Butterhead cv. Cobham Green. The unusual amino acids octopine or nopaline, characteristic of crown galls incited by *A. tumefaciens* strains ACH5 and C58, respectively, were synthesised by the resulting tumours. The same workers also used an engineered strain, GV3111, of *A. tumefaciens* harbouring the cointegrate vectors pTiB6S3, pMON120 or pMON200, or the binary vector pMON505. Plasmid MON200 carried the chimaeric neomycin phosphotransferase (*npt*II) gene with a *nos* promoter and terminator (*nos.npt*II.*nos*). Explants transformed by GV3111 with either pMON200 or pMON505 produced callus on medium supplemented with 50 mg l^{-1} kanamycin sulfate; shoots were regenerated from transformation experiments using the disarmed pTiB6S3. These pioneering transformation studies provided the initial evidence that the lettuce genome could be manipulated using *A. tumefaciens*-mediated gene delivery. However, at that time, a reliable transformation system was not available for economically important Crisphead cvs.

Other workers transformed the lettuce cv. Kayser using *A. tumefaciens* strain LBA4404 harbouring the binary vector pTRA415 (Enomoto et al. 1990). The vector carried the *npt*II gene, with the constitutive CaMV 35S promoter, and the *β*-glucuronidase (*gus*) reporter gene. The latter was driven by either the CaMV 35S promoter, or by the stress or salicylic acid-inducible pathogenesis-related (PR) 1a protein-encoding gene promoter from tobacco. GUS activity increased 3–50 fold in PR-*gus* transformed plants, indicating that the transgene was expressed normally under the regulated control of the PR 1a promoter. Again, cotyledon explants excised from young seedlings were the source material for inoculation by *Agrobacterium*.

Several workers, in addition to Michelmore et al. (1987), exploited vectors constructed by the agrochemical company Monsanto in early studies of lettuce transformation. For example, the cv. Lake Nyah was transformed by inoculating cotyledons from axenically grown seedlings with *A. tumefaciens* carrying the cointegrate vector pMON200 (Webb 1992). Transgenic plants of the lettuce cv. South Bay were generated using *A. tumefaciens* strain A208 carrying an engineered Ti plasmid, pTiT37SE, or the binary vectors pMON9749 or pMON9793 (Torres et al. 1993). Transformed plants were selected on culture medium containing 50–100 mg l^{-1} kanamycin sulfate. Histochemical studies for GUS activity demonstrated expression of the *gus* gene in transgenic plants, with PCR and Southern hybridisation analyses confirming integration of the transgene into the genome of transgenic plants.

Initially, Crisphead cvs. of lettuce were difficult to transform. Importantly, Curtis et al. (1994a, 1995) developed a reliable, genotype-independent protocol for *A. tumefaciens*-mediated transformation of 13 lettuce cvs., including Crisphead cvs. Cotyledons excised from 7-day-old seedlings were inoculated with *A. tumefaciens* strain 0065 or 1065 with the binary vector pMOG23 derived from pBIN19. The binary vector carried the chimeric *nos.npt*II.*nos* gene and a CaMV 35S *gus*-intron reporter gene on the T-DNA. Strain 1065 also harboured pTOK47 with extra copies of the Ti plasmid virulence genes *virB*, *virC* and *virG*, conferring supervirulent properties on the bacterium. This supervirulent bacterial phenotype was essential for transforming some cvs. such as Reflex (Curtis et al. 1994a). In general, a 1 : 10 (v : v) dilution of an overnight bacterial suspension yielded more kanamycin-resistant shoots compared to a suspension diluted 1 : 1 with liquid MS-based medium lacking growth regulators, or an undiluted bacterial inoculum, especially when cotyledons were inoculated with strain 0065. These results confirmed that the bacterial density in the inoculum was important with respect to transformation, as suggested earlier by Michelmore et al. (1987). Minor modifications of the original protocol, together with detailed experimental notes relating to the technique, were presented by Davey et al. (2001). *Agrobacterium*-mediated gene delivery to genetically transform lettuce is now routine in many laboratories and is based upon these published, well established methods. Indeed, the ease of transforming lettuce with *Agrobacterium* was emphasised by Wroblewski et al. (2005), who used *L. sativa* cvs. Valmaine and Mariska and the wild lettuce *L. serriola* (LS102) in transient *gus* expression assays. The laboratory strain C58C1 of *A. tumefaciens*, which has been used routinely in many laboratories for at least two decades to effect gene transfer into target plants, was found to be the most reliable strain for delivering genes to lettuce.

7 Introduction of Agronomically Important Genes into Lettuce

Efforts have been made to introduce specific characteristics into lettuce using recombinant DNA-transformation technology, some of the results of which

are discussed in the following sections. Since weeds and viruses cause severe crop losses, it is not surprising that they have been prime targets for genetic manipulation in this leafy vegetable.

7.1 Induction of Male Sterility

Male sterile plants are important as pollen recipients in the production of hybrid plants by sexual hybridisation. In experiments to induce male sterility in lettuce, a pathogenesis-related β-1,3-glucanase gene linked to a tapetum-specific promoter, A9, was cloned into the binary vector pBIN19 and the latter introduced into *A. tumefaciens* carrying pGV2260, prior to transformation of the lettuce cv. Lake Nyah (Curtis et al. 1996b). Transgene expression resulted in dissolution of the callose wall of developing microspores, leading to male sterility in all the transgenic plants. In contrast, plants regenerated from uninoculated cotyledons of seedlings were fertile, since their microspores developed the normal elaborate surface ornamentation, as revealed by scanning electron microscopy. This approach, albeit with other transgenes, may be useful in generating male sterile plants for hybrid seed production in lettuce, eliminating the necessity to remove pollen from the stigmatic surface of recipient plants to avoid self-pollination prior to application of donor pollen.

7.2 Herbicide Tolerance and Virus Resistance

Herbicide resistance has been introduced into lettuce not only to generate resistant plants per se, but also to exploit this trait to investigate gene expression associated with plant development. As weeds compete with field-grown lettuce (Anderson 1996), the introduction into this crop of resistance to broad-spectrum herbicides will simplify weed management. Bialaphos, a tripeptide antibiotic synthesised by the Gram-positive soil bacterium *Streptomyces hygroscopicus*, is a non-selective herbicide, with phosphinothricin (PPT), an analogue of L-glutamic acid and two L-alanine residues, being the active moiety. *Streptomyces*, through expression of its *bar* gene encoding bialaphos resistance, has evolved mechanisms to neutralise the toxicity of its own products. PPT, synthesised chemically as glufosinate ammonium, is an irreversible inhibitor of glutamine synthetase, the enzyme that detoxifies ammonia produced during nitrate reduction, photorespiration and amino acid degradation in plant cells. Application of PPT to plants results in the accumulation of ammonia to phytotoxic concentrations, inducing death of non-transformed tissues.

The *bar* gene was introduced into the lettuce cv. Evola by *Agrobacterium*-mediated transformation of seedling cotyledons using *A. tumefaciens* strains 0310 and 1310 (Mohapatra et al. 1999). The latter strain carried the hypervirulent pTOK47 in addition to the binary vector on which the *npt*II and *bar* genes were cloned. Primary transformed shoots were selected on regeneration medium supplemented with kanamycin sulfate. Interestingly, the hyperviru-

lent pTOK47 in strain 1310 resulted in multiple insertions of T-DNA into some regenerated plants, whereas strain 0310 gave single gene inserts in all plants analysed by DNA–DNA hybridisation. Axenic seedlings grew on medium supplemented with glufosinate ammonium at 5 mg l^{-1}, while glasshouse-grown plants were resistant to the herbicide when the latter was administered as a spray at 300 mg l^{-1}. Stable expression of the *bar* gene was observed in the second seed generation. The *npt*II and *bar* genes segregated in a Mendelian fashion in some plant lines in the first seed generation, with herbicide resistance segregating in the expected ratio in the second generation in most transgenic plants. This investigation confirmed that herbicide resistance can be introduced into lettuce and that its expression is stable in subsequent seed generations. Although sexual hybridisation has been used to transfer sulfonylurea resistance into cultivated lettuce from the wild species, *L. serriola* (Mallory-Smith et al. 1990), sexual incompatibility and lack of availability of herbicide resistance in the gene pool restricts further introgression of herbicide tolerance to transformation-based approaches.

Virus resistance has been introduced into lettuce using a coat protein-mediated approach. Pang et al. (1996) transferred the nucleocapsid (N) protein gene of the lettuce tospovirus, tomato spotted wilt virus (TSWV), into two lettuce breeding lines developed by the Asgrow Seed Company. Transgenic plants, expressing the N protein gene, were protected against TSWV. In studies with lettuce infectious yellow virus (LIYV), plants of both lettuce and tobacco were transformed for resistance to this virus using the same constructs (Falk 1996). However, although LIYV resistance was detected readily in transgenic tobacco following analysis of a limited number of plants, LIYV resistance was not observed in transgenic lettuce plants, in spite of numerous attempts to detect gene expression. Other workers have performed similar experiments in attempts to generate virus resistant plants. For example, Dinant et al. (1993) introduced a LMV coat protein (LMV-CP) gene into LMV-susceptible lettuce. However, the transgene gave only poor resistance to this virus, although its expression conferred LMV resistance, with heterologous resistance to potato virus Y, in tobacco. Loss of virus resistance was more pronounced in lettuce during subsequent seed generations, an observation made by others. In this respect, Gilberton (1996) provided evidence that 39 first seed generation (T1) lettuce plants transformed by *Agrobacterium* carrying either a LMV-CP gene, or an untranslatable LMV-CP gene between the T-DNA borders, were resistant to LMV when inoculated mechanically with the virus but, subsequently, only eight of the 39 LMV-resistant first seed generation (T1) plants produced LMV-resistant T2 seed progeny after selfing. Dinant et al. (1997) also introduced the coat protein gene from LMV strain O (LMV-O) into the virus-susceptible lettuce cvs. Girelle, Jessy and Cocarde. Several transformed plants accumulated detectable concentrations of LMV coat protein. As in other examples of potyvirus sequence-mediated protection, some plants exhibited complete resistance, but in others this resistance was not sustained, leading to later development of symptoms of virus infection. The efficiency of this strategy to

induce LMV resistance was believed to be related to the developmental stage of the transgenic plants at the time of their inoculation with the virus.

A novel application for explant-derived shoots and in vitro-grown seedlings has been the development of a simple and efficient procedure to assess the resistance of lettuce cvs. to LMV. Excellent correlation was found between those lettuce cvs. already known to be LMV-resistant and their resistance following virus inoculation of in vitro grown material (Mazier et al. 2004). Since LMV is one of the most destructive viruses of lettuce and endive globally (Dinant and Lot 1992), this simple approach with its advantages of reduced space requirements and improved environmental safety, especially in the handling of recombinant viruses and genetically engineered plants, should prove attractive to researchers and breeders world-wide.

Studies were performed involving the cloning of genes for resistance to downy mildew (*Dm*), in attempts to understand the molecular basis for resistance to *B. lactucae* in lettuce (Okubara et al. 1997). These investigations, involving the generation of 192 primary transformed plants of the lettuce cv. Diana using *A. tumefaciens*-carrying constructs with the maize *Ac* transposase and *Ds*, showed that several *Dm* genes are located in four clusters in the lettuce genome, with *Dm3* being in the largest cluster containing at least nine genes. More information relating to *Dm* genes is discussed by Jeuken and Lindhout (2002).

7.3 Modification of Plant Morphology and Physiology for Drought and Cold Tolerance

The lettuce cv. Lake Nyah was the target for the *rolAB* genes from *A. rhizogenes* (Curtis et al. 1996a). Transgenic, kanamycin-resistant plants exhibited more extensive root development than their non-transformed counterparts. Leaf explants from the transgenic plants were also more responsive in terms of root and callus production and in their increased response to auxin supplied exogenously in the culture medium. The authors suggested that such transgenic plants may be more drought-tolerant, while expression of the *rolAB* genes may increase the endogenous concentration of auxin which, in turn, could reduce the incidence of leaf russett spotting in these plants. The development of crops capable of high yields under drought, salinity and cold conditions was the focus of investigations by Pileggi et al. (2001). These authors improved previously reported transformation procedures for lettuce using the cv. Grand Rapids with a mutated P5CS gene for delta 1-pyrroline-5-carboxylate synthase, a bifunctinal enzyme that catalyses two steps of proline biosynthesis in plants, the mutated gene being insensitive to feedback inhibition by proline. These authors concluded that increased concentrations of proline act like an osmoprotectant that could confer resistance to drought, salinity and cold on transgenic plants. Transgenic lettuce plants that were obtained in these studies were resistant to freezing.

Abdel-Kader (2001) studied drought-induced stress in the cvs. Longifolia-Baladi and Crispa-Mignonette, with drought inducing lipid peroxidation and catalase activity, but decreasing the protein content and peroxidase and esterase activities. Treatment of plants with gibberellic acid alleviated the effects of drought. Other authors have targeted drought and cold tolerance in lettuce. For example, Vanjildorj et al. (2005) over-expressed the *Arabidopsis thaliana ABF3* gene, which encodes a transcription factor for the expression of abscisic acid responsive genes, in the lettuce cv. Chongchima. Transgenic plants were morphologically normal, set seed and exhibited greater tolerance than wild-type plants to drought and cold stress.

7.4 Disease Resistance, Taste and Vitamin Content

Whilst most studies have focused on the transformation of *L. sativa*, Kisiel et al. (1995) used the wild-type *A. rhizogenes* strain LBA9402 to transform leaf explants from axenic seedlings of *L. virosa*. The latter species was the target since it is used as a traditional medicine because of its analgesic and sedative properties. Such properties are attributed to the presence of sesquiterpene lactones, which accumulate mainly in the latex of roots and aerial parts of the plant. Transformed roots were induced and cultured to maximise biomass production and to facilitate analysis of secondary products. Eight sesquiterpene lactones were isolated from transformed roots.

The cloning of genes from lettuce has been instigated with the aim of modifying the sesquiterpene lactone profile of this plant (Bennett et al. 2002). In lettuce, these compounds include components of latex, such as lactucin, and the induced phytoalexin, lettucenin A. Thus, sesquiterpene lactones represent excellent targets for genetic manipulation to enhance phytoalexin response for disease resistance and to change the profile of these latex-expressed compounds to enhance resistance to insect pests and to reduce bitterness. The germacrene A synthases, *LTC1* and *LTC2*, are prime targets for modified expression by sense and anti-sense technologies in attempts to change these profiles.

Other workers have focussed attention on reducing the bitterness of cultivated lettuce. *Richadella dulcifica*, a shrub native to tropical West Africa, synthesises the sweet and taste-modifying protein, miraculin. In a novel series of experiments, Sun et al. (2006) cloned the miraculin gene from the pulp of berries of *Richadella* and introduced the gene driven by the CaMV 35S promoter into the lettuce cv. Kaiser through transformation with *A. tumefaciens* GV2260. Expression of the gene in transgenic plants resulted in the accumulation of significant concentrations of the sweet-enhancing protein. As miraculin acts at extremely low concentrations, it is acceptable as a sweetener for diabetics. It will be important, in future experiments, to determine the effects of other sweet or taste-modifying proteins in lettuce, such as thamatin, monellin, mabinlin, pentadin, brazzein and curculin, since genes for these proteins have been cloned and sequenced and their expression already monitored in other plants (Faus 2000).

Vitamin E includes a group of compounds, the tocopherols, that are lipid-soluble antioxidants, with tocopherol existing in the α, β, γ and δ isoforms. The relative vitamin E potencies of these isoforms are 100%, 50%, 10% and 3%, respectively. In experiments to improve the tocopherol composition of lettuce, Cho et al. (2005) expressed a cDNA encoding γ-tocopherol methyltransferase from *A. thaliana* in the lettuce cv. Chungchima. Stable inheritance and expression of the transgene in transformed plants increased enzyme activity and conversion of γ-tocopherol to the more potent α form. As Cho et al. (2005) emphasised, conversion of γ-tocopherol to α-tocopherol in major food crops could increase their value and importance in human health, since vitamin E reduces the risk of several serious disorders, including cardiovascular disease and cancer, in addition to slowing ageing and enhancing the function of the immune system.

7.5 Accumulation of the Trace Elements Iron and Zinc in Lettuce

The iron storage protein, ferritin, which is responsible for sequestering intracellular iron present within plants, has been a target for genetic manipulation in lettuce (Goto et al. 2000). In tobacco, over-production of ferritin reduced oxidative stress and improved tolerance to pathogens by sequestering free iron which would normally produce iron-mediated Fenton oxidants, such as hydroxyl radicals (OH; Deak et al. 1998). Transgenic lettuce plants were generated with enhanced iron accumulation and increased growth (Goto et al. 2000). In these studies, the binary plasmid BG1, carrying the CaMV 35S promoter driving the soybean ferritin cDNA with the nptII gene as a selectable marker, was transferred into *A. tumefaciens* strain LBA4404. Lettuce plants transformed with this construct accumulated 1.2- to 1.7-fold more iron than wild-type plants. Transgenic plants also showed enhanced growth rates, attributed to a reduction of iron-mediated Fenton oxidants, such as hydroxyl radicals.

The accumulation of other elements has been investigated in lettuce. Zuo et al. (2002) expressed a mouse metallothionein mutant β-cDNA in the lettuce cv. Salinas 88 following *Agrobacterium*-mediated gene delivery. The concentration of zinc in transgenic plants increased to 400 $\mu g\, g^{-1}$ dry weight, this being considerably greater than in wild-type plants. Since zinc is an essential element with a multiple role in human nutrition, its deficiency severely impairs organ function, emphasising the importance of this research in terms of health. However, it will be interesting to observe consumer reaction to the expression of this animal gene in a leafy vegetable.

7.6 Nitrate Accumulation by Lettuce

Nitrate is the major source of nitrogen for higher plants, with nitrate reductase catalysing the first step in its assimilation. Lettuce often accumulates concen-

trations of nitrate that exceed the maximum concentrations permitted by the European Community. Consequently, nitrate concentration is one parameter that governs the marketability of the crop in Europe, especially in winter when plants accumulate nitrate because of low light conditions. Reduction of nitrate accumulation in leaves of lettuce has been a target for genetic manipulation. Curtis et al. (1999) introduced the nia2 cDNA for nitrate reductase from tobacco driven by the 35S promoter into the lettuce cv. Evola. However, none of the transgenic plants showed any reduction in nitrate content compared to wild-type plants at harvest, although plants with nitrate concentrations slightly less than those of wild-type plants were observed at the mid-stage of cultivation. It was proposed that the lack of reduction of nitrate in leaves of lettuce heads at maturity might be related to a decline in activity of the 35S promoter.

In order to attempt to explain the results of Curtis et al. (1999), Dubois et al. (2005) reinvestigated nitrate accumulation in the lettuce cv. Jessy, using a similar 35S::nia2 construct carried on a binary vector in *A. tumefaciens*. In a population of 50 transgenic plants, none showed reduced nitrate concentration compared to wild-type plants, in spite of nitrate reductase activity from the transgene. However, 28% of the transgenic plants had bleached leaves similar to the phenotype of tobacco and potato plants transformed with the same gene. Dubois et al. (2005) showed that silencing directed against the nitrate reductase mRNA from the transgene occurred early in plant development and, in some plants, this transgene-specific silencing extended to the homologous endogenous nitrate reductase mRNA. Nitrate reductase mRNA in lettuce is under strict expression control. Clearly, this approach to reduce nitrate accumulation in lettuce has limitations because of transgene silencing resulting in chlorosis and eventual death of the transgenic plants.

7.7 Senescence in Lettuce: Prolonging Shelf-Life

Lettuce is a leafy vegetable that undergoes rapid leaf deterioration after harvesting, resulting in a limited shelf-life when it is sold as a salad crop. Consequently, attempts have been made to delay this senescence process. For example, the *T-cyt* gene, also known as the *ipt*, *tmr* or gene 4, from the T-DNA of *A. tumefaciens*, was introduced on the T-DNA of the binary vector pMOG23 into the lettuce cv. Saladin (Curtis et al. 1999). The gene codes for isopentenyl phosphotransferase, which is involved in cytokinin biosynthesis in plant cells transformed to tumorous growth during the induction of crown gall disease. The *Agrobacterium* strain used for transformation also carried pTOK47 for supervirulence. Interestingly, kanamycin-resistant shoots were initiated from inoculated cotyledon explants only when sites were deleted within the promoter of the *T-cyt* gene, probably because *T-cyt* over-expression with the intact promoter was phytotoxic to target plant tissues. Kanamycin-resistant shoots grown in vitro exhibited several phenotypes, such as gall production, dwarfism and hyperhydricity (vitrification). However, rooted kanamycin-resistant plants

recovered from their abnormal phenotypes after transfer to the glasshouse and could be self-pollinated to set viable seed. Transgenic plants exhibited increased cytokinin and chlorophyll contents in their leaves compared to non-transformed plants. These results suggested that the introduction of the *ipt* gene into lettuce could provide an approach to delay senescence in this leafy vegetable, possibly reducing the necessity for elaborate and costly post-harvest controlled environmental conditions to prolong shelf-life in harvested plants.

In later work, the *ipt* gene under the control of the senescence-specific promoter SAG12 from *A. thaliana* (P_{SAG12}-*IPT*) was introduced into the lettuce cv. Evola, with plants homozygous for the transgene exhibiting significantly delayed post-harvest leaf senescence (McCabe et al. 2001). The transgene was activated only during senescence, particularly when senescence commenced in the outer (lower) leaves, initiating cytokinin biosynthesis. Subsequently, the latter inhibited leaf senescence, simultaneously attenuating activity of the P_{SAG12}-*IPT* gene, preventing the overproduction of cytokinin. Heads harvested from transgenic plants retained chlorophyll for up to 7 days longer in their outer leaves, compared to leaves of heads from non-transformed plants. Apart from retardation of leaf senescence, mature 60-day-old plants were morphologically normal, with no significant differences in head diameter or fresh weight of leaves or roots.

Since ethylene is a plant hormone known to influence many developmental processes, including fruit ripening and senescence, Kim and Botella (2004) studied the effects of this gas on leaf senescence in lettuce. Cotyledons from 6-day-old seedlings of the cvs. LE126 and Seagreen were transformed by *A. tumefaciens* carrying a 35S-*gus* construct, or a construct with the ethylene mutant receptor *etr1-1* under the SAG12 promoter, this senescence-specific promoter being the same as used by McCabe et al. (2001). Expression of the *etr1-1* gene had a dramatic effect on shoot regeneration from cultured cotyledon explants, with root formation from 34% of the explants and a delay in callus and shoot initiation. A limited number of explants (2.86%) eventually regenerated shoots. Such results confirmed that ethylene insensitivity conferred by *etr1-1* modifies the normal regeneration pattern in lettuce.

Transgenic P_{SAG12}-*IPT* lettuce plants generated by McCabe et al. (2001) were the experimental material used to compare the metabolites synthesised by these transformed plants with the metabolites synthesised by non-transformed plants, using ionisation-mass spectrometry (APCI-MS) and linked gas chromatography (GC-APCI/EI-MS; Garratt et al. 2005). During post-harvest storage, heads of transgenic plants showed a 3-fold increase in the concentrations of acetaldehyde, ethanol and dimethyl sulfide. Increase in the latter compound was paralleled by an accumulation of reactive oxygen species. These investigations will form the basis for future studies of the physiology of these transgenic plants. Such studies also emphasise the importance of detailed metabolite profiling of plants following transgene insertion, since the integration of a specific gene to modify one or more specific traits my also affect other biosynthetic pathways. Investigations have also been reported that provide background

knowledge for further experiments on oxidative stress and antioxidant defence systems in lettuce (Abdel–Kader 2001).

7.8 Plant Proteinase Inhibitors in Lettuce

Plant proteinase inhibitors that act on animal or microbial proteases play a role in inhibiting proteolytic enzymes from pests and pathogens. In order to investigate the role of the proteinase inhibitor II, *SaPIN2a* from *Solanum americanum*, Xu et al. (2004) generated transgenic lettuce plants with *SaPIN2a* driven by the CaMV 35S promoter. These authors found that heterogeneously expressed *SaPIN2a* inhibited endogenous protease activity in transgenic plants, indicating that *SaPIN2a* regulates proteolysis. This gene could be exploited to protect foreign protein production in transgenic plants.

7.9 Synthesis of Recombinant Antibodies in Lettuce

Experiments have been conducted to develop a simple system to produce recombinant antibodies in lettuce (Negrouk et al. 2005). Importantly, the technology exploits commercially available lettuce, negating the requirement for plant growth facilities and minimising costs. In the protocol reported, mature harvested heads were dipped in a suspension of *A. tumefaciens* strain C58C1 carrying the appropriate construct, prior to vacuum infiltration. The heads were rinsed in water and incubated on wet paper in closed transparent boxes at 20−26 °C, with a 16-h photoperiod, for 3–4 days. Subsequently, the leaves were homogenised for protein extraction to produce 20−80 mg of functional antibody per kilogram of fresh lettuce leaf tissue in less than 7 days. Humanised IgG1 k anti-tissue factor antibody (hOAT) was one of the antibodies produced using this approach. This simple procedure should be amenable to scale-up for a range of lettuce cvs. and products.

8 Inactivation of Gene Expression in Transgenic Lettuce

Gene insertion by *Agrobacterium*-mediated transformation is reproducible and is the procedure of choice in lettuce, but transgene expression may be inconsistent (Davey et al. 2001). Consequently, the choice of promoters is crucial for stable gene expression. Additionally, gene copy number and the extent of DNA methylation influence transgene expression in this leafy vegetable, as in other plants (Finegan and McElroy 1994).

In an evaluation of gene expression in the lettuce cv. Evola, plants were transformed with the CaMV 35S-*bar* gene, but selected by their kanamycin resistance through expression of the *npt*II gene on the same T-DNA as the *bar* gene. Sixteen percent of T1 plants, 22% of T2 plants and 11% of T3 plants

transformed with the CaMV 35S-*bar* gene were resistant to glufosinate ammonium at 300 mg l^{-1} compared with 63% of T1, 83% of T2 plants and 99% of T3 plants transformed with a *petE-bar* construct in which the *bar* gene was driven by the –784 plastocyanin promoter from pea (McCabe et al. 1999b). Interestingly, NPTII protein, as determined by ELISA, was absent in 29% of the herbicide-resistant *petE-bar* T2 lines, indicating inactivation of the CaMV 35S promoter on the same T-DNA as an active petE promoter. Southern hybridisation confirmed the presence of the transgenes in kanamycin-resistant plants.

Other studies also used the constitutive CaMV 35S promoter to express transgenes in lettuce (Enomoto et al. 1990; Curtis et al. 1994a, b; Pang et al. 1996; McCabe et al. 1999a), with some studies utilising the promoters *nos* (Michelmore et al. 1987; Yang et al. 1993; Curtis et al. 1994a, 1996a, b; McCabe et al. 1999a), *Mac* (Curtis et al. 1994b), *petE* (McCabe et al. 1999b) and ACT1 (McCabe et al., unpublished data). Use of the tobacco pathogenesis-related protein gene promoter PR1a resulted in *gus* gene expression following induction by salicylic acid (Enomoto et al. 1990). In other investigations, the *Mas* promoter resulted in root-specific expression (Curtis et al. 1994b). A tapetum-specific promoter, A9, was used in the induction of male sterility in transgenic lettuce (Curtis et al. 1996b). A 48-fold increase in zeatin riboside equivalents was detected in primary leaves of 60-day-old lettuce plants of the cv. Evola transformed with the *ipt* gene from *A. tumefaciens* linked to the senescence-specific promoter, P_{SAG12} from *A. thaliana* (McCabe et al., unpublished data).

In reviewing the literature, it is clear that the effects of transgene copy number on expression in lettuce and in other plants is often ambiguous and sometimes contradictory, even in the same target plants (Hobbs et al. 1993). T-DNA integration into lettuce is often complex (Michelmore et al. 1987; McCabe et al. 1999a). In an investigation of the integration, expression and inheritance of a CaMV 35S-*gus*-intron gene and a *nos.npt*II.*nos* gene on the same T-DNA in the lettuce cv. Raisa, McCabe et al. (1999a) reported that there was no clear correlation between gene copy number and transgene expression. T-DNA integration into the lettuce genome was complex following gene delivery by the supervirulent *A. tumefaciens* strain 1065. Truncation of the right side of the T-DNA, including the right border, was observed in T1 plants, while complex T-DNA integration patterns did not always correlate with low transgene expression. About 30% of plants with several T-DNA insertions exhibited high gene expression in the T1 generation, which was maintained at least to the T4 generation in some cases. Transgene expression was lost or completely inhibited in the T2 generation in weakly expressing plants. GUS activity showed a 50-fold difference, while a 16-fold variation in NPTII protein concentration was detected in T1 generation plants from different selected, kanamycin-resistant parental plants.

Post-transcriptional gene silencing was accelerated by increased transgene dosage in lettuce transformed with a tomato spotted wilt virus N (TSWV N) coat protein gene driven by the CaMV 35S promoter, TSWV N protein accumu-

lation decreasing 2-4 times faster in homozygous than in hemizygous plants (Pang et al. 1996). Other authors have reported a 3:1 segregation of kanamycin resistance to sensitivity amongst T1 plants, indicating single active T-DNA loci (Michelmore et al. 1987; Enomoto et al. 1990; Dinant et al. 1997). Southern analysis revealed that highly rearranged T-DNA insertions are common in transgenic lettuce. Fourteen of 16 lettuce plants transformed by *A. tumefaciens* harbouring binary or co-integrate vectors carrying a *nos.npt*II.*nos* gene, had rearranged T-DNA inserts (Michelmore et al. 1987).

DNA methylation is known to be correlated with transgene silencing, but little information is available for transgenic lettuce. Treatment of transgenic lettuce seedlings with the demethylating agent 5-azacytidine appeared to alleviate silencing of the CaMV 35S-*gus* gene in transgenic plants of the cv. Raisa (McCabe et al. 1999a) but, in the same experiments, reactivation of transgene activity did not occur in seedlings from a transgenic line in which the same *gus* gene was silenced. Promoters may be susceptible to methylation which may also account for transgene silencing. For example, the CaMV 35S promoter has about three times more methylation sites (59 sites kb^{-1} DNA) compared with the –784 bp *petE* promoter (18 sites kb^{-1}), which could account for the susceptibility of the CaMV 35S promoter to inactivation (McCabe et al. 1999b).

Overall, several factors probably contribute to transgene silencing in lettuce, including the complexity of the lettuce genome (Michelmore 1996). Its high proportion of heterochromatic or repetitive DNA is likely to increase transgene inactivation (Stam et al. 1997). Lettuce genomic DNA cannot be digested fully with common restriction endonucleases, such as *Sst*II, *Pst*I and *Pvu*II, which contain CNG in their recognition sequence, indicating that the lettuce genome is highly methylated. The insertion of transgenes into highly methylated regions of the genome is associated with transgene methylation and inactivation in *Petunia* (Pröls and Meyer 1992) and this may also occur in lettuce. The enhancement of transformation in lettuce by supervirulent plasmids, such as pTOK47, may be related to insertion of multiple T-DNA copies at different plant genomic loci, increasing the probability of T-DNA integrating at a site in the plant genome which is not susceptible to methylation and, consequently, not to gene silencing (McCabe et al. 1999a).

9 Plastid Transformation in Lettuce

To date, most transformation experiments with lettuce and, indeed, other plants have involved the introduction of genes into the nucleus. Interest in plastid transformation has gained momentum, since it offers several advantages compared to nuclear transformation. Such advantages include lack of gene silencing and positional effects because of targeted integration of foreign genes into the plastome, the potential for high foreign gene expression and, importantly in most plants, transgene containment because of the maternal in-

heritance of cytoplasmic genomes (Daniell et al. 2002; Maliga 2003; Skarjinskaia et al. 2003). As Lelivelt et al. (2005) indicated, it has taken a decade to extend plastid transformation from tobacco to other plants. In their studies, these authors employed PEG-induced DNA uptake into isolated leaf protoplasts of the lettuce cv. Flora to generate fertile, homoplasmic plants. The vector used for transformation targeted genes to the *trnA–trn1* intergenic region of the lettuce plastome, with the *aadA* gene for resistance to spectinomycin as a selectable marker. Importantly, Lelivelt et al. (2005) showed spectinomycin resistance and heterologous gene transcription in first seed generation transgenic (T1) plants, following self-pollination of primary regenerants. Additionally, crossing of transgenic plants with male sterile wild-type lettuce confirmed that antibiotic resistance was not transmitted by pollen. As the authors emphasise, this leafy vegetable system could provide excellent production and delivery for edible human therapeutic proteins.

10 Harvested Lettuce: Biotechnological Approaches to Maintain Quality, Shelf–Life and Safety

The viability of lettuce seed, whether harvested from genetically manipulated or wild-type plants, is generally limited to months when stored at room temperature. Interesting experiments have been conducted on the longevity of cryogenically stored seeds, using lettuce as a model (Walters et al. 2004). Cryogenic storage prolonged the viability of lettuce seeds, with half-lives projected as more than 500 years and 3400 years for freshly harvested seed stored in the vapour and liquid phases, respectively, of liquid nitrogen. However, the benefit of such low temperature storage was lost if seeds were stored initially at 5 °C, during which time the initial stages of ageing commenced. Nevertheless, cryogenic storage may be important in the future long-term conservation of elite lines of both wild-type and genetically engineered plants.

Whilst the effort to address issues relating to the harvested crop has become the topic of detailed investigations, as discussed later in this section, it is clear that these issues will be relevant to transgenic lettuce, once the latter has been approved for human consumption. Since lettuce deteriorates rapidly on harvesting, there has been considerable investment of effort and finance in attempts to maintain the quality and shelf-life of cut material, with an extensive and relevant recent literature. Fresh cut tissues experience severe injury that increases respiration, stimulating quality deterioration. Wounding also increases the synthesis of phenylalanine ammonia lyase and phenolic compounds, such as chlorogenic acid, that contribute to tissue browning (Kang and Saltveit 2003). Exposing cut lettuce heads and leaves of iceberg lettuce to 1-methylcyclopropene decreased the accumulation of phenolic compounds and tissue discolouration (Saltveit 2004). The same research group demonstrated that wound-induced accumulation of phenolic compounds and

browning in the Romaine lettuce cv. Longifolia was reduced by exposure to *n*-alcohols (Choi et al. 2005).

Although fresh produce, particularly leafy vegetables, makes a significant contribution to a healthy diet, it is clear that the number of outbreaks of illness caused by foodborne pathogens has increased during the past three decades (Sivapalasingam et al. 2004). These authors reviewed the sources of such outbreaks, including those attributed to the consumption of lettuce. General concern relating to this issue prompted investigations to reduce health risks from uncooked vegetables. Several approaches have been reported that focus on the reduction of microbial activity on the surface of lettuce leaves. For example, Garcia et al. (2003) assessed the effectiveness of ozone in combination with chlorine, as well as the quality of the water used in the commercial processing of lettuce, to reduce surface microbes and prolong shelf-life. Beltrán et al. (2005) also reported that washing with ozonated water extends the shelf-life of fresh cut lettuce, whilst retaining sensorial qualities, control of browning and no detrimental reduction of antioxidants. Other workers investigated the use of calcium lactate washing for salad-cut Iceberg lettuce (Martin-Diana et al. 2005), the efficiency of chlorine dioxide gas (Lee et al. 2004) and chlorine with peroxyacetic acid as sanitisers (Beuchat et al. 2004; Lang et al. 2004) in killing *Listeria monocytogenes*, *Escherichia coli* 0157:H7 and *Salmonella typhimurium*, with chlorine dioxide gas being useful during storage and transport to improve the microbial safety of lettuce. McKellar et al. (2004) discussed a warm chlorinated water treatment with oxygen-permeable film packaging to extend the shelf-life of ready-to-use lettuce, with potential for development as a standardised automated procedure.

Reports have also detailed assessments of the exposure of lettuce to ultraviolet-C radiation which, at the appropriate dose, could reduce microbial loading without affecting the quality of Lollo Rosso lettuce (Allende and Artés 2003). Yaun et al. (2004) adopted a similar approach by investigating the effects of ultraviolet energy on *Salmonella* species and *E. coli* 0157:H7 on the surfaces of apples, lettuce and tomatoes. They concluded that this approach may be beneficial in protecting the safety of fruits and vegetables in conjunction with good agricultural practices and manufacturing processes. Similarly, Gómez-Lopez et al. (2005) assessed the effects of intense light pulses, rich in UV-C, to decontaminate the surfaces of vegetables, such as lettuce. Investigations also focused on the survival of viruses, including hepatitis A, in modified atmosphere packaged lettuce (Bidawid et al. 2001) and in fresh produce, including lettuce, fennel and carrot. In the latter case, washing did not guarantee any substantial reduction in viral contamination. The time of modified atmosphere packaging following harvesting was also assessed in relation to the maintenance of quality of Romaine lettuce (Kim et al. 2005a), together with the effect of the initial oxygen concentration and the oxygen transmission rate of the packaging film on leaf quality (Kim et al. 2005b). Del Nobile et al. (2006) discussed the importance of correct packaging for Iceberg and Romaine lettuce cvs. following experiments with three plastic films, two polyolefins and

a biodegradable film, combined with storage of harvested heads at 5 °C. The parameters monitored included the composition of the package headspace, leaf colour and the oxygen, carbon dioxide and water permeability of the wrapping films. Over a 10-day period, the lowest respiratory activity was for lettuce heads packaged with a polyolefin film.

11 Concluding Remarks

Since lettuce is such an important fresh vegetable globally, it is not surprising that considerable effort has been directed, and will continue to be focused, on maximising the yield and durability of this crop. Molecular approaches have considerable relevance in the selection of the most appropriate germplasm for incorporation into breeding programmes. The amenability of lettuce to shoot regeneration from explants, tissues and isolated protoplasts, facilitates the application of in vitro-based technologies, such as somatic hybridisation and transformation, to underpin conventional breeding. Long-term transgene expression may be compromised in certain lettuce cultivars. However, the use of the appropriate constructs and transformation procedures should reduce the incidence of this problem. Biotechnological approaches are also relevant to lettuce production and it is likely that producers and consumers will become more dependent upon these to control the rapid deterioration of lettuce, and other leafy vegetables, following harvesting.

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III.5 Chickpea

J.C. Popelka and T.J.V. Higgins[1]

1 Introduction

1.1 Importance

Amongst the grain crops, grain legumes (also known as pulses or food legumes) rank third behind cereals and oilseeds in world production. With a worldwide production of 55×10^6 t, pulses represent an important dietary constituent for humans and animals and play an often under-estimated role as break crops that fix nitrogen. Legumes associate with nitrogen fixing bacteria and play a central role in low input agricultural production systems, particularly on small-scale farms (Graham and Vance 2003). Grain legumes are cultivated mainly in developing countries where they accounted for 61.3×10^6 ha in 2002, compared to 8.5×10^6 ha in developed countries. Chickpea (*Cicer arietinum* L.) ranks fourth among the pulses on a global scale, behind soya, peanut and common bean. However, chickpea, also known as garbanzo bean, can be the most important crop at a regional level, especially in semi-arid areas of the world.

This ancient crop probably originated over 7000 years ago in Turkey and spread from there to the Middle East, South Asia and North Africa, where it became an important crop. The small-seeded desi-type chickpea now accounts for about 85% of world production (7.8×10^6 t) and is the principal type grown in India, Pakistan, Iran, Afghanistan and Ethiopia. The less common, large-seeded Kabuli type is grown in the Middle East, India, Mexico as well as in North America, Australia and Spain. Chickpeas are mostly consumed as a mature pulse (cooked whole, dehulled or as flour), but are also served as a vegetable (immature shoots and seeds). Seeds average about 20% protein, 55% carbohydrate and 5% fat and represent a basic food crop in many developing countries. In India, especially, they have a high economic value. Similar to other legumes, proteins of chickpeas are high in lysine, but low in methionine and cysteine. However, combined with cereals they result in a well balanced diet of energy and protein.

Chickpea is a classic low-input crop that often completes its lifecycle in drought and heat stress with no more input than seeds and labour.

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1.2 Crop Constraints

The average yield of chickpea is about 0.8 t ha^{-1} (Sharma and Lavanya 2002), but its estimated yield potential is 5 t ha^{-1}. Drought stress, poor management practices and diseases are the main yield limiting factors in chickpeas. Fungal diseases, such as *Ascochyta* blight, *Rhizoctonia* root rot, *Pythium* rot, *Fusarium* wilt and white mold, as well as bacterial blight and certain viruses can cause considerable damage to the crop. The exudation of malic and oxalic acids from granular hairs covering leaves, stems and pods, make chickpea less susceptible to direct damage from aphids and other insects. However, damage due to the pod borer *Helicoverpa armigera* is a major threat. Stored chickpeas are highly susceptible to bruchid beetle attack (*Callosobruchus maculatus*, *C. chinensis*). Germplasm with some degree of resistance to bruchids has been identified, but it appears to be correlated with undesirable physical characteristics of the seeds, such as dark colour, roughness, altered chemical composition and thickness of the seed coat (Schalk 1973; Ahmed et al. 1991, 1993; Pacheco et al. 1994).

The goals of current breeding programmes are to increase productivity by upgrading the genetic potential of germplasm and by reducing the impact of abiotic and biotic factors, such as diseases, insects, drought and cold. Singh (1997) produced a detailed review of breeding achievements and specific breeding goals in chickpea. Classic breeding techniques may be limited by the availability of desired traits in chickpea germplasm or the linkage of desired traits with undesired characteristics. For instance, dark colour, roughness, altered chemical composition and thickness of the seed coat may make chickpeas resistant to bruchids, but also less desirable for human consumption. Biotechnology, particularly genetic transformation, offers the advantage of introducing unlinked resistance genes into elite germplasm and has the potential to complement existing breeding programmes.

Two popular strategies for gene transfer to plants (Potrykus 1990; De Block 1993; Songstrad et al. 1995; Barcelo and Lazzeri 1998) are the *Agrobacterium* method (Zupan and Zambryski 1997; Gheysen et al. 1998) and direct DNA introduction by micro-particle bombardment (Sanford et al. 1987). The efficient production of transgenic plants requires stringent selection procedures supported by a selectable marker gene that confers resistance to agents such as antibiotics or herbicides. The pre-requisites for successful application of gene technology in plants have been outlined earlier (Popelka et al. 2004).

2 Advances in Chickpea Tissue Culture

An efficient tissue culture protocol is the basis for successful genetic transformation. It is characterised by easy and uncomplicated ways to obtain tissue

Table 1. In vitro culture systems for chickpea and their predicted suitability for genetic transformation. *EA* Embryonic axes, *gS* germinated seeds, *hyp* hypocotyl, *Imm cot* immature cotyledon, *CLS* cotelydon-like structures. *ABA* Abscisic acid, *B5* Gamborg et al. (1968), *BA* N6-benzyladenine, *BAP* benzylaminopurine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *GA3* gibberellic acid A3, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *K* kinetin, *MS* Murashige and Skoog (1962), *NAA* α-naphthalene acetic acid, *2,4,5-T* 2,4,5-trichlorophenoxyacetic acid, *TDZ* thidiazuron, *Z* zeatin, *2-iP* 2-isopentenyladenine

Genotype	Explant	Important media components	Result	Reference	Predicted suitability
Nabin	Hyp	Pre-soaking seeds with B5 and BA	Shoots	Islam et al. (1999)	Moderate
ICC 640	Imm cot	B5; 2,4-D; 2,4,5-T; NAA; IAA; BA; K; Z; ABA	CLS	Shri and Davis (1992)	Moderate
BG 362, 329, 267, 256 and C 235	EA	MS; B5; BA	Shoots	Polisetty et al. (1997)	Moderate
Nabin	Internode	MS; B5; 2,4-D; BAP; NAA; K; IAA	Callus, shoots	Huda et al. (2000)	Low
C 235	EA of gS	MS; 2-iP; TDZ; K; GA3; IBA; NAA	Shoots	Jayanand et al. (2003)	High

explants with the capacity to produce a large number of independent, healthy and fertile plants. In chickpeas, two distinct regeneration strategies have been described. The first is via somatic embryogenesis, e. g. by induction of embryogenic callus growth with auxin-type growth regulators. The second is *via* organogenesis, e. g. by induction of multiple shoot formation with cytokinin-type growth regulators. In chickpea, different genotypes and explants including hypocotyls, cotyledons and embryonic axes have been cultured. Furthermore, a wide range of tissue culture media have been tested (Table 1). In several early attempts, regeneration of shoots was achieved, but the number of shoots per explant was low and this limited the likelihood of recovering transgenic shoots. Jayanand et al. (2003) described an efficient protocol for the regeneration of whole chickpea plants using embryonic axes after removal of the shoot and root tips as well as the axillary bud. Culture on medium containing low concentrations of thidiazuron (TDZ), 2-isopentenyladenine and kinetin yielded an average of 40 shoots per responding explant. The formation of vigorous roots on in vitro-generated shoots can be a further hurdle. Rooting in vitro has been developed by Fratini and Ruiz (2003) and Jayanand et al. (2003) and, for many situations, may be a suitable alternative to the grafting technique (Murfet 1971) used in several transformation protocols (Krishnamurthy et al. 2000; Sarmah et al. 2004; Senthil et al. 2004; Polowick et al. 2004; Sanyal et al. 2005).

3 Advances in Chickpea Transformation

Early transformation experiments which relied on callus cultures failed due to poor shoot regeneration but demonstrated the potential of *A. tumefaciens* as a transformation vector for chickpea (Islam et al. 1994). The first report of successful chickpea transformation (Fontana et al. 1993) after co-cultivation of embryonic axes with *A. tumefaciens* included molecular evidence for the transgenic nature of at least two independent plants. Genes encoding β-D-glucuronidase (GUS) and neomycin phosphotransferase II (*npt*II) were expressed and the transgenes were transmitted at least to the T_2 generation. Subsequently, using similar experimental protocols (Table 2), the formation of multiple shoots from different genotypes and the production of primary transgenic plants was reported (Kar et al. 1996; Krishnamurthy et al. 2000). Multiple shoot formation was achieved on MS medium supplemented with 6-benzylaminopurine (BAP; Krishnamurthy et al. 2000), BAP and α-naphthalene acetic acid (NAA; Kar et al. 1997) or BAP, NAA and kinetin (Fontana et al. 1993). Transgenic plants were selected via multiple cycles in vitro on media containing kanamycin (Fontana et al. 1993; Kar et al. 1996) or phosphinothricin (Krishnamurthy et al. 2000). Transformation frequencies and reproducibility in these early breakthroughs were low and limited their practical applicability. However, both transformation frequency and reproducibility have been improved recently in four separate studies (Polowick et al. 2004; Sarmah et al. 2004; Senthil et al. 2004; Sanyal et al. 2005), enabling the routine application of transformation technology to chickpea.

The four protocols are compared in Table 3 and it can be seen that they are very similar. Senthil et al. (2004) and Sanyal et al. (2005) pre-germinated seeds for 2 days or 20 days on medium containing TDZ or BAP, respectively, while the other authors imbibed the seeds in water overnight before preparation of explants (Polowick et al. 2004; Sarmah et al. 2004). All four protocols rely on embryonic axes as explant source, either halved and still attached to the cotyledon (Fig. 1A; Sarmah et al. 2004), sliced longitudinally (Fig. 1B; Polowick et al. 2004; Senthil et al. 2004) as described earlier for *Pisum sativum* L (Schroeder et al. 1993) or as an excision with exposed cells of the L2 layer (Sanyal et al. 2005). In addition, Sanyal et al. (2005) performed a pre-conditioning of explants for 24 h prior to cocultivation (Table 3; Sanyal et al. 2005). Multiple shoots were induced by the growth regulator BAP (Fig. 1C) and Senthil et al. (2004) further supplemented all culture media with TDZ in decreasing concentrations with time (Table 3). Selection was commenced early during the shoot induction phase (Polowick et al. 2004; Sarmah et al. 2004; Sanyal et al. 2005) with constant or increasing kanamycin concentrations during the tissue culture process [200 mg l^{-1} (Sarmah et al. 2004), 50–150 mg l^{-1} (Polowick et al. 2004), 100–200 mg l^{-1} (Sanyal et al. 2005)]. Further evidence for the transformation of embryonic axes was obtained by testing for the transient expression of the screenable marker gene encoding GUS (Fig. 1D). Using phosphinothricin (PPT) as selection agent, Senthil et al. (2004) started the selection process at

Table 2. Approaches to genetic transformation of chickpea. All genotypes are desi types unless otherwise indicated. *At Agrobacterium tumefaciens*, *CNs* cotyledonary nodes, *EA* embryonic axes, *MPB* micro-particle bombardment, *NPE* not possible to estimate, *PGIP* polygalacturonase-inhibiting protein gene. *gus* or *uid*A: Gene encoding β-glucuronidase, *bar*: gene encoding phosphinothricin acetyl transferase, *cry1*Ac: gene for crystal protein toxin from *B. thuringiensis*, *npt*II: gene encoding neomycin phosphotransferase II, *αAI*1: gene for α-amylase inhibitor 1 from *P. vulgaris*

Genotype	Explant	Method of gene transfer	Expressed genes	Evidence provided	Reproducibility and robustness	References
Local ecotype	EA	*At* (LBA 4404)	*npt*II, *uid*A	Gus expression in T_0; gene integration and expression in T_0 (Southern and Western)	Low	Fontana et al. (1993)
ICCV 1, ICCV 6 Desi	EA	*At* (LBA 4404)	*npt*II, *uid*A	Gus expression in T_0; gene integration and expression in T_0 (Southern and Western)	Low	Kar et al. (1996)
ICCV 1, ICCV 6	EA	MPB	*npt*II, *cry1*Ac	*Cry1*A(c) expression in T_0; transmission to T_1 (PCR)	Low	Kar et al. (1997)
Turkey, Chafa	EA	*At* (EHA 101 and C58C1)	*npt*II, *pat, gus*	Gus expression in T_0; transmission to T_1 (PCR)	Low	Krishnamurthy et al. (2000)
P 362, P 1042, P 1043	EA	*At* (EHA 101)	*npt*II, *uid*A, *bar*	Gus expression in T_0; transmission to T_1 (PCR)	Low	Tewari-Singh et al. (2004)
Semsen	EA	*At* (AGL1)	*npt*II, *αAI*1	Transmission and expression in T_1 (Southern and western blot); functionality of *αAI*1	Good	Sarmah et al. (2004)
ICCV5 (Kabuli), H208, ICCL87322, K850	Sliced EA	*At* (AGL1)	*bar*, PGIP, *uid*A	Transmission and expression of GUS gene in T_1–T_3	Good	Senthil et al. (2004)
CDC Yuma (Kabuli)	Sliced EA	*At* (EHA105)	*uid*A, *npt*II	Transmission and expression of GUS gene in T_1–T_3	Good	Polowick et al. (2004)
C 235, BG 256, Pusa 362, Pusa 372	L2 layers of CNs	*At* (LBA4404)	*uid*A, *npt*II, *cry1Ac*	Transmission and expression of *npt*II and *cry1Ac* in T_1; functionality of *cry1Ac*	Good	Sanyal et al. (2005)

Table 3. Comparison of four robust transformation protocols for chickpea. *AS* Acetosyringone, *B5* Gamborg et al. (1968), *BAP* 6-benzylaminopurine, *bm* basal medium, *cef* cefotaxime, *IBA* indole-3-butyric acid, *kan* kanamycin, *kin* kinetin, *MES* 2-(*N*-morpholino)ethanesulfonic acid, *MS* Murashige and Skoog (1962), *NAA* α-naphthalene acetic acid, *PPT* phosphinothricin, *TDZ* thidiazuron, *tim* timentin, *vit* vitamins

	Sarmah et al. (2004)	Senthil et al. (2004)	Polowick et al. (2004)	Sanyal et al. (2005)
Explant pre-culture	Over-night imbibition	Germination for 2 days	Over-night imbibition	20-day-old seedlings
Germination medium	Water	MS-bm with B5 vit, pH 5.7, 10 µM TDZ	Water	MS-bm, 1 mg l^{-1} BAP
Explant preparation	Halved embryonic axes	Longitudinal slices of embryonic axes (4–5 per embryo)	Longitudinal slices of embryonic axes (5–6 per embryo)	Exposed and pre-conditioned[a] tissue after transverse excision of main root, shots and shoot buds, exposing L2 layer
Infection	In liquid *Agrobacterium* suspension for 1 h	In liquid *Agrobacterium* suspension for 35 min and 150 rpm	In liquid *Agrobacterium* suspension for 1–2 h	In liquid *Agrobacterium* suspension combined with 60 s sonication and 20 min vacuum treatment
Co-culture	3 days at 24 °C, 16 h light	4 days at 22 °C, dark	4 days at 25 °C, 16 h light	48 h at 24 °C, 16 h light
Medium	B5-bm, 10 mM MES, 1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 100 µM coniferyl alcohol, pH 5.8	MS-bm, B5-vit, 10 µM TDZ, pH 5.7	B5-bm, 100 µM AS, pH 5.7	MS-bm, 100 µM AS, 1 mg l^{-1} BAP, 200 mg l^{-1} L-cysteine, 100 mg l^{-1} DTT
Wash transfer	4× with water, blotted	None	Minimum 1 h in 300 mg l^{-1} tim solution	In MS-bm, 1 mg l^{-1} BAP, 500 mg l^{-1} cef or carbenicillin
Shoot initiation	2 weeks	3 weeks	2 weeks, then many cycles of 3 weeks	15 days
Medium	MS-bm, 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} kinetin, 0.05 mg $l1^{-1}$ NAA, 10 mM MES, 200 mg l^{-1} kan, 150 mg l^{-1} tim	MS-bm, B5-vit, pH 5.7, 5 µM TDZ, 500 mg l^{-1} cef	B5-bm, 150 mg l^{-1} tim, 3 mg l^{-1} BAP, 50 mg l^{-1} kan	MS-bm, 1 mg l^{-1} BAP, 100 mg l^{-1} kan, 500 mg l^{-1} cef, 0.02 g l^{-1} silver nitrate

Table 3. (continued)

	Sarmah et al. (2004)	Senthil et al. (2004)	Polowick et al. (2004)	Sanyal et al. (2005)
Shoot elongation or proliferation	10–14 days	3× to 4× 4-week subcultures	3 cycles of 3 weeks each	2× 15 days
Medium	MS-bm, 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} kin, 10 mM MES, 200 mg l^{-1} kan, 150 mg l^{-1} tim	MS-bm, B5-vit, 2.5 µM TDZ, 500 mg l^{-1} cef, 8.9 µM BAP, 0.1 µM NAA, 2.5 mg l^{-1} PPT	MS-bm, B5-vit, 1 mg l^{-1} BAP, 50–75 mg l^{-1} kan	MS-bm, 1 mg l^{-1} BAP, 100 mg l^{-1} kan, 0.02 g l^{-1} silver nitrate
Shoot elongation and multiplication	Minimum of six cycles of 10–14 days	3–4 weeks	None	2× 15 days
Medium	MS-bm, 0.1 mg l^{-1} BAP, 0.1 mg l^{-1} kin, 10 mM MES, 200 mg l^{-1} kan, 150 mg l^{-1} tim	MS-bm, B5-vit, 8.9 µM BAP, 0.1 µM NAA, 2.5 µM TDZ, 500 mg l^{-1} cef, 2.5 mg l^{-1} PPT	None	MS-bm, 1 mg l^{-1} BAP, 150–200 mg l^{-1} kan, 0.02 g l^{-1} silver nitrate
Rooting	In vitro shoots rooted for 2 weeks or grafted	Shoots of 1.5–2.0-cm for 3–4 weeks or grafted	Until roots form (1–3 weeks)	In vitro shoots rooted or grafted
Medium	MS-bm, 1 mg l^{-1} IBA, 10 mM MES	MS-bm, B5-vit, 2.5 µM NAA, 2.5 µM TDZ, 500 mg l^{-1} cef	B5-bm, 0.18 mg l^{-1} NAA, 150 mg l^{-1} kan	None
Transfer to greenhouse	Rooted shoots or grafted shoots on rootstocks with a silicon ring	Rooted shoots or grafted shoots on rootstocks	Rooted shoots	Rooted shoots or grafted shoots on rootstocks
Transgenic plants per seed	0.72%	2.0–13.3% (4–5 explants per seed)	3.1% (5–6 explants per seed)	5.18–13.2% (not clear)
Time required	6–9 months	5–6 months	4.5–13 months	Not specified

[a] Explants were 24 h pre-cultured on solidified MS-bm, 1 mg l^{-1} BAP, 4 µM L-glutamine and L-arginine, 200 mg l^{-1} L-cysteine.

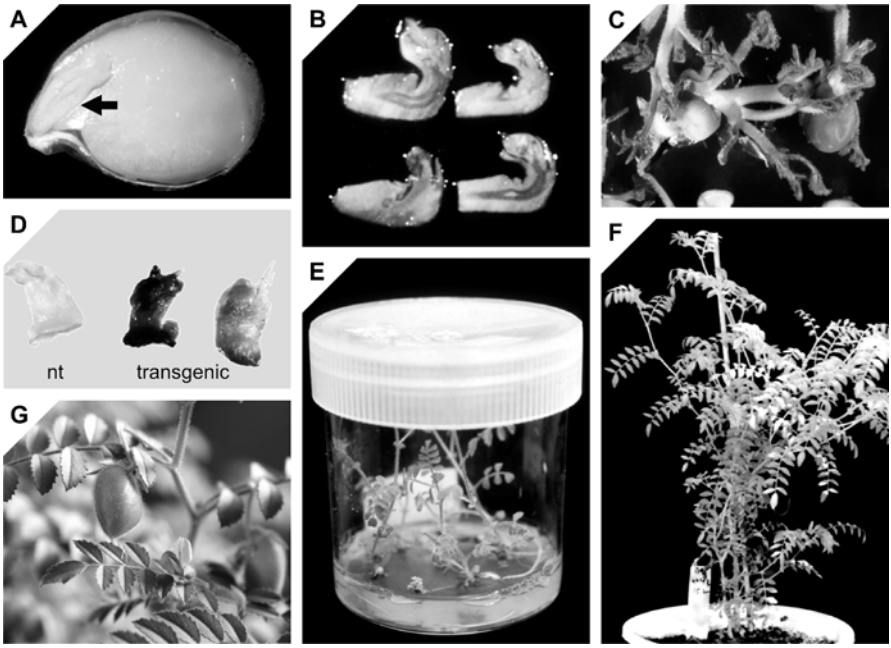

Fig. 1. Transformation of chickpeas. **A** Halved chickpea cotyledon with attached embryonic axis (*arrow*). Sarmah et al. (2004) used this explant after removal of the ends of the shoot and root. **B** Sliced embryonic axes (Schroeder et al. 1993) were used by Polowick et al. (2004) and Senthil et al. (2004). **C** Multiple shoots induced on halved embryonic axes. **D** Transient GUS expression in embryonic axes of chickpeas (*nt* non-transformed control explant). **E** Shoots surviving the selection process induce roots on rooting medium. **F–G** Transgenic chickpea plants established in the glasshouse are normal in phenotype and fertile

a later stage, with 2.5 mg l^{-1} PPT. Surviving shoots were either transferred to rooting medium (Table 3; Fig. 1E; Polowick et al. 2004; Sarmah et al. 2004; Senthil et al. 2004) or grafted onto seedlings (Sarmah et al. 2004; Senthil et al. 2004; Sanyal et al. 2005) and finally transferred to soil in the glasshouse (Fig. 1F,G). The four systems all appear equally useful and have the following important elements in common, namely: (1) mature seeds (imbibed or pre-germinated) are the preferred explant source, (2) embryonic axes contain the target tissue, (3) submersion of explants in liquid *Agrobacterium* suspension followed by several days of co-culture on semi-solid medium, (4) frequent sub-cultures on selective medium for shoot initiation, elongation and rooting and (5) transfer of rooted shoots to soil in the glasshouse. In summary, many different genotypes can now be transformed, including both desi and Kabuli types; and the slight differences in growth regulator type and concentration between the three reports may be only a reflection of the different starting cutivars or lines.

4 Application of Transformation Technology to Chickpea and Its Potential

It is expected, with these reliable transformation protocols available, that a number of potentially useful genes will be introduced into chickpeas in the near future, providing excellent opportunities for plant improvement against insect pests and other constraints, such as quality related traits (White et al. 2000; Wang et al. 2003). Besides the large family of *Bacillus thuringiensis*-derived genes (Krattiger 1997), there are a significant number of useful genes available from other organisms including higher plants (Schuler et al. 1998). The latter includes genes for lectins (Murdock et al. 1990), diverse proteases (Ryan 1990), protease inhibitors (Hilder et al. 1987) and α-amylase inhibitors (Shade et al. 1994).

So far, there have been three reports of trangenic chickpeas expressing genes for protection against insect pests. Transgenic chickpea plants produced with the biolistic transformation approach expressed the bacterial *cry*1Ac gene from *B. thuringiensis* (Kar et al. 1997). Insect feeding trials with one primary transgenic plant demonstrated an inhibitory effect on growth of larvae of the chickpea pod-borer *H. armigera* (Kar et al. 1997). Transmission to T_1 progeny was demonstrated, although further analysis has not been reported. The *Agrobacterium* method was used to introduce a seed-specific α-amylase inhibitor (α *AI*1) gene from *Phaseolus vulgaris* L (Sarmah et al. 2004). Stable transmission and expression of the transgene in subsequent generations was demonstrated (Table 2). The high level of expression of the α*AI*1 gene protected chickpea seeds from insect damage by severely inhibiting the development of cowpea weevils (*C. maculatus*) and adzuki bean weevils (*C. chinensis*; Sarmah et al. 2004). Finally, Sanyal et al. (2005) tested the toxicity of T_0 and T_1 plants expressing the *cry1Ac* gene. In bioassays, larvae of *H. armigera* ceased feeding on transgenic chickpea leaves after 2 days and showed high mortality after weight decreases of 40–90%. In T_1, many lines showed complete protection against the insects (Sanyal et al. 2005).

While progress in genomics research will continue delivering interesting and useful genes, intellectual property rights, regulatory approval as well as biosafety concerns will determine the future. It will be important to ensure that biosafety regulations and regulatory compliance systems are in place in each of the countries using the technology before regulatory agencies are faced with requests for release of the transgenic crop. Risk assessments for non-target species associated with the crop (Romeis et al. 2004) will also need to be concluded. Regulatory packages are in place for pesticides and will similarly be applied to transgenic plants expressing insecticidal genes (Hill and Sendashonga 2003). Further, issues relating to food safety, labelling, traceability, trans-border movement and trade must be addressed concurrently, as technological advances described here mean that transgenic chickpeas with useful genes are now in the pipeline for small-scale field trials and potentially for broad-acre release.

5 Conclusion and Outlook

The prospects are now excellent for the genetic improvement of chickpeas using gene technology. There are at least four closely related transformation protocols from which to choose and all the indications are that the systems are robust and reproducible. It is possible to predict that genes for protection against major insect pests, such as pod borer, will soon be installed in chickpea germplasm for use in varieties adapted to local conditions around the world. These will be followed by other genes including those for other biotic stresses as well as genes that will overcome abiotic stresses such as drought, soil acidity and frost. Similarly, transformation protocols are available for other important legumes, such as soybean (Olhoft and Somers 2004), groundnut (Yang et al. 1998; Li et al. 2000), lupin (Tabe and Molvig 2006), cowpea (Popelka et al. 2006) and garden pea (McPhee et al. 2004).

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III.6 Common Bean and Cowpea

F.J.L. Aragão[1] and F.A.P. Campos[2]

1 Introduction

Both common bean, *Phaseolus vulgaris* L., and cowpea, *Vigna unguiculata* (L.) Walp., are among the most important grain legumes for direct consumption in tropical and sub-tropical countries of Latin America, Africa and Asia. The world annual production of common bean and cowpea is about 19×10^6 t and 3×10^6 t, respectively (FAOSTAT 2006). These two species are cultivated by resource-poor farmers who lack the ability to purchase and apply agrochemicals on a regular basis. Despite their nutritional importance, productivity has been declining in some regions. The main limiting factors are poor agronomic practices, diseases, insects, nutritional deficiencies, soil and climate constraints, lack of improved varieties and weed competition.

The development and distribution of enhanced germplasm has ensured improved cowpea production. Conventional breeding programmes of the International Institute for Tropical Agriculture (Nigeria), the Brazilian Agricultural Research Corporation (Embrapa, Brazil) and the United States Agency for International Development-sponsored Bean/Cowpea Collaborative Research Support Program (Fatokun et al. 2002; Hall et al. 2003) have been developing and delivering varieties with enhanced disease and insect resistance, greater drought resistance and other important agronomic traits.

Current efforts in the identification of molecular markers for various traits and the construction of genetic linkage maps will be instrumental for the development of tools for marker-assisted selection in breeding programmes, as well as subsequent map-based cloning of various genes (Quédraogo et al. 2002; Kelly et al. 2003). While classic breeding will continue to bring benefits to producers, it is unlikely to provide all the solutions for improving the crops to meet the needs of farmers and commercial production. Therefore, it is important to introduce agronomically useful traits into common bean and cowpea by breeding and genetic engineering. Genetic engineering may accelerate the production of plants with useful traits.

The use of genetic engineering to incorporate useful genes into farmer-preferred varieties and elite breeding lines is dependent on the availability

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of: (1) tissue culture techniques to generate organogenic/embryogenic cells and (2) efficient genetic transformation systems, in which transgenic plants can be produced readily. This Chapter discusses the technologies developed to genetically modify *P. vulgaris* and *V. unguiculata*. Earlier reviews written by Christou (1994, 1997) and Nagl et al. (1997) have dealt with this subject. Readers are also referred to recent publications by Konowicz et al. (1997), Aragão and Rech (2001), Timko (2002), Somers et al. (2003), Svetleva et al. (2003) and Popelka et al. (2004), in which some aspects of common bean and cowpea biotechnology are discussed.

2 Common Bean (*Phaseolus vulgaris*)

2.1 Tissue Culture and Genetic Transformation

Multiple shoots are formed in the peripheral regions of the apical meristem (Aragão and Rech 1997). Induction of shoot formation in bean meristems can be achieved by culturing the mature embryos in the presence of cytokinins such as kinetin, zeatin and benzylaminopurine (BAP; Kartha et al. 1981; Martins and Sondahl 1984; McClean and Grafton 1989; Franklin et al. 1991; Malik and Saxena 1992; Mohamed et al. 1992, 1993; Aragão and Rech 1997). Compounds such as thidiazuron (TDZ) and *N*-(2-chloro-pyridyl)-*N*'-phenylurea (CPPU) that possess cytokinin-like effects have also been investigated (Mohamed et al. 1992). Cruz de Carvalho et al. (2000) employed the transverse thin cell layer (tTCL) method to optimize the frequency of shoot regeneration without an intermediate callus stage. The same authors also showed that the addition of 10 μ M silver nitrate ($AgNO_3$) to the medium with BAP enhanced the number of shoots that developed per explant and increased shoot elongation.

Early efforts to transform bean demonstrated its susceptibility to *Agrobacterium* and some tissues, such as callus, leaves, meristems, cotyledon and hypocotyl, have been transformed (Lippincott et al. 1968; McClean et al. 1991; Franklin et al. 1993; Becker et al. 1994; Lewis and Bliss 1994; Brasileiro et al. 1996; Nagl et al. 1997). Mariotti et al. (1989) reported the production of transgenic bean plants using *Agrobacterium*, but there was no molecular evidence for genetic transformation or progeny analysis. Transient gene expression using either electroporation or PEG-mediated protoplast transformation has also been demonstrated (Crepy et al. 1986; Bustos 1991; Leon et al. 1991; Giovinazzo et al. 1993). Dillen et al. (1995) electroporated intact tissues to introduce and express the *gus* gene in bean embryonic axes. However, transgenic bean plants were not obtained due to difficulties in regenerating plants.

During the past two decades, efforts to achieve an efficient methodology for bean transformation were not successful due, mainly, to the lack of an efficient tissue culture system to regenerate plants from transformed cells, despite numerous attempts being made to regenerate plants from several types of isolated

cells and tissues. Although no satisfactory results have been achieved, some workers have described shoot organogenesis (through multiple shoot induction) of the apical and axillary meristems from bean embryonic axes (McClean and Grafton 1989; Malik and Saxena 1992; Mohamed et al. 1992, 1993). These tissue culture systems, coupling with particle bombardment, allow the recovery of transformed bean plants (Aragão et al. 1996).

Particle bombardment can be used to transform plant cells where a culture system for regeneration is not available. The status of *Phaseolus* and *Vigna* tissue culture and transformation was reviewed by Nagl et al. (1997). Early efforts to produce transgenic beans using particle bombardment demonstrated the feasibility to introduce and express genes of interest in tissues (Genga et al. 1991; Aragão et al. 1992, 1993). Russel et al. (1993) were the first to report the production of transgenic navy bean (cv. Seafarer) plants, using an electrical particle acceleration device, but the frequency of transformation was low (0.03%) and variety-limited. In addition, the tissue culture protocol was time-consuming, involving several temperature treatments and transfer of the bombarded embryos to different media before recovery of transgenic shoots.

Several studies confirmed that transgenic shoots could be regenerated following particle bombardment of apical meristems of embryonic axes, as was first demonstrated in soybean (McCabe et al. 1988). In *P. vulgaris*, bombardment of meristematic cells revealed that foreign genes could efficiently reach the superficial cell layers, demonstrating the feasibility of producing transgenic bean using this method (Aragão et al. 1993). Later, Kim and Minamikawa (1996) reported the recovery of transgenic bean plants (cv. Goldstar) by bombarding embryonic axes. We also developed a transformation system for routine regeneration of transgenic bean plants based on the development of an efficient tissue culture protocol for multiple shoot induction, elongation and rooting (Aragão et al. 1996; Aragão and Rech 1997). The morphology of the explants used during bombardment may greatly influence the successful generation of transgenic plants (Aragão and Rech 1997). In some varieties, only the central region of the opical meristem was exposed as a target for particle bombardment. As de novo shoot differentiation occurred in the peripheral layers of the meristematic ring in bean embryos cultured on medium with cytokinins (McClean and Grafton 1989; Franklin et al. 1991; Malik and Saxena 1992; Aragão and Rech 1997), this limited the number of meristematic cells that could be targeted by microparticle-coated DNA, leading to decreased transformation efficiency.

Recently, Liu et al. (2005) described an *Agrobacterium*-mediated transformation system of kidney bean by using sonication and vacuum infiltration. Among 18 combinations of transformation procedures, *Agrobacterium*-mediated transformation combined with 5 min sonication and 5 min vacuum infiltration were optimal, resulting in the highest transformation efficiency.

2.2 Introduction of Useful Traits into Common Bean

Since the first report on *P. vulgaris* transformation in 1993, a few research groups have transformed bean to introduce useful traits. Our group has introduced agronomically important genes in *Phaseolus*, such as the *be2s1* gene to improve the methionine content of the seeds. The transgene was stably integrated and correctly expressed in homozygous R_2 to R_6 generations. In two of the five transgenic lines, the methionine content was significantly increased by 14% and 23% compared to that in non-transformed plants (Aragão et al. 1999).

Russel et al. (1993) introduced into bean the *bar* gene that confers resistance to the herbicide phosphinothricin and the coat protein gene from the bean golden mosaic geminivirus (BGMV) in an attempt to produce virus-resistant plants. The introduced *bar* gene was shown to confer strong resistance to the herbicide under glasshouse conditions. However, transgenic bean plants expressing the BGMV coat protein gene did not exhibit virus resistance (D. Maxwell, University of Wisconsin, personal communication). In order to produce bean plants resistant to BGMV, antisense *Rep-TrAP-REn* and *BC1* genes from the virus under the control of the CaMV 35S promoter were transferred into bean. Two transgenic lines were generated which showed delayed and attenuated viral symptoms (Aragão et al. 1998). The transgenic bean lines are being used in our bean-breeding programme to evaluate gene expression in different genetic backgrounds under glasshouse and field conditions. Recently, we tested in the field transgenic common bean plants containing the *bar* gene resistant to the herbicide glufosinate ammonium (Aragão et al. 2002). We generated high BGMV-resistant plants by expression of a mutated *AC1* viral gene (Faria et al. 2006) and using interfering RNA (RNAi) (F.J.L. Aragão and J.C. Faria, unpublished data). The transgenic bean lines were introduced into the breeding programme and are being tested under field conditions.

Using an *Agrobacterium*-mediated transformation system, transgenic lines expressing the group 3 *lea* (*l*ate *e*mbryogenesis *a*bundant) protein gene from *Brassica napus* were produced. Transgenic kidney bean plants demonstrated enhanced growth ability under salt- and water-deficit stress conditions (Liu et al. 2005).

3 Cowpea (Vigna unguiculata)

3.1 Tissue Culture

The earliest attempts to develop the tissue culture system of cowpea were reported by Kartha et al. (1981), who showed plant regeneration from isolated shoot apical meristems in the absence of exogenously supplied growth regulators. Plant regeneration could also be achieved by the presence of low concen-

trations (0.1−0.001 µM) of benzyladenine (BA) either alone or in combination with low concentrations (0.1−0.001 µM) of naphthaleneacetic acid (NAA). The regeneration frequencies diminished drastically with increasing concentrations of BA or NAA. Later, Brar et al. (1997) developed a micropropagation protocol through shoot tip multiplication by manipulation of cytokinins and auxins in the culture media. Most shoots were obtained by culturing shoots in MS medium (Murashige and Skoog 1962) containing 22.2 µ M BA and 0.05 µ M NAA.

The first attempt to induce de novo organogenesis in cowpea was reported by Muthukumar et al. (1995), who obtained organogenic calli by culturing primary leaves in B5 basal medium supplemented with 0.8 µM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Upon transfer of calli to the basal medium supplemented with BA (5.0 µ M), shoots were regenerated from calli at a low frequency (21%) and the mean number of shoots per explant was also low. De novo organogenesis in cowpea was also described by Pellegrineschi et al. (1997), who used cotyledons and embryonic axes isolated from immature seeds as explants. In this study, a new basal medium was formulated instead of MS medium, taking into account the mineral salt composition of cowpea embryos. In addition, the effects were evaluated of growth regulators, carbohydrate sources, casein hydrolysate, putrescine and thymine additions. Direct organogenesis in hypocotyls and cotyledons was promoted by cytokinins such as BA, kinetin and zeatin, but none of the auxins tested was found to promote plant regeneration. Of 18 cowpea varieties tested, five responded positively, although these responsive varieties varied greatly in terms of shoot formation frequency and mean number of shoots per explant. The de novo origin of the shoots was demonstrated by histological analysis. Cotyledons from immature seeds were also used as explants for inducing indirect organogenesis by Anand et al. (2001). Organogenic callus was obtained by culturing the explants in MS basal salts supplemented with B5 vitamins, putrescine, BA (8.8 µ M) and kinetin (9.2 µ M). Shoot formation was achieved by transferring callus to medium with a lower concentration of kinetin (0.46 µ M) than that used previously by Pellegrineschi (1997). The shoots elongated in culture medium containing BA and gibberellic acid A3 (GA_3) and rooting was obtained on medium with indole-3-butyric acud (IBA). However, the number of shoots per explant was low, which was similar to the results reported by Pellegrineschi (1997). Choi et al. (2003) also attempted to induce organogenesis from immature cowpea embryos by incubating the explants in MS medium containing increasing concentrations of BA. The highest frequency of shoot organogenesis (65.5%) was observed at 4.4 µ M BA.

As for *Vigna* species, cotyledonary nodes seem to be promising explants for inducing organogenesis. The axillary junction of the cotyledon and the embryo axis contain cells that are competent for regeneration and hence can be useful targets for gene delivery. Van Le et al. (2002) reported the regeneration of fertile cowpea plants from thin cell layer explants of cotyledonary nodes by the use of TDZ, an auxin analogue. Brar et al. (1999a) also established a regeneration

protocol that involved an initial exposure of the explants to a high concentration of BA, followed by transfer to a medium containing reduced BA to induce shoot regeneration. The protocol was tested with several cowpea genotypes and the regeneration capacity was shown to be highly variable. Thus, of 36 genotypes tested, only 17 responded favourably and these genotypes exhibited variable regeneration frequencies (1–11%) and the number of shoots per explant was generally low. For the variety Early Scarlet, both the regeneration frequency and number of shoots per explant were significantly increased by the addition of ethylene inhibitors, such as silver nitrate, to the induction and regeneration media (Brar et al. 1999b). This observation suggests an important role for ethylene in the process of in vitro morphogenesis in cowpea and may also explain the low regeneration frequencies in previous studies.

Considerable progress has been achieved in the induction and development of somatic embryos in leguminous plants, especially in those economically important species such as soybean (Lakshmanan and Taji 2000). However, reports dealing with cowpea are scarce and there are no protocols that could be applied to a wide range of cowpea genotypes. Furthermore, as far as we know, there is no report on somatic embryogenesis in the refereed scientific literature that has been validated by another laboratory.

It seems that in cowpea somatic embryogenesis could be induced in young meristematic tissues, such as immature embryos and developing leaves. For example, Kulothungan et al. (1995) demonstrated that the incubation of primary leaf explants in MS medium supplemented with 2 mg l^{-1} 2,4-D resulted in the formation of shiny friable embryogenic callus that, upon transfer to liquid medium with an identical concentration of 2,4-D, generated globular embryos that progressively matured into more advanced developmental stages. Plant recovery at a very low rate (3.2%) was achieved by transferring cotyledonary stage embryos to a medium containing a lower concentration of 2,4-D. Later, the same group introduced several changes is this protocol in order to increase the frequency of plant regeneration (Anand et al. 2000). The changes included inducing embryogenic callus in MS medium supplemented with 2,4-D (6.75 µ M) and establishing a suspension culture by transferring the callus to a liquid medium containing 2.26 µ M 2,4-D. Embryo development and maturation were achieved by reducing the concentration of 2,4-D to 0.05 µM and adding mannitol (3.0%) and abscisic acid (ABA; 5 µ M) to the medium. Conversion into plants with a frequency of 21.8% was achieved by transferring mature embryos to semi-solid MS medium supplemented with 2 µ M zeatin, 3% mannitol and 5 µ M ABA. Upon transfer to soil, only 8–10% of the plants survived. At present, it is not known whether this protocol can be successfully applied to other cowpea genotypes. Attempts have also been made to induce somatic embryogenesis from protoplasts isolated from immature embryos. Several reports have shown successful isolation and culture of cowpea protoplasts (Jha and Roy 1980; Steinbiss and Li 1983). Gill et al. (1987) reported callus formation from hypocotyl-derived protoplasts isolated from cowpea and two other legume species, but attempts to induce organogenesis were unsuccessful.

Li et al. (1995) attempted to induce somatic embryogenesis from protoplasts isolated from immature cotyledons of cowpea. Callus derived from protoplasts grown in liquid MS medium supplemented with 0.9 µ M 2,4-D, 5.37 µ M NAA and 2.2 µ M BA was transferred to a semi-solid medium consisting of MS minerals and B5 organic components, supplemented with 500 mg l^{-1} NaCl, 500 mg l^{-1} casein hydrolysate, 9.0 µ M 2,4-D and 2.2 µ M BAP. A limited number of the calli (5%) was shown to be embryogenic. For embryo development, embryogenic calli were transferred to a semi-solid medium consisting of MS minerals, B5 organic components, 0.57 µ M IAA, 2.3 µ M kinetin, 2% sucrose and 3–5% mannitol. In this medium, embryo differentiation was observed, but only a proportion of the embryos developed into cotyledon-stage embryos. Embryo maturation and germination were achieved by omitting mannitol from the medium. The rate of conversion from immature to mature embryos was low, while the rate of conversion from embryos to plants was 10–15%.

3.2 Genetic Transformation

Garcia et al. (1986, 1987) were the first to investigate whether cowpea was susceptible to *A. tumefaciens* infection and demonstrated the formation of crown gall tumours following the inoculation of cowpea plants with *A. tumefaciens* strain LBA1010 harbouring octopine-type or nopaline-type Ti plasmids. These authors also demonstrated that foreign genes could be stably introduced and expressed in cowpea cells. Leaf discs from primary leaves were inoculated with *A. tumefaciens* harbouring a Ti plasmid that contained a kanamycin resistance gene. After the leaf discs were cultured in selective medium, kanamycin-resistant callus was obtained and transformation was confirmed by the detection of nopaline synthase activity and Southern blot analysis. As the interest of these studies was to investigate the interaction between cowpea mosaic virus and cowpea plants, attempts were not made to regenerate transgenic plants. The transformed calli obtained maintained their antibiotic resistance phenotype for more than one year, thus indicating that the kanamycin resistance gene can be exploited as a marker in the selection of transformed cowpea cells. Mature cowpea embryos were used as a target for genetic transformation by Penza et al. (1991), who were able to regenerate putatively transgenic plants following co-cultivation of mature embryos excised from mature seeds with *A. tumefaciens* strain C58 carrying a plasmid containing the *gus* gene under the control of the 35S CaMV promoter. The putatively transformed plants were subjected to Southern blot analysis, but conclusive prove of transgene integration was not presented.

The first successful production of transgenic cowpea plants was reported by Muthukumar et al. (1996). These authors co-cultivated detached cotyledons explants with *A. tumefaciens*, followed by transfer of the explants to a selective organogenic medium, and recovered rifampycin-resistant shoots. From 17 rooted shoots, six survived after acclimation to soil. Four of these plants

were grown to maturity and set seed, but none of the seeds germinated and no evidence of transgene transmission to the progeny was obtained. Recently, Popelka et al. (2006) developed an *Agrabacterium*-mediated system presenting a frequency of transformation of 1–3 transgenic plants per 1000 explants. Transgenic lines transmitted the transgene (*bar* gene) to their progenies following Mendelian laws.

Methods of cowpea transformation via direct DNA delivery have also been used. Penza et al. (1992) demonstrated transient expression of a reporter gene in mature cowpea embryos following electroporation. GUS activity in the embryos could be detected up to seven days after electroporation. The same group (Akella and Lurquin 1993), following electroporation of mature embryos, increased the number of cowpea seedlings expressing the reporter gene in a variety of tissues, including apical meristem. These authors suggested that transgenic plants could be obtained from electroporated embryos if DNA integration occurred in these cells.

Genetic transformation and recovery of transgenic plants of several legume species have been reported following particle bombardment (Aragão et al. 2000, 2002; Somers et al. 2003), with the recovery of putative transgenic cowpea plants by this transformation method being reported first by Ikea et al. (2003). The embryonic axis from mature seeds was used as the target for transformation and the tissues were bombarded with the vector ptjk142 that contained the *gus* reporter and the *bar* genes, both driven by the CaMV 35S promoter. However, the regeneration method employed to recover putative transgenic plants was not described. The bombarded embryo axes gave rise to plants, the latter setting seeds after transfer to soil. Expression of *gus* and *bar* genes was followed over three generations, but Mendelian transmission of the transgenes could not be confirmed conclusively.

In most legume species, both plant regeneration and transformation are highly genotype-dependent, regeneration is a slow process and the frequency of transformation is often low (Somers et al. 2003). As is evident from this discussion, cowpea is not an exception and we are still far from developing regeneration and transformation protocols that could be used to obtain of transgenic cowpea lines on a routine basis. Several experimental approaches to deliver DNA into target cells (i. e. embryogenic/organogenic cells) have been developed for several legume species (Somers et al. 2003; Chandra and Pental 2003). It is reasonable to assume that these approaches may make it easier to devise genotype-independent protocols for cowpea transformation. Developing variety-independent, easy to use and efficient regeneration protocols, via either somatic embryogenesis or organogenesis, will certainly be a more demanding task. It appears that, in legumes, somatic embryogenesis is feasible in a relatively easy manner, especially in young meristematic tissues, such as immature embryos and developing leaves (Lakshmanan and Taji 2000). As far as cowpea is concerned, this was confirmed by the studies of Kulothungan et al. (1995) and Anand et al. (2000) who established embryogenic cell suspensions from these explants. More effort should be made in order to determine the

culture conditions for achieving differentiation from these cell suspensions. Regeneration of shoots from cotyledonary nodes or from other meristematic explants has been achieved in several legume species, including *Vigna* species (Saini and Jaiwal 2002), but little effort has been devoted to cowpea.

4 Future Prospects and Limitations

Biotechnological tools to complement traditional breeding have the potential to accelerate the generation of new varieties carrying genes with desired agronomic traits, such as those that are difficult to find in the primary or secondary gene pool. In a scenario of increasing population, farming must become more productive, especially in tropical and sub-tropical areas, if these regions are to avoid increasing poverty and dependence on food imports and further environmental degradation. In Brazil, common bean has been increasingly cultivated by large farmers. This implies that development of cowpea and common bean varieties will need to be targeted to adapt to more intensive cultivation. The introduction of genes for viral, insect, bacterial and fungal resistances and drought tolerance in common bean and cowpea would dramatically increase productivity in many countries and reduce costs, safety hazards and environmental risks (Ehlers and Hall 1997; Graham and Ranalli 1997). In addition, herbicide resistance and manipulation of plant morphological and phenotypic characteristics can facilitate agronomic management, increasing grain yield and quality.

To date, the genetic manipulation of cowpea and common bean is still not trivial and regeneration and transformation systems need to be improved. The possibility of combining *Agrobacterium* and particle bombardment systems has been proposed. This may lead to a high efficiency of the *Agrobacterium* system to integrate the T-DNA in a susceptible host and the capacity to introduce new genes into living cells by the particle bombardment process. Our studies demonstrate that it is possible to combine *Agrobacterium* and particle bombardment systems to transform both common bean (Brasileiro et al. 1996) and cowpea. The micro-wounds caused by particle bombardment can enhance the frequency of *Agrobacterium*-mediated transformation. However, we were unable to recover transgenic bean plants. Further investigation will be necessary in order to confirm the practical utility of these combined techniques for bean and cowpea transformation.

Plants of several members of the genera *Phaseolus* and *Vigna* (reviewed by Nagl et al. 1997) have been regenerated de novo from undifferentiated cells originated from differentiated cells. The extrapolation of these technologies would be a breakthrough regarding the development of a transformation system to accelerate the generation of new transgenic commercial varieties. The ideal system would be the regeneration of fertile mature plants through somatic embryogenesis or organogenesis, preferentially from mature embryonic

axes, especially as the latter are preferred for transformation by *Agrobacterium*, intact tissue electroporation and particle bombardment.

Other important constraints in the transformation system based on bombardment of meristematic tissues of embryonic axes is the difficulty of having an efficient selection for transformed cells. In this case, only a few cells from the meristem are transformed. Although antibiotics such as kanamicin have been used as a selective agent (Aragão et al. 1996), this selection is not efficient. Recently, we developed a novel system for selecting transformed meristematic cells based on the use of imazapyr, an herbicidal molecule capable of systemically translocating and concentrating in the apical meristematic region of the plant. This selectable marker, coupled with an improved multiple particle bombardment protocol, resulted in a significant increase in the recovery of fertile, transgenic material compared with standard soybean transformation protocols (Aragão et al. 2000). This selection system is being successfully used to introduce genes into dry bean and cowpea (unpublished data).

In our experience, only one out of 20 independent transgenic plants obtained has the potential to be introduced into a breeding programme to generate a commercial variety. Consequently, a bean transformation technology that would allow selection of transgenic, meristematic cells and regeneration of fertile plants should accelerate the development of bean varieties with improved agricultural characteristics.

Studies on the behaviour of transgenic bean plants under field conditions have been carried out by the Embrapa Biosafety Network. In these assessments, the interactions of transgenic plants with other plants and micro-organisms have been evaluated. Moreover, the stability of foreign genes and factors related to their interactions with the complex physiology of these plants submitted to natural stress in tropical areas is also being evaluated. An increased understanding of common bean and cowpea genomes associated with contributions from biotechnology will provide an opportunity for breeders to accelerate the development of new varieties with valuable agricultural traits.

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III.7 Carrot

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1 Introduction

Carrot (*Daucus carota* L. subsp. *sativa*), a member of the family Apiaceae, is grown world-wide for its edible taproot. Carrot roots are marketed as fresh whole or baby carrots, and are used after processing in canned foods, soups and juice, and in frozen products. Nutritionally, carrots are high in β-carotene (provitamin A) as well as vitamins B_1 and C, and are a good source of dietary fiber. Carrot is a biennial plant, which flowers after exposure to a period of cold temperature (vernalization), generally during the second year of growth. Premature flowering (bolting) in the first year of growth can occur in some cultivars grown in certain geographic regions. High temperatures (30–35 °C) during growth may result in lighter-colored roots with reduced levels of β-carotene, whereas low temperatures (5–10 °C) early in the season may result in poor seedling establishment. The optimal temperature range for carrot growth and root development is 15–20 °C (Ammirato 1986). In addition to environmental stresses, biotic agents such as fungi, viruses, phytoplasmas, and nematodes can cause significant losses in yield and quality of carrots world-wide (Ammirato 1986; Agrios 1997).

The development of carrot cultivars through traditional breeding methods has been a major effort since the 1980s and has resulted in significant improvements to yield and quality (Simon 1984; Ammirato 1986; Peterson and Simon 1986). Root shape, length and color, smooth skin, flavor, early maturity, and resistance to various diseases are high priorities. Most of these genetic improvements in carrot require long-term effort, and many are multigenic traits and thus are not amenable to genetic engineering. In contrast, disease resistance and several other traits are monogenic. Much of the carrot acreage currently comprises of F_1 hybrids, produced by using a system of cytoplasmic male sterility that makes crossing of inbred lines achievable and economical (Allard 1960; Peterson and Simon 1986; St. Pierre and Bayer 1991). Inbred lines with proprietary genes introduced by genetic engineering techniques can therefore be marketed under licensing agreements.

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2 Applications of Biotechnology to Carrot Improvement

At the present time, there are no transgenic carrot cultivars commercially available on the market. Techniques in biotechnology provide the opportunity to enhance disease resistance in carrot, create herbicide-resistant plants, and modify the carotenoid biosynthetic pathway to produce novel carotenoid compounds. These approaches are discussed in more detail in this chapter. Most of the efforts to date have involved single-gene introductions coupled with antibiotic or herbicide resistance as a selection agent. Multiple pathogenesis-related proteins have been introduced into carrot plants to enhance resistance to fungal diseases (Tigelaar et al. 1996). Carrot is readily amenable to tissue culture and *Agrobacterium*-mediated transformation;and transgenic plants can be recovered within a 6-month time-frame (Chen and Punja 2002). There have also been significant advances in using carrot for metabolic engineering of enhanced stress-resistance and production of designer compounds, as carrot has many advantages over other easily transformed plants, including tobacco and potato (Marquet-Blouin et al. 2003; Kumar et al. 2004). Much of the metabolic engineering has been in the bio-medical industry where genetically engineered carrots have been used to produce antibodies and viral antigens, which can be utilized as edible vaccines (Bouche et al. 2003, 2005; Marquet-Blouin et al. 2003). The protocols used to achieve transgenic plants are described in more detail in this chapter.

2.1 Tissue Culture Methods

Carrot is a model system for use in tissue culture, and extensive research has been conducted in a number of areas, including somatic embryogenesis, bioreactor scale-up of suspension cultures, protoplast culture and fusion, and somaclonal variation (Ammirato 1986, 1987; Zimmerman 1993; Komamine et al. 2005). For somatic embryogenesis, the most commonly used media are Murashige and Skoog (MS; Murashige and Skoog 1962), Uchimya and Murashige (Scott and Draper 1987), and B5 media (Thomas et al. 1989), containing 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin or benzyladenine (BA; Ammirato 1986). Transfer of proembryonic masses to liquid medium can result in highly embryogenic synchronous cultures (Ammirato 1986). In the absence of auxin, somatic embryos continue to develop into plants. Protoplasts can be isolated from these suspension cultures or somatic embryos, or from mature plant tissues. Several genes associated with the early development of somatic embryos have been isolated (Takahata et al. 2004) but the detailed genetic understanding of this process is poorly understood. A number of variants have been observed in long-term tissue cultures of carrot and also after selection, including altered morphological characteristics, resistance to specific amino acid analogues and antibiotics (Sung and Dudits 1981; Ammirato 1986), and resistance to the herbicide glyphosate (Murata et al. 1998).

Protoplast fusion studies using carrot have resulted in somatic hybrids with altered characteristics (Sung and Dudits 1981; Ammirato 1986). However, neither artificial selection in tissue culture nor somatic hybridization studies have yielded significant improvements to the presently grown cultivars of carrot.

The response of different carrot cultivars to tissue culture conditions and the frequency of recovery of embryogenic calli were evaluated by Gilbert et al. (1996). Using epicotyl explants from in vitro-grown carrot seedlings and a range of 2,4-D concentrations (0.5, 2.0 or 4.5 μM) in MS medium, a frequency of 55–81% of embryogenic callus formation was recorded after 2–4 months of incubation in the presence of 4.5 μM 2,4-D for four carrot cultivars (Gilbert et al. 1996). Therefore, carrot cultivar may be a variable in transformation experiments as a result of the different responses to tissue culture conditions. Scale-up of somatic embryos in suspension cultures can be readily achieved, providing a method to recover large numbers of plants, especially following *Agrobacterium*-mediated transformation (Chen and Punja 2002).

2.2 *Agrobacterium*-Mediated Transformation of Carrot

Carrot roots have been inoculated with wild-type *A. rhizogenes* to produce transformed root tissue (Guivarc'h et al. 1999), from which plants could be regenerated via somatic embryogenesis (Tepfer 1985). Although these plants had altered root and leaf morphological features, they were used to quantify the rates of transfer of introduced genes through successive generations. *A. rhizogenes* was also used to introduce the maize transposable element *Ac* into carrot (Van Sluys and Tempe 1989). Upon regeneration of plants, the characteristic hairy root phenotype developed, but it did not impede study of *Ac* activity. Carrot hairy root cultures have also been utilized to study host–mycorrhizal interactions in vitro (Bécard and Fortin 1988).

Scott and Draper (1987) utilized proembryogenic carrot suspension cells and inoculated them with non-oncogenic *A. tumefaciens* strain C58C1 carrying the plasmid pGV3850::1103 with a chimaeric gene of *nos-npt*II (neomycin phosphotransferase) that confers kanamycin resistance. Plants were regenerated from transformed cells via somatic embryogenesis in the presence of 100 mg l^{-1} kanamycin; transformation efficiency was estimated to be 62–74%. Southern blot analysis showed that copy number ranged from one to eight. Western blot analysis confirmed the expression of the *npt*II gene and indicated that there was a single, full-length polypeptide (Scott and Draper 1987).

In a study by Wurtele and Bulka (1989), carrot callus cells were transformed with several strains of *A. tumefaciens* that possessed the same C58 chromosomal background. Each strain contained the binary vector pGA472 comprising the *nos-npt*II construct. Callus pieces (1 month old) were divided into 0.5 g (fresh-weight) aliquots, mixed with a suspension of *A. tumefaciens*, cocultivated for 3 days, and transferred to regeneration medium (MS medium

containing 1 mg l^{-1} 2,4-D). Plants were regenerated via somatic embryogenesis on medium containing 300 mg l^{-1} kanamycin. The effects were studied of *p*-hydroxybenzoic acid, acetosyringone and mechanical wounding of the cells at the time of cocultivation. The results showed that these treatments had little or no effect on the timing or frequency of kanamycin-resistant clump formation (Wurtele and Bulka 1989). Southern blot analysis, using the binary vector as a probe, showed that the transgenic plants contained 1–15 copies of the introduced gene (Wurtele and Bulka 1989).

Carrot hypocotyl segments from sterile 1-week-old seedling were used as the explant source by Thomas et al. (1989). The explants were preincubated on tissue culture medium (B5 with 9 μM 2,4-D) for 2 days prior to infection with *A. tumefaciens* LBA4404 containing a CaMV (cauliflower mosaic virus) 35S-GUS construct on a binary vector (pRGUSII). The plasmid also contained the *npt*II gene for kanamycin resistance. Results showed that explant preincubation was essential, since no transformation occurred in the absence of the preculture treatment (Thomas et al. 1989). Different carrot varieties were surveyed, and it was found that production of kanamycin-resistant calli was variety-dependent and ranged from 0.9% to 5.8%. The transformed calli (up to 1 cm diam.) were used to establish suspension cultures, and transformed plants were regenerated via somatic embryogenesis. Southern blot analyses confirmed that selected lines were transformed, with one to three copies of the *β*-glucuronidase (GUS) gene (Thomas et al. 1989).

Pawlicki et al. (1992) studied the factors influencing *A. tumefaciens*-mediated transformation of carrot. The parameters evaluated were carrot variety, age and type of explants, cocultivation and precultivation times, and effect of acetosyringone. Explants from five cultivars were cocultivated with non-oncogenic *A. tumefaciens* strain C58C1 carrying the plasmid GSTRN943 or pGSGluc1. Both plasmids contained a chimeric *npt*II gene, and pGSGluc1 also contained the GUS gene. Petioles, cotyledons, hypocotyls, and roots were used as explant sources and were compared for their transformation efficiency. Cocultivation periods were varied from 1 day to 7 days, and the precultivation periods (1, 2, 3, or 7 days, or 1 month) prior to infection and the presence or absence of acetosyringone (100 μM) were examined. Plants were regenerated from embryogenic calli (with 100 mg l^{-1} kanamycin), and rooted plants were potted and transferred to the glasshouse. The results from this study showed that transformation efficiency was dependent on carrot cultivar and ranged from zero to46.7%. Explant type was also found to be important. Petiole explants were more conducive to transformation compared to cotyledon, hypocotyl, or root explants. The age of the explants was also found to be a variable; a 3- to 4-week-old seedling was optimal. Cocultivation periods of 2 or 3 days gave higher transformation frequencies than periods of 1 or 7 days. Lastly, it was found that a preculture period or the presence of acetosyringone did not influence transformation efficiency. Transformation was confirmed by histochemical detection of GUS activity in transformed cells and by Southern hybridization analysis (Pawlicki et al. 1992). Guivarc'h et al. (1993) reported

that the presence of acetosyringone significantly increased the transformation efficiency of cells in carrot root disks as measured by GUS expression.

Carrot root disks were utilized by Gogarten et al. (1992) to study the effect of an antisense construct containing a vacuolar H^+ adenosine triphosphatase (ATPase) subunit cDNA. The *npt*II gene was used as a selectable marker. Kanamycin-resistant calli were grown in liquid MS medium containing 2,4-D and BA. Shoots and roots were induced on semi-solid MS media containing appropriate growth regulators, and plantlets were recovered.

In a study by Hardegger and Sturm (1998), GUS expression driven by the 35S promoter was shown to be high in transgenic carrot root tissues. The explants used for cocultivation were hypocotyls, and calli were produced on B5 medium with 0.5–2.0 mg l^{-1} α-naphthaleneacetic acid and 0–0.5 mg l^{-1} BA. *Agrobacterium* strain GV3101 was found to be more efficient than LBA4404 in the frequency of transformation, giving an average frequency of 20%.

Direct DNA uptake by carrot protoplasts by chemical and electrical means has been utilized to study gene expression, for example, the effects of promoters, enhancer elements, and introns on transcription efficiencies. These studies were made possible by coupling sequences of interest with the coding regions of reporter genes, such as the firefly luciferase (*lux*), *npt*II, chloramphenicol acetyl transferase (*cat*), and GUS (Bower and Birch 1993; Rasmussen and Rasmussen 1993). A gene for herbicide resistance was introduced into carrot via direct gene transfer (Dröge et al. 1992). The resultant transgenic plants contained the phosphinothricin-*N*-acetyltransferase (PAT) gene and were shown to be resistant to the herbicide L-phosphinothricin.

2.3 *Agrobacterium* Transformation Protocol for Carrot

A protocol to recover transgenic carrot plants with enhanced resistance to fungal pathogens and the herbicide phosphinothricin has been described from studies conducted by Gilbert et al. (1996) and Chen and Punja (2002). Seeds were surface-sterilized by dipping in 70% ethanol for 1 min followed by soaking in a 20% solution of bleach, containing 0.05% v/v Tween 20, for 15 min and then rinsing three times in sterile distilled water. Seeds were geminated on half-strength MS medium with 0.25% sucrose and 0.8% agar (pH 5.8) under a 16-h photo-period provide by cool-white fluorescent lamps (16 h day^{-1}) for 3–4 weeks. Petiole explants (0.5–1.0 cm long) were used for infection with disarmed *A. tumefaciens* strain LBA 4404 (or another suitable strain) containing the appropriate gene constructs for selection for resistance to kanamycin (*npt*II), hygromycin (*hpt*) or phosphinothricin (PPT; *bar*). Overnight cultures of *A. tumefaciens* were centrifuged at 650 *g* for 15 min and the pellet resuspended in 10% strength MS medium with 200 μM acetosyringone, 2% glucose, and 1% sucrose (pH 5.8) to an optical density at 600 nm (OD_{600}) of 0.3 (approx. 1×10^8 cells ml^{-1}). For infection, freshly cut petiole explants were immersed in the bacteria for 10 min, blotted dry, and transferred to MS medium contain-

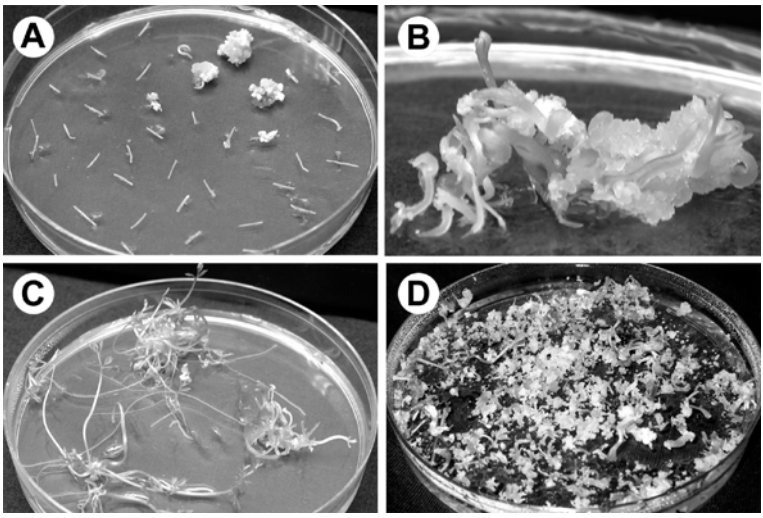

Fig. 1. Tissue culture and selection of transformed calli and plants of carrot. **A** Petiole explants infected with *Agrobacterium* and plated onto selection medium containing 1 mg l^{-1} phosphinothricin showing callus formation on about 10% of the explants after 2–4 weeks. **B** Development of somatic embryos on putatively transformed callus on selection medium after 2–4 months. **C** Development of plantlets on selection medium with 10 mg l^{-1} phosphinothricin. **D** Planting of embryogenic suspension cultures for plantlet regeneration. Diameter of Petri dishes = 9 cm

ing 3% sucrose and 1 mg l^{-1} 2,4-D for 3 days of cocultivation in the dark. Following this, the explants were rinsed in sterile water to remove excess *A. tumefaciens* and transferred to MS medium with 300 mg l^{-1} Timentin (30:1 ticarcillin:clavulanic acid; SmithKline Beecham) or 100 mg l^{-1} carbenicillin, to inhibit bacterial growth, and with the appropriate selection agent (25 mg l^{-1} kanamycin, 25 mg l^{-1} hygromycin or 1 mg l^{-1} PPT). After 2–4 weeks of incubation in the dark (Fig. 1A), explants were transferred to new medium with an increased selection regime (100 mg l^{-1} kanamycin, 100 mg l^{-1} hygromycin or 10 mg l^{-1} PPT). Transfers were made to new medium with selection for 2–4 months until somatic embryos developed (Fig. 1B). Embryos were transferred to growth regulator-free medium and incubated in the light until plants developed (Fig. 1C), or suspension cultures were initiated in liquid MS medium with 0.25 mg l^{-1} 2,4-D with appropriate selection (50 mg l^{-1} kanamycin or hygromycin or 5 mg l^{-1} PPT). Suspension cultures were maintained through subculture every 2–4 weeks. Plating of embryogenic clusters onto growth-regulator free MS medium gives rise to plantlets within 2 weeks (Fig. 1D). Plantlets with roots can be transferred into sterile potting medium (Soil mix 4 (Sunshine, Surrey, British Columbia), containing 55–60% Canadian *Sphagnum* peat moss, perlite, dolomitic limestone (for pH adjustment) and gypsum, and placed in a growth chamber under high humidity with a 16 h photo-period until ac-

climatized. Confirmation of transformation includes RT-PCR, Southern and western analyses (Gilbert et al. 1996; Chen and Punja 2002).

Factors that can influence transformation frequency (percent confirmed transgenic plants in relation to the original number of explants infected) include carrot cultivar, strain of *Agrobacterium* and bacterial concentration, duration of infection and cocultivation time (Gilbert et al. 1996). The average transformation frequency can range from 0.5% to 12% and is generally in the range 3–5% (Chen and Punja 2002).

2.4 Herbicide–Resistant Carrot Plants

Using the protocol described in Sect. 2.3, Chen and Punja (2002) developed carrot plants resistant to the herbicide Liberty (containing glufosinate-ammonium or L-phosphinothricin) by introducing the *bar* gene (which encodes phosphinothricin acetyltransferase) from *Streptomyces hygroscopicus*. The presence of the gene was confirmed by PCR, using specific primers; and the three carrot cultivars showed varying degrees of resistance to herbicide sprays, ranging from low (visible injury) to excellent (normal-appearing plants; Fig. 2). The results demonstrate the utility of genetic engineering technology to develop herbicide-resistant carrot plants. A safety evaluation of the PAT protein in transgenic plants has shown the enzyme to be highly specific, non-allergenic, rapidly degradable and with no adverse effects to humans (Hérouet et al. 2005).

Fig. 2. Comparison of a non-transformed control carrot plant of the cv. Nantes (*left*) with a transgenic plant expressing the phosphinothricin acetyltransferase (*bar*) gene (*right*). Both plants were sprayed with a 0.4% aqueous solution of the herbicide Liberty (containing glufosinate-ammonium). Note the excellent growth of the transgenic plant compared to the control. *Bar* 5 cm

2.5 Fungal Disease–Resistant Carrot Plants

The availability of cloned genes encoding for pathogenesis-related (PR) proteins, antifungal proteins (AFPs), and a number antimicrobial compounds, derived from a range of sources (Lamb et al. 1992; Cornelissen and Melchers 1993; Kamoun and Kado 1993; Van den Elzen et al. 1993), provides opportunities to enhance the tolerance of carrot to a broad spectrum of fungal pathogens after *Agrobacterium*-mediated transformation. Enhanced disease resistance has been reported in a range of plants as a result of genetic engineering methods (Punja 2001). In carrot, a number of genes have been introduced to enhance resistance to fungal pathogens. These include chitinases, glucanases, thaumatin-like protein, osmotin, and lysozyme (Punja and Raharjo 1996; Tigelaar et al. 1996; Melchers and Stuiver 2000; Takaichi and Oeda 2000; Chen and Punja 2002; Punja 2005).

Chitinases are a well-characterized group of PR proteins. Their expression in plants is increased by pathogen attack and certain abiotic stresses (Samac and Shah 1991; Punja and Zhang 1993). They accumulate intracellularly in the central vacuole (basic chitinases) or extracellularly in the intercellular space (acidic chitinases; Linthorst et al. 1990; Broglie et al. 1991). Chitinases catalyze the hydrolysis of chitin, a substrate that is not found in plants, but is a component of the cell walls of many fungi (Benhamou et al. 1993). Thus, exposure of fungal cells to chitinases has been shown to cause them to lyse (Mauch et al. 1988; Punja and Zhang 1993). In order to determine whether enhanced expression of chitinases in plants could also have a similar effect on fungi *in planta*, different chitinase-encoding genes were introduced into carrot under control of the 35S promoter. These included chitinases cloned from petunia (Linthorst et al. 1990), tobacco (Linthorst et al. 1990), and bean (Broglie et al. 1991). Transgenic lines of the carrot cvs. 'Golden State' and 'Nanco', containing either an acidic chitinase gene from petunia or a basic chitinase gene from tobacco, were regenerated from transformed calli after *A. tumefaciens*-mediated transformation and multiplied using a cell suspension culture system as described earlier. Plants were evaluated for their response to disease at various times during their growth, ranging from 4 months to 12 months after transplanting. The pathogens used were *Alternaria radicini*, *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotium rolfsi*, and *Thielaviopsis basicola*.

In general, significant differences were not apparent until 4 days after inoculation for most of the pathogens tested. In the transgenic line expressing the acidic petunia chitinase, statistical analyses of the data did not show a significant difference between transgenic and non-transgenic plants in several experiments. In the transgenic line expressing the basic tobacco chitinase, lesion size was significantly ($P = 0.01$) smaller in transgenic plants of both 'Golden State' and 'Nanco' at 4 days and 6 days after inoculation with *B. cinerea* (Fig. 3). With *R. solani* and *S. rolfsii*, lesion development was significantly reduced in the transgenic cultivar 'Nanco' at all evaluation times (2, 4, 7 days), whereas in 'Golden State', a significant reduction was seen only

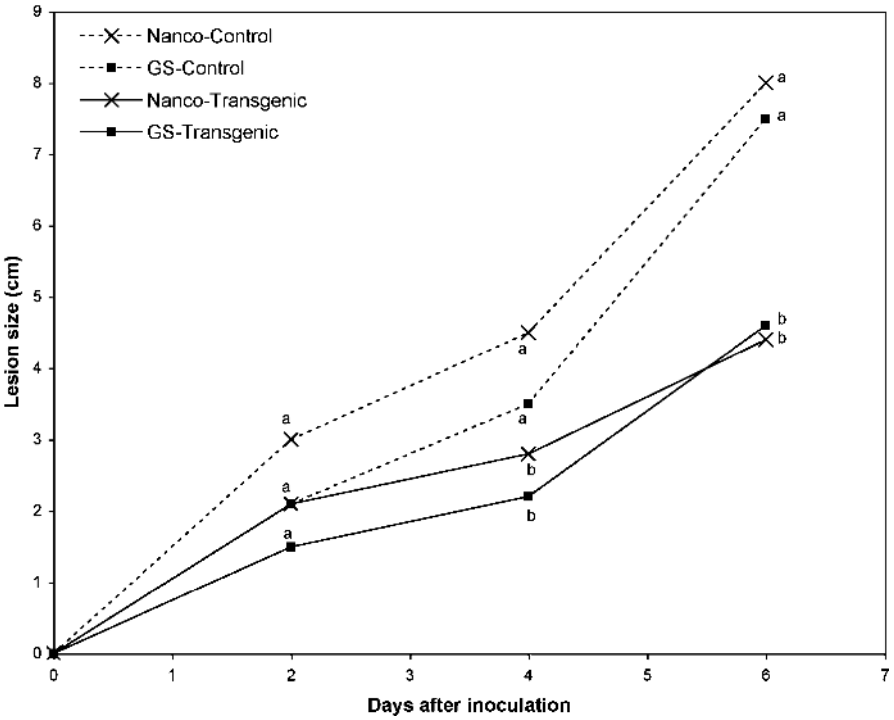

Fig. 3. Lesion development due to *Botrytis cinerea* on the foliage of control and transgenic plants of carrot cultivars Golden State (*GS*) and Nanco expressing a basic tobacco chitinase gene. Lesions were significantly ($P = 0.01$) smaller at 4 days and 6 days after inoculation (least significant diference test) in both cultivars

after 7 days. The greater reduction in lesion size at later evaluation times is suggestive of a cumulative effect of the antifungal activity due to the expressed chitinases. Thus, chitinase expression in transgenic carrot plants has the potential to delay the appearance of disease symptoms and result in lower overall disease incidence.

Thaumatin-like proteins (TLPs) belong to the PR-5 group of pathogenesis-related proteins and exert antifungal activity by altering plasma membrane permeability, by affecting signal transduction cascades, and by hydrolyzing polymeric β-1,3-glucans in the fungal cell walls (Koiwa et al. 1997; Grenier et al. 1999). A thaumatin-like protein from rice was expressed in the carrot cultivars 'Nanco' and 'Nantes' under control of the maize ubiquitin promoter and plants were evaluated initially for tolerance to *B. cinerea* and *Sclerotinia sclerotiorum* (Chen and Punja 2002). Four transgenic lines showed enhanced tolerance to foliar infection under controlled environment conditions. In a subsequent study, Punja (2005) evaluated two transgenic carrot lines of 'Nanco' expressing TLP against six fungal pathogens. The results indicated that enhanced tolerance

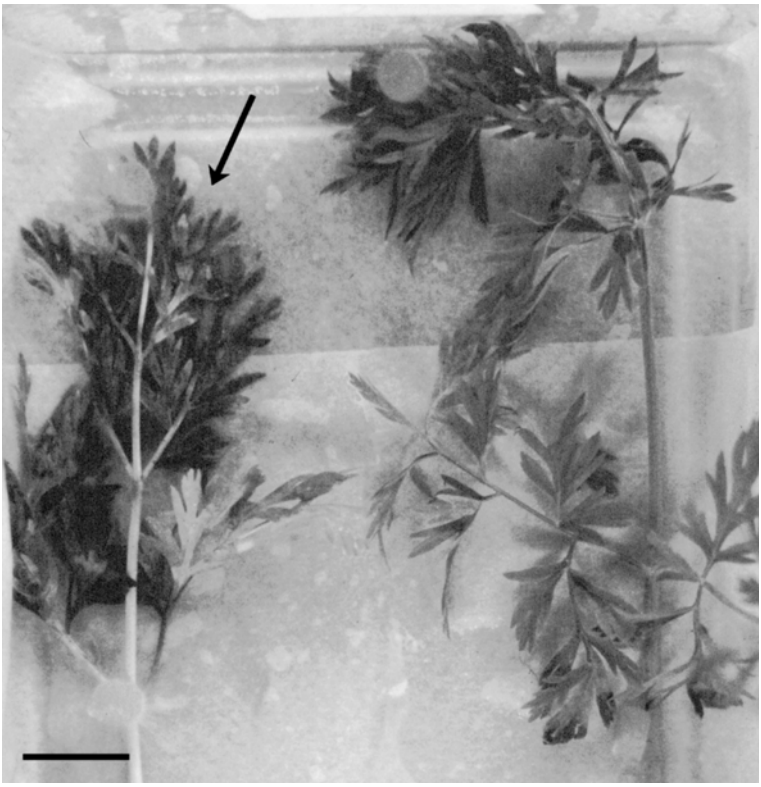

Fig. 4. Control (*left*) and transgenic (*right*) carrot leaves expressing a thaumatin-like protein inoculated with *Rhizoctonia solani*. Note differences in extent of foliar infection and necrosis. *Arrow* indicates necrotic leaf. *Bar* 3 cm

to foliar infection was observed for several species of *Alternaria*, *B. cinerea*, *R. solani*, *S. rolfsii*, and *S. sclerotiorum*. The response of transgenic and control carrots to infection by *R. solani* is shown in Fig. 4.

Several antifungal proteins from tobacco were constitutively expressed in transgenic carrot plants using constructs containing cDNAs and genomic fragments after *Agrobacterium* transformation (Tigelaar et al. 1996; Melchers and Stuiver 2000). The genes encoded AP 24 (PR-5, osmotin), chitinase, and glucanase. Transformed lines were regenerated and self-pollinated, and seed was used for field trials conducted over 2 years. Selected lines showed increased resistance against four pathogens: *A. dauci*, *A. radicini*, *Cercospora carotae*, and *Erysiphe heraclei*. The most promising lines were those expressing a combination of a chitinase and a glucanase gene, and they exhibited broad-spectrum tolerance (Tigelaar et al. 1996; Melchers and Stuiver 2000). A human lysozyme gene was constitutively expressed in two carrot cultivars and some of the transgenic lines showed enhanced resistance to *E. heraclei* and *A. dauci* (Takaichi and Oeda 2000).

2.6 Modification of the Carotenoid Biosynthetic Pathway

Carrot roots are a well known source of carotenoids, especially α- and β-carotenes, which serve as provitamin A (retinol) compounds. Lee (1986) reported that the carotene content of roots increased gradually up to 100 days after seeding, reaching a maximum at 135 days (at harvest). In carrot roots, carotenoid levels range over 5–35 $\mu g\,g^{-1}$ ($mg\,kg^{-1}$) for α-carotene and 11–640 $\mu g\,g^{-1}$ for β-carotene (Van den Berg et al. 2000). In addition, lutein can be found at up to 20 $\mu g\,g^{-1}$.

Engineering of the carotenoid biosynthetic pathway has been achieved in a number of plant species, including rice (Ye et al. 2000), canola (Shewmaker et al. 1999), tomato (Romer et al. 2000) and tobacco (Mann et al. 2000). The most well known example is "golden-rice", expressing novel β-carotene in the endosperm (Ye et al. 2000). In addition, canola producing novel β-carotene and potatoes with enhanced β-carotene concentrations were developed through

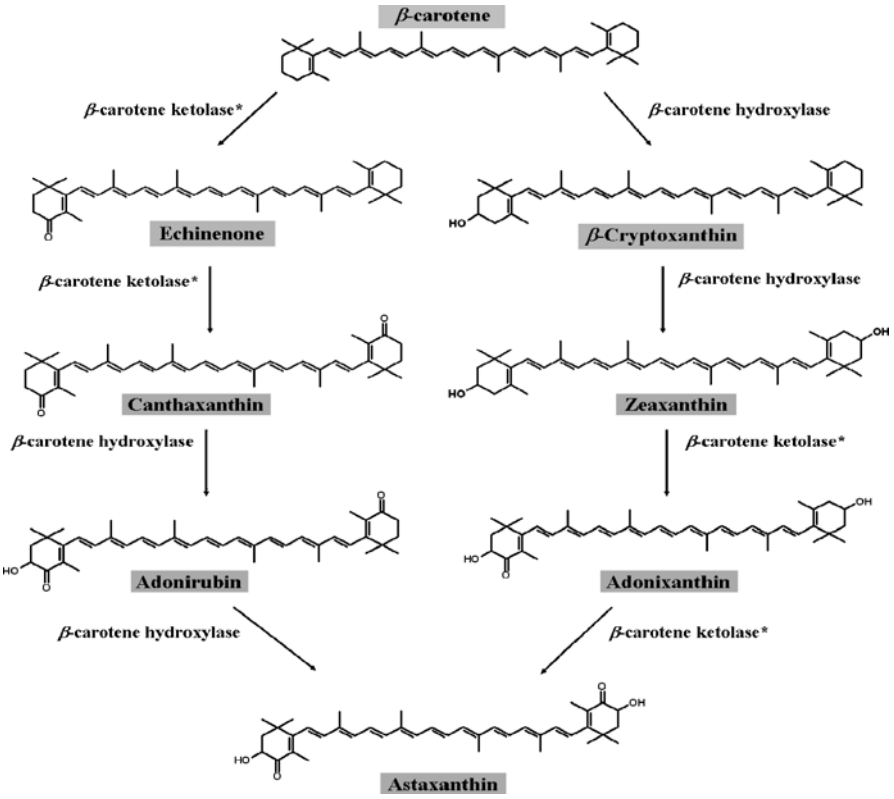

Fig. 5. Pathway for ketocarotenoid production from β-carotene, utilizing ketolase and hydroxylase enzymes. Intermediate products are shown. Adapted from Giuliano et al. (2000) and Ladygin (2000)

expression of a heterologous phytoene synthase gene (Shewmaker et al. 1999; Ducreux et al. 2004).

In tobacco, engineering of an algal β-carotene ketolase gene resulted in conversion of β-carotene to the ketocarotenoid astaxanthin (Mann et al. 2000). The conversion of β-carotene to astaxanthin first requires two ketolation steps to produce canthaxanthin by a ketolase enzyme (Fig. 5). A hydroxylase gene then converts canthaxanthin to astaxanthin through hydroxylation (Fig. 5). If hydroxylation occurs first, an intermediary product zeaxanthin is produced, which can be converted by ketolase to form astaxanthin. Both astaxanthin and canthaxanthin are used to supplement the diets of cultured salmon and trout to achieve the characteristic pink-red color of these fish. These ketocarotenoids, including zeaxanthin, are also strong antioxidants and enhance tolerance of plants to environmental stresses, including UV light (Gotz et al. 2002), and surpass the antioxidant activity of vitamins C and E in humans (Naguib 2000).

In our laboratory, we are attempting to engineer an algal (*Haemotococcus pluvialis*) β-carotene ketolase gene and an *Arabidopsis* β-carotene hydroxylase gene into carrot roots to convert β-carotene into ketocarotenoids. This could potentially provide an alternate source of these valuable compounds. The *Agrobacterium*-mediated transformation protocol described earlier was used

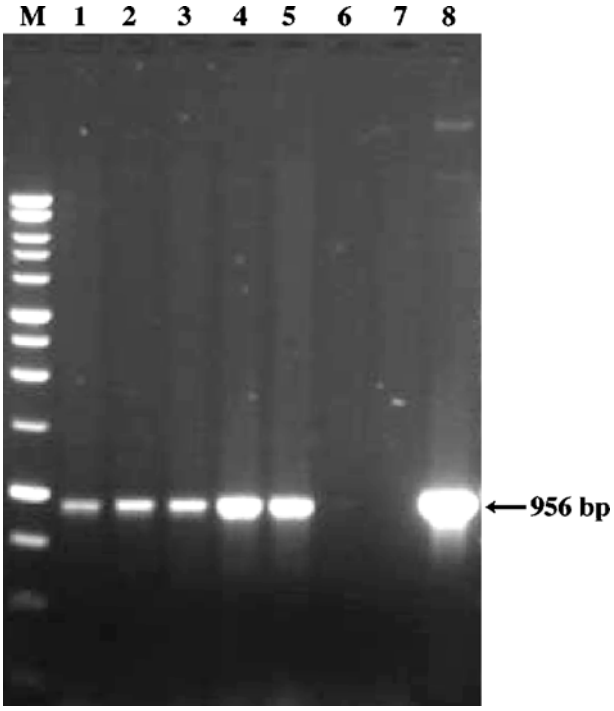

Fig. 6. Detection of the algal ketolase gene in transgenic carrot plants using PCR. *Lanes 1–5* DNA from five different transgenic lines, *lanes 6*, 7 no DNA, *lane 8* positive control-pCambia plasmid containing the ketolase gene. *M* Molecular weight markers

and selection for resistance to phosphinothricin was employed. Transgenic plants expressing the algal β-carotene ketolase gene have been obtained (Fig. 6). The protein was targeted to the root chromoplasts/leaf chloroplasts using the Rubisco transit peptide. In addition, a range of root-specific and constitutive promoters is currently being evaluated for expression in carrot tissues (see below). The phenotype remains to be characterized with regard to the actual rate of conversion of β-carotene, but the results to date suggest the possibility for modifying the carotenoid biosynthetic pathway in carrots.

2.7 Evaluation of Promoters for Transgenic Expression

An essential aspect of the engineered expression of novel proteins in carrots is the use of suitable promoters to achieve either constitutive or root-specific expression. In previous studies, either the 35S promoter or maize ubiquitin promoter was utilized (Gilbert et al. 1996; Chen and Punja 2002). In on-going work to enhance the resistance of carrots to fungal diseases and to engineer novel carotenoids in carrot roots, we are assessing the expression levels of GUS in carrot tissues as a marker to quantify promoter strength. The promoters under investigation are summarized in Table 1. Prelimary results from six of the different promoter constructs (*mas* and the 'super-promoter' were not tested) in both leaf and root tissue indicated that *Arabidopsis* ubiquitin promoter (*ubi-q*) has the highest activity in the leaves, followed by maize ubiquitin, 35S, *rolD*, and double 35S, with little to no activity detectable with the *ags* promoter (Fig. 7A). The promoter strength in the root tissue was strongest with the *ubi-q*, followed by 35S, *ubi-1*, *rolD*, double 35S, and *ags* (Fig. 7B).

Table 1. Promoters evaluated for expression in carrot tissues

Promoter	Tissue expression	Source
Cauliflower mosiac virus 35 S	Constitutive	Odell et al. (1985)
Double cauliflower mosiac virus 35 S (psm)	Constitutive	Kay et al. (1987)
Arabidopsis ubiquitin (*ubi-q*)	Constitutive	Norris et al. (1993)
Maize ubiquitin 1 (*ubi-1*)	Constitutive	Christensen et al. (1992)
Agrobacterium rhizogenes agropine synthase (*ags*)	Root-specific, wound-inducible	Bandyopadhyay et al. (1989)
A. rhizogenes mannopine synthase (*mas*)	Callus- and root-specific, wound-inducible	Ellis et al. (1984)
A. rhizogenes rooting loci gene *rolD*	Root-specific	Leach and Aoyagi (1991)
Super promoter (*A. tumefaciens*, octopine synthase + *mas*)	Constitutive	Ni et al. (1995)

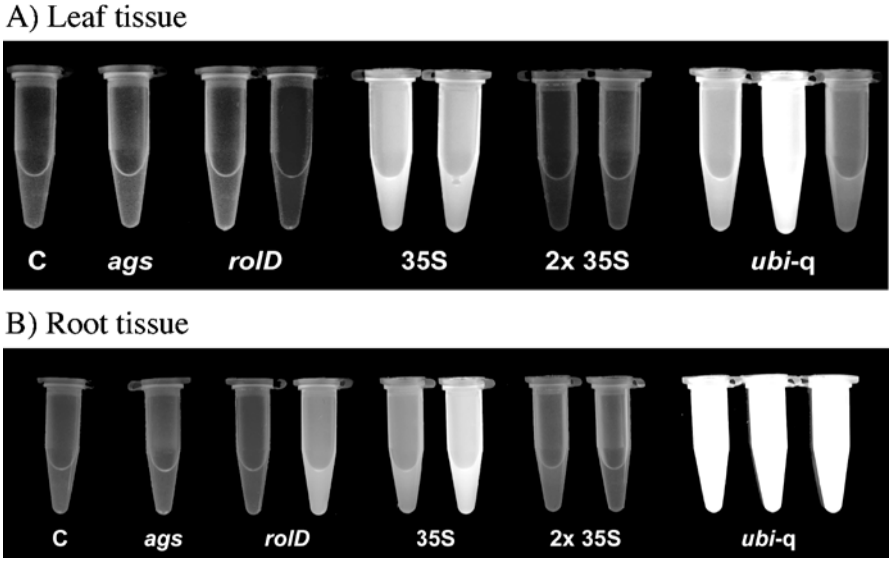

Fig. 7. Fluorogenic measurement of GUS activity from extracted carrot protein of transgenic plants. Aliquots were taken from a MUG assay of 10 μg of total soluble protein and the reaction was stopped after 10 h. **A** Measurement from leaf tissue (*C* non-transformed carrot leaf protein). **B** Measurement from root tissue (*C* non-transformed root protein). The GUS activity due to the expression of the different promoters is shown (see Table 1 for promoter sources)

3 Future Prospects

It is evident from all of the published work on carrot transformation conducted to date that several different protocols are available. The variables that can affect the frequency of transformation include carrot cultivar, explant source, bacterial strain, tissue culture medium, and growth regulators. In spite of this, recovery of transgenic carrot plants has been reported from several laboratories. Surprisingly, however, there are few reports of the introduction of potentially useful agronomic traits in carrot, with the exception of fungal disease resistance and herbicide resistance.

Although breeding efforts have provided, and will continue to provide, superior cultivars for commercial use, there are many unexploited opportunities for future targets in genetic engineering of carrot. The feasibility of introducing virus resistance, resistance to insect pests, tolerance to environmental stress, and modification of the carotenoid biosynthetic pathway, as well as the ratio of various sugars for quality enhancement, has yet to be explored. Confirmation of expression of the desired trait under field conditions and transmission of the introduced genes to the progeny will be required, as well as regulatory approval for widespread use.

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Section IV Root Crops

IV.1 Potato

F. Börnke, U. Sonnewald, and S. Biemelt[1]

1 Introduction

Potato is the world's fourth most important food crop, being surpassed in total production only by wheat, corn and rice. Sterility and tetraploidy, in conjunction with a high degree of heterozygosity, greatly reduce the efficiency of traditional methods for potato breeding. Despite these limitations, conventional breeding programs have been extremely effective in delivering new cultivars for a diversity of end uses. Breeding aims are, for instance, yield, earliness, disease resistance and starch quality. In the case of yield, the harvest index of potato (ratio of dry weight of harvestable organs to the dry weight of the entire plant) has been increased from 0.09 in wild species to 0.81 in modern cultivars (Inoue and Tanaka 1978). Although such improvements have been both dramatic and revolutionary, they are also time-consuming and slow. The emergence of molecular assisted breeding and plant transformation technologies offers the possibility of manipulating metabolism using a more rapid, targeted approach. Many of the recent advances in molecular biology are now being applied to crop improvement; and potato is no exception. Since potato can be propagated vegetatively every efficiently, novel traits can be directly introduced into commercial lines without the need for subsequent breeding. This review summarizes the tools available to genetically engineer useful traits in potato and gives some representative examples for the application of biotechnology in potato improvement.

2 Economic Importance

Potatoes are grown in almost every country of the world. Today, the annual worldwide production of potatoes exceeds 300×10^6 t (FAOSTAT 2006). In 2004, China was by far the biggest potato producer (75×10^6 t), followed by the Russian Federation (35×10^6 t) and India (25×10^6 t). Although potato production in Europe has declined since the early 1960s, this decline has been more than offset by the growth in Asia, Africa and Latin America. The majority of potato production is used for human consumption (50–60%); the rest is used as an-

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imal feed, for industrial products, or as seed tubers. Consumption per capita in developing countries is rapidly increasing and has reached 14 kg year^{-1}, but is still only a fraction of per capita consumption of 86 kg year^{-1} in Europe or 63 kg year^{-1} in North America, suggesting that ample room exists for continued increases in consumption.

3 Genetic Engineering

3.1 Potato Transformation

For genetic engineering to be effective in delivering new potato cultivars, four essential and interacting components are needed. These include: (1) an efficient transformation system to deliver foreign genes, (2) suitable selection and regeneration systems to produce transgenic plants, (3) appropriate *cis*-regulatory elements for precise transgene expression and (4) suitable genes that have been characterized and which can impart favorable traits.

Potato is one of the few crop species naturally susceptible to infection by *Agrobacterium tumefaciens*, the causative agent of crown gall disease. Thus, *Agrobacterium*-mediated gene transfer has been the method of choice to deliver foreign genes into potato since it was first applied in 1986 (Ooms et al. 1986). The current knowledge concerning transfer of DNA from *A. tumefaciens* into plants, as well as a history of *A. tumefaciens*, was reviewed recently by Zupan et al. (2000) and will not be discussed in detail here. *Agrobacterium*-mediated transformation has been employed with several different potato cultivars (Deblock 1988; Sheerman and Bevan 1988; Hoekema et al. 1989; Beaujean et al. 1998), using leaf (Deblock 1988; Rochasosa et al. 1989; Visser et al. 1989), stem (Visser et al. 1989; Newell et al. 1991; Beaujean et al. 1998), or tuber discs (Sheerman and Bevan 1988; Hoekema et al. 1989).

Despite its general applicability, transformation by *Agrobacterium* remains a relatively rare event. In the case of potato, only 0.2% of regenerated shoots represent transgenic events under non-selective conditions, which can be boosted to a modest 4.5% using super-virulent *Agrobacterium* strains (de Vetten et al. 2003). Therefore, the gene of interest is usually linked to a selectable marker gene. The most widely used selection gene in potato transformation is neomycin phosphotransferase (*npt*II; Bevan et al. 1983), which gives the transgenic tissue resistance against the antibiotic kanamycin and some other related antibiotics. Also, genes conferring herbicide resistance have been used to generate transgenic potato plants. Acetohydroxyacid synthase (AHAS), catalyzing the first step of the synthesis of branched-chain amino acids, is the target enzyme for a number of herbicides. A S653N mutation in the AHAS gene results in an increased tolerance to imidazoline herbicides and a mutated form of the enzyme has been used as a selection marker during transformation of a number of commercially important potato varieties (Andersson et al. 2003).

Although the stable integration of such genes makes it possible to identify the rare transformed cells and to regenerate plants from them, their presence in the transgenic plants after transfer from tissue culture complicates the regulation process and negatively affects public acceptance of the final products. Thus, effort has focused on the development of alternative selection systems. One category of alternative marker genes enables the transformed cell to thrive on an otherwise non-metabolizable carbon source. For instance, externally added mannose is converted to mannose-6-phosphate, which most plants cannot metabolize further and which accumulates to inhibitory levels. Therefore, mannose-6-phosphate isomerase can be used as a selectable marker as this enzyme converts mannose-6-phosphate to fructose-6-phosphate which is readily metabolized through glycolysis (Bojsen 1993). Mannose selection has been shown to be successful for a range of plant species (Joersbo 2001), including potato (Thorbjornsen et al. 2002; Rung et al. 2004). Following a similar idea, transgenic potato plants have been generated using a selection marker based on detoxification of 2-deoxyglucose (Kunze et al. 2001). 2-Deoxyglucose is a glucose analogue that, following hexokinase-mediated phosphorylation to 2-doxyglucose-6-phosphate, severely impairs plant growth due to multiple effects on metabolism. Detoxification is achieved by over-expression of a specific 2-deoxyglucose-6-phosphate phosphatase from baker's yeast (Kunze et al. 2001).

Although development of alternative selection systems will be important to meet consumers' expectations and to allow multiple transformation of elite lines, the ultimate goal is the production of marker-free transgenic crops. Usually, this can be achieved by either co-transformation, followed by subsequent out-crossing of the marker gene, or by the use of site-specific excision systems allowing removal of unwanted selection markers after passage through tissue culture (Hare and Chua 2002). However, these methods are often not only inefficient and time-consuming but also are not applicable to vegetatively propagated species, such as potato. Recently, marker-free transgenic potato plants have been produced by using a hyper-virulent *Agrobacterium* strain in combination with an efficient PCR method for the selection of transgenic events (de Vetten et al. 2003). Because this system does not require genetic segregation or site-specific DNA-deletion systems to remove marker genes, it may provide a reliable and efficient tool for generating transgenic potato plants for commercial purposes.

In an elegant attempt to reduce even further the transfer of foreign DNA fragments into transgenic potato plants, Rommens et al. (2004) replaced the *Agrobacterium* T-DNA of the binary transformation vector by a plant-derived transfer DNA. Plant-transferred DNAs (P-DNAs) were uncovered through database searches and PCR analyses for sequences that resembled T-DNA borders. P-DNAs of a number of species, including tomato and potato, have been shown to support DNA transfer from *Agrobacterium* to plant cells (Rommens et al. 2004). A potato P-DNA supported almost double the potato transformation efficiency of a conventional T-DNA (Rommens et al. 2004).

3.2 Transgene Expression

Depending on the specific question, the introduced foreign gene must be expressed in a predictable and desired manner. Nevertheless, the most commonly used promoters drive constitutive expression throughout the entire plant, such as the cauliflower mosaic virus (CaMV) 35S promoter (Franck et al. 1980) or the 34S FMV promoter (Richins et al. 1987). Both promoters have been used to engineer pathogen-resistant potato cultivars, but they are of limited use when it comes to the manipulation of metabolic pathways. Since the potato tuber represents the harvestable organ of the crop, genetic engineering of output traits is focused on this part of the plant. Thus, tuber-specific expression of the transgene is required for a number of applications. The most widely used promoters for potato tuber-specific expression of transgenes are the patatin B33 promoter (Rochasosa et al. 1989) or the granule-bound starch synthase (GBSS) promoter (Rhode et al. 1990). These promoters combine tight tissue specificity with high-level expression.

Most of the promoters currently used have been isolated following a detailed expression analysis of cDNA clones. To accelerate promoter discovery, tagging strategies for the isolation of genomic sequences directing transgene expression have been followed in *Arabidopsis*, tobacco and potato (Lindsey et al. 1993). To this end, promoterless β-glucoronidase (GUS) reporter gene constructs are randomly introduced into the plant's genome via *Agrobacterium*-mediated gene transfer. This allows the random fusion of the GUS gene with regulatory sequences of native genes, leading to expression of the fusion gene in a number of distinct cell types. Following histochemical characterization of GUS expression, the desired genomic fragments can be isolated and tested in transgenic plants. Although promoters conferring cell- or tissue-specificity are versatile tools for biotechnological applications, chemical regulation of transgene expression offers the opportunity to switch on and off at will the synthesis of gene products. This feature is especially important if toxic substances or pharmaceutical products are to be synthesized. A number of chemical-inducible expression systems have been described for plants (Padidam 2003). For use in potato, the ethanol-inducible *alc*-gene switch has been best characterized (Junker et al. 2003, 2004).

4 Goals of Potato Biotechnology

In general, the goals of plant biotechnology do not differ very much from those of classic potato breeding. They can be divided into attempts to optimise input and output traits. Input traits refer both to increased resistance towards abiotic and biotic stresses and to strategies to increase yield and improve post-harvest characteristics. Attempts to engineer output traits include the production of foreign proteins for pharmaceutical and technical use, the

production and modification of endogenous and novel polymers for food and non-food applications and the synthesis of low molecular weight compounds, including vitamins, essential amino acids and pharmaceutically relevant secondary plant products.

4.1 Approaches to Improve Starch Quantity and Quality

Starch is the principle constituent of the potato tuber. Besides its importance as a staple in human and animal diets, it is also used as a renewable raw material for a wide range of industrial applications (Slattery et al. 2000; Roper 2002). Starch is a relatively simple polymer composed of glucose molecules that are linked in two different forms. Amylose, which makes up 20–30% of potato tuber starch, is an essentially linear molecule in which the glucose units are joined end-to-end by $\alpha(1 \rightarrow 4)$ linkages. Amyolopectin is the major component of potato tuber starch and is a much larger branched molecule, in which about 5% of the glucose units are joined by $\alpha(1 \rightarrow 6)$ linkages. Potato starch is unique among commercial starches in that it possesses a high level of phosphate groups that are covalently linked to the C3 and C6 positions of the glucose monomers. The physico-chemical properties of starch, such as gelatinization temperature, retrogradation and viscosity, which are important for technical uses, are mainly influenced by the amylose to amylopectin ratio. High-amylose starches are used in fried snack products to create crisp and evenly brown snacks, as gelling agents and in photographic films, whereas high amylopectin starches are useful in the food industry to improve uniformity, stability and texture and in the paper and adhesive industries (Slattery et al. 2000).

Therefore, attempts to both increase the accumulation of starch and to modify its structural properties by molecular means have received considerable attention.

4.1.1 Attempts to Increase Starch Quantity

In the case of potato, yield can be regarded as a function of the starch content of the potato tuber. The biochemical pathways leading to starch formation are well documented and key enzymatic steps have been identified (Fernie et al. 2002; Geigenberger 2003). Briefly, in growing potato tubers, sucrose delivered through the phloem is cleaved by sucrose-synthase to fructose and uridine-diphosphoryl-glucose pyrophosphorylase (UDP-glucose), which are converted to hexose-phosphates by the action of UDP-glucose-pyrophosphorylase and fructokinase, respectively. Hexose-phosphates are then imported into the amyloplast and used for starch synthesis via plastidial phosphoglucomutase, ADP-glucose pyrophosphorylase (AGPase) and various isoforms of starch synthase. Recently, this general scheme was challenged by a study concluding that starch biosynthesis, at least in source leaves of potato, starts with the synthesis of ADP-glucose in the cytosol (Baroja-Fernandez et al. 2004). However, a large

body of literature argues against this proposal (discussed by Neuhaus et al. 2005).

To increase the efficiency of the pathway and thus increase starch accumulation, molecular strategies have concentrated mainly either on the modulation of sucrose catabolism (Sonnewald et al. 1997; Trethewey et al. 1998), or on the plastidial starch synthetic pathway (Stark et al. 1992; Tjaden et al. 1998a). Expression of yeast invertase within the apoplast of transgenic tubers led to a dramatic reduction in tuber sucrose contents and a corresponding large increase in glucose content. Although an increase in size was observed in the transgenic tubers, an increase in yield was largely compensated by a simultaneous reduction in the number of tubers per plant (Sonnewald et al. 1997). To further stimulate hexose utilization in invertase-expressing plants, a bacterial glucokinase was co-expressed with invertase. Despite massive accumulation of hexose-phosphates, the transgenic tubers showed no increase in starch synthesis but were characterized by an induction of glycolysis and a massive partitioning of carbon into respiration (Trethewey et al. 1998). The reason for this dramatic change in partitioning remains obscure, but it clearly demonstrates the flexibility of plant metabolism and highlights the necessity for a detailed understanding of the underlying factors that involved in its regulation.

In an attempt to increase the activity of the starch synthetic pathway, Stark et al. (1992) over-expressed de-regulated bacterial AGPase in the potato variety Russet Burbank. The data revealed a 30% increase in starch in the transgenic tubers. However, this effect was lost upon transformation of a different potato cultivar (Sweetlove et al. 1996). In contrast to chloroplasts where ATP required for starch production is made during photosynthesis, starch synthesis in potato tubers relies on the import of ATP from the cytosol into the amyloplast mediated by an ATP/ADP-translocator. The ADP/ATP-translocator was determined to be an important control point of starch synthesis and over-expression of this protein in transgenic potato tubers led to a 48% increase in starch formation, indicating that energy supply might be one factor limiting starch accumulation (Tjaden et al. 1998b). Recently, an increase in the concentration of adenylates in potato tubers was achieved through antisense repression of a plastidial adenylate kinase (Regierer et al. 2002). As a result, starch content was increased by about 60% in the transgenic plants and tuber yield was 85% above that of the wild-type control (Regierer et al. 2002).

4.1.2 Engineering Starch Quality

As stated above, one of the major factors influencing the physico-chemical properties of starch is the amylose to amylopectin ratio. Hence, considerable effort has been aimed at changing the ratio towards one or the other direction. The synthesis of amylose is accomplished through the activity of a particular isoform of starch synthase, GBSS, and antisense inhibition of this gene leads to amylose-free potato starch (Visser et al. 1991). Amylose-free potato starch has

improved paste clarity and stability and can be expected to find applications both in the food industry and in paper manufacture. Large-scale field trials with amylose-free transgenic potatoes have been conducted in Europe and this crop is currently going through the regulatory approval process.

An important property of starches that are used in food products is their freeze–thaw stability. Although, in this respect, amylose-free starches represent a considerable improvement over conventional starches, for most applications they still require chemical modification. A potato starch with excellent freeze–thaw stability was developed recently through a combined antisense approach. The simultaneous down-regulation of three starch synthase genes, GBSS, starch synthase II and starch synthase III, in potato tubers led not only to amylose-free starch, but also to a reduced length of amylopectin chains (Jobling et al. 2002).

High-amylose starches have also been of great interest (Jobling 2004). Interestingly, potato tubers over-expressing an amyloplastidial ATP/ADP-transporter displayed not only higher starch content but also altered starch structure (Tjaden et al. 1998b). Starch isolated from these transgenic tubers was found to have greatly elevated amylose content and a modified crystal structure (Geigenberger et al. 2001). Recently, an innovative approach to increase amylose content involved the inhibition of starch-branching enzyme A (SBE A) activity, the enzyme responsible for introducing $\alpha 1 \rightarrow 6$ linkages into amylopectin (Jobling et al. 2003). The authors of this study expressed a single-chain antibody targeted against the active centre of SBE A, thereby neutralizing its activity. They found that immunomodulation increased the amylose content of starch granules from about 20% in wild-type tubers to 74% in the best transgenic line, exceeding the concentrations of amylose achieved by conventional antisense strategies (Jobling et al. 2003).

Potato starch has the highest phosphate content amongst commercial starches; and this is, in part, responsible for the high swelling power and stable paste properties of this starch. Recently, the enzyme responsible for the incorporation of phosphate groups into starch was identified from potato (Lorberth et al. 1998) and was shown to encode a glucan-water dikinase (Ritte et al. 2002). Antisense repression of this enzyme in potato resulted in a dramatically reduced starch phosphate content and an increase in amylose content (Lorberth et al. 1998).

4.2 Production of Novel Carbohydrates in Transgenic Potato Tubers

In addition to attempts at manipulating the contents and properties of endogenous carbohydrates, there have been several successful approaches for the production of novel carbohydrates in transgenic potato tubers. However, for most of the examples reported so far, the potato tuber serves as a model system to achieve proof-of-concept rather than being the final target crop. Thus, only a brief overview is given here.

Fructans are a diverse group of polysaccharides that derive from sucrose and contain one or more β-linked fructose units. Due to their health-promoting properties, fructans are of growing interest as functional food ingredients (Ritsema and Smeekens 2003). Fructans are normally isolated from plants with low agronomic value, such as the Jerusalem artichoke (*Helianthus tuberosus*) and chicory. Thus, there is great interest to transfer fructan-synthesizing enzymes into crop plants. Only two fructosyltransferases are needed to synthesize fructans in plants, namely, sucrose:sucrose-fructosyl transferase (SST) and fructan:fructan-fructosyl transferase (FFT). Combined expression of SST and FFT from *Cynera scolymus* in transgenic potato tubers resulted in the production of the full range of fructans found in *C. scolymus*, demonstrating the potential of transgenic plants to produce fructans (Hellwege et al. 2000).

Recently, the production of another sugar was achieved in transgenic potato tubers that is of potential importance for the food industry. The sucrose isomer isomaltulose (Palatinose; 6-*O*-α-D-glucopyranosyl-D-fructose) is an excellent sweetener since it shares many of the properties of sucrose but is non-metabolizable and non-cariogenic (Börnke et al. 2001, 2002). A gene encoding a sucrose isomerase (*pal*I) which catalyzes the conversion of sucrose into isomaltulose has been isolated from the bacterium *Erwinia rhapontici* (Börnke et al. 2001). Expression of the *pal*I gene within the apoplast of transgenic tubers led to a nearly quantitative conversion of sucrose into palatinose. Despite the soluble carbohydrates having been altered within the tubers, growth of *pal*I-expressing transgenic potato plants was indistinguishable from wild-type plants. Therefore, expression of a bacterial sucrose isomerase provides a valid tool for high-level palatinose production in the storage tissues of transgenic crop plants (Börnke et al. 2002). Other examples for the production of novel carbohydrates in transgenic tubers are cyclodextrins (Oakes et al. 1991) and trehalose (Goddijn et al. 1997).

4.3 Engineering Resistance

Insect pests and diseases caused by fungal, viral and bacterial pathogens pose a considerable threat to crops. Despite the use of sophisticated crop protection strategies, global loss because of pathogens is estimated to be 12% of potential crop production (James et al. 1992). Hence, since the advent of plant transformation during the 1980s, disease resistance was recognized as a primary target for plant genetic engineering. Several crop varieties with agronomically useful levels of resistance to a number of different pests have been generated through gene transfer, and were among the first transgenic plants to be introduced into the market place (Shah et al. 1995).

Potato is particularly subject to virus diseases, in part because vegetative propagation allows a virus several years to accumulate over successive generations. Genetic engineering of virus-resistant plants has exploited new genes derived from viruses themselves in a concept referred to as "pathogen-derived

resistance" (PDR; Scholthof et al. 1993). The mechanisms underlying PDR can be broadly classified into those that are dependent on gene-silencing events and those that are not (Baulcombe 1996). The first genetically engineered plant virus resistance was reported by Abel et at. (1986). In this study, a cDNA clone of the tobacco mosaic virus (TMV) coat protein (CP) was expressed in transgenic tobacco plants, and plants that accumulated coat protein developed no disease symptoms or showed delayed symptom development when inoculated with TMV. This resistance was referred to as coat protein-mediated resistance (CP-MR). Subsequent studies showed that CP-MR is effective against almost all classes of viruses in many different plants, including potato. For example, reduced symptoms, movement and titers were observed in potato plants expressing the CP of potato virus X (PVX; Hoekema et al. 1989), potato leaf roll virus (PLRV; van der Wilk et al. 1991), potato virus Y (PVY; Kaniewski et al. 1990) and potato carla-virus (PVS; MacKenzie et al. 1991). Most of these studies were carried out under controlled environmental conditions but unfortunately the results obtained did not translate well to plants grown under field conditions (Kaniewski and Thomas 2004). Therefore, several other virus genes have been used to engineer resistance, including the replicase, movement protein and protease genes (Kawchuk 2002). Transgenic potato plants expressing the full-length replicase gene of PLRV showed full resistance to the homologous PLRV isolate in growth chamber experiments (Lawson et al. 2001). Field experiments later showed that some of the replicase-expressing lines remained virus-free throughout the growing season (Thomas et al. 2000). The non-specificity of resistance in these lines was confirmed by field exposure of each line to 64 different PLRV isolates collected throughout the United States.

A general drawback of PDR is the possibility of recombination events between an invading virus and the transgene, or in case of transgenic coat protein, the transencapsidation of viral genomes. Therefore, strategies have been devised to engineer virus resistance using non-viral genes (Kawchuk 2002). These involve the expression of ribosome-inactivating proteins (RIPs) and ribonuleases specific for double-stranded RNAs. Furthermore, the identification of host proteins necessary for virus infection will open new possibilities to engineer resistance. For example, the CP of PVY was shown to interact with a subset of DNA J-like chaperones from tobacco in a yeast two-hybrid screen. Transgenic tobacco plants overexpressing a DNA J-mutant still capable of interacting with the PVY CP, but devoid of its J-domain, showed a dramatic and durable increase in resistance towards PVY (Hofius et al. 2003).

Progress has also been made in engineering resistance to insect pests that attack potato. The Colorado potato beetle (CPB) is the most destructive pest of potato; and, without its control using insecticides, complete defoliation of potato fields can occur. Transgenic potato lines expressing the *Bacillus thuringiensis* var. *tenebrionis* cryIIIA delta-endotoxin gene displayed protection against damage by all insect stages in the laboratory and gave dramatic levels of protection at multiple field locations (Perlak et al. 1993). Transgenic

potato cultivars resistant to CPB were commercialized in the United States in 1996. Later, CPB resistance was combined with resistance against PLRV or PVY, respectively, and cultivars displaying double resistance were commercialized under the name NewLeafPlus and NewLeafY by Monsanto in 1998. Although commercially and agronomically successful, NewLeaf varieties were withdrawn from the market in 2001 since several potato processors banned genetically modified crops from their processing chain (Kaniewski and Thomas 2004).

Resistance to the tuber moth (*Phtorimaea operculella*) in potato plants expressing the *cryV Bt* transgene has also been reported (Douches et al. 1998). Other strategies to engineer insect resistance in transgenic plants include the over-expression of proteinase inhibitors (for a recent example, see Abdeen et al. 2005).

In contrast to the success in the production of commercially useful insect- and virus-resistant potatoes, the production of fungus-resistant varieties with useful levels of resistance has been more limited. Potato late blight, caused by the oomycete pathogen *Phytophtora infestans*, is the most devastating potato disease in the world. Although chemicals targeted against *P. infestans* provide reasonable levels of disease control, world-wide losses are estimated to still exceed U.S. $\$ 3 \times 10^9$ year^{-1} because of lateblight and costs of control measures. In the past, breeders relied on wild *Solanum* species as a source for dominant resistance (*R*) genes against *P. infestans*. Unfortunately, classic transfer of resistance from wild *Solanum* species to cultivated potato is not easy because of differences in ploidy and endosperm balance number. However, the recent cloning of a number of *R* genes from potato (Ballvora et al. 2002; Song et al. 2003; van der Vossen et al. 2003) provides a new source for developing late blight-resistant cultivars through genetic engineering. Transgenic potato plants containing either the *RB* or the *Rpi-blb1* locus from *S. bulbocastosum* displayed broad spectrum late blight resistance (Song et al. 2003; van der Vossen et al. 2003). Another strategy to control fungal diseases is the expression of antifungal proteins (Shah 1997). Though often successful under artificial conditions in the laboratory, single antifungal protein genes have until now failed to provide agronomically useful levels of resistance. Recently, transgenic potato plants expressing a defensin protein derived from alfalfa were shown to display robust resistance against *Verticillium dahliae* under glasshouse conditions as well as under field conditions (Gao et al. 2000).

4.4 Potato as a Production System for Pharmaceutical Proteins

Within the past two decades, plants have emerged as a competitive force for the expression of pharmaceutical proteins. The main advantages of plants compared to fermentation-based microbial systems are the low production costs, the proteins folding and assembling correctly and the eukaryotic post-translational modifications being performed. In addition, any potential health

risk arising from contamination with human pathogens or oncogens can be excluded.

Since the first pharmaceutical protein, a human growth hormone, was produced in transgenic tobacco plants (Barta et al. 1986), a vast number of recombinant proteins, including antibodies, vaccines, hormones and growth regulators, have been successfully produced in different plant species (for recent reviews, see Ma et al. 2003; Twyman et al. 2003; Fischer et al. 2004).

Potato tubers were shown to be suitable for the expression of different recombinant proteins like antibodies (Artsaenko et al. 1998), technical enzymes such as glucanases (Dai et al. 2000), or for protein polymers such as synthetic spider silk proteins (Scheller et al. 2001). However, potato plants have been mainly exploited as a model system for the production of subunit vaccines. Most vaccines are still injected, although oral application enhances the chance of increased immune response. Therefore, the idea of using vegetables or fruit crops that can be eaten uncooked or partially processed as "edible" vaccines becomes quite attractive. The Norwalk virus capsid protein was the first vaccine candidate expressed in potatoes, which were orally delivered to mice to determine their immunogenicity (Mason et al. 1996). This study provided proof of concept and indicated the potential usefulness of potato as an edible vaccine. In following studies, other targets such as the hepatitis B surface antigen (Richter et al. 2000) or the *Escherichia coli* heat-labile enterotoxin subunit B (LT-B) (Mason et al. 1998; Lauterslager et al. 2001) were successfully produced in potato tuber and proven to be immunogenic when administered orally to mice. The latter was the first vaccine used in clinical trials with volunteers revealing that consumption of raw potatoes which contained 0.3–10.0 mg of LT-B resulted in a significant level of protection against challenge with the toxin (Tacket et al. 1998). Yu and Langridge (2001) published the production of a multivalent recombinant protein in potato designed for protection against acute gastroenteritis caused by rotavirus and enterotoxigenic *E. coli* (ETEC). To this end, the immunodominant epitope of the murine rotavirus enterotoxin NSP4 was C-terminally fused to the cholera toxin (CT) subunit A, and the ETEC was linked to the CT-B subunit in a N-terminal fusion. Cholera toxins were shown to possess distinct carrier and adjuvant properties; and fusion to antigens strongly stimulated immune response in animal test systems (Holmgren et al. 1993).

Recently, potato plants have been evaluated for production of novel vaccine candidates such as the structural proteins VP6 (Yu and Langridge 2003) and L1 (Biemelt et al. 2003) of the murine rotavirus and the human papillomavirus (HPV 16). Both proteins assembled into virus-like particles and induced the formation of specific antibodies when fed to mice. However, extensive codon optimisation and introduction of the translational enhancer Ω of the tobacco mosaic virus (Gallie et al. 1987) were necessary to achieve expression of the HPV 16 L1 protein. Interestingly, expression of a HPV16 L1 sequence adapted for codon usage of mammalian cells resulted in the accumulation of L1 protein, up to 0.5% of total soluble protein (Biemelt et al. 2003). Quite recently, the

synthesis of the Gag structural protein of the simian immunodeficiency virus (SIV_{mac}) in transgenic potato plants paved the way for the development of plant-based vaccines against SIV and HIV infections (Kim et al. 2004). However, as for other examples, the expression level (0.006–0.014% total soluble protein) was very low. The low protein yield is one of the major limitations of plant-based production systems and needs further improvement. Current strategies to achieve high yield of recombinant proteins relate to both efficiency of transgene expression and protein stability; and they were recently summarized (Twyman et al. 2003; Fischer et al. 2004; Biemelt and Sonnewald 2006).

4.5 Postharvest Performance

Although commonly consumed fresh, potato tubers are versatile and can be used frozen, fried or dehydrated amongst other foods. The strong all-year-round demand for potato tubers requires roughly 70% of the autumn potato crop to be placed into medium- to long-term storage. Unlike other major food crops, potato is stored in a fully hydrated, highly perishable form. This renders the potato tuber particularly prone to postharvest losses, which are estimated to amount to about 10–15% of the harvested crop in the United States, but can be as high as 30% (Suttle 2004). Thus, maintenance of postharvest market quality is of prime importance to producers and processors. Tuber deterioration during storage can result from both disease-related and physiological processes, as well as from mechanical injuries during harvest and handling.

Mechanical injury during harvest or storage causes areas of the tuber to develop discolored patches (blackspots) which negatively impacts potato storage and processing quality. The discoloration of the damaged tissue results from enzymatic production of complex phenols catalyzed by polyphenol oxidases (PPOs). Accordingly, antisense inhibition of PPOs in transgenic potato plants leads to the inhibition of enzymatic browning in harvested potato tubers (Bachem et al. 1994). However, PPOs are involved in the activation of defenses against pests and pathogens; and global antisense inhibition of PPOs has been shown to increase the susceptibility of transgenic plants (Thipyapong et al. 2004). To circumvent this problem, specific repression of a PPO isoform that is predominantly expressed in mature tubers has been achieved. Although these plants displayed reduced enzymatic browning, they showed no signs of increased susceptibility towards the fungal pathogen *P. infestans* (Rommens et al. 2004).

Of the physiological processes affecting postharvest tuber quality, unregulated sprouting is one of the most important. Sprouting is accompanied by many physiological changes, including increases in reducing sugar content, respiration, water loss and glycoalcaloid content. All of these changes are detrimental to processing quality. To prevent sprouting, tubers are either treated with synthetic sprout inhibitors or are kept at low temperatures. Low-temperature treatment leads to the accumulation of reducing sugars, making them unsuitable for

processing. An unacceptable dark and bitter-tasting product is formed at high frying temperatures because of the Maillard reaction that takes place between reducing sugars and amino groups. The cause of cold-induced sweetening is thought to be an imbalance between starch breakdown and glycolytic activity. Consequently, sucrose is formed via sucrose-phosphate synthase and subsequently hydrolyzed by invertases, yielding the reducing sugars glucose and fructose (Sowokinos 2001). To overcome cold-induced hexose accumulation by molecular means, two general strategies have been envisaged: (1) inhibition of starch degradation and (2) reduction in the activity of invertase(s). Overexpression of a deregulated AGPase protein from *E. coli* led to an increased rate of starch synthesis in transgenic potato tubers (Stark et al. 1992). Cold storage of these tubers revealed that hexose accumulation was largely inhibited (Stark et al. 1996). The reason for the observed decrease in hexose accumulation has been speculated to be the higher starch biosynthetic capacity of the tubers. Antisense inhibition of a glucan–water dikinase (GWD) led to a reduced phosphate content of potato starch (see above) and a concomitant decrease in starch turnover. As a further consequence, cold-governed hexogenesis was inhibited (Lorberth et al. 1998). Although successful in the reduction of cold-induced sweetening, both approaches suffer from considerable shortcomings because either starch quantity or quality were altered.

In a more direct approach to inhibit sucrose-derived formation of hexoses, the expression of a cold-inducible vacuolar invertase (VI) isoform was inhibited by antisense technology (Zrenner et al. 1996). Although VI activity was reduced to approximately 10% of wild-type activity in cold-stored transgenic potato tubers, hexose accumulation was reduced by only 34% in these lines. This was explained by either the presence of additional invertase isoforms that may contribute to hexose accumulation in the cold, or by an excess of enzymatic activity of the cold-inducible VI isoform. In plants, invertase activity is controlled at the transcriptional level as well as post-translationally by the interaction of the enzyme with an invertase inhibitor protein. To achieve stronger repression of VI activity in the cold and to circumvent possible limitations posed by isoform-specific antisense, Greiner et al. (1999) expressed an invertase inhibitor from tobacco under the control of the 35S promoter in transgenic potato. As a result, cold-induced hexose accumulation was reduced by up to 75% without any effect on tuber yield. The processing quality of cold-stored transgenic tubers was improved greatly without changing starch quality and quantity, an important prerequisite for the practical application of this technology.

As an alternative to cold storage, sprouting of potato tubers can be inhibited by treatment with chemical sprout inhibitors, such as chloropropham and maleic hydrazide. Although very effective, their application is problematic due to environmental concerns and thus might become restricted. Hence, genetic engineering of tuber metabolism was considered to be a promising approach for sprout control (Sonnewald 2001). One strategy to delay sprouting relies on the inhibition of carbon supply of the developing sprout. In this context, it was

shown that removal of cytosolic pyrophosphate through over-expression of an *Escherichia coli* pyrophosphatase (PPase) under the control of a chimeric ST-LS1/35S promoter delayed the sprouting of transgenic tubers (Hajirezaei and Sonnewald 1999). Sprouting of wild-type tubers started 4 months after storage at room temperature, whereas transgenic tubers did not sprout even after a prolonged storage period of 2 years. The reason was thought to be a reduced sucrose export and inhibition of sucrose utilization in PPase-expressing tubers (Hajirezaei and Sonnewald 1999). Therefore, cell-specific removal of pyrophosphate might enable the regulation of sucrose use. To test this hypothesis and to accelerate potato tuber sprouting, Farré et al. (2001) expressed the *E. coli* PPase gene under the control of the tuber-specific patatin B33 promoter. The rational behind this experiment was to increase the availability of sucrose in storage parenchyma cells without affecting sucrose utilization in the phloem or in meristematic tissue (i. e. the growing sprout). Accelerated sprouting was expected from the assumption that sucrose availability would restrict sprout growth. Indeed, transgenic tubers did begin to sprout 6–7 weeks earlier than wild-type tubers (Farré et al. 2001). This is of special interest for the production of seed tubers, where it is desirable to rapidly induce tuber sprouting to permit the sale and planting of fall-harvested potatoes in markets of the southern hemisphere.

5 Conclusions and Future Outlook

In summary, potato has been the subject of numerous biotechnology-based efforts during the past 15 years. The initial focus has been primarily in the area of pest and virus resistance as well as improving starch, but as more potentially useful genes become available, this range of traits is likely to increase in the immediate future. In addition, improvements in methodology will allow for more precise manipulations of metabolism and for the minimization of transfer of foreign DNA. However, public acceptance of transgenic food crops, such as the potato, will be an important consideration. The efficacy and safety of all transgenic plants and products need to be diligently pursued to enable the continued use of this powerful and important technology.

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IV.2 Cassava

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1 The Crop and its Importance

Cassava (*Manihot esculenta* Crantz) is a member of the family Euphorbiaceae. Cassava is grown in (sub)tropical countries between 30°N and 30°S and from sea level up to 1,800 m, covering a total cultivated area of over 13×10^6 ha (El-Sharkawy 2004). The crop is grown mainly for its tuberized roots, of which 80% of the dry matter is starch. In Africa and Asia, the leaves are also used for animal and human consumption. Cassava is the most important source of carbohydrates after rice, sugarcane and maize, in the (sub)tropics (El-Sharkawy 2004), feeding over 600×10^6 people. The annual world production has been estimated to be over 45×10^6 t of dry roots, with 70% consumed by humans either directly or after cooking or processing, while the remaining 30% is used for animal feed and as source for industrial starch. With economic development, cassava is expected to be utilized increasingly as a source of industrial starch in the near future (Sriroth et al. 2001). Currently, cassava plays a main role in food security in many developing countries, as it can be grown under conditions where other crops fail to produce because of soils with low fertility or acidity. Cassava has a flexible harvest time, it can also withstand periods of drought and it recovers relatively rapidly after being damaged. Because cassava is grown under adverse conditions, the annual yield of cassava world-wide is less than 10% of the yield under optimal conditions.

The cassava root is not a tuber as, for example in potato, and therefore has no direct reproductive function (Ceballos et al. 2005). Most probably its function is to serve as a pool of carbohydrates to reinitiate vegetative growth after the plant has been damaged. As a consequence, cassava roots have a very short shelf life. The roots start to deteriorate within 2 days after harvest and show brownish discoloration that ends with disruption of the organs. Post-harvest deterioration, together with the pressure of cyanogenic glycosides, is the main problem that limits full commercialization of the crop.

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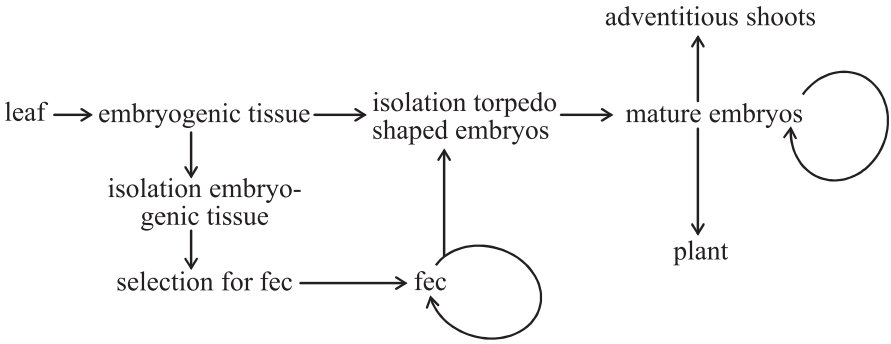

Fig. 1. Overview of the different regeneration systems and their linkage used for genetic modification of cassava

The cyanogenic glycosides release cyanide after enzymatic hydrolysis, which can cause intoxication.

Compared to other crops, cassava breeding is still in its infancy. As a consequence, the genetic difference between landraces and improved breeding lines is smaller than those crops with a longer breeding history. In view of this, landraces still play an important role in cassava breeding. Cassava breeding is not an easy task because the plant is a heterozygous, allotetraploid crop, making it difficult to identify parents with good breeding value and to identify recessive traits (Ceballos et al. 2005). In addition, many genotypes do not flower well and/or possess reduced male fertility. This makes the production of hybrid seeds cumbersome. At CIAT (Columbia) and IITA (Nigeria), the two main cassava breeding stations in the world, an average fertility rate has been estimated to be only 0.6 seeds per pollination (Ceballos et al. 2005). Despite these problems, breeding has resulted in significant progress in the genetic improvement of cassava, especially in yield, low cyanide content, architecture and resistance or tolerance to diseases and pests (Ceballos et al. 2005). However, breeding cannot provide the solution to some problems, as the desired genetic variation is not present in the cassava gene pool. It is therefore anticipated that genetic engineering may be an important approach for cassava improvement. This review focuses on the latest research and development of genetic modification of cassava. It provides an overview of the different regeneration systems employed to produce genetically modified plants (Fig. 1) and the first genetically modified plants with improved characteristics.

2 Regeneration Methods Combined with Genetic Modification

Regeneration of plants can be classified as non-adventitious and adventitious. In non-adventitious regeneration, plants develop from existing meristems, while in adventitious regeneration plants develop from newly formed meris-

tems. Adventitious regeneration can follow two different developmental pathways, namely, somatic embryogenesis (SE) or shoot organogenesis. SE is defined as the process in which a bipolar structure, with no vascular connection with the parental tissue, is formed through a series of stages characteristic of zygotic embryo development. In organogenesis, a unipolar structure is formed that, with its vascular system, is connected to that of the parental tissue. Different non-adventitious regeneration systems in cassava have been reviewed previously (Raemakers et al. 1997). Therefore, this review focuses on adventitious regeneration systems which have been used to obtain genetically modified plants.

2.1 Somatic Embryogenesis

The process of SE can be considered as direct and indirect. In direct SE, embryogenic divisions lead directly to a bipolar somatic embryo, while in indirect SE embryogenic divisions lead to structures that do not develop further but instead break up into new embryogenic units. In some crops, both types of SE can occur simultaneously by choosing the correct explant or the proper culture conditions. In cassava, both SEs also exist, but each requires specific culture conditions for induction and maintenance.

2.1.1 Direct Somatic Embryogenesis

Direct SE in cassava was first described by Stamp and Henshaw (1982, 1987a, b) and Szabados et al. (1987), using zygotic embryos or immature leaves. Explants were cultured on a medium supplemented with 2–16 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic tissue appeared on the adaxial surface of the explants, often near the midvein and the cut surface, after 8 days of culture (Stamp and Henshaw 1987a). Torpedo shaped somatic embryos developed within 15 days, directly from the embryogenic tissues. These embryos could be isolated from the explants and transferred to medium supplemented with 0.01 mg l^{-1} 2,4-D and 0.1 mg l^{-1} benzyladenine (BA), where they developed into mature embryos with a distinct hypocotyl and large green cotyledons (Fig. 2a). Continued subculture of isolated mature somatic embryos on the same medium supported their further development into plants. Mature somatic embryos can also be used for initiation of secondary somatic embryos (SE). This can be achieved by culturing mature embryos on the same medium as used for primary SE.

The same protocol has been used by other researchers to initiate SE from leaves in different genotypes in Asia, Africa and Latin America (Mathews et al. 1993; Raemakers et al. 1993a, b, c, 1997; Schöpke et al. 1993; Konan et al. 1994a, b; Sofiari et al. 1997; Ma 1998; Groll et al. 2001; Joseph et al. 2001; Danso et al. 2002; Ma and Xu 2002; Ihemere 2003). Primary SE has been described in at least 62 genotypes and secondary SE in more than 30 genotypes. It has been

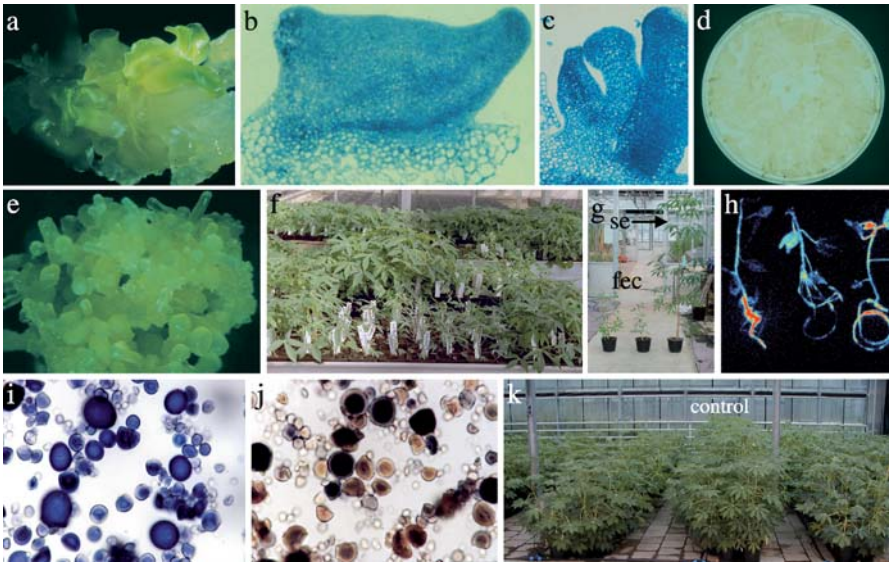

Fig. 2. Regeneration and transformation in cassava. **a** Mature somatic embryos derived from leaf, **b** somatic embryo induced by NAA (note horizontal attachment to explant), **c** somatic embryo induced by 2,4-D (note vertical attachment to explant), **d** friable embryogenic callus, **e** torpedo-shaped embryos regenerated from FEC, **f** R60 plants grown in the glasshouse, **g** plants regenerated from different FEC lines of different ages in the genotype TMS60444 (*fec* derived from FEC, *se* derived from primary somatic embryos; note the differences in size), **h** luciferase positive in vitro plant, **i** starch granules of control plants stained with iodine, **j** starch granules of amylase-free plants stained with iodine, **k** wild-type and amylase-free cassava plants grown in the glasshouse

reported that both types of SEs can be enhanced by adding 1–5 µM $CuSO_4$ to the embryo induction medium containing auxin (Danso et al. 2002).

In addition to 2,4-D, dicamba and picloram, secondary SE can also be induced by α-naphthaleneacetic acid (NAA; Sofiari et al. 1997). NAA induced SE differs from that induced by 2,4-D, dicamba and picloram. Secondary SE induced by NAA are initiated much faster and the newly initiated somatic embryos are attached horizontally to the original explant (Fig. 2b). Also, mature somatic embryos can be obtained within 2 weeks without the need to transfer to the second medium. For somatic embryos induced by other growth regulators, it usually takes 1–2 weeks more than those induced by NAA and the newly initiated somatic embryos are attached vertically to the original explant (Fig. 2c). A second medium is needed for the development of mature torpedo-shaped somatic embryos.

In the original protocol described by Stamp and Henschaw (1982, 1987a, b) and Szabados et al. (1987), only a few mature somatic embryos developed into shoots on the medium supplemented with 0.1 mg l^{-1} BA. It was found later that greater BA concentrations increased the number of mature somatic embryos capable of forming shoots (Raemakers et al. 1993b, c). The regener-

ated shoots were fleshy, thickened, fasciated and did not form roots initially but became normal in appearance after prolonged culture on hormone-free medium. Mathews et al. (1993) obtained normal shoots at high frequency from mature somatic embryos after desiccation.

The protocols for initiation of primary and secondary SE and the germination of mature somatic embryos into plants have been well established. To date, it has been successful in more than 90% of the genotypes tested.

2.1.2 Indirect SE

Taylor et al. (1996) reported that direct-induced embryogenic tissue grown on specific medium, in conjunction with the selection for a specific type of embryogenic callus, leads to the formation of friable embryogenic callus (FEC) that possessed the characteristics of indirect SE. The embryogenic units in FEC consist of 10–10 cells and are not enclosed by an epidermis. Furthermore, it does not differentiate beyond the pre-globular stage (Fig. 2d) and, if cultured in the presence of high concentrations of auxin, new FEC units can be continuously initiated, perhaps from the surface of the older units. FEC cultures were initiated for the first time in TMS60444, which is currently grown on a small scale in some regions of Nigeria. Since then, FEC from other cassava genotypes has also been reported. In our laboratory, FEC has been initiated in six of the 12 cassava genotypes, while 14 of the 22 tested genotypes responded in ILTAB, United States (Taylor et al. 2001). At CIAT in Columbia, FEC has been obtained in four cassava genotypes (Taylor et al. 2005). Some genotypes have taken 3 months of selection and subculture before a pure FEC culture can be obtained, but the time required by other genotypes can be longer (up to 9 months). Plants can be regenerated by culturing FEC first on a medium supplemented with 1 mg l^{-1} NAA, followed by subculture to the new medium at 3–4 weeks intervals. In general, FEC of most genotypes formed torpedo-shaped somatic embryos within 2–4 weeks, while 3–5 weeks are required in some genotypes, e. g. TMS60444 (Fig. 2e). These embryos develop into plants after culture on a medium containing BA. Using this procedure, plants from six cassava genotypes have been obtained in our laboratory. Taylor et al. (2001) tested various FEC lines of 14 genotypes, eight of which regenerated plants.

In summary, efficient protocols for FEC initiation are scarce, as it is genotype-dependent and time-consuming. This is in contrast to primary and secondary SE, which are relatively easy and require only a few weeks. To date, FEC initiation and plant regeneration have been described in less than 15 genotypes.

2.2 Organogenesis

Organogenesis in cassava is less well described than SE. Shoot organogenesis was first reported by Tilquin (1979) using stem explants. In spite of considerable efforts, this could not be repeated. Organogenic structures, which were

described as foliose structures, were observed occasionally together with somatic embryos when explants were cultured for initiation of SE (Stamp and Henshaw 1982, 1986, 1987a, b). However, none of the foliose structures developed into plants. Plant development was obtained when the protocol developed for SE was adjusted. Ma and Xu (2002) and Mussio et al. (1998) obtained adventitious shoots when leaves were cultured for a few days on medium with auxin, followed by transferred to the medium containing cytokinins. Raemakers et al. (1997) reported both adventitious shoots and (secondary) SE when primary somatic embryos were cultured on a medium supplemented with 10 mg l^{-1} NAA and 1 mg l^{-1} BA. Both protocols were tested, but only a few shoots were obtained per explant. The capacity of shoot regeneration was markedly improved using the protocol developed by Li et al. (1998), who cultured mature somatic embryos, cut into 0.5 cm^2 pieces, on a medium supplemented with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} indole-3-butyric acid (IBA), where up to 60% of explants formed shoots. In the genotype M.Col22 8.7, shoots/plants were obtained. Organogenesis was also reported in three other tested genotypes. Zhang et al. (2001a) showed that addition of $AgNO_3$ to the medium increased both the percentage of responsive explants and the number of shoots per explant.

3 Quality of Plants Derived from Different Regeneration Systems

One of the prerequisites for a regeneration system to be used for genetic modification is its ability to produce high-quality plants. This paragraph summarizes the available information on this aspect of the three described regeneration systems (primary and secondary SEs, FEC, shoot organogenesis).

3.1 Primary and Secondary SE

In our laboratory, cassava plants have been obtained via secondary for the genotypes M.Col22, Adira 4, R60, TMS60444, Gading and line 11. When multiplied in vitro, most of the plants grew well and showed no obvious phenotypic differences compared with the control plants. In M.Col22 where a total of 485 embryo-derived plants were evaluated in vitro, one plant exhibited variegated leaves, which was also observed sporadically in Adira 4. The embryo-derived plants of M.Col.22, Adira 4 and R60 were grown in the glasshouse. In the first generation, significant differences were observed between the plants derived via secondary SE and control. There was also wide variation between plants originating from one particular regenerated plant. However, most differences disappeared after plants were multiplied using stem cuttings (Raemakers et al. l997). The phenotype of of Adira 4 and R60 plants derived from secondary embryos (Fig. 2f) was similar to that of control plant.

3.2 Friable Embryogenic Callus

In our laboratory, plants derived from FEC lines of TMS60444, M7, Adira 4, R60 and Thai5 were investigated in culture and glasshouse conditions. To do this, plants were regenerated from 2–6 months (young FEC) and after 14–18 months (old FEC lines) FEC lines by culturing 100 mg of FEC on maturation medium. Young FEC lines of TMS60444, Adira 4, M7 and Thai5 gave rise to about 1000 torpedo shaped embryos and 250 embryos in R60 (Table 1). With respect to old FEC lines, TMS60444 and M7 formed over 1000 torpedo-shaped embryos, but less than 500 embryos per 100 mg of FEC were obtained in other genotypes. It was observed that 40–80% of the torpedo-shaped embryos could develop into mature somatic embryos. In TMS6044, about 75% of the mature somatic embryos derived from young FEC lines developed into plants, but the frequency of plant regeneration from embryos of other genotypes was lower (30–50%). In general, embryos derived from old FEC exhibited a reduced ability to develop into plants.

More than 1000 FEC-derived plants were regenerated from TMS60444, Adira 4, R60, M7 and Thai5. These plants were multiplied by subculture at 3- to 5-month intervals. After 3–5 subcultures, 95% of the regenerated plants originating from young FEC lines of TMS60444, Adira 4 and M7 could be recovered, whereas only 45% and 70% of the plants were recovered from R60 and Thai5, respectively. In Adira 4 and R60, less than 30% of the original plants derived from old FEC were recovered. The remainder were lost because the cuttings did not develop shoots. In general, the survival rate of plants derived from old FEC was lower than those regenerated from young FEC.

Plant characterization revealed that all M7 plants were phenotypically normal regardless of FEC age. In TMS60444, all plants from young FEC and 95%

Table 1. Regeneration potential of young and old FEC lines and the quality of the regenerated plants in five cassava genotypes. *Y* Young (FEC maintained 2–6 months before being used for regeneration), *O* old (FEC maintained 14–18 months before being used for regeneration)

Genotype	Regeneration				Assessment of quality of plants (in vitro)			
	Somatic embryos[a]		% Plants[b]		% Survival[c]		% Normal[d]	
	Y	O	Y	O	Y	O	Y	O
TMS60444	> 1000	> 1000	75	40	95	60	100	95
Adira 4	> 1000	200	50	30	95	25	75	0
R60	200	100	50	30	45	20	0	0
M7	> 1000	> 1000	50	30	95	50	100	100
Thai5	> 100	400	30	30	70	40	75	50

[a] Number of somatic embryos regenerated from 100 mg of FEC.
[b] Percentage of somatic embryos that regenerated into plants.
[c] Percentage of the original regenerated plants which survived in vitro multiplication.
[d] Percentage of normal-looking plants (compared to control propagated plants).

plants from old FEC were normal. In contrast, normal plants were not observed in R60. The percentage of normal/abnormal plants in Adira 4 and Thai5 was intermediate. Most of the abnormal plants exhibited reduced vigour (reduced height, thin stems, and elongated internodes). Furthermore, abnormalities such as branched or bushy shoots, deformed or zig-zag stems or dwarfism observed in plants regenerated from older FEC was more severe than those from young FEC.

Plants derived from FEC lines of TMS60444, Adira 4, R60, M7 and Thai5 were transferred to the glasshouse (Fig. 2f). It was observed that the survival rate of plants derived from young FEC was > 90%, which was comparable with that of control plants, whereas only 20–70% of plants derived from old FEC survived. Plant growth is affected by FEC age and genotype. In TMS60444, Adira 4 and M7, growth of plants from young FEC and was comparable with that of control plants in terms of plant height and fresh weight (foliage and roots) after 7 months. However, R60 plants from young FEC were significantly smaller and their fresh weight was less than that of the control (Fig. 2g). Similar reduced growth was also observed in plants regenerated from old FEC of all genotypes. Further characterization of regenerated plants revealed that all genotypes except TMS60444 and R60 possessed the expected chromosome number of 2*x*, whereas 60% of TMS60444 plants derived from old FEC and 67% of R60 plants from young FEC had the chromosome number of 4*x*. Plants derived from young and old FEC of TMS6044 and Adira 4 and young FEC of R60 were also analyzed using AFLP. All TMS60444 and R60 plants showed

Table 2. Size and fresh weight of plants regenerated from young and old FEC lines in five cassava genotypes after 7 months of growth in the glasshouse. *Young* FEC maintained for 2–6 months before being used for regeneration, *Old* FEC maintained 14–18 months before being used for regeneration, *Control* in vitro propagated plants transferred to the glasshouse. *No. of plants* Number of independent regenerated plants

Genotype	Age	No. of plants	Height (cm)	Fresh weight (g)	
				Foliage	Roots
TMS60444	Control	40	199	306	215
	Young	140	223	347	238
	Old	40	182	193	148
R60	Control	18	358	619	229
	Young	30	192	100	33
	Old	45	135	125	27
Thai5	Control	12	225	268	250
	Young	29	127	154	100
Adira 4	Control	42	307	428	167
	Young	42	258	416	185
	Old	59	174	146	43
M7	Control	9	223	342	247
	Young	26	237	277	243

the same banding pattern as the control plants. However, the pattern differed between control plants of Adira 4 (results not shown), although growth of both plants was comparable under glasshouse conditions (Table 2).

4 Genetic Modification of Cassava

4.1 Genetic Modification via Primary/Secondary SE

Transgenic cassava plants have been regenerated from explants inoculated with *Agrobacterium* or direct gene transfer by particle bombardment via primary or secondary SE. Raemakers et al. (1997) used both methods in cassava transformation, during which inoculated or bombarded mature somatic embryos were cultured on selection medium containing kanamycin. Explants gave rise to secondary SE and about 1% of the newly formed embryos were shown to contain cells expressing the *gus* gene encoding β-glucuronidase (GUS). Later, the experiments were repeated using the *luc* gene encoding luciferase as a reporter, with the same results. Excision of the *luc* positive sectors and their subculture for secondary SE formation resulted in the loss of *luc* activity, which was also reported by Schöpke et al. (1993). Based on the results of histological studies reported by Stamp (1987) and Raemakers et al. (1997), this phenomenon could be explained by the possibility that both primary and secondary SE might be initiated from multiple cells located inside the tissues which were difficult to transform. However, regeneration of transgenic cassava via primary and secondary SE has been reported in several laboratories. Sarria et al. (2000) inoculated leaf explants with a wild-type *Agrobacterium* strain carrying *gus* and *pat* encoding phosphinotricin acetyltransferase. Transformed cells expressing *pat* were resistant to the herbicide Basta, when inoculated explants were cultured on the selection medium containing Basta. From 100 inoculated explants, 42 Basta resistant plants were regenerated, 30% of which were shown to be GUS-positive in their leaves. Fifteen herbicide-resistant plants were selected, after the leaves of GUS-positive plants were tested for Basta resistance. These plants were grown in the glasshouse, where they were further tested by spraying with Basta. The results showed that only one plant was completely resistant to the herbicide. Southern analysis indicated that this herbicide-resistant plant also carried an introduced *pat* gene that, however, was not detected in the other 14 plants.

Apart from the wild-type strain, the disarmed *Agrobacterium* strains containing the *npt*II gene have also been used for cassava transformation (Ihemere 2003; Siritunga et al. 2004; Siritunga and Sayre 2004). The transformation was conducted by inoculating mature somatic embryos with *Agrobacterium*, followed by culture on selection medium with 75 mg l^{-1} paromomycin. Cell clumps containing torpedo-shaped embryos forming on inoculated explants after 6 weeks were transferred to the selection maturation medium. Of the in-

oculated explants, 1–2% gave rise to paromomycin-resistant somatic embryos and 35–50% of the embryos developed into plants. PCR analysis revealed that 15% of the plants carried the transgene.

4.2 Genetic Modification Using FEC

FEC is an important material for cassava transformation. Genotypes TMS60444, Bonoua Rouge, M.Col1505 and Adira 4 have been transformed using FEC (Taylor et al. 2005). In general, FEC was first cultured in liquid medium in the absence of selective agent for 1–3 weeks after particle bombardment or inoculation with *Agrobacterium*, followed by transfer to semi-solid selection medium for another 2–6 weeks, during which time transgenic FEC was selected. Selection of transgenic FEC was based either on culture color (yellowish embryogenic units selected among white embryogenic units) or differential growth of colonies of FEC on selection medium or expression of the *luc* gene.

It has been reported that 100 mg FEC inoculated with the *Agrobacterium* strain LBA4404(pTOK233) could give up to 300 colonies resistant to 10 mg l^{-1} kanamycin or 0.5 mg l^{-1} hygromycin (Schreuder et al. 2001). Of all the colonies analyzed, 35% were shown to contain GUS-positive cells, while 60% of the GUS-positive lines selected on 10 mg l^{-1} kanamycin regenerated plants compared to 20% of the lines selected on 0.5 mg l^{-1} hygromycin. It was shown that 40% of the GUS-positive lines gave rise to GUS-positive plants, while 15% formed only GUS-negative plants; 55% regenerated both GUS-positive and GUS-negative plants. The number of escapes was reduced significantly after the antibiotic-resistant colonies were cultured on medium containing a higher concentration of the selection agents (results not shown). In another study, Gonzalez et al. (1998) inoculated FEC with the *Agrobacterium* strain ABI(pILTAB188) carrying a gene for paromomycin resistance. Yellow embryogenic paromomycin-resistant FECs and with a size of 0.5−1.5 mm were selected 5–6 weeks after inoculation. Twenty to 180 yellow FEC units were recovered from 0.5 ml settled cell volume of inoculated tissue. Using the same selection system, 50–300 yellow units were selected from bombarded FEC (Schöpke et al. 1996; Taylor et al. 2001). These yellow FECs were transferred to new selection medium, where 33% of them were resistant to paromomycin (Schöpke et al. 1996) and 65% of the paromomycin-resistant FEC was shown to be GUS-positive. Plants were also regenerated from 10–40% of the GUS-positive, paromomycin-resistant FEC (Taylor et al. 2001).

The selection protocol based on detoxification of herbicides and antibiotics, as described previously, is a negative selection. Selection based on the expression of *pmi* that encodes phosphomannose isomerase is a positive system, as this enzyme can catalyze the conversion of mannose to useful plant carbohydrates, as a result allowing transgenic plants expressing *pmi* to grow in the presence of mannose. Zhang et al. (2000a, b) employed this positive selection system, in which FEC of TMS60444 was transformed by particle bombardment

or inoculation with *Agrobacterium* carrying the construct with the *pmi* gene. FEC was cultured in liquid medium containing 4% mannose and 1% sucrose. After 4 weeks, FEC was transferred to semi-solid medium supplemented with 2% mannose, where 664 mannose-resistant yellow calli were selected and 178 of these were developed into FEC colonies. More than 80% of these colonies were shown to be GUS-positive; five colonies regenerated GUS-positive plants.

A not-stringent selection system based on the expression of the *luc* gene has also been employed in cassava transformation. Raemakers et al. (1996) reported plant regeneration from FEC cultures of TMS6044 transformed with the *luc* gene using particle bombardment. Verification of putative transformants showed that 1–5% of the plants were luciferase positive (Fig. 2h). A similar study was also conducted by Munyikwa et al. (1998), but the frequency of regenerated plants with luciferase activity was considerably greater (> 50%). In both studies, not more than three of the transgenic lines per bombarded Petri dish were obtained. We found that *Agrobacterium*-mediated transformation was more efficient than particle bombardment, as the former resulted in more than 300 transgenic lines using the same amount of tissue for transformation (unpublished results).

4.3 Genetic Modification Using Organogenesis

Apart from FEC, organogenic cultures have also been used for cassava transformation. This has been demonstrated in M.Col22, where transgenic tissues and plants were selected by expression of *npt*II, *hph* and *pmi* after the explants were transformed using particle bombardment or *A. tumefaciens* (Li et al. 1996; Zhang et al. 2000a, b). In cases where mature somatic embryos were used for transformation, inoculated or bombarded embryos were first cultured on a medium without selective agent for 2–6 days, after which cultures were transferred to medium containing the appropriate selective agent. Cultures usually gave rise to calli, together with small shoot primordia resistant to the selection agent.

Li et al. (1996) inoculated 563 explants with the *Agrobacterium* strain LBA4404(pBinGusint) and 1753 explants with LBA4404(pTOK233). The former, cultured on medium with 20 mg l^{-1} gentamycin, gave rise to 27 shoots, three of which were shown to be GUS-positive. The latter explants cultured in the presence of 15 mg l^{-1} hygromycin formed 30 shoot primordia, six of which were GUS-positive. However, some plants appeared to be chimeric (Li et al. 1996). Using particle bombardment, 77 plants were regenerated from 1130 bombarded explants (Zhang et al. 2000a). However, a large fraction of the generated plants were escapes. The escapes were thought to be due to the selection phase, in which prolonged culture on selection medium may have inhibited shoot formation, whereas a reduced selection period favored the occurrence of escapes. The frequency of escapes was later reduced to 40% by culture on selection containing 7.5 mg l^{-1} hygromycin for the first 10 days,

followed by transfer to medium with a hygromycin concentration of 10 mg l^{-1} during organogenesis (Zhang et al. 2000a). However, in another study, a similar stepwise procedure of selection with a more stringent regime (first 10 days at 7.5 mg l^{-1}, followed by 15 mg l^{-1} hygromycin) resulted in 85% escapes (Zhang et al. 2000b). Transgenic plants were also selected by culturing bombarded tissues on the medium supplemented with 10 mg l^{-1} mannose. Some explants (4%) regenerated shoots and 8% of these shoots were shown to possess transgene (GUS) activity.

5 Transfer of Agronomical Useful Genes to Cassava

5.1 Altering Starch Content and Composition

Cassava is one of the most efficient producers for starch. Under optimal conditions, the crop can produce more than 100 t ha^{-1} $year^{-1}$ of tuberous roots, of which 75–80% is starch. Starch consists of the two glucan polymers, amylose and amylopectin. In cassava, the amylose content ranges from 14% to 24% (Moorthy and Ramanujan 1986). Amylose is essentially a linear polymer of 100–1,000 α(1-4) linked glucosyl residues with a molecular weight of 0.5 1.0E6. Amylopectin is much bigger with a molecular weight of several millions and is much more branched. Amylose and amylopectin possess different physicochemical properties that allow the crop to be utilized for different applications.

5.1.1 Manipulating Starch Content

The conversion of glucose-1-phosphate to ADP-glucose by the enzyme ADP-glucose pyrophosphorylase (AGPase) is thought to be the rate-limiting step in starch biosynthesis. In cassava, expression of AGPase gene has been down- and up-regulated. Down-regulation can be accomplished by expression of the antisense AGPase gene under control of the CaMV35S promoter. This was demonstrated in TMS60444, where FEC cultures were bombarded with particles carrying the chimeric gene and transgenic plants were selected based on the presence of luciferase activity (Munyikwa et al. 1998). Twenty-four transgenic plants were analyzed and shown to possess 1–7 insertions of the transgene. Starch accumulation in these plants was also analyzed by growing the plants on a medium containing 6% sucrose, where the plants deposited starch in their stems (Salehuzzaman et al. 1994). Sections of stem tissue from 16 plants were stained with iodine, four of which did not show visible reduction in the starch content, although both the cortex and the epidermis contained large amounts of black/blue starch granules. Another three plants showed no starch staining in the cortex and a reduced staining in the epidermal region. The extent of starch staining in the remaining plants was intermediate.

Up-regulation of starch biosynthesis was reported by expressing a bacterial AGPase gene under the control of the tuber-specific patatin promoter (Ihemere 2003). Somatic embryos of TMS711773 inoculated with *Agrobacterium* carrying the AGPase gene gave rise to seven independent transformed plants via secondary SE. All transgenic plants showed increased shoot and root biomass when grown in the glasshouse and the starch content was 2-fold higher compared with that of wild-type plants (Ihemere 2003). However, a further study is required of plant performance under field conditions.

5.1.2 Manipulating Starch Composition

Biosynthesis of amylose is controlled by the action of granule-bound sucrose synthase (GBSS1). In order to generate transgenic plants with altered starch composition, FEC cultures of TMS60444 were bombarded with constructs carrying an antisense *gbss1* gene under the control of the potato *gbss1* promoter and the *luc* gene (Raemakers et al. 2005). In a total of 390 dishes bombarded, 84 luciferase-positive FEC lines were selected and 58 lines were shown to regenerate luciferase positive plants. Southern analysis revealed that these transgenic plants possessed 2–8 transgene insertions. The levels of transcript and protein of GBSS were also dramatically reduced and the amylase content decreased (Raemakers et al. 2005). Results of starch analysis for these transgenic plants revealed that starch in wild-type plants were stained black/blue (Fig. 2i), whereas starch in amylose-free transgenic lines stained brown (Fig. 2j). These findings showed that seven of the 55 analyzed plants were comparable with the amylose-free potato mutant, whereas 35 plants had an amylose content comparable with the wild type and 14 plants were intermediate.

All 55 transgenic plants were grown in the glasshouse, where plants with amylose were shown to produce amylase-containing starch in the tuberous roots, but only two of seven amylose-free plants synthesized amylase-free starch in their tuberous roots. The two amylase-free plants were multiplied and grown for another year in the glasshouse. As shown in Fig. 2k, the control plants were larger in size and produced more tuberous roots (results not shown), compared with the transgenic lines. The stability of starch/water mixtures was determined by measuring the viscosity, opacity and freeze–thaw stability, as this is an important parameter for potential application. The viscosity of starch from wild-type plants was shown to increase with time by about 3-fold, whereas the viscosity of starch from transgenic lines was relatively constant. Opacity is an indicator of starch retrogradation, whereby the glucan chains reassociate and precipitate in solution. The opacity of starch from the two transgenic lines was less than that of control plants, even after prolonged storage at 4 °C. It was observed that a limited amount of syneresis was present in these gels after 1 week of storage. Freeze–thaw stability was evaluated by measuring syneresis and clarity of the paste following several cycles of freezing and thawing. The

results showed that amylose-free starch was stable under different conditions compared with wild-type starch (Raemakers et al. 2005).

5.2 Virus Resistance against Cassava Mosaic Disease

Cassava mosaic disease (CMD) is caused by whitefly-transmitted cassava mosaic gemini viruses (CMV). At present, six African and two Indian CMV have been described (Legg and Fauquet 2005). The most important viruses are African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV) and Indian cassava mosaic virus (ICMV). Recombination between viruses is common.

Two research groups reported the production of transgenic cassava resistant to viruses. One employed antisense RNAs targeting virus replication genes to protect the plants against CMD (Zhang et al. 2005). This was achieved by inoculation of TMS60444 FEC lines with *Agrobacterium* carrying the antisense RNAs. In 41 independent transformed plants selected, most showed reduced viral DNA replication of two ACMV isolates in detached leaves. When whole plants were challenged with ACMV using particle bombardment, symptom development was reduced and attenuated. It was speculated that virus resistance was caused by post-transcriptional gene silencing. The strategy of other groups was to over-express a viral gene responsible for viral replication in transgenic TMS60444 plants (Chellappan et al. 2005). The results of plant characterization revealed that four of 18 transgenic plants showed increased resistance against CMD. The authors also hypothesized the important role of post-transcriptional gene silencing, leading to reduced viral replication.

5.3 Reducing Cyanide Content

Cassava contains toxic amounts of cyanogenic glycosides (linamarin and lotaustralin), which can be found in leaves, stems and roots. The cyanogenic glycosides in plants may play a role in defense against herbivores. Cyanogenesis that can be initiated by wounding starts after linamarin is released from cell vacuoles. The cyanogenic glycosides are hydrolyzed by linamarase, located in cell walls, to release acetone cyanohydrin, which can either decompose spontaneously at temperatures above 35 °C into acetone and cyanide or enzymatically by hydroxynitrile lyase (McMahon et al. 1995). Cyanide in tuberized roots and leaves of cassava can be removed either by the fermentation process using linamarase containing microorganisms, or by soaking, rinsing and baking. Poorly processed cassava can contain toxic concentrations of acetone cyanohydrin that cause several disorders. The severity of the disorders depends on the level of intake of acetone cyanohydrin, the nutritional quality of the other food and the health of the consumer.

Two strategies have been employed to reduce cyanide toxicity in cassava foods. One is to over-express the hydroxynitrile lyase gene in tuberized roots

(Siritunga et al. 2004). This strategy is based on the observation that cassava tuberized roots possessed no detectable hydroxynitrile lyase activity or protein (White et al. 1998). This enzyme is known to catalyze the conversion of acetone cyanohydrin to cyanide. Siritunga et al. (2004) postulated that cassava tuberized roots could be detoxified by over-expression of the hydroxynitrile lyase gene in transgenic plants. To test this hypothesis, cassava was transformed using *Agrobacterium* with the cassava hydroxynitrile lyase gene driven by the 35S promoter. During transformation, about 2500 somatic embryos were inoculated and cultured on selection medium containing paromomycin. A total of 77 paromomycin-resistant transformants were obtained, three of which were described in detail (Siritunga et al. 2004). While one transgenic plant possessed three copies of the T-DNA, the other two had only one copy. Further analysis showed that the concentrations of hydroxynitrile lyase protein and enzyme activity in leaves of the three transgenic plants increased by 1.2- to 2.3-fold and by 1.4- to 2.3-fold, respectively, compared to wild-type plants. The hydroxynitrile lyase activity in roots of glasshouse-grown transgenic plants was also increased by 8- to 13-fold, while the cyanohydrin content in transgenic plants was 3-fold less than that in wild-type plants.

The second strategy of cyanide reduction in cassava is to block cyanide production by inhibition of linamarin synthesis in transgenic plants expressing antisense genes involved in linamarin production. This was reported by Siritunga and Sayre (2004), who inoculated mature somatic embryos with *Agrobacterium* carrying the antisense cytochrome P450 genes, *cyp79d1* and *cyp79d2*, under the control of the leaf-specific *cab* promoter. The cyp79d1 and cyp79d2 products catalyze the first step of linamarin biosynthesis. Inoculated embryos were cocultivated on selection medium in the presence of 75 mg l^{-1} paromomycin, where embryos gave rise to paromomycin resistant secondary SE after 4 weeks. Five transgenic embryo-derived plants were analyzed and shown to possess transgenes inserted at one to three locations in the genome. While the cyp79d1 and cyp79d2 transcripts were not detectable in leaves of two plants, the transcript levels in the other three plants were 20–80% of that of the wild type. Furthermore, the linamarin content in transgenic leaves was reduced up to 94%. In rootas, the linamarin content was reduced to less than 1% of the control, although the level of cyp79d1 and cyp79d2 transcripts in all transgenic plants was not affected (Siritunga and Sayre 2004).

5.4 Insect Resistance

Several insect pests can significantly reduce the yield of cassava. These pests include mites, white fly, thrips, mealy bugs, hornworms and stem borers. Although resistance to some of these pests has been found in wild species, there are several pests for which resistance genes are not available in the cassava gene pool. Attempts have been made to produce insect resistant cassava using genetic engineering. This has been demonstrated in the study of Taylor et al.

(2005), who generated transgenic TMS60444, CM3306-4 and SM1219-9 plants expressing the *cryIAb* gene. Currently, these transgenic plants are being tested in the glasshouse. The results against hornworm appear to be encouraging. However, it was considered that stronger expression of *cryIAb*, especially in stems, is required to provide full protection against stem borer (Taylor et al. 2005).

6 General Conclusions

The different pathways of regeneration described here have been used for successful production of transgenic cassava. Plant regeneration via primary/secondary SE is technically simple, almost genotype-independent and generally capable of producing high-quality plants. However, information regarding the mechanisms underlying the SE and organogenic events is limited. The main drawback of this system is the relatively low efficiency and reproductibility that prevents routine transformation of cassava. Furthermore, the occurrence of chimeric transformed plants is problematic, which is especially true for plants produced via second SE. This is probably due to the formation of secondary SE that are derived from coordinated division of epidermal and subsurface cells, as a result giving rise to embryos of multi-cellular, multi-layer origin (Stamp 1987; Raemakers et al. 1997).

The FEC transformation system can lead to the formation of escapes, but the frequency of producing chimeric transformed plants is much lower. This is because the selection of transformed cells occurred during proliferation of FEC, in which transformed cells can divide independently from their neighboring cells. In the case of secondary SE and the organogenic systems, once the cells are activated for regeneration, they develop in a linear way into somatic embryos or adventitious shoots. Only when the somatic embryos or adventitious shoots are derived from single cells, are completely transformed plants produced. However, the FEC system is technically complicated. It requires highly skilled workers, is strongly genotype-dependent and can produce low-quality plants. The quality of plants can be addressed by using young cultures, but it can still be affected by the plant genotype.

The organogenic and secondary SE systems can be considered as direct regeneration, with indirect regeneration for the FEC system. This differential mode of regeneration explains the pros and cons for using these systems for genetic modification of cassava. One might expect that an intermediate new system arises from the existing two regeneration modes might combine the advantages of both systems. Such a system was described by Groll et al. (2001), who obtained embryogenic cultures where the embryos developed up to the globular stage before they broke up in new embryogenic propagules. However, information is not available on genetic modification using this system.

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IV.3 Sweet Potato

T. Shimada and M. Otani[1]

1 Introduction

Sweet potato, *Ipomoea batatas* (L.) Lam., is the seventh most important food crop in the world, and in the developing countries the fifth after rice, wheat, maize and cassava (Table 1; CIP 2004). The cultivation area of this plant species is located mainly at low latitudes in South America, South Asia and South Africa and spreads as far as some regions in Europe and the United States.

The sweet potato was domesticated in tropical America more than 5000 years ago. Although prehistoric transmission to the Pacific islands has been suggested (Yen 1982), it is well documented that sweet potato was brought to the Old World by European exportation at the end of the fifteenth century. Then it was introduced into China in the late sixteenth century and spread through Asia and Africa during the seventeenth and eighteenth centuries.

Sweet potato is a member of the morning glory family (Convolvuceae), is an autohexaploid ($2n = 6x = 90$) and has storage roots. It propagates vegetatively, via shoots sprouting from roots. Its productivity is very high; the average of yield in Asia is 17 t ha^{-1}, as high as 24 t ha^{-1} in Japan. Moreover, it does not require a large amount of fertilizers and other agricultural chemicals and is rather tolerant to environmental stresses. Therefore, the sweet potato is one of the most important crops for securing a stable food supply and has potential to alleviate some global environmental problems in the twenty-first century.

Sweet potato is not only a good source of energy, supplying starch and other carbohydrates, but is also a nutritive food containing calcium, iron and other minerals and vitamins, particularly vitamins A and C. Recently, a multinational team has been working to screen and evaluate the orange-fleshed sweet potato varieties rich in β-carotene. In the Sub–Sahara region where vitamin A-related diseases are widespread, they have propagated in an attempt to eliminate childhood blindness caused by vitamin A deficiencies (CIP 2004). In Japan, the research on sweet potato has been designed to develop alternative utilization. New cultivars have been developed, for example, a cultivar that allows utilization of sweet potato tops (foliage and petiole) as human food (not only as livestock feed) and cultivars with a high carotene content and a high anthocyanin content.

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Table 1. World sweet potato production (modified from CIP 2004)

Production region	Number of countries	Production ($\times 10^3$ t)	Area ($\times 10^3$ ha)	Yield ($\times 10^3$ t ha^{-1})	Rank[a]
Asia[b]	32	126,198	7,226	17	4
Africa	39	6,957	1,519	5	11
Latin America	31	1,850	247	7	13
Europe	5	78	18	12	15[c]
United States	1	604	34	18	12
World	111	135,756	9,046	15	7
Industrial countries		1,892	102	19	12
Developing countries		133,865	8,944	15	5

[a] On a fresh-weight basis out of 21 major food crops.
[b] Production in China is 117,848,000 t year^{-1}, the highest production in the world. Yield in Japan is 24 t ha^{-1}.
[c] In Portugal ranks 9.

Despite the hardy nature of sweet potato, there is ample scope for improvement. Sweet potato is also threatened by a number of biological and abiological stresses, especially severe damage from viral and bacterial diseases and pests. The average yield in Africa is 5 t ha^{-1} in fresh weight tubers, which is 60% below that in other developing countries and less than 20% of the yield in Japan (Table 1). However, the conventional breeding program based on sexual hybridization of sweet potato is limited by its shy flowering and cross-incompatibility. Novel approaches such as somatic hybridization and genetic transformation, especially the latter, being incorporated into sweet potato breeding may be effective to overcome such limitations.

This chapter gives an outline of the progress made in the development and application of biotechnology for the agronomic improvement of sweet potato. An efficient and reproducible system for the plant regeneration system from cultured tissues or cells, somatic hybridization and gene transformation are discussed. Moreover, we make a brief reference to the molecular works in sweet potato.

2 Tissue Culture

2.1 Plant Regeneration from Cultured Tissues

At present, successful regeneration of whole plants from transformed cells is essential for plant transformation. Because of the low frequency and genotypic dependence in plant regeneration from cultured tissue, sweet potato is well known as a recalcitrant crop to tissue culture. To overcome this difficulty in tissue culture, a lot of effort has been exerted. Work on the tissue culture

of sweet potato before 1981 was reviewed by Henderson et al. (1984). Plant regeneration from cultured tissues of sweet potato has been achieved in some tissue materials, such as root tuber, leaf, stem and petiole. The frequency of plant regeneration in sweet potato was relatively low in the early 1980s (Carswell and Locy 1984, Templeton-Somers and Collins 1986).

The embryogenic callus is defined as cultured tissue that generates somatic embryos and then regenerates plantlets from the somatic embryos under the given culture condition. There has been much improvement since Liu and Cantliffe (1984) and Jarret et al. (1984) succeeded in somatic embryogenesis and embryogenic callus production using shoot meristem tissues (Table 2). Jarret et al. (1984) obtained embryogenic calli from shoot tips of nine genotypes on medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), and the frequency of embryogenic calli was 10–31% of cultured explants. Komaki et al. (1991) examined the effect of 2,4-D concentration on embryogenic callus induction using shoot tips of 70 cultivars; and 1.1 $mg\,l^{-1}$ or 2.2 mg^{-1} 2,4-D was effective for embryogenic callus formation, but apparent genotypic differences were observed and the frequency of embryogenic callus formation varied from 0% to 100% of explants cultured. Otani and Shimada (1996) obtained embryogenic calli from 11 cultivars tested at relatively high frequencies (50–94.5%) by adding various auxin-like plant growth regulators to the medium, although genotypic differences still remained. Out of eight auxin-like plant growth regulators tested, 4-fluorophenoxyacetic acid (4FA), picloram and dicamba at 0.5 $mg\,l^{-1}$ to 1.0 $mg\,l^{-1}$ were effective for embryogenic callus induction in sweet potato meristem tissues (Fig. 1). Shimonishi and Karube (1996) reported that naphthaleneacetic acid (NAA) at 2 $mg\,l^{-1}$ was effective for embryogenic callus production in genotypes recalcitrant to 2,4-D-containing medium. They also reported the effectiveness of picloram on embryogenic callus induction. Recently, Kwon et al. (2002) reported that 1 mgl^{-1} 2,4-D gave the highest embryogenic callus induction rate in major Korean cultivars. The effect of auxin on the embryogenic callus formation from shoot meristem tissues showed a strong genotypic dependence; in some cultivars 2,4-D was effective, but in other cultivars picloram, dicamba or 4FA was more effective. The proper choice of auxins would be important for conquering such genotypic dependence in sweet potato.

Plant regeneration from tissues and organs besides the shoot tip has also been reported. Sawada et al. (1990) succeeded in somatic embryogenesis and plant regeneration using cultured stem explants of cultivar 'Tosabeni', a variant of 'Kokei 14', on medium supplemented with 0.2–2.0 mgl^{-1} 2,4-D and 5 $mg\,l^{-1}$ abscisic acid (ABA). Gosukonda et al. (1995a, b) reported that thidiazuron (TDZ) was effective for shoot regeneration from petiole explants of cultivar 'PI318846-3' and up to 77% of cultured explants formed shoots. Plants have also been regenerated from leaf explants by the same protocol using zeatin riboside instead of TDZ (Dessai et al. 1995). Adventitious shoots were regenerated directly from the explants from both petioles and leaves of 18 out of 27 genotypes.

Table 2. Summary of published studies concluded on induction of embryogenic callus in sweet potato. *EC* Embryogenic callus, *MS* MS medium (Murashige and Skoog 1962)

Cultivar/genotype	Target tissue (size)	Basal medium used	Plant growth regulators (mgl^{-1})	Observations/ remarks (%)	Reference
'White Star', 'GaTG 3'	Leaf, shoot tip, stem and root explants	LS	2,4-D (1.0)	EC (22–23% in leaf explants)	Liu and Cantliffe (1984)
9 cultivars 'PI8458', etc.)	Shoot tips	MS	2,4-D (0.3 or 3.0)	EC (10–31%)	Jarret et al. (1984)
70 cultivars ('White Star', etc.)	Apical domes	MS	2,4-D (1.1 or 2.2)	EC (0–100%)	Komaki et al.(1991)
'White Star'	Apical domes (0.2–1.0 mm)	Modified MS	2,4-D (2.2)	EC (>80%)	Cantliffe (1993)
7 cultivars ('Kokei 14', etc.)	Shoot tips (0.5 mm)	MS	2,4-D (0.2)	EC (0–70%)	Liu et al. (1993a)
7 cultivars ('Yu shu 34', etc.)	Shoot tips	MS	2,4-D (0.5–5.0)	EC (20.0–37.5%)	Tan et al. (1993)
10 cultivars ('Duclos 11', etc.)	Lateral buds (0.5–1.0 mm)	MS	2,4-D (2.2)	EC (0–17.0%)	Cavalcante et al. (1994)
11 cultivars ('Kokei 14', etc.)	Shoot apices (0.5–0.7 mm)	LS	Dicamba (0.5–1.0) 4FA (0.5–1.0) picloram (0.5–1.0)	EC (50.0–94.5%)	Otani and Shimada (1996)
9 cultivars ('Hi-Starch', etc.)	Shoot tips (0.3–0.5 mm)	MS	NAA (1–5)+GA_3 (1.0–10.0) 2,4-D (0.05–1.0) picloram (0.5–2.0)	EC	Shimonishi and Karube (1996)
4 cultivars ('Zami', etc.)	Shoot apical meristems	LS	2,4-D (1.0)	EC (78–86%)	Kwon et al. (2002)

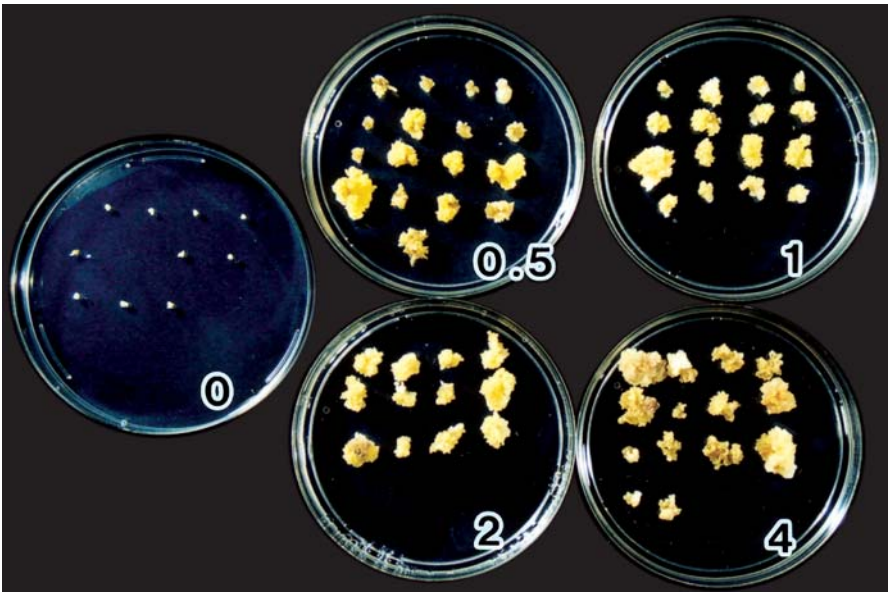

Fig. 1. Effect of picloram on embryogenic callus formation from shoot meristem tissues. *Numbers* indicate the mean concentration (mgl^{-1}) of picloram in each medium

Further, the frequency of plant regeneration from callus culture has also been improved. Otani et al. (1996) reported that the presence of either ABA or silver nitrate ($AgNO_3$) in the callus induction medium promoted shoot regeneration and the optimum concentration was 2 $mg\,l^{-1}$ for both ABA and $AgNO_3$, the frequency of plant regeneration from leaf-derived callus being 70.0% and 73.3%, respectively. Wang et al. (1999) reported that the combination of 0.05 $mg\,l^{-1}$ 2,4-D and 0.5 mgl^{-1} kinetin in the callus induction medium was useful for efficient plant regeneration from leaf- and petiole-derived callus.

2.2 Protoplast Culture and Somatic Hybridization

In sweet potato, the protoplast is an attractive source for the production of somatic hybrids among sexually incompatible cultivars or wild relatives and for transformation. For studies on the isolation and culture of sweet potato protoplasts before 1991, see Sihachakr and Ducreux (1993). Perra and Ozias-Akins (1991) succeeded in plant regeneration from petiole protoplasts of two cultivars. Belarmino et al. (1994) reported plant regeneration from stem and petiole protoplasts of sweet potato cv. 'Shirosatsuma'. The protoplast-derived plants showed morphological characters similar to those of the donor plant. Dhir et al. (1998) also succeeded in plant regeneration from petiole protoplasts of 'Jewel' via somatic embryogenesis; and regenerated plants showed normal leaf morphology and growth habits same as 'Jewel'. Wang et al. (1998a) obtained

several regenerated plants from petiole- and embryogenic callus-derived protoplasts.

A few works on somatic hybridization in sweet potato have been reported in the past decade. Wang et al. (1997) obtained somatic hybrid plants between cross-incompatible cultivars, 'Koganesengan' and 'Bitambi'. The hybrid plants had the expected chromosome number, $2n = 12x = 180$, and showed slow growth and low pollen fertility. For somatic hybridization between sweet potato and wild relatives, Liu et al. (1993b) succeeded in producing somatic hybrids between sweet potato cv. 'Kokei 14' and *I. triloba* by PEG-mediated protoplast fusion, though they did not confirm the characteristics of the hybrid clearly. Another report on inter-specific hybridization describes how somatic hybrids between sweet potato cv. 'Bitambi' and *I. triloba* were obtained and the hybrid plants showed intermediate morphology and a chromosome number of 45–65 (Wang et al. 1998b). Further, Belarmino et al. (1996) reported asymmetric protoplast fusion between sweet potato and *I. trifida* or *I. lacunosa* irradiated with X-rays. They obtained hybrid plants from one combination, cv. 'Shirosatsuma' and *I. trifida*, confirmed by isozyme analysis.

To date, somatic hybrid plants have not been analyzed in detail for their characters, such as root system and progeny. Further research is needed to obtain hybrid plants between cross-incompatible genotypes.

3 Transformation System

3.1 Genetic Transformation of Sweet Potato

Reports on the transformation of sweet potato are listed in Table 3. By using the direct gene delivery system, transgenic plants were regenerated from transformed protoplasts via electroporation (Okada et al. 2001) and from bombarded cells of suspension cultures (Okada et al. 2002), respectively. Also, an *Agrobacterium*-mediated transformation system has been established in several laboratories. Dodds et al. (1991) and Otani et al. (1993) obtained transgenic sweet potato plants integrated with Ri T-DNA and/or *gus*A and *npt*II genes by using *A. rhizogenes*, although those transgenic plants showed abnormal morphology due to integrated Ri T-DNA. Transgenic plants have been produced successfully by co-cultivation of *A. tumefaciens* with storage roots (Newell et al. 1995), leaf disks (Moran et al. 1998), stem segments (Song et al. 2004) and embryogenic callus (Gama et al. 1996, Otani et al. 1998).

The promoter is an important factor for the expression of the introduced foreign genes. In sweet potato, CaMV35S promoter has been used commonly in transformation studies. However, the expression level of a fatty acid desaturase gene from tobacco (*NtFAD3*) driven by the CaMV35S promoter in transgenic sweet potato plants was lower than that driven by a modified CaMV35S promoter, El2Ω (Mitsuhara et al. 1996), on the basis of not only Northern blot

Table 3. Summary of published studies on the transformation of sweet potato

Cultivar/ genotype	Target tissue	Method used	Genes	Observations/ remarks	Reference
Not stated	In vitro whole plant	*A. rhizogenes*	Synthetic sequence	Transgenic plants	Dodds et al. (1991)
'Jewel', 'TIS-70357'	Leaves and petioles	Particle bombardment	*nptII*, *gusA*	Transformed calli	Prakash and Varadarajan (1992)
Five cultivars ('Chugoku 25', etc.)	Leaves	*A. rhizogenes* 15834, etc.	*nptII*, *gusA*	Transgenic plants	Otani et al.(1993)
'Jewel'	Storage roots	*A. tumefaciens* LBA4404	*nptII*, *gusA*, cowpea trysin inhibitor, snow- drop lectin	Transgenic plants	Newell et al. (1995)
'White Star'	Embryogenic callus	*A. tumefaciens* EHA101	*nptII*, *gusA*	Transgenic plants	Gama et al. (1996)
'Kokei 14'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt*, *gusA*	Transgenic plants	Otani et al. (1998)
'Jewel'	Leaves	*A. tumefaciens* C58C1	*nptII*, *cryIIIA*	Transgenic plants	Moran et al. (1998)
'Beauregard'		Electroporation, particle bombardment	GFP	Transformed calli	Lawton et al. (2000)
'Kokei 14', 'Beniazuma'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt, NtFAD3*	Transgenic plants	Wakita et al. (2001)
'Kokei 14'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt*, *GBSSI*	Transgenic plants	Kimura et al. (2001)
'Chikei 682-11'	Mesophyll protoplasts	Electroporation	*hpt*, SPFMV-S coat protein	Transgenic plants	Okada et al. (2001)
'Nanging 51-93'	Embryogenic cell suspension	Particle bombardment	*hpt*, SPFMV-S coat protein	Transgenic plants	Okada et al. (2002)
'Kokei 14'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt*, *bar*	Transgenic plants	Otani et al. (2003a)
'Beniazuma'	Stem	*A. tumefaciens* EHA105	*nptII*, *hpt*, *gusA*	Transgenic plants	Song et al. (2004)
'Yulmi'	Embryogenic callus	Particle bombardment	*nptII*, *SOD*, *APX*	Transgenic plants	Lim et al. (2004)
'Lizixiang'	Embryogenic cell suspension	*A. tumefaciens* LBA4404	*nptII, oryzacystain-I (OCI)*	Transgenic plants	Jiang et al. (2004)
'Kokei 14'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt*, mouse *adiponectin* cDNA	Transgenic plants	Berberich et al. (2005)
'Kokei 14'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt*, spermidine synthase *(FSPD1)*	Transgenic plants	Kasukabe et al. (2006)
'Kokei 14'	Embryogenic callus	*A. tumefaciens* EHA101	*hpt, SBEII* RNAi construct	Transgenic plants	Shimada et al. (2006)

analysis, but also fatty acid composition (Wakita et al. 2001). It is necessary to use an appropriate promoter for each gene in a transformation study of sweet potato. Moreover, promoters of organ-specific genes would be forceful, for example promoters of sporamin and β-amylase which are expressed specifically in the storage root of sweet potato (Ohta et al. 1991).

3.2 Transgenic Sweet Potato with Agronomic Importance

3.2.1 Biotic Stresses

Some transgenic plants have been developed to withstand biotic stresses, such as weeds (Otani et al. 2003b), insects (Newell et al. 1995; Moran et al. 1998) and virus diseases (Okada et al. 2001; Table 3).

Herbicides are effective for weed control in the field production of sweet potato. Otani et al. (2003b) introduced the phosphinothricin acethltransferase (PAT, *bar*) gene for herbicide resistance. Transgenic sweet potato plants exhibit bialaphos resistance (Figs. 2, 3, 4) and also show resistance to the commercial herbicide, Herbie Liquid (Meiji Seika Kaisha Ltd, Japan). Genetic engineering of herbicide tolerance in sweet potato may make weed control weeds more convenient and more economical during cultivation.

The sweet potato weevil is the major sweet potato insect pest worldwide, especially in developing countries. To date, two reports have been published on transformed plants with insect-resistant genes. Newell et al. (1995) introduced cowpea trypsin inhibitor and snowdrop lectin genes into sweet potato genome, but they did not confirm the insect tolerance of regenerated transgenic plants.

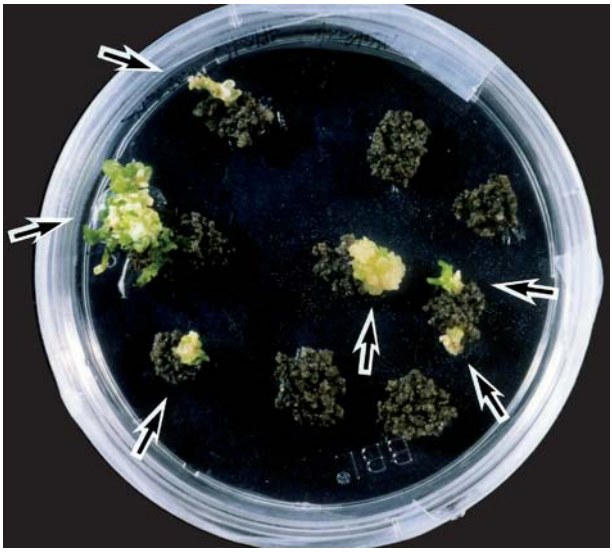

Fig. 2. Hygromycin-resistant somatic embryos and embryogenic callus formation from *Agrobacterium tumefaciens*-infected embryogenic callus. *Arrows* indicate hygromycin-resistant somatic embryos and embryogenic calli

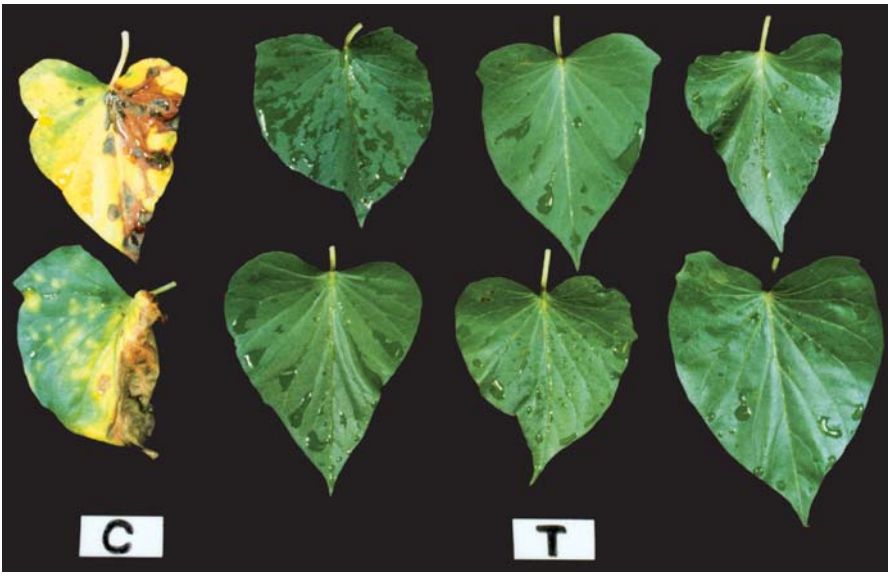

Fig. 3. Leaves of transgenic sweet potato plants showing bialaphos resistance. *C* Untransformed plants, *T* independent transgenic plants

Fig. 4. Root tubers of a transgenic sweet potato plant which has the *bar* gene. *C* Untransformed plant, 6 transgenic plant

Moran et al. (1998) regenerated transgenic plants possessing the *cryIIIA* gene and observed the transgenic sweet potato plants to be less affected by weevils than the untransformed control plants in field tests.

Viruses are another main constraint to sweet potato production and a sweet potato feathery mosaic virus (SPFMV) is the most widespread in the world. Okada et al. (2001) produced the transformed sweet potato cv. Chikei 682-11 with the coat protein cDNA of SPFMV and observed the transformants to be highly resistant to SPFMV. Recently, in Kenya transgenic lines of sweet potato variety CPT560 transformed with SPFMV coat protein gene were tested in the field under controlled conditions to evaluate virus resistance, yield of storage roots and other characters (Gichuki et al. 2003).

3.2.2 Abiotic Stresses

Abiotic stresses such as low temperature, drought and early frost are also a serious problem for sweet potato production. We introduced a fatty-acid desaturase gene to increase the content of unsaturated fatty acid for low-temperature tolerance and succeeded in modifying the fatty acid composition in transgenic sweet potato plants (Wakita et al. 2001). We are now examining the low-temperature tolerance of these transgenic sweet potato plants.

Oxidative stress is one of the major damaging factors in plants exposed to environmental stresses. Damage from multiple environmental stresses would be alleviated by manipulation of antioxidatives in chloroplasts. Lim et al. (2004) produced transgenic sweet potato plants over-expressing CuZn superoxide dismutase (SOD) and ascorbate peroxidase (APX), which are toxic reactive oxygen species (ROS)-scavenging enzymes. Those leaves and whole plants appeared to have a stronger tolerance to paraquat (methyl viologen)-mediated oxidative stress than non-transgenic plants. Studies are in progress to investigate their tolerance under various stresses, including low temperatures.

3.2.3 Starch Quality

Starch is the main product of sweet potato and provides important food processing and industrial materials. Amylose and amylopectin are the main polysaccharide components of most natural starches and the amylose:amylopectin ratio is the important factor in the textural properties of starch. A new sweet potato variety that contains starch with amylose-free or low amylose content would develop new industrial applications. We introduced the full-length sense cDNA of granule-bound starch synthase I (*GBSSI*), which is one of the key enzymes to catalyze the formation of amylose, a linear α(1,4)D-glucan polymer, from ADP-glucose, for the modification of starch structure. Of the 26 transgenic plants independently regenerated, one lacked amylose in the storage roots (Kimura et al. 2001). The amylose-free transgenic sweet potato plants were also successfully obtained more efficiently by inhibition of sweet potato *GBSSI* gene expression through RNA interference (Otani et al. 2003a).

3.3 Experimental Protocol for *A. tumefaciens*-Mediated Transformation

3.3.1 Embryogenic Callus Induction from Shoot Meristem Tissues

Step 1. Culture in vitro plants of sweet potato on LS medium (Linsmaier and Skoog 1965) supplemented with 3% sucrose and 0.25% Gelrite at 26 °C under a 16-h photoperiod at 38 μmol m^{-2} s^{-1} from daylight fluorescent tubes.

Step 2. Place shoot meristems (0.5 mm diam.) of in vitro plants onto LS medium for embryogenic callus induction, which is LS medium supplemented with 1 mg l^{-1} 4-fluorophenoxyacetic acid (4FA; Aldrich; or picloram), 3% sucrose and 0.32% Gelrite. Keep cultures at 26 °C in the dark for 45 days.

Step 3. Maintain embryogenic calli by subculture on the same fresh medium every month. Use calli subcultured at least three times for the transformation.

3.3.2 Transformation

Step 1. Culture *Agrobacterium tumefaciens* for 2–3 days at 26 °C on Luria broth (LB; Sambrook et al. 1989) supplemented with 50 mg l^{-1} kanamycin, 50 mg l^{-1} hygromycin B and 1.5% (w/v) agar. Then, transfer the colony of bacteria to liquid LS medium and shake at 100 rpm for 30 min in the dark at 26 °C.

Step 2. Soak the embryogenic calli in a bacterial suspension for 2 min and blot dry with sterile filter paper to remove excess bacteria.

Step 3. Then, transfer the calli onto co-culture medium, which is LS medium supplemented with 1 mg l^{-1} 4FA (or picloram), 10 mg l^{-1} acetosyringone (Aldrich), 1% glucose, 3% sucrose and 0.32% Gelrite, and culture for 3 days in the dark at 23 °C.

Step 4. Wash the infected calli four times with sterile distilled water supplemented with 500 mg l^{-1} carbenicillin and then transfer onto selection medium, which is LS medium supplemented with 1 mgl^{-1} 4FA (or picloram), 25 mg l^{-1} hygromycin B, 500 mg l^{-1} carbenicillin, 3% sucrose and 0.32% Gelrite. Cultures were carried out in the dark at 26 °C.

3.3.3 Selection and Plant Regeneration

Step 1. After 2 weeks of culture on the selection medium, wash the calli again as described above and then transfer to fresh selection medium. Subculture the calli on fresh medium every 2 weeks.

Step 2. After 60 days of culture on the selection medium, transfer the calli onto the somatic embryo formation medium, which is LS medium supplemented with 4 mg l^{-1} ABA, 1 mg l^{-1} gibberellic acid (GA_3), 3% sucrose and 0.32% Gerlite, and culture at 26 °C under a 16-h photoperiod at 38 µmol m^{-2} s^{-1} from daylight fluorescent tubes.

Step 3. After 21 days of culture on the somatic embryo formation medium, transfer the somatic embryos formed from hygromycin-resistant calli onto the plant formation medium, which is LS medium supplemented with 0.05 mg l^{-1} ABA, 3% sucrose and 0.32% Gerlite for germination.

4 Molecular Genetics

4.1 Storage Proteins

The pioneer works in molecular genetics on sweet potato were performed on the genes encoding two major proteins, sporamin and β-amylase, which specifically accumulate in the storage root (Nakamura 1992). They cloned and characterized their genes to study the molecular mechanisms regulating the formation of storage roots. The specific expression of a chimeric gene with a sporamin promoter in transgenic tobacco and potato has been observed and the promoters of sporamin and β-amylase genes are used to express the desired proteins in storage roots to improve their qualities and yield (Ohta et al. 1991).

4.2 Genes for Sucrose Metabolism in Sweet Potato Storage Root

Biochemical and molecular research on the starch biosynthesis has been started for developing the tuberous root of sweet potato. Genes that affect sucrose cleavage and related metabolisms may play a central role in the control of storage root development, storage sink strength and photosynthate partitioning.

ADP-glucose pyrophosphorylase (AGPase) plays a central role in starch biosynthesis in both photosynthetic and non-photosynthetic plant tissues. It catalyzes the first step of starch biosynthesis: the production of ADP-glucose and inorganic phosphate from glucose 1-phosphate and ATP. Plant AGPase is characterized as a heterotetramer composed of two large subunits and two small subunits encoded by different genes. In sweet potato, cDNAs encoding both large and small subunit AGPase have been isolated and characterized (Bae and Liu 1997, Noh et al. 2004). They introduced cDNAs of the small subunit AGPase into tobacco plants. The transgenic tobacco overexpressing the small subunit of AGPase were partially male sterile. Starch synthesis via AGPase and cell proliferation may work together in tuber formation in sweet potato root (Bae et al. 2001).

4.3 Storage Root Induction

Sweet potato develops two types of roots: fibrous roots and storage roots. There is not enough physiological and molecular information on the conditions that induce storage root formation in sweet potato.

The expression sequence tag (EST) approach has been used in profiling the genes with enhanced expression in specific tissues. You et al. (2003) identified 22 differentially expressed genes in the early developmental stage of fibrous roots and storage roots. They suggested that those genes involved in gene regulation, signal transduction and development were possibly related to the storage root induction processes in sweet potato.

Moreover, the development of sweet potato storage roots coincides with starch accumulation. Li and Zhan (2003) used ESTs to investigate the enzymes involved in sucrose metabolism and its immediate downstream biochemical reaction in the storage root and in fibrous roots. They found that SuSy (sucrose synthase) is the most active gene at the enlarging and starch-accumulating stages of sweet potato storage roots.

Further, MADS-box genes have been cloned in many plant species and their role as homoeotic genes that control floral organ development are well established (Coen and Meyerowits 1991). It is very interesting that new MADS-box genes were isolated from pigmented and tuber-forming root tissue in sweet potato (Kim et al. 2002). These MADS-box genes from sweet potato were found to be expressed preferentially in toot tissues; and these genes were suggested to play an important role in tuber initiation in sweet potato.

4.4 Retrotransposons in Sweet Potato

Numerous plant retrotransposons (transposable genetic elements) have been characterized (Kumar and Bennetzen 1999). The LTR retrotransposons possess long terminal repeats and have been identified in many plant genomes. However, only three families (Tnt1, Tto1, Tos17) have been demonstrated to include elements that are capable of transposition. Genetic alterations (mutations) are believed to be likely results from the activity of retrotransposons, the majority of which can be activated by stresses, for example, from viruses and tissue culture. In the sweet potato genome, Tahara et al. (2004) isolated an active LTR retrotransposon that can be activated by tissue culture. They used the LTR retrotransposon to identify sweet potato cultivars by insertion polymorphism (Ooe et al. 2004).

5 Concluding Remarks

As mentioned above, the biotechnology of sweet potato has developed very quickly during the past decade. Although transformation of sweet potato re-

mains cumbersome, labor-intensive and genotype-dependent, some transgenic plants of sweet potato with agronomic importance have been produced in several countries. Transgenic sweet potato resistant to viruses and weevils should contribute to increased yield and food security in developing countries in the near future.

Genetic engineering of starch can be used to improve the quality of sweet potato starch for the development of new dietary and industrial products. We have succeeded in producing transgenic sweet potato plants having amylose-free starch. By manipulating the genes controlling the synthesis of starch, such as granule-bound starch synthase I (*GBSSI*), branching enzyme and debranching enzyme genes, we can obtain novel starches with modified amylose:amylopectin ratios for various uses.

Further, because of the high production yield of its biomass, sweet potato would be a cost-effective alternative to microbial and animal systems as a target of 'molecular farming' for the production of various biomolecules, such as high-value pharmaceutical polypeptides, industrial enzymes and biodegradable plastics (Goddjin and Pen 1995).

For the further improvement of multi-gene-controlled traits, such as yield, the accumulation of starch in storage roots and post-harvest properties, considerable progress in biotechnology is expected in the next 10 years.

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IV.4 Sugar Beet

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1 Introduction

Beet sugar and cane sugar constitute 25% and 75%, respectively, of the world sucrose production of about 145×10^6 t year^{-1}. Sugar beet (*Beta vulgaris* ssp. *vulgaris* var. *altissima* Döll) is grown mainly in Europe, producing 28×10^6 t beet sugar (hereof 20×10^6 t in the EU), North America (4.0×10^6 t) and Asia (2.5×10^6 t). For many years, beet sugar yields have been increasing steadily, from 5 t sugar ha^{-1} in the mid-1950s to 8–9 t ha^{-1} in the EU today (Ahlfeld 2005). Further progress is needed to maintain the competitiveness of the sugar beet industry, which is under increasing pressure, notably from major cane sugar exporting countries. As a result, the EU sugar regime is being reformed, which is expected to lead to a considerable reduction in beet sugar production; in 2006 the decrease was about 19% (Anon 2006). However, such a decline might be diminished by an increasing demand for bioethanol. Due to its large biomass production capacity, it has been estimated that 5.7 m^3 ha^{-1} bioethanol can be produced from sugar beet, compared with 2.6 m^3 ha^{-1} from wheat (Anon 2005a).

In this chapter, the use of biotechnology for the production of improved sugar beet varieties is reviewed, along with studies on its potential impact on the environment and human health. The transgenic traits that have received most interest may be assessed by the number of EU notifications for field trials with genetically modified (GM) sugar beet under Directive 90/220/EEC (Table 1). Tolerance to broad-spectrum herbicides is the main focus (71% of the notifications), while virus resistance, particularly to rhizomania, is also an important target (19%). Other GM traits include tolerance to drought and fungi, synthesis of fructans and enzymes. Notifications for GM beet constitute 18% of the total number of notifications under this directive.

After the implementation of the new directive regarding deliberate release into the environment of GM organisms (2001/18/EC), the number of sugar beet notifications so far is three. Presently no GM sugar beet varieties are available commercially.

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Table 1. Most studied transgenic traits in sugar beet, indicated by the number of EU notifications for GM sugar beet field trials under Directive 90/220/EEC (in force from October 1991 to October 2002) where the indicated main trait occurs (Anon 2005b). *BNYVV* Beet necrotic yellow vein virus, *BWYV* beet western yellows virus, *BYV* beet yellows virus

	Belgium	Denmark	Finland	France	Germany	Greece
Glyphosate tolerance	8	19	4	29	6	1
Glyfosinate tolerance	4		1	27	13	1
Sulphonylurea tolerance				3		
BNYVV tolerance	1	1		16	7	
BWYV tolerance				3		
BYV tolerance						
Other traits				8	1	

	Ireland	Italy	Netherlands	Spain	Sweden	UK	Total
Glyphosate tolerance	4	12	23	15	5	24	150
Glyfosinate tolerance		16	15	9	5	11	102
Sulphonylurea tolerance			1				4
BNYVV tolerance		10	10		1	3	49
BWYV tolerance			11			3	17
BYV tolerance			2			1	3
Other traits		2	15			3	29

2 Production of Transgenic Sugar Beet

Genetic transformation of sugar beet started in the mid-1980s but was hampered by the generally low frequency of cells with competence for both transformation and regeneration, along with poor selection efficiencies. Although significant progress has been achieved, transformation frequencies are still relatively low compared with those for other crop species (Joersbo 2003). Many combinations of explant types and gene transfer techniques, in addition to optimised/new selection systems, have been studied, leading to an array of protocols for the production of transgenic sugar beet plants. However, reproducibility amongst different laboratories tends to be modest (D'Halluin et al. 1992; Snyder et al. 1999; Ivic and Smigocki 2003), indicating that several factors and/or mechanisms determining the outcome of transformation experiments remain to be elucidated.

2.1 Transformation of Protoplasts

Protoplasts, being devoid of cell walls, are particularly suited for direct gene transfer by methods such as electroporation (Fromm et al. 1985). In sugar beet, after protoplasts were treated with short high-voltage pulses in the presence of

plasmid DNA (pDNA), transient expression was detected of a chloramphenicol acetyltransferase reporter gene (Joersbo and Brunstedt 1991). In order to enhance DNA uptake, a direct gene transfer method was developed, based on a sub-lethal treatment with ultrasound as a means to promote plasma membrane permeability. This resulted in a 7- to 15-fold increase in transient expression compared with electroporation (Joersbo and Brunstedt 1992). These gene transfer techniques are suitable for rapid assessments of gene construct functionality and relative effect of regulatory elements, such as promoters, and also for stable transformation. However, only transgenic callus is obtained (Lindsey and Jones 1989) if the morphogenic potential of the employed protoplasts is not sufficient for full regeneration into transgenic shoots.

Hall et al. (1996) devised a method for enriching protoplast preparations specifically for totipotent cells derived from stomatal guard cells. Protoplasts were prepared from blended de-ribbed leaf material, purified thoroughly by gradient centrifugations and had division frequencies of about 50% compared with 0.01–0.5% for unfractionated leaf protoplasts. Transfer of pDNA encoding phosphinothricin acetyltransferase was performed by the addition of polyethylene glycol. Plant regeneration occurred from glufosinate-resistant calli within 4 weeks, producing about 20 transformed plants per gramme leaf material. Five plants were single-copy transformants suitable for breeding purposes.

2.2 Transformation of Cells

Cell cultures of sugar beet gradually tend to lose competence for regeneration by irreversible dedifferentiation. However, D'Halluin et al. (1992) were able to produce embryogenic callus induced on cotyledons, hypocotyls, petioles and true leaves of 2- to 3-month-old seedlings grown in the dark at high concentrations of 6-benzylaminopurine (BAP). Callus induction was strongly genotype-dependent, with no response in 20–30% of the tested lines. Callus was finely chopped and co-cultured with *Agrobacterium tumefaciens* carrying a bialaphos resistance or a mutant acetolactate synthase gene. Transgenic sugar beet plants resistant to commercial concentrations of the corresponding herbicides were selected. The production time of transgenic shoots ready for transfer to the glasshouse could be up to 2 years and this long culture period may be responsible for the observed aberrant phenotypes.

Following a similar protocol using hypocotyl segments, Snyder et al. (1999) induced embryogenic callus which was subjected to biolistic gene transfer. Transgenic shoots appeared in 16–33% of the treated plates, most of which showed evidence of multiple-copy DNA insertions and molecular rearrangements. In a related study, Ivic and Smigocki (2003) used leaf discs of a breeding line, also incubated in the dark at high concentrations of BAP, from which friable embryogenic callus was harvested. Using particle bombardment for transformation, kanamycin-resistant sugar beet callus lines were obtained, displaying complex integration of the heterologous DNA.

2.3 Transformation of Tissues

Sugar beet tissues are, in general, susceptible to *Agrobacterium* (Krens et al. 1988). Following inoculation of 20 sugar beet lines with a supervirulent *A. tumefaciens* strain, D'Halluin et al. (1992) reported tumour formation in 19 lines. Thus, the recalcitrance of sugar beet may not be caused by lack of competence for DNA uptake and integration, but rather to a low number of morphogenic cells and possibly also to restricted access to such cells if they are embedded in large numbers of non-morphogenic cells.

To transform a tissue type believed to be enriched in regenerable cells, Lindsey and Gallois (1990) used shoot-bases of in vitro propagated sugar beet shoots for co-cultivation with *A. tumefaciens* harbouring a *npt*II gene; and they obtained kanamycin-tolerant shoots. Similar results have also been reported by Konwar (1994) and Hisano et al. (2004). Leaves have also been employed as explants. Thus, Norouzi et al. (2005) co-cultivated leaf explants derived from tissue-cultured shoots with *A. tumefaciens* and obtained regeneration of transgenic shoots from cells around the main vein of the explants. Ivic-Haymes and Smigocki (2005) incubated leaf disks of greenhouse-grown sugar beet plants for 7 weeks before biolistic transformation. After 5 months, GUS-positive shoots were obtained from transgenic calli. However, due to costs and risk of somaclonal variation, in vitro culture periods prior to transformation should be minimised. Cotyledons of in vitro germinated seedlings can be produced within 1 week and they have, in contrast to the report by D'Halluin et al. (1992), been found to be suitable for transformation by several authors (Fry et al. 1991; Steen and Pedersen 1993; Krens et al. 1996; Mannerlöf et al. 1997; Snyder et al. 1999; Joersbo et al. 1998, 2000). This is probably because the transition zone between the cotyledon and hypocotyl possesses a high de novo regeneration capacity (Krens et al. 1996). Compared with other sugar beet transformation protocols, cotyledon transformation by *A. tumefaciens* seems to be more reproducible and is extensively studied, although transformation frequencies in most cases are modest, in the range of 0.1–1.0%, based on the percentage of transgenic shoots per cotyledon.

Unlike *A. tumefaciens*, *A. rhizogenes* transformation of sugar beet tissue, such as petioles, readily gives transgenic differentiated structures (hairy roots). This provides a rapid test system for studying the expression of stably integrated foreign genes in roots (Ehlers et al. 1991), for promoter analysis (Dimmer et al. 2004) and for host–parasite interactions between cyst nematodes and sugar beet root cells (Cai et al. 1997, 2003).

2.4 Selection of Transgenic Tissues and Shoots

After transformation, the selection system should favour the growth of transgenic shoots while suppressing that of non-transgenic cells. Kanamycin selection, used for many plant species as a selectable marker (Fraley et al. 1983), has

also been employed for sugar beet; but the high natural tolerance to aminoglucoside antibiotics has caused low selection efficiencies, resulting in a considerable fraction of non-transgenic shoots, the so-called escapes (Lindsey and Gallois 1990, Hisano et al. 2004). Konwar (1994) found only 0–35% of the regenerated kanamycin-tolerant shoots were transgenic and kanamycin was reported to impair root formation of the selected sugar beet shoots.

Using the herbicide glyphosate as a selective agent appeared to give more stringent selection, as 71% of the selected shoots transferred to the glasshouse displayed no or minor visible effects after leaf application of a commercial dose of glyphosate (Mannerlöf et al. 1997). In contrast, phosphinothricin selection of transformed embryogenic callus gave an efficiency of only 30% (D'Halluin et al. 1992). In order to obtain efficient selection, several new systems were devised (Joersbo 2001). Among those, mannose selection was found to be particularly useful for sugar beet, as the selection efficiencies and rooting frequencies were 5- to 7-fold higher compared with kanamycin selection, being 88% and 89%, respectively (Joersbo et al. 1998).

During selection, transgenic regenerable cells must be able to grow and differentiate in a micro-environment of dying non-transgenic cells. Lindsey and Gallois (1990) speculated that dark or even black necrotic sugar beet tissue, which is often observed during antibiotic or herbicide selection, may inhibit regeneration of transgenic cells due to release of toxic substances. In comparing kanamycin selection with mannose selection using the same transformation protocol, Joersbo et al. (1998) found that the latter gave about 10-fold higher

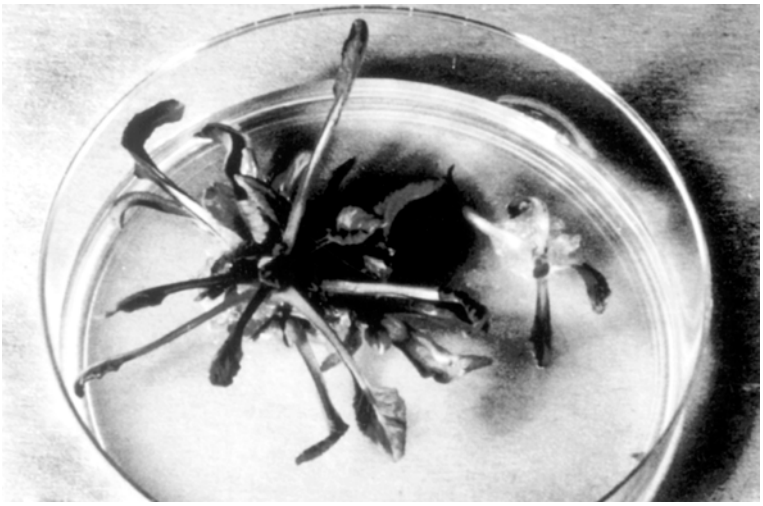

Fig. 1. Sugar beet transformation using mannose selection. The transgenic shoot (*left*) converts mannose to an easily metabolisable substance, enhancing growth, while the non-transgenic shoot (*right*) is starved, gradually losing vigour. The selection system which also works in several other important crops, such as rice and wheat, eliminates possible risks of spread of antibiotic resistance genes to the environment

transformation frequencies, which might be attributed to the much slower deterioration of non-transgenic cells, reducing the possible formation of growth inhibitors (Fig. 1). Therefore, the selection system has a major impact not only on the number of non-transgenic shoots, but can also be critical for the emergence of transgenic shoots (Aragao and Brasileiro 2002).

3 Herbicide Tolerance

Efficient weed control is of paramount importance in sugar beet cultivation as this crop competes poorly in the early growth stages, until at least 8–10 true leaves. Thus, insufficient weed control can reduce yield by 50–100% (Schweizer and May 1993; Scott 1997; May 2001; May and Wilson 2006). Weed control programmes in conventional sugar beet varieties exploit the modest levels of natural tolerance to several selective post-emergence herbicides (such as metamitron, chloridazon, ethofumesate, desmedipham, phenmedipham, triflusulfuron-methyl), which are employed in various mixtures. The programmes may also include a pre-emergence treatment with a broad-spectrum herbicide, such as glyphosate (Coyette et al. 2002).

Conventional weed control programmes are associated with some difficulties. The post-emergence herbicides must be applied 3–5 times at certain rather specific intervals after sowing in order to control weeds while they are small and most sensitive. Such strict regimes are more vulnerable to delays of scheduled sprayings (Coyette et al. 2002). Also, these programmes may be insufficient for the control of difficult weeds, such as volunteer potatoes and weed beet, and the herbicides constitute a relative high cost to farmers (May 2003). Moreover, some of the herbicides (i. e. triflusulfuron-methyl) may cause transient growth inhibition of young sugar beet plants with a concomitant sugar yield reduction of several percent (Jørgensen 1997; Wilson 1999; Mitchell 2000). Therefore, to overcome these problems, sugar beet resistant to broad-spectrum herbicides has been developed by genetic transformation (D'Halluin et al. 1992; Mannerlöf et al. 1997), as no natural tolerance to such herbicides has been observed in this species.

3.1 Glyphosate Tolerance

Glyphosate, the active ingredient of 'Roundup' and many other formulations, is a broad-spectrum, environmentally preferable herbicide which accumulates in meristematic zones. This herbicide inhibits the biosynthesis of aromatic amino acids through binding to 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) at micromolar concentrations (Steinrücken and Amrhein 1980). EPSPS is only present in plants, bacteria and fungi and is not found in mammals, which may contribute to the extremely low mammalian toxicity of glyphosate (Tomlin 2003).

Table 2. Economics of glyphosate-tolerant sugar beet, giving an estimation of the economic benefits (ave. 111 € ha^{-1}) of growing glyphosate-tolerant sugar beets compared with conventional sugar beets in various European countries (from Gianessi et al. 2003)

Country	Conventional herbicides		Glyphosate		Savings
	kg a.i. ha^{-1}	€ ha^{-1}	kg a.i. ha^{-1}	€ ha^{-1}	€ ha^{-1}
Belgium	4.1	201	1.5	83	118
Denmark	2.5	178	1.2	78	100
France	2.7	170	1.9	90	80
Germany	3.7	240	1.7	87	153
Italy	3.1	140	2.2	96	44
Netherlands	2.4	226	1.7	87	139
Spain	3.1	200	2.2	96	104
UK	3.2	205	1.9	90	115
Average	3.2	197	1.9	86	111

Glyphosate is highly effective in controlling major weeds infesting sugar beet fields, usually at application levels significantly below those of conventional selective herbicides (Table 2). Thus, the glyphosate dosages required to suppress wide-spread weed species like *Galium aparine*, *Lamium purpureum*, *Polygonum persicaria*, *Solanum nigrum* and *Stellaria media* were 1.4- to 16.0-fold lower than with conventional herbicides (Madsen and Jensen 1995). Also, difficult weeds like *Chenopodium album*, *Solanum tuberosum* and *Urtica urens* are well controlled by normal glyphosate dosages (Tenning 1998).

The strategies for the production of glyphosate tolerant crops have been to transform either with a construct resulting in overproduction of wild-type EPSPS (e. g. from *Petunia*), or with a gene encoding a modified EPSPS enzyme significantly less inhibited by glyphosate (e. g. CP4 from *Agrobacterium*; Hinchee et al. 1993). This may be supplemented with a glyphosate oxidoreductase (GOX) gene (from *Achromobacter* sp.) encoding an enzyme catalysing the conversion of glyphosate to herbicidally inactive compounds (Barry et al. 1992). Mannerlöf et al. (1997) transformed sugar beet with both an CP4-EPSPS and a GOX gene, driven by an enhanced cauliflower mosaic virus 35S promotor or a figwort (*Scrophularia nodosa*) mosaic virus promotor. They produced 260 independent transformants that displayed varying degrees of glyphosate tolerance, of which at least two were agronomically useful (Fig. 2). Analysis of genomic DNA showed a clear negative correlation between the T-DNA copy number and glyphosate tolerance. The two lines with the highest tolerance both contained single T-DNA inserts.

3.1.1 Agricultural Performance of Glyphosate Tolerance

The agricultural performance of glyphosate-tolerant sugar beet was compared with conventional varieties, using conventional herbicides, with respect to root and sugar yield, fungal disease susceptibility and juice purity, but no diffe-

Fig. 2. Transgenic herbicide-tolerant sugar beet allows more efficient weed control and gives higher yields. *Top* Sugar beet field not treated with herbicides. *Bottom left* Glyphosate-tolerant sugar beet treated with glyphosate. *Bottom right* Non-transgenic sugar beet treated with the same amount of glyphosate (from Mannerlöf 1997, with permission)

rences were found (Thomsen 1999, 2000). Comparisons of yields of glyphosate-tolerant sugar beet treated with glyphosate or conventional herbicides have been conducted in several countries, covering a range of different climatic conditions and agricultural practices. Generally, root yields have been found to be several percent higher using glyphosate compared with conventional herbicides, while the sugar content seems unaffected. The highest increase was observed in Nebraska (USA), where glyphosate-treated herbicide-tolerant sugar beet (using $2 \times 0.84\,\mathrm{kg\,ha^{-1}}$ glyphosate) gave a 15% higher sugar yield

per hectare compared with conventional herbicides (Wilson et al. 2002). The two programmes had similar efficiency on weed control, leaving 1–3 final weed plants m^{-2}. Also, compared with hand-weeded control plots (no final weeds), glyphosate treatment increased sugar yield by 8% per hectare.

In trials in North Europe (Belgium, Denmark, Finland, The Netherlands, UK), Brants and Harms (1998) tested glyphosate-tolerant sugar beet lines with three applications each of 0.7, 1.1, 1.4 or 2.2 kg ha^{-1} glyphosate and found an increase in root fresh weight of approx. 5% compared with conventional treatments, irrespective of the amount of glyphosate applied. Similar increases of yield have been reported from Denmark (Thomsen 2000), France (Richard-Molard and de Garambe 1998), Germany (Schäufele et al. 1998; Bückmann et al. 2000), The Netherlands (Wevers 1998) and the United Kingdom (Dewar et al. 2000) using a total of about 2.2 kg ha^{-1} glyphosate in 2–3 sprayings. This generally resulted in superior weed control (often zero weed ground cover) compared with conventional herbicides, suggesting that lower concentrations of glyphosate may be sufficient. Thus, Wevers (1998) observed that 2×0.72 kg ha^{-1} or even 2×0.54 kg ha^{-1} glyphosate gave efficient weed control on fields with predominantly sensitive weed species (without *U. urens*, *P. convolvulus*, *L. purpureum*). In Ireland (Mitchell 2000) and Poland (Jassem 2000), glyphosate treatment resulted in only slight yield increases, compared with conventional herbicides.

3.1.2 Flexibility of Glyphosate Application

Current selective herbicides are most effective on weeds that are small (cotyledon to early true-leaf stage), while the effect of glyphosate is much less dependent on developmental stage. Studies have been performed to find the optimum timing for glyphosate applications. Dewar et al. (2000) found that, when the first glyphosate spraying was performed at the 2–4 leaf stage, sugar yields were significantly higher compared with first sprayings at the 8–10 or 12–14 leaf stages, where the weed ground cover had reached almost 100%. Similar results were obtained by Wilson et al. (2002), who reported maximum sugar yields with the first glyphosate application somewhat later, at the 4–6 leaf stage (where average weed height was 10 cm), whereas a later first-time application at the 8–10 leaf stage gave about 15% lower sugar yield (weed height 25 cm). This is in contrast to Bückmann et al. (2000), who found that the time for the first glyphosate application was unimportant, even up to the 16 leaf stage.

Despite the different observations that may be attributed to varying weed densities/species, it seems preferable to apply the first glyphosate spray before competition from weeds becomes important, that is, at the 4–8 leaf stage of sugar beet. This implies considerably more flexibility in weed control programmes based on glyphosate, as the applications of conventional herbicides should be accomplished within a time-frame of 3 days for optimal timing (Wevers 1998). The delay of the scheduled sprayings (e. g. due to adverse weather

conditions) may, in order to control the more developed weeds, lead to a need for greater herbicide rates (Coyette et al. 2002), resulting in growth depression or damage to the crop (Fischer and Petersen 2002).

3.1.3 Reduced Herbicide Input

Although the dosage of herbicide required to obtain adequate weed control depends on several factors, such as weed infestation levels and species, it is generally observed that glyphosate is needed in a lower amount compared with conventional sugar beet herbicides [measured as kilograms of active ingredient (kg a.i.) per hectare]. Coyette et al. (2002) calculated the average use rate of conventional herbicides in major sugar beet-growing EU countries to be 3.2 kg a.i. ha^{-1}, while the average requirement for glyphosate would be 1.9 kg a.i. ha^{-1} (Table 2). There is a tendency that the reduction in herbicide use would be more pronounced in countries, as well as in fields (Champion et al. 2003), where the herbicide requirements are highest. Wevers (1998) suggested that carefully optimised timing for glyphosate sprayings might further reduce the required herbicide input. Assuming a market penetration of glyphosate tolerant sugar beet of 56%, the estimated total reduction compared with conventional herbicides would be 1.2×10^6 kg $year^{-1}$ and 1.9×10^6 kg $year^{-1}$ in a 100% glyphosate-tolerant market.

3.1.4 Economics of Glyphosate Tolerance

The economic consequences of the higher yield, increased flexibility and reduced herbicide input of glyphosate-tolerant sugar beet were estimated by May (2003), suggesting total savings of 230 € ha^{-1} compared with conventional sugar beets grown in the United Kingdom at 2003 prices. A large proportion of this amount (120 € ha^{-1}) was due to the lower herbicide usage, assuming application of 2.2 kg ha^{-1} glyphosate. However, Dewar et al. (2002) suggested that 1.1–1.4 kg ha^{-1} glyphosate may be sufficient in the United Kingdom, which would reduce costs correspondingly. In addition, the lower application number (2×, compared with 4–5× conventionally) was calculated to save 25 € ha^{-1}. Higher yields, estimated at 5%, would result in savings of 75 € ha^{-1}, calculated as the value of the area saved when used for an alternative crop. Other considerable savings would be on weed beet control (15 € ha^{-1}) and on reduced subsoiling, as glyphosate sprayings can await suitable soil conditions and thus induce less damage on soil structure (15 € ha^{-1}). An assumed technology fee of 40 € ha^{-1} to cover the higher price of glyphosate tolerant seeds is included in the total savings.

Gianessi et al. (2003) presented similar calculations for more sugar beet growing countries in the EU, including only the costs of herbicides, their application and the above-mentioned technology fee. Based on Coyette et al. (2002) and assuming glyphosate dosages of 1.2–2.2 kg ha^{-1} and conventional herbi-

cide mixtures of 2.4–4.1 kg a.i. ha^{-1}, the average savings would be 111 € ha^{-1}, ranging from 44 € ha^{-1} in Italy to 153 € ha^{-1} in Germany (Table 2).

3.2 Glufosinate Tolerance

Glufosinate, sold in formulations as 'Basta' and 'Liberty', is a racemate of DL-phosphinothricin, of which only the L-isomer has herbicidal activity. Like glyphosate, it is a broad-spectrum post-emergence herbicide with low mammalian toxicity (Tomlin 2003). L-Phosphinothricin, initially identified as the active moiety of the tripeptide bialaphos (L-phosphinotricinyl-L-alanyl-L-alanine) is a potent inhibitor of glutamine synthetase, which is critical for the assimilation of ammonia (Hinchee et al. 1993).

Genes encoding enzymes capable of detoxifying L-phosphinothricin by N-acetylation have been isolated from *Streptomyces hygroscopicus* (Thompson et al. 1987) and *S. viridochromogenes* (Wohlleben et al. 1988). The genes, 87% identical at the nucleotide sequence level, are referred to as *bar* (for bialaphos resistance) and *pat* (for phosphinothricin acetyltransferase), respectively, and the encoded enzymes as BAR and PAT, respectively (Wehrmann et al. 1996), although sometimes both gene products are designated PAT.

D'Halluin et al. (1992) transformed sugar beet with a *bar* gene driven by the 35S or TR2′-promotors and both constructs gave Basta tolerance under glasshouse conditions after spraying with 0.80 kg ha^{-1} glufosinate-ammonium (4.0 l ha^{-1} Basta).

3.2.1 Agricultural Performance of Glufosinate Tolerance

Like glyphosate tolerance, glufosinate-tolerant sugar beet has been tested in a number of different environments and similarities with respect to weed control efficiency and, to a lesser extent, to yield increases have been reported for the two weed control strategies. Wevers (1998) found that 2–3 sprayings of 0.72–2.12 kg ha^{-1} glyphosate or 0.40–0.80 kg ha^{-1} glufosinate both resulted in good weed control, although glyphosate was more effective against perennial weeds like *C. arvense* and *S. nigrum* and it eliminated all late weeds.

Loock et al. (1998) reported a multi-locational trial investigation in Europe and the United States and found that there was no indication of any harmful effects of the transformation event leading to glufosinate tolerance, so that no differences were observed between transgenic hybrids and their non-transgenic counterparts. Also, no differences were found in the yields of glufosinate-tolerant plants when treated with glufosinate compared with conventional herbicides. Weyens et al. (1998) produced transgenic sugar beet plants which under glasshouse conditions showed tolerance to up to 4.0 kg ha^{-1} glufosinate-ammonium. This amount is 6–8 times the average field application rate. In contrast, Büttner et al. (1998) found that high dosages of glufosinate-ammonium (2.4 kg ha^{-1}) resulted in leaf discolouration, structural damage of

leaf tissue and a sugar yield reduction of about 25%. At agricultural relevant dosages, the glufosinate-tolerant sugar beet treated with glufosinate or conventional herbicides gave the same sugar yields which, however, were about 5% below untreated controls (cultivated in pots).

Wilson et al. (2002) reported that the sugar yield of glufosinate-tolerant sugar beet treated with glufosinate was about 5% below that of untreated hand-weeded control plots, but 3–4% above the plots treated with conventional herbicides. Jassem (2000) also found that glufosinate-tolerant varieties gave higher sugar yield (1–4%) when treated with glufosinate compared with a conventional herbicide treatment.

4 Disease and Pest Resistance

In addition to the ubiquitous requirement for weed control, diseases and pests are also very important in sugar beet as they can cause major economic losses and render cultivation unprofitable in infested areas. The major diseases and pests are beet necrotic yellow vein virus, beet western yellows virus, beet yellows virus and several fungal diseases, as well as nematodes (Asher and Hanson 2006; Dewar and Cooke 2006; Stevens et al. 2006). Although several disease resistance genes exist in the gene pool of closely related *Beta* species, they may be difficult to breed or may be associated with undesirable side-effects. Therefore, gene technology has also been employed to facilitate resistance breeding.

4.1 Rhizomania

Rhizomania is a disease caused by beet necrotic yellow vein virus (BNYVV) which is transmitted in the soil by the widely distributed fungus *Polymyxa betae*. The disease leads to decreased root yields and sugar contents which, depending on infestation levels, can be reduced by 60–80% in susceptible varieties (Asher 1993; Stevens et al. 2006). The spread of the disease continues, as measures to diminish long-distance transport of infested soil may not have been sufficiently effective. Thus, breeding companies have undertaken to produce tolerant varieties by conventional breeding programmes, using both the sugar beet gene pool as well as wild *Beta* species such as *B. maritima* as sources of resistance. However, concern about spread of the more aggressive P-pathotype, which can infect varieties tolerant to the common BNYVV pathotypes, type A and B, along with reported resistance-breaking by A-type BNYVV isolates (Liu et al. 2004), renders an alternative approach desirable.

Genetic transformation with viral coat protein genes has resulted in resistance to several virus diseases and Mannerlöf et al. (1996) demonstrated this mechanism is also operative in sugar beet in relation to BNYVV. They observed that transformed plants, expressing the BNYVV coat protein gene driven by the 35S promotor, grown both in glasshouse and field, showed a clear

reduction of virus multiplication even though the amount of expressed coat protein was very low, less than 0.02% of the total soluble protein. Similarly, Mechelke and Kraus (1998) found that field trials with sugar beet hybrids, combining both transgenic resistance and classic tolerance, gave higher sugar yields under very heavy infection pressure, compared with non-transgenic classic tolerant varieties. However, under moderate infection levels, the sugar yields were similar.

Another target for obtaining virus tolerance is the proteins involved in viral cell-to-cell movement. By transformation of sugar beet with genes encoding such proteins in mutated versions, tolerance to fungus-mediated infection with BNYVV was obtained (Lauber et al. 2001). Candidates for new commercial transgenic sugar beet varieties, resistant to BNYVV types A, B and P, are being tested officially (Anon 2004).

4.2 Nematodes

Beet cyst nematode (*Heterodera schachtii*) is the most important nematode pest of beet which can cause significant yield reduction, in some fields up to 50%. The economic losses can be reduced by crop rotation and, to some extent, by resistant cruciferous green manure crops (white mustard or oil radish) which can increase the rate of decline of populations of beet cyst nematodes (Cooke 1993; Dewar and Cooke 2006). Available nematode-tolerant varieties may be advantageous to grow on heavily infested soils, giving yields in the range 10–15% above those of non-tolerant varieties, but on less-infested soil the opposite is found. Moreover, the tolerant varieties generally suffer from high rates of bolting, low juice purity and increased susceptibility to mildew and root tumours (e. g. Thomsen 2002), indicating the difficulties of breeding for this trait.

Tolerance genes present within the *B. vulgaris* gene pool are not sufficiently effective, but complete resistance has been found in the related wild beet species *B. procumbens*, *B. patellaris* and *B. webbiana*. However, only interspecific crossings with *B. procumbens* has resulted in introgression (Jung et al. 1998). Thus, a fragment of chromosome 1 carrying the resistance gene $Hs1^{pro\text{-}1}$ has been translocated to cultivated sugar beet. The undesirable accompanying wild-beet DNA sequences, encoding leaf- and root-tumours and a multi-top phenotype, have been difficult to eliminate by back-crossing, partially due to the limited stability of the nematode resistance (Sandal et al. 1997). The $Hs1^{pro\text{-}1}$ gene has been successfully cloned, the first gene conferring resistance to nematodes, and transformed into sugar beet by *A. rhizogenes* (Cai et al. 1997). On all transgenic root cultures, the number of developed nematode females was significantly reduced, several to the level of the resistant translocation line.

An alternative approach has been to employ an unrelated resistance gene, namely the sweet potato trypsin inhibitor gene encoding sporamin. This has been reported to confer insect-resistance in transgenic tobacco and cauliflower,

while it has been demonstrated to be harmless to human beings and animals after cooking. Its potential as an anti-feedant for beet cyst nematodes was shown by Cai et al. (2003), studying sugar beet root cultures transformed by *A. rhizogenes* with a *SpT1-1* sporamin gene under the control of the 35S promoter. Growth and development of female nematodes were significantly inhibited in 60–70% of the hairy root clones, depending on trypsin inhibitor activity.

5 Other Traits

Transgenic resistance to the destructive foliar fungus *Cercospora* is in progress (Kuykendall and Upchurch 2004) and, in the future, resistance to other fungal diseases and traits like bolting control may possibly also be introduced into sugar beet by genetic engineering. Moreover, the high cellular capacities for biosynthesis and storage of low molecular weight carbohydrates may possibly be further exploited commercially by redirecting metabolic flow into other related substances of higher value. Such substances could be fructans which are used currently as a low-calorie sweetener and bulking agent with beneficial effects on human health (Sévenier et al. 2002). Thus, Sévenier et al. (1998) introduced a 1-sucrose:sucrose fructosyl transferase gene from *Helianthus tuberosus* into sugar beet and found that the stored saccharides were almost completely converted into low molecular weight fructans. Likewise, a similar gene from onion expressed in sugar beet has resulted in effective conversion of sucrose into complex onion-type fructans, without loss of storage carbohydrate content (Weyens et al. 2004).

6 Safety and Environmental Impact

Consumer acceptance of transgenic plants has for several years been low in Europe, particularly compared with North America. Thus, a consumer survey in the late 1990s showed that, on average, 44% of European consumers perceived genetic engineering to be a serious risk, compared with 14% in North America (Hoban 2002). Consumer scepticism has prompted numerous risk assessment studies on human health and the environment, seeking to provide scientific data for possible precautions for the commercial production of genetically modified crops, including transgenic sugar beet.

Bennett et al. (2004) made a life-cycle assessment comparing human health and environmental impacts of conventional sugar beet growing regimes with those that might be expected if/when herbicide tolerant beets are commercialised. They found that the latter would be less harmful due to lower levels of herbicide use and field operations.

6.1 Safety

Tenning (1998) described the assessments presented in an application according to EU Directive 90/220 (Environmental Risk Assessment) and EU Regulation 258/97 (Novel Food) to obtain clearance for the possible market introduction of a glyphosate-tolerant sugar beet variety. The transgenic line was subjected to molecular characterisation with respect to the inserted genes and the transgene expression level was found to be at ca. 0.6% of the total soluble protein. Studies on expression level stability indicated that the glyphosate tolerance was stable over generations and various climatic conditions. Concerning toxicity and allergenicity, no effects were observed from the ingested proteins, which were found to be degraded by gastric and instestinal juices within minutes. In addition, 18 different chemical components of beet were analysed and it was established that the transgenic line was substantially equivalent to conventional sugar beet.

Sugar from transgenic beet, obtained by standard purification steps of the manufacturing process, has also been investigated, using a transgenic line expressing the BNYVV coat protein gene (Klein et al. 1998). Chromosomal DNA in the raw juice (70 °C) was degraded within minutes due to nuclease activity. This and the following steps, including liming, filtration and crystallisation, reduced the amount of DNA by a factor of $> 10^{14}$. The viral coat protein could be detected in the raw juice of transgenic beet but at a lower concentration than in conventional BNYVV-infected beets. In the final sugar, no transgene products could be detected. The authors concluded that the sugar from conventional and transgenic beet was indistinguishable or substantially equivalent.

A safety assessment of the mannose selection system, originally developed for sugar beet, showed that the *E. coli* phosphomannose isomerase gene product has no significant homology to any known toxin or allergen, is not toxic to mice and degrades rapidly. Moreover, it does not affect the glucoprotein profiles or yield or nutritional composition of transgenic plants (Privalle et al. 2002).

6.2 Environmental Impact on Biodiversity

The impact on farmland wildlife biodiversity of growing glyphosate-tolerant sugar beet and other GM crops and the associated agricultural practice has been the subject of a large study in the UK from 2000 to 2002, the so-called Farm-Scale Evaluation (Firbank et al. 2003). Farm trial sites were selected representatively with a high proportion in major sugar beet growing area of East Anglia and each was split in half and subjected to either a conventional or a glyphosate weed control strategy. Mean timings for the first glyphosate application were 49 days after sowing, which was 34 days after the first conventional treatment. As a result, the weed ground cover was higher in the glyphosate plots (up to approx. 10–15%) than in the conventional plots (approx. 5%) in the beginning of the growth period, but after the second glyphosate spraying (on average 70 days after sowing) the effect was reversed so that late-season weed ground cover was

lowest in the glyphosate-treated plots, being approx. 5% compared with approx. 10% in the conventional plots (Champion et al. 2003). The authors observed a positive correlation between the weed seedling density (counted before the first glyphosate treatment) and the dosages of post-emergence conventional herbicides applied by farmers, reaching a maximum of 4.04 kg a.i. ha^{-1} and divided into up to 4 treatments, compared with up to 2.16 kg ha^{-1} glyphosate in 1–2 treatments.

A closer analysis of the weed populations in the Farm–Scale Evaluation showed that the above-ground weed biomass a month before harvest was 3- to 4-fold lower in the glyphosate-treated fields, predominantly caused by reduced biomasses of the major weed species *Chenopodium album*, *Poa annua*, *Polygonum aviculare* and *Veronica persica* (Heard et al. 2003a). With regard to weed diversity, the authors observed that, before glyphosate application, there were notably fewer species in the conventional half-fields than in the corresponding glyphosate half-fields, but after glyphosate spraying no differences were apparent in diversity or in the relative survival of dicotyledons and monocotyledons. It appears therefore that diversity was stable in the short term (Heard et al. 2003b).

The biodiversity of the fauna was also assessed in the Farm–Scale Evaluation. Butterflies and bees, as well as herbivores on the weeds, were less abundant in glyphosate-treated half-fields. Other larger groups of invertebrates and ground-dwelling predators showed no overall differences in numbers totalled over the year (Firbank et al. 2003). With regard to birds, the authors noted that the 32% less seed rain in glyphosate-treated crops could reduce food supplies for wintering birds, but they speculated that the actual effects of commercial glyphosate-resistant beet cropping will depend on the national strategy for crop harvesting.

In conclusion of the Farm–Scale Evaluation studies, Firbank et al. (2003) found that there was no evidence that treatment effects had arisen because the crops had been produced using genetic modification as opposed to conventional breeding. Rather, the differences could be explained entirely by the effects of the two different herbicide regimes, that is the more efficient weed control by glyphosate compared with conventional herbicides. In a related study, Volkmar et al. (2003) investigated biodiversity (important epigeous insect predators) in fields with glufosinate-tolerant sugar beet. They found that the relatively late applications of Liberty had a positive effect on the population of phytophagous carabids due to stronger weed infestation early in the season. Over a 3-year period, no evidence of any negative influence of the Liberty plots compared with conventional plots was observed.

6.3 Transgenic DNA Spread and Horizontal Gene Transfer to Bacteria

As antibiotic resistance genes have often been used as selectable markers for plant transformation, acquisition of such (functional) genes from transgenic

plants by plant-associated bacteria and subsequent transfer to human or animal bacterial pathogens has been one of the main concerns for the large-scale use of transgenic crops (WHO 1993). However, addressing this issue, efficient selection methods devoid of such genes have become available, such as mannose selection (see Sect. 2.4). This system relies on a selectable gene (a phophomannose isomerase gene) involved in carbohydrate metabolism. Moreover, it is obtained from *E. coli*, a bacterium ubiquitous in the human intestine (Joersbo et al. 1998).

Gebhard and Smalla (1998) studied transgenic DNA spread to naturally competent bacteria (*Acinetobacter* sp. strain BD413) and demonstrated their ability to take up and to integrate transgenic sugar beet DNA, encoding a functional *npt*II gene, either in the form of purified genomic DNA or homogenates of leaves. However, these experiments were conducted using optimised laboratory protocols which may be substantially different from field conditions.

In a study on the availability of free DNA from transgenic sugar beet in the soil as a prerequisite for horizontal gene transfer to bacteria, Gebhard and Smalla (1999) observed long-term persistence (up to 2 years) of amplifiable fragments of transgenic sugar beet DNA, including a *npt*II gene, in the field (silt loam) where the beet had been grown and deposited. The authors assumed that part of the extracted DNA had been protected inside cells of decaying plant residues and would not be available for possible uptake by bacteria. Naked genomic DNA, extracted from transgenic sugar beet and mixed with non-sterile soil from the field, showed a similar, although reduced, persistence. After plating on selective media, kanamycin-resistant soil bacteria were found, but they did not contain construct-specific sequences, indicating that the kanamycin resistance did not originate from the transgenic sugar beet DNA.

In addition to residual vegetative parts, pollen might also be a source for transgenic sugar beet DNA in fields (Meier and Wackernagel 2003). Observations 5 months after harvest showed that, in plots with flowering plants, all soil samples contained transgenic amplifiable DNA fragments, while no such DNA fragments were found in soil samples from root crop plots. At a distance of 50 m from the flowering plants, 30–70% of the plots contained amplifiable DNA fragments after a period of one year.

6.4 Crop-to-Wild Spread of Transgenic DNA

In addition to the spread of antibiotic resistance genes from transgenic plants, a concern is that transgenic crops may hybridise with wild relatives, resulting in the transfer of transgenes into the gene pool of natural populations, potentially acquiring unintended properties such as enhanced invasiveness (Colwell et al. 1985; Mikkelsen et al. 1996).

Sugar beet is a biennial wind-pollinated species and the sugar-containing tap root is produced in the vegetative stage of the first year, while the reproductive stage is in the second year, following a vernalisation period. Therefore,

only a very small number (usually below 0.1%) of the plants produce flowers (bolters) in commercial non-seed sugar beet cultivation and common practice is to remove them, further diminishing pollen dispersal. Therefore, separation distances from such fields with genetically modified beet may not be required, as the risk of the utilised portion of the crop being affected by cross-pollination is exceedingly small (Ingram 2000).

However, in seed production areas, mainly in northern Italy and south-west France, the situation is different. Here, pollen-producing male plants are grown in rows along with male-sterile female plants (often in a ratio of 2:6) from which hybrid seeds are harvested (Bornscheuer et al. 1993). Sugar beet pollen can be spread over considerable distances by the wind (1000 m; van Raamsdonk and Shouten 1997) and this may be of particular relevance in those cases where the transgenic trait is present in the male rather than in the female plants. If a breeding strategy with transgenic female plants is pursued, minimising transgenic pollen spread, contamination by wild beet pollen may lead to transgenic 'weed beet' seed which, in the case of herbicide resistance, will not be eradicated by spraying with the corresponding herbicide (Bartsch and Schmidt 1997; Eastham and Sweet 2002) and should then be manually removed, like conventional bolters.

Saeglitz et al. (2000) examined pollen spread using non-transgenic male-sterile sugar beet bait plants, planted at various distances from a central plot of 30 pollen-producing transgenic sugar beet plants. They found that an average of approx. 50% of the seeds harvested from the bait plants within 9 m from the pollinators were transgenic, while at 300 m transgenic offspring were not detected. The transgenic pollen spread correlated with the main wind direction. The authors noted that the use of bait plants ensured that only viable pollen was detected as opposed to physical traps (sticky surfaces). However, this may not reflect natural conditions in the field where transgenic pollen has to compete with pollen produced by the potential recipient plants.

6.5 Environmental Impact of Pollen Spread

It is well established that cultivated sugar beet can hybridise with the wild relative sea-beet (*B. vulgaris* ssp. *maritima*), producing 'weed beets' that are able to bolt and flower in only one growth season. If such seeds are present in appreciable quantities in commercial sugar beet seed, more weed control effort is required by farmers. The gene pool of sea-beet has for many years been exploited by breeders as a source of disease resistance against *Cercospora* and *Rhizomania* (Bosemark 1993). Gene flow in the crop-to-wild direction has been studied by Bartsch et al. (1999) in the proximity of sugar beet seed production fields in north-east Italy. They found that two alleles (out of about 30) common in cultivars, but otherwise typically quite rare in wild beets, were present in unusually high frequencies in the sea-beet populations, demonstrating introgression of genes from cultivated beet to wild beet. Interestingly,

a slight increase in the genetic variability in these wild beet populations was observed. The authors concluded that a century of crop-to-wild gene flow in that area had a limited evolutionary effect on the wild beet populations, due to the limited establishment of domesticated genes.

Pohl-Orf et al. (2000) noted that, in order to be of biological relevance, gene flow should be assessed in the context of ecological consequences, rather than just describing the occurrence of the phenomenon. Therefore, the authors studied transgenic rhizomania tolerance as an example of transgenic disease resistance, regarded to be of more ecological significance than herbicide tolerance, especially in non-agricultural habitats. They hybridised transgenic sugar beet tolerant to rhizomania with sea-beet and produced transgenic and non-transgenic isogenic offspring lines which were compared with a non-transgenic rhizomania-tolerant cultivar with regard to weed competitiveness, hibernation, bolting and seed formation at field sites with or without rhizomania virus infestation. No significant differences were found amongst the three genotypes, independent of virus presence or weed competition level. The authors suggested that this was most likely due to the wild beet genetic background of the cultivars; and they concluded that an addition of a transgenic virus-tolerance trait does not appear to result in an ecological advantage over naturally tolerant genotypes.

In a similar study, Bartsch et al. (2001) produced hybrids between transgenic rhizomania-resistant sugar beet and the closely related species Swiss chard (*Beta vulgaris* ssp. *vulgaris* var. *vulgaris* Lain) and monitored biomass production at a low and high rhizomania infestation level at different degrees of weed competition. Compared with the non-transgenic non-rhizomania tolerant Swiss chard control and the non-transgenic hybrid, the transgenic hybrid demonstrated superior performance (about 20% of biomass production) over all weed competition levels, but only at high-background rhizomania infestation. The transgenic hybrid had a much lower bolting rate, suggesting that flowering and hence gene flow by pollen would be decreased under usual field conditions, increasing the biosafety of this particular genotype.

7 Conclusion

Genetic engineering of sugar beet can significantly improve its agronomic performance by increasing the efficiency and flexibility of weed control and potentially by enhancing resistance to major diseases such as rhizomania and cyst nematodes. Also, the large biosynthetic capacity of sugar beet roots may be exploited for the production of other compounds.

Transgenic herbicide-resistant sugar beet has been found not to damage human health or the environment, although the higher weed control effectiveness compared with conventional herbicides may decrease biodiversity in late season. The risk of crop-to-wild spread of transgenes is exceedingly small for

the commercial root crop and in seed production areas, appropriate separation distances and breeding strategies can minimise pollen spread.

So far, no transgenic sugar beet is grown commercially, although breeding companies have for some years been ready to launch such varieties. This is related to the present negative public perception in Europe of this technology, notably for products intended for human consumption.

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IV.5 Radish

I.S. Curtis[1]

1 Introduction

Radish (*Raphanus sativus* L. var. *longipinnatus* Bailey) is a popular crop cultivated mainly for its enlarged hypocotyl and taproot, whilst its leaves and seeds have culinary and medicinal uses, respectively. The crop is generally classified into two groups based on the size of the edible root. One group is the small-rooted, short-season radish used mainly in salads; and the other is the large-rooted type (weighing up to 40 kg) that exhibits a great diversity of phenotypes in terms of skin and flesh colour. The latter is the most widely grown root crop in the Far East (Curtis 2004a). In spite of the considerable importance of the crop, the production of transgenic radish plants has lagged behind other crops due to its recalcitrance in culture. Genetic engineering of radish is an attractive area of research to both accelerate the production of novel and desirable germplasms and allow recently identified, medicinally valued chemicals to be manipulated to over-production and thus establish the radish as a major healthy crop.

2 Research in Tissue Culture

2.1 Progress in the Regeneration of Shoots from Cultured Explants

To date, the production of transgenic plants through tissue culture has been achieved in many major crops of the world, including members of the Brassicacae. However, the production of transgenic radish (a member of the Brassicacae) has eluded scientists for many years due to the recalcitrance of cultured cells/plant organs to regenerate shoots (Lichter 1989; Matsubara and Hegazi 1990; Jeong et al. 1995; Takahata et al. 1996). In the mid-1990s, the discovery that ethylene may act as a negative regulator towards the regeneration of shoots from mustard (*Brassica juncea* L.; Pua and Lee 1995), prompted researchers to modify culture media to improve the production of shoots from radish. The inclusion of L-α-2-aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis, at 10 μM with 20 μM or 30 μM silver nitrate (an inhibitor of ethy-

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lene action) in N1B2 shoot regeneration medium significantly improved the regeneration of Chinese radish hypocotyl explants (Pua et al. 1996). In the same study, the use of putrescine at 10–25 mM with 30 μM silver nitrate or AVG improved the regeneration of shoots from hypocotyls explants to 40%. More recently, in a separate study, the use of excised cotyledons in the presence of 10 μM AVG to the regeneration medium CR, improved the production of shoots to 60% in the commercial Korean ecotype 'Jin Ju Dae Pyong' (Curtis et al. 2004). Although there has been no report on the production of transgenic radish plants in culture, the recent improvements in tissue culture media should allow transformants to be produced in the near future.

2.2 Phenotypic Assessment of Tissue Culture–Derived Plant

Plants derived from tissue culture can often exhibit genetic changes, such as alterations in chromosome number, which can lead to reductions in plant fertility (Lee and Phillips 1988). Hence, it is important to assess the usefulness of a new shoot regeneration system for the production of phenotypically normal plants. In our own study, 144 regenerants (74 from 1-month-old, 70 from 3-month-old cultures) derived from hypocotyl and cotyledon explants were compared with a population of 62 seed-derived plants (Curtis et al. 2004). The tissue culture-derived plants exhibited earlier bolting and flowering times and reduced pollen viability compared to wild-type plants. However, plants derived from older cultures (3 months) produced larger seed weights compared with plants derived from 1-month-old cultures and wild-type plants. In order to determine whether the greater seed weights produced by plants derived from older cultures was due to ploidy, true leaves from 21-day-old R1 (seed progeny derived from regenerated R0 plants in culture) plants from tissue culture (1- and 3-month-old cultures) and wild-type plants were excised, chopped and the nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) before being analysed by flow cytometry using a PAS-type flow cytometer (Mitsubishi, Tokyo, Japan). Leaves from 1-month-old cultures (R1 generation) and wild-type plants produced a major peak at 100 fluorescence units (which corresponds to the chromosome complement of diploid plants). However, studies from a random population of ten R1 lines derived from 3-month-old cultures showed that plants from three R1 lines produced a major peak at 120 units. Together with chromosome number data from root-tip squashes, it was found that plants derived from older cultures exhibited greater genetic variability (50–80% of cells having 20–40 chromosomes) compared to the wild type (85–90% with diploid number 18 chromosomes per cell) and plants derived from 1-month-old cultures (78–88% diploid). This study emphasised that using shoots from explants which have been in culture for 3 months should be avoided when designing a tissue culture-based transformation system, as such plants exhibit more genetic instability compared with 1-month-old cultures.

3 Genetic Transformation

3.1 Transgenic Plants Via the Floral–Dip Method

Due to the inability to regenerate transformed cells into shoots in culture, alternative procedures were sought to create transgenic radish. One such approach *in planta* is useful for the transfer of foreign genes into plants, as such a procedure does not require knowledge of complex tissue culture systems and can be performed by workers with basic skills. In *Arabidopsis thaliana* L., the first report on the production of transgenic plants *in planta* was by vacuum infiltration (Bechtold et al. 1993). This technique involves the submergence of a recently flowering plant into a container containing a surfactant (usually Silwet L-77), an energy source (sucrose) and the plant soil-borne pathogen *Agrobacterium tumefaciens*. Transgenic plants were produced from the progenies of plants dipped into the inoculation due to the transformation of the ovules (Ye et al. 1999; Desfeux et al. 2000). This system of transformation has been simplified with the omission of a vacuum and is referred to as floral-dipping. It was first established for the production of transgenic plants in *A. thaliana* (Clough and Bent 1998) and subsequently in *Brassica rapa* L. ssp. *chinensis* (Qing et al. 2000) and *Medicago truncatula* (Trieu et al. 2000). Due to the taxonomic closeness of *Arabidopsis* and radish, experiments were conducted to establish the value of floral-dipping for the production of transgenic radish plants by investigating the importance of the type of surfactant (Silwet L-77, Pluronic F-68, Tween 20), its concentration (0%, 0.01%, 0.05%, 0.1%) and the developmental stage of the plant at the time of floral-dipping (Curtis and Nam 2001). This study formed the platform for the first report on the production of transgenic radish plants, which has subsequently been described in detail (Curtis 2004a, b). In this investigation, *A. tumefaciens* strain AGL1 (Lazo et al. 1991) was used, carrying pCAMBIA3301 with β-glucuronidase (*gus*A) and bialaphos resistance (*bar*) genes under the control of the cauliflower mosaic virus (CaMV) 35S promoter located between T-DNA border fragments, due to its ability to transform seedling explants of the commercial Korean radish ecotype 'Jin Ju Dae Pyong' in culture. The presence of a surfactant in the inoculation medium was critical in the production of transformed seeds, as treatments without the chemical failed to yield any transformants. Silwet L-77 was the most effective of the surfactants, especially when used at a concentration of 0.05% on plants exhibiting a primary bolt with many immature floral buds (optimal transformation frequency of 1.4%). The stage at which a plant was dipped into the inoculation medium was also a critical factor in the transformation of radish. Plants which exhibited a tertiary bolt failed to produce transformed seeds, whilst younger plants with secondary bolts yielded transformants at a frequency of 0.2% in the presence of 5.0% sucrose and 0.05% Silwet L-77. This report clearly identified that radish plants of 'Jin Ju Dae Pyong' produced the highest yield of transformed seeds when primary bolted plants were dipped into an inoculation medium containing 0.05% Silwet L-77 and 5.0% sucrose.

3.2 Molecular Studies of Transgenic Plants

Since the initial breakthrough on the production of transformed radish by floral-dipping, studies were performed to understand the mechanism of production of transformed seeds. Thus, all seeds from floral-dipped plants were carefully recorded in terms of their origin (location of silique on the bolt, derived plant). Both of the published reports on the transformation of radish by floral-dipping (Curtis and Nam 2001; Curtis et al. 2002) revealed that a high proportion of transformed seeds (50–60%) had an identical T-DNA insertion pattern (such plants are termed siblings; Fig. 1). It appeared that these groups of sibling transformants originated from the same bolt. In comparison, molecular studies in *A. thaliana* L., revealed that all transformed seeds from floral-dipped plants produced independent hemizygotes (Clough and Bent 1998). However, studies using *M. truncatula* showed a similar response to radish in the number of transformed siblings at a frequency of 77–87% (Trieu et al. 2000). Although the true mechanism of how radish seeds are transformed during floral-dipping is currently unknown, it is proposed that both *M. truncatula* and radish may follow a common route. Trieu et al. (2000) postulated that *Agrobacterium* cells may transform meristematic cells which are responsible for the development of floral bolts. Hence, a transformed meristematic cell would carry the same integrated T-DNA into cells for the whole developing bolt. Thus, as the ovule is the site of *Agrobacterium*-mediated transformation (Ye et al. 1999; Desfeux et al. 2000), all ovules developing from this initial bolt would carry the same inserted T-DNA. In our studies in radish, from several thousands of flowers stained for transgenic activity after floral-dipping (in this

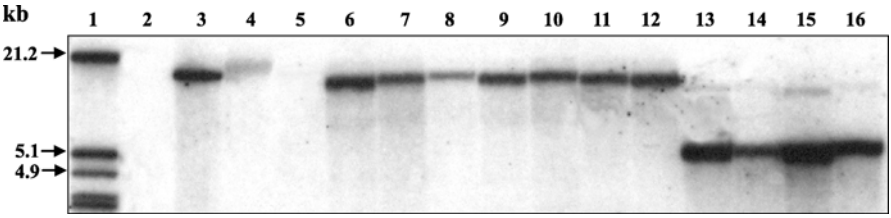

Fig. 1. A typical Southern blot analysis of genomic DNA from T1 transformed radish plants produced by floral-dipping, showing the prominence of sibling transformants. DNA was extracted from young leaves (Dellaporta et al. 1983) and digested with *Hin*dIII to reveal the number of copies of the *gus*A gene integrated into the genome of transformed plants. Following electrophoresis in a 0.8% (w/v) agarose gel, DNA fragments where transferred to a nylon membrane by alkaline transfer. After pre-hybridization, the membrane was hybridized overnight with a digoxigenin (DIG)-labelled *gus*A gene fragment as probe. *Lane* 1 *Hin*dIII/*Eco*RI-digested λDNA, *lane 2* DNA from wild-type plant, *lane 3* binary vector pCAMBIA3301 cut with *Hin*dIII, *lane 4* uncut DNA from a transformed plant, *lane 5* DNA from a non-transformed plant, *lanes 6–12* DNA from six T1 plants which were derived from seeds which originated from the same primary bolt of a single floral-dipped plant, *lanes 13–16* DNA from four T1 plants which were derived from seeds originated from a different floral-dipped plant also located from a single primary bolt

case by GUS histochemical staining), none has shown that mature pollen was transformed.

4 Practical Applications

4.1 Designing Late–Flowering Korean Radish

In South Korea, 85% of the land surface devoted for root crops is for the cultivation of radish. However, the cultivation of Korean ecotypes is rather restricted due to their sensitivity to cold temperatures, which causes the crop to alter its growth habit from a vegetative rosette to one that forms a stem (or bolt) which results in the root becoming shrunken and inedible. Despite intensive breeding programmes, which commenced in the 1950s and involved transferring the late-flowering trait from Japanese ecotypes into commercial Korean ecotypes by conventional breeding, all resulting crosses produced plants with poor quality roots or were prone to bolting during the hot summer season (Lee 1987). For this reason, the development of a gene transfer system for radish is highly desirable for the transfer of late-flowering inducing genes into commercial Korean ecotypes.

The *Arabidopsis GIGANTEA* (*GI*) gene (Fowler et al. 1999; Park et al. 1999) was considered a target for inducing late-flowering into radish, as *gi* mutants of *Arabidopsis* exhibit photoperiod-insensitive flowering and disruption of circadian rhythms (Koornneef et al. 1983). Hence, a down-regulation in the expression of the native *GI* gene in radish may delay the onset of flowering. Thus, an investigation was made to determine whether native *GI* expression in radish could be 'switched off' by expressing an antisense *GI* gene from *Arabidopsis* using the floral-dip method (Curtis et al. 2002). This transformation study used *A. tumefaciens* strain AGL1 carrying the binary vector pCAMBIA3301 as described earlier (see Sect. 3.1). From a total of 1462 seeds harvested from 25 floral-dipped plants, 16 transformed plants (T1 generation) were screened *in solium* using herbicide spraying and by GUS histochemical staining. These 16 transformed plants were also Southern blot-positive for both *gus*A and *bar* marker genes and were allowed to grow to maturity and produce seed (T2 generation). Eleven of the 16 transformed T2 lines were randomly selected for phenotypic analysis. All antisense-*GI* transformed lines showed a significant delay in bolting and flowering times compared with the wild type and transformed lines carrying only the selectable *gus*A and *bar* marker genes (positive control). The longest delay in time to bolting exhibited by a T2 antisense-*GI* line compared to wild-type plants was 17 days. This same T2 antisense-*GI* line also showed a delay to anthesis (time of first flower opening) compared to wild-type plants by 18 days. Despite ten of the 11 T2 antisense-*GI* lines exhibiting a significant reduction in plant height compared to the wild type, the report provided evidence that down-regulating the expression of *GI* in radish

can have a significant effect on delaying the onset of bolting and flowering, which may allow the cropping season to be extended into the autumn season.

4.2 Medicinal Properties of Radish

4.2.1 Early Studies

One of the first reports on the medicinal value of radish was published by K.M. Nadkarni in 1927, who found that seeds of radish could be used as an emmenagogue and in the treatment of gonorrhoea (Nadkarni 1927). In the same study, it was also reported that consumption of the root was beneficial for the treatment of haemorrhoids, syphilis and urinary complaints. However, at this time, there was no indication of which chemicals were involved in conferring these medicinal properties. Recent studies using clinical trials and a bacterial antimutagenic assay may have revealed two chemicals (peroxidase and isothiocyanates, respectively), which identify the radish root as an important source of medicinal compounds.

4.2.2 Peroxidase

Hyperlipidemia, a condition associated with an excess of fat in the blood, is the main cause for atherosclerosis and coronary heart diseases. Although a number of hypolipidemic drugs are available on the market, many have potential side-effects. It is generally known that the intake of fruits and vegetables containing oxidant compounds can reduce the incidence of coronary artery diseases. Peroxidase, a protein containing Fe^{2+} heme and present in rich amounts in radish roots, is an oxido-reductase capable of scavenging harmful free radicals. For these reasons, consumption of radish roots may help in treating hyperlipidemia. Clinical trials have been conducted using hyperlipidemic mice fed with different purities of peroxidase from radish juice, crude radish peroxidase and horseradish peroxidase (Wang et al. 2002). In a sample of ten female mice per treatment, the peroxidase significantly reduced the levels of total serum cholesterol (32–37%), triglyceride (37–49%), blood glucose (17–23%) and lipid peroxidation in the small intestine (35–47%) and liver (40–47%) compared with mice fed without peroxidase. PAGE of esterase isoenzymes in mice showed that peroxidase in the diets caused a reduction in the expression or activity of esterase, confirming a modification in the lipid metabolism of treated mice. Although further research is necessary using human trials, this preliminary study suggests that peroxidase may help in reducing the chance of hyperlipidemia.

4.2.3 Isothiocyanates

Organic isothiocyanates are highly reactive compounds capable of inducing antimicrobial, antimutagenic and anticarcinogenic activities. Such chemicals

are not readily found in cruciferous crops (such as cabbage, cauliflower, kales), but wounding of these plants rapidly increases their production. It appears, therefore, that wounding plant cells activates the enzyme myrosinase to convert glucosinolate into isothiocyanates. Recent studies investigated whether wounding a cruciferous plant, such as radish, could increase the production of isothiocyanates and whether such chemicals confer antimutagenic activity (Nakamura et al. 2001). From eight cultivars of radish tested, a correlation was observed between the potency of antimutagenicity (as determined by UV-induced mutation assay of *E. Coli* B/r WP2) and the amount of 4-(methylthio)-3-butenyl isothiocyanate (MTBITC; the major isothiocyanate in radish roots) in *n*-hexane extracts. However, there were varietal differences, in that the less popular varieties 'Karami' and 'Momoyama' produced 5- and 2-fold, respectively, more MTBITC compared to the commercial variety 'Aokubi'. Further studies revealed that the amounts of total isothiocyanate in grated roots were increased 7-fold compared to diced roots measured after 30 min of cooking. Although this study utilized only a bacterial antimutagenic assay, if further results demonstrate that MTBITC is potent in mammalian and human cell mutation assays, then one of the carcinogenic properties of radish would be known.

4.2.4 Improving the Medicinal Properties of Radish

Through the development of a gene transfer system for radish, it is now possible to over-express potential pharmaceutical genes in this crop. Over-expression of genes involved in the production of peroxidase and MTBITC may further improve the medicinal value of radish. In addition, due to the large size of the radish plant, the production of transgenic plants expressing antibodies or synthetic vaccines to be used orally in passive immunization may also be of considerable benefit, which was recently reviewed in several plant systems (Daniell 2003). Overall, future research may demonstrate that the radish may become globally a very important medicinal crop.

5 Conclusions

The development of a gene transfer system for radish is still in its infancy and, at present, has only been successful for the commercial Korean ecotype 'Jin Ju Dae Pyong'. Despite the inability to produce transgenic radish in culture, recent improvements in the shoot regeneration of seedling explants may allow other important germplasms to be manipulated in vitro. Establishment of a gene transfer system for a wide range of radish genotypes should allow further improvements in the production of novel germplasms, which can aid both cropping and improved medicinal value.

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Section V Herbs and Spices

V.1 Sweet and Hot Peppers

D.A. Brummell and R. Pathirana[1]

1 Introduction

The fruit of *Capsicum* spp (peppers) is a New World horticultural crop of the family Solanaceae. Peppers are now widely cultivated and eaten both as a vegetable and as a spice. Five major species have been domesticated (*C. annuum* L., *C. frutescens* L., *C. chinense* Jacquin, *C. baccatum* L., *C. pubescens* Ruiz & Pavon), of which *C. annuum* is the most widely cultivated worldwide and has been bred to produce numerous large-fruited sweet cultivars. The other four cultivated species are important crops mainly in the northern half of Latin America and the Caribbean (Ochoa-Alejo and Ramirez-Malagon 2001). *C. frutescens* and *C. pubescens* require tropical conditions for growth, but the other three cultivated species are adapted to a temperate climate. Peppers show tremendous diversity in both genetics and phenotype, reflected in a considerable range in shape, colour, flavour, pungency and aroma of the fruit and in an extremely variable plant habit (Smith et al. 1987). Genetically, *C. annuum*, *C. frutescens* and *C. chinense* are related and can be used for interspecific breeding, although there are substantial hybridisation difficulties. *C. baccatum* and *C. pubescens* are more divergent and will not hybridise with the other three species or with each other (Smith et al. 1987). There are at least 18 other *Capsicum* species which have not yet been exploited by man and which may be a valuable resource for the discovery of trait genes, such as for resistance to particular diseases. However, recombinant DNA technology may be necessary in order to transfer such genes into cultivated species, since successful examples of embryo or anther culture and somatic hybridisation are rare (Morrison et al. 1986; Mityko et al. 1995) and have not so far been able to reliably overcome the problems of interspecific incompatibility.

Peppers can be categorised into two groups, sweet (non-pungent) and hot, but come in a large variety of shapes, sizes and colours. Sweet peppers are most commonly of the bell type (large, smooth, thick fleshed, crisp texture), whereas hot chilli peppers are usually of the longer, slender, tapering but more irregular Anaheim chilli shape (Smith et al. 1987). Fresh sweet peppers are grown throughout the world and are major crops in China, Mexico, Turkey and other countries (Table 1). Production doubled between 1995 and 2004 in both China and Mexico and has substantially increased in Turkey. China

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Table 1. Production of fresh chillies and green peppers by country (FAOSTAT 2006)

Country	2004 production (t)	1995 production (t)
China	12,031,030	5,492,180
Mexico	1,853,610	918,490
Turkey	1,700,000	1,080,000
Indonesia	1,100,510	1,589,980
Spain	1,006,000	790,500
United States	978,890	642,800
Nigeria	720,000	612,000
Egypt	467,430	250,550
South Korea	410,280	316,350
Italy	364,110	325,120

Table 2. Value of exported fresh chillies and green peppers by country (FAOSTAT 2006)

Country	2004 value (U.S. $ $\times 10^3$)	1995 value (U.S. $ $\times 10^3$)
Netherlands	798,313	512,562
Spain	675,032	361,372
Mexico	576,690	221,276
United States	126,234	56,778
Canada	106,103	11,353
Israel	105,507	0
France	54,168	10,829
South Korea	49,002	510
Turkey	46,196	23,276
Belgium	42,630	0

produces sweet peppers mainly for domestic consumption and the export trade is dominated (in value terms) by high-quality produce from the Netherlands, Spain and Mexico (Table 2). Fresh raw sweet peppers are a good balanced source of essential nutrients, containing proteins, minerals, sugars and lipids, as well as being a good dietary source of vitamins C and E, several B-complex vitamins and the vitamin A precursor α-carotene (Hanson et al. 2004). Sweet peppers are a staple vegetable in many regions, such as China, Korea and Central America. Hot chilli pepper is an important crop for consumption and export by tropical countries (Table 3) and is now the most widely used spice in the world (Ochoa-Alejo and Ramirez-Malagon 2001). Hot peppers are dried and crushed as paprika and chilli powder or flakes, or are used as ingredients in pepper sauces, curry powder and pickles. Extracts of pigments from red peppers are also used as red food colorants in sausages, cheeses, butter and processed foods (Ochoa-Alejo and Ramirez-Malagon 2001). The hot taste of chilli peppers is due to the presence of a group of alkaloids known as capsaicinoids, which recent studies indicate may have anti-cancer properties (Surh 2002). The growing popularity of hot chillies both as a food crop and a traded commodity suggests peppers will be of increasing commercial importance. This chapter reviews

Table 3. Production of dry chillies, peppers and allspice by country (FAOSTAT 2006)

Country	2004 production (t)	1995 production (t)
India	1,100,000	810,000
China	235,000	175,000
Bangladesh	138,000	52,670
Ethiopia	116,000	105,000
Pakistan	90,400	135,880
Vietnam	78,500	73,500
Hungary	70,000	40,790
Myanmar	70,000	31,500
Mexico	55,000	53,800
United States	54,900	0

the use of biotechnology as applied to *Capsicum* spp. However, the use of genetic engineering to improve peppers has been hampered by the extreme difficulty of developing reliable transformation procedures and, consequently, the biotechnology of peppers lags significantly behind that achieved in other crops.

2 Cell, Tissue and Organ Culture

Pepper plant regeneration has been attempted for many purposes, but has proved to be much more difficult than in other Solanaceous crops, which are generally amenable to these processes. In pepper, methods appear to be cultivar-specific and not reproducible to other genotypes.

2.1 Isolation of Protoplasts and Plant Regeneration

Protoplasts can be useful tools for plant transformation, somatic hybridisation or for the induction of mutations and the selection of desirable mutants. Leaf tissue-derived protoplasts have been used for callus induction and whole plant regeneration in pepper (Saxena et al. 1981; Díaz et al. 1988; Prakash et al. 1997). Mannitol at concentrations of 0.4–0.6 M (Saxena et al. 1981; Murphy and Kyle 1994) or CPW13M medium (Díaz et al. 1988) were used for plasmolysing the cells and mixtures of Cellulase (1–2%), Macerozyme (0.25–0.4%) and Pectolyase (0.25%) to digest the cell wall. Browning of callus tissue was overcome by the use of ascorbic acid and polyvinylpyrrolidone in the media and incubation of protoplasts in the dark (Prakash et al. 1997). Controlling the density of the protoplasts to between 5×10^4 ml^{-1} and 1×10^5 ml^{-1} seems to be important in inducing division and subsequent callusing. Although plant regeneration from protoplasts is feasible, it has not so far been used to obtain somatic hybrids, somaclonal variants, induced mutants or transgenic plants.

2.2 Cell Cultures

Cell cultures of *Capsicum* spp have been established mainly for the purpose of studying secondary metabolite production, which is discussed elsewhere in this chapter. A prerequisite for the successful establishment of cell cultures is the production of callus on solid media supplemented with plant growth regulators (PGRs; Santos-Diaz and Ochoa-Alejo 1994; Sudha and Ravishankar 2003; Martinez-Juarez et al. 2004). Holden and Yeoman (1994) demonstrated that cell clones of *C. frutescens* arising from the same culture can have different growth rates and metabolic activity. Santos-Diaz and Ochoa-Alejo (1994) were able to select cell lines tolerant of high osmoticum in the culture medium. Considering the high variability in metabolic activity of different cell lines, it is important to select cell lines before using them for the production of secondary metabolites.

2.3 Anther Culture for Generating Doubled Haploids

Haploids offer several advantages for breeding, including the isolation of recessive alleles and the generation of unique recombinants from hybrids. Production of homozygous, doubled haploid lines from F_1 hybrids in one step through anther and microspore culture provides a unique opportunity to produce new cultivars and breeding lines of self-pollinated crops. A review of haploid induction of peppers in vitro has been published by Vagera (1990). When anthers are cultured in vitro, the normal developmental pathway of pollen can be altered to produce either callus or embryos. The stage of the floral bud when the anthers are at the late uninucleate or preferably the early haploid mitotic stage (anthers 2.3–3.5 mm in length, corolla same size or slightly longer than calyx) should be used for cold treatment (see below; Sibi et al. 1979; Morrison et al. 1986; Mityko et al. 1995; Kim et al. 2004; Nowaczyk and Kisiala 2006), but at the time of culture it is critical that they are at the late stage of spore development and that the cultures are incubated at elevated temperature (35 °C; Morrison et al. 1986; Kim et al. 2004). In pepper, embryogenesis occurs by division of the vegetative nucleus and not the generative nucleus (Sibi et al. 1979; Barcaccia et al. 1999; Kim et al. 2004). Unlike many other crops where earlier stages of anther development are used successfully for doubled haploid production, younger flower buds (1.5–2.5 mm in length) of *Capsicum* spp fail to produce embryos and shoots (Novak 1974).

It has long been known that pollen embryogenesis is induced by subjecting the anthers to a shock treatment, especially cold. Morrison et al. (1986) selected *C. annuum* cv. Emerald Giant for high androgenetic response and used it in a cross with *C. chinense*. To successfully produce doubled haploids from this F_1 hybrid, they had to resort to a double-layer culture medium incorporating charcoal. In this study and one by Munyon et al. (1989), all the regenerated plants were confirmed as spontaneously doubled haploids. Cytological analysis

and/or flow cytometry in other studies have revealed that about half the plants show spontaneous doubling of chromosomes (Barcaccia et al. 1999) or are a mixture of haploids, diploids, tetraploids and aneuploids (Gyulai et al. 2000). Another important factor for successful anther culture is the age of the donor plant, with anther response declining with the increasing age of the donor plant (Kristiansen and Andersen 1993). In general, the culture process is in two stages, where a higher level of PGR is first used to induce the microspores into an embryogenic developmental pathway and then the embryogenic callus tissue is cultured onto media with a lower concentration of the same or a different PGR. Lefebvre et al. (1995) used segregating doubled haploid populations to produce an integrated molecular linkage map, using randomly amplified polymorphic DNA and restriction fragment length polymorphic markers. Although the methodology has apparently been optimised for doubled haploid production in pepper, the potential use of the technology to develop interspecific hybrids with a combination of characters derived from different species of *Capsicum* is very limited.

2.4 Virus Elimination through Meristem Culture and In Vitro Grafting

Peppers are severely affected by viral infection; and meristem culture is one approach to producing virus-free material from infected clones. Using a filter paper bridge on liquid Murashige and Skoog (MS) medium supplemented with benzyladenine (BA), Madhuri and Rajam (1993) induced multiple shoots from apical meristems of *C. annuum* cv. Bhivapuri, which is susceptible to viral infections. Shoots were rooted on solid media and acclimatised in the glasshouse to produce whole plants. Difficulty in using this method for mature pepper plants was subsequently overcome by Katoh et al. (2004) by micrografting apical meristems of virus-infected plants to hypocotyls of virus-free root-stocks of in vitro-grown seedlings. Resulting plants were shown to be virus-free by ELISA and bioassays. The other advantage of this approach is that PGRs, which may induce somaclonal variation (Anu et al. 2004), are not used during the culture process.

2.5 Embryo Rescue for Production of Interspecific Hybrids

Interspecific hybrids of *C. annuum* and *C. frutescens* are difficult to germinate due to incompatibility, but this can be overcome by embryo rescue (Hossain et al. 2003). These authors found beneficial effects of supplementing basal MS medium with casein hydrolysate, yeast extract, coconut water, gibberellin (GA) and naphthalenacetic acid (NAA) to rescue immature hybrid embryos, 28–33 days after pollination. Very limited attention has been paid to this technique for pepper, but it has tremendous potential for interspecific hybridisation.

3 In Vitro Plant Regeneration and Genetic Transformation

Pepper plant regeneration from cultured tissue explants has been achieved through both organogenesis and somatic embryogenesis. Plant regeneration through organogenesis was successful when young embryonic or expanded cotyledon, hypocotyl or leaf explants were used. Optimisation of in vitro regeneration procedures for plant transformation using the *Agrobacterium tumefaciens* system is the major target, but pepper transformation has been a challenge for many laboratories. Liu et al. (1990) and Zhu et al. (1996) used direct regeneration from cotyledonary and leaf explants in MS basal medium supplemented with BA and indole-3-acetic acid (IAA). Liu et al. (1990) demonstrated transformation in the shoot buds using the GUS assay, but were unable to regenerate whole plants.

The first method suitable for large-scale pepper transformation was developed by Engler et al. (1993), based on a patent application submitted in 1991. Their method used explants from young embryonic or expanded cotyledons, which were infected with *Agrobacterium*. Transformed shoots were regenerated in the dark at 28 °C, followed by transfer of explants to a medium incorporating silver thiosulfate (an ethylene inhibitor). Elongation of shoot buds was conducted on a medium supplemented with GA_3 and with reduced concentrations of BA. Stepwise increase of light intensity is another salient feature of this novel method. The method was originally developed using a selectable marker gene for chlorsulfuron resistance, since selection based on the neomycin phosphotransferase *npt*II gene required very high concentrations (250 mg l^{-1}) of kanamycin and the widely used 50 mg l^{-1} concentration resulted in the regeneration of non-transformants. Induction of shoots directly in the explant material without passage through a callus phase was critical. The original disclosure described the generation of only a few transformed lines, but the procedure was subsequently used to develop a large population of primary transformants (Harpster et al. 2002a).

Lee et al. (1993), using zeatin as the cytokinin, were able to induce shoot buds after co-cultivation with *Agrobacterium* and achieved shoot elongation by transferring the shoot buds to a medium with a concentration of auxin lower than in the induction medium. The use of more responsive leaf base and petiolar explants with high BA levels enabled Zhu et al. (1996) to achieve an antibiotic-resistant bud formation frequency of 62%. Devising a three-step regeneration protocol with transfer of shoot buds first to a medium supplemented with GA_3 and in 10 days to another with GA_3 and ABA followed by a rooting medium incorporating NAA enabled them to overcome the general problem of lack of elongation of induced buds in pepper, but only five putative transgenic lines were produced. This protocol was, however, applied to only one Chinese variety (Zhong Hua No. 2). Cai et al. (2003) improved on these methods to successfully transform *C. annuum* var. Longunt by inducing *vir* genes of *Agrobacterium* in acetosyringone and using high cytokinin/auxin media for selection and induction, lower PGR levels for regeneration, and media

containing only an auxin as PGR for shoot bud elongation and rooting. For the variety Pusa jwala, Manoharan et al. (1998) devised a successful protocol based on pre-culture of cotyledonary explants for 2 days on a MS-based medium supplemented with thidiazuron (TDZ) and co-cultivation on the same medium for another 2 days, followed by transfer to selection medium. Elongation and rooting was achieved in one step by the transfer of shoot buds to a low-auxin medium. They managed to produce four transgenic plants from 200 cotyledonary explants using this procedure. Using the same cultivar, Shivegowda et al. (2002) reported better plant regeneration in zeatin-supplemented media compared with those using TDZ and BA. They reported three stable transformants from 85 cotyledonary explants.

Li et al. (2003) further improved the transformation protocol based on direct shoot regeneration of leaf discs by including a water extract of pepper seedlings in all the media, a 2-day pre-conditioning of explants in induction media and a 2-day delay of selection after co-cultivation. They incorporated $AgNO_3$ and GA_3 in the medium for elongation of shoot buds. These authors reported a transformation rate of 41% among 409 plants regenerated from four cultivars on rooting media with 50 mg l^{-1} kanamycin, which is the highest rate of transformation reported so far. Nevertheless, the very high interaction effects of different *Agrobacterium* strains (five strains used) with nine pepper genotypes show that genotype and strain specificity need to be considered carefully before initiating any large-scale transformation experiments.

In order to overcome the low frequency of transformed shoots in direct regeneration systems, Lee et al. (2004) selected shoots derived from callus formed on cotyledonary explants on media supplemented with zeatin and NAA and reported better discrimination of non-transformed shoots, although regeneration rates from callus were much lower. Moreover, Anu et al. (2004) have shown the occurrence of somaclonal variation in selfed progenies of *C. annuum* when organogenesis is based on callus induced in explants. Hence, this pathway needs to be considered with caution when the transformation is aimed at improving an agronomic trait in an already established cultivar. In attempts to improve shoot elongation, which is one of the main barriers for pepper transformation, recent studies have shown the positive effect of phenylacetic acid (Husain et al. 1999) and of a synthetic epimer of brassinolide, 24-epi-brassinolide (Franck-Duchenne et al. 1998) when using the direct regeneration pathway. These may contribute in future to further improvements in transformation efficiencies.

Combining scanning electron microscopy with expression of a green fluorescent protein (GFP) reporter gene, Wolf et al. (2001) elegantly showed that adventitious shoot regeneration in pepper occurs in the epidermal layers surrounding the cut surface, whereas *Agrobacterium*-mediated transformation is limited to the vascular areas. Most of the bud-like structures did not have an organised meristem. Linking regeneration and transformation in the same cells seems to be the major difficulty in the transformation of pepper using direct regeneration from seedling-based explants. So far, transformation at-

tempts have not been reported using direct somatic embryogenesis, already established in pepper using both solid cultures (Harini and Lakshmi Sita 1993; Binzel et al. 1996) and liquid cultures (Buyukalaca and Mavituna 1996). It would be interesting to see whether further improvement in transformation frequencies could be achieved using this pathway of regeneration. However, shoot production is often problematic, due to the failure of apical shoot meristems to develop normally (Steinitz et al. 2003). In at least some varieties, the problem of recalcitrant morphogenesis can be improved by inverting the explants on the medium (Kumar et al. 2005). Changing the polarity of the tissue also seems to help in the subsequent elongation of short buds (enhanced by growing in complete darkness), which is another major obstacle to successful plant regeneration in pepper.

4 Plant Improvement through Genetic Engineering

Pepper disease resistance has been a more attractive target for biotechnology than fruit quality traits (Table 4). Peppers are susceptible to a wide range of pathogens (viral, fungal, bacterial, insect, nematode) which can severely limit yield and quality. Conventional breeding has developed many improved pepper cultivars, but sources of resistance genes do not exist for all pathogens or are not introgressible, so traditional methods cannot provide solutions to every pathogenic problem. The possibility of inserting a single gene from a sexually incompatible wild species in a single step into elite varieties to provide resistance without compromising agricultural traits and without the need for extensive back-crossing is very attractive (Wilson 1993). Biotechnology has allowed dramatic improvements in the resistance of some crops to viral infection (Zaitlin and Palukaitis 2000) and may be similarly useful in pepper. However, pepper has proven to be a difficult crop to genetically transform. The low shoot regeneration rate in vitro combined with a low efficiency of transformation by *Agrobacterium* has hindered the development of reliable transformation procedures, which are rarely consistent or repeatable and in any case are not reproducible in other cultivars. These factors mean that, even now, populations of primary transformants are usually very small despite substantial investments of time and effort.

4.1 Virus Resistance

Viral diseases can be a significant problem for peppers, which are susceptible to cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), tobacco etch virus (TEV), tomato mosaic virus (ToMV), potato virus Y (PVY), tomato spotted wilt virus (TSWV) and pepper mild mottle virus (PMMV) among others (Ochoa-Alejo and Ramirez-Malagon 2001). Viral diseases are very difficult to control and generally are combatted either by chemical insecticides to kill

Table 4. Examples of transgenic populations of peppers and the target agricultural trait. CMV Cucumber mosaic virus, CP coat protein, T_0 primary transformant, TMV tobacco mosaic virus, ToMV tomato mosaic virus

Promoter	Transgene	Agricultural traits	Plant selection	Primary population	Inheritance	Field or glasshouse	References
35S	CMV satellite RNA	CMV resistance	Kanamycin	4	T_1	G	Lee et al. (1993), Kim et al. (1997)
35S	CMV satellite RNA	CMV resistance	Kanamycin	2	T_1	G	Dong et al. (1995)
35S	*CMV-CP*	CMV resistance	Kanamycin	5	T_0	No	Zhu et al. (1996)
35S	*OsMADS1*	Dwarfism, early flowering	Kanamycin	5	T_0	G	Kim et al. (2001)
35S	*CMV-CP* + *TMV-CP*	Multiple virus resistance	Kanamycin	49	T_4	F	Cai et al. (2003)
35S	*CaCel1*	Fruit firmness	Chlorsulfuron	57	T_1	G	Harpster et al. (2002a)
35S	*Bt cry1Ac*	Insect resistance	Phosphoinothricin	19	T_0	G	Kim et al. (2002)
OsrbcS	*Bt cry1Ac*	Insect resistance	Phosphoinothricin	19	T_0	G	Kim et al. (2002)
35S	*CMV-CP* + *ToMV-CP*	Multiple virus resistance	Kanamycin	12	T_1	G	Shin et al. (2002a)
35S	*NtTsi1*	Pathogen resistance	Kanamycin	15	T_1	G	Shin et al. (2002b)
35S	*TMV-CP*	TMV resistance	Kanamycin	12	T_1	G	Lee et al. (2004)
35S	*CcPPI1*	Virus resistance	Kanamycin	6	T_1	No	Lee et al. (2004)

insect vectors of the disease or by breeding programmes to introduce natural resistance genes into crops. Pepper crop losses due to viral infection can be high and yield losses of 50–70% have been reported in epidemic years (Dong et al. 1995). Resistance to TMV has largely been achieved through traditional breeding methods, but no natural resistance to CMV has been identified. The first attempts at biotechnological improvement in pepper were aimed at developing resistance to CMV to reduce the reliance on agrochemicals for crop protection.

4.1.1 Virus Resistance from Satellite RNA Genes

Pathogen-derived resistance to viruses relies on the expression of functional or non-functional virus-derived genes as recombinant transgenes in plants (Wilson 1993). This approach has been taken using viral coat protein, replicase and movement protein genes, defective-interfering RNA (DI-RNA), and using transgenes derived from viral satellite RNA (Baulcombe 1996). In addition to the genomic RNA species, some viruses possess an extra RNA, termed a satellite RNA, which alone is not infectious and is completely dependent on their helper virus for replication and packaging (Francki 1985; Simon et al. 2004). Satellite RNAs are not necessary for the multiplication of the helper virus, but the presence of a satellite RNA can modify the symptoms caused by its helper virus, in some cases inducing more severe or even altered symptoms but more often causing their attenuation (Tien and Wu 1991). One of the earliest uses of satellite RNA-mediated resistance was the expression of cDNAs of virus satellite RNAs of CMV and tobacco ringspot virus (TRSV) as transgenes in plants (Gerlach et al. 1987; Harrison et al. 1987). This was found to attenuate the symptoms of infection and reduce viral replication, probably by the transgenic RNA competing or acting as decoy RNA to directly inhibit some aspect of the viral infection cycle (Baulcombe 1996).

In a preliminary report, Dong et al. (1995) used *Agrobacterium* infection to transform cotyledonary petioles of the pepper cultivar 89-1 with a plasmid containing (an unspecified) CMV satellite RNA gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Only two transgenic pepper lines were obtained, together with a population of seven transgenic tomato lines created at the same time. RNA gel blot analysis showed that some of the transgenic tomato plants accumulated the transcript of the CMV satellite RNA. A primary pepper transformant (T_0) was allowed to self-pollinate and the progeny (T_1) were examined for disease resistance. Plant growth was more vigorous and CMV symptoms were less in the transgenic line; and the time from inoculation to the appearance of the first visible symptoms was delayed from approximately 13 days to 24 days.

Four independent transformants expressing a cDNA transgene of CMV $I_{17}N$ satellite RNA under the control of the 35S promoter were also created using cotyledon explants in hot pepper variety Golden Tower (Lee et al. 1993).

A detailed characterisation of T_1 progeny derived by self-fertilisation from two of the T_0 primary transformants appeared subsequently (Kim et al. 1997). In plants of the segregating T_1 generation shown by PCR to possess the kanamycin selectable marker gene, RNA gel blot analysis detected the accumulation of a transcript derived from the satellite RNA transgene. Plant resistance to two CMV strains (shown previously not to have satellite RNA) was assayed by harvesting pepper leaf discs at various intervals after mechanical inoculation and grinding them in phosphate buffer. Extracts were then inoculated onto the leaves of the local lesion host *Chenopodium amaranticolor*, to allow an accurate measurement of the biological activity of particular virus inocula. The data showed an approximately four-fold reduction in lesion number on the local lesion host, implying significantly reduced virus accumulation on the transgenic pepper plants challenged with either CMV strain. After inoculation, vegetative growth of the transgenic pepper plants was substantially improved, relative to controls, and viral symptom severity was reduced.

Despite these successes, the lack of suitable satellite RNAs for some viruses and concerns about possible mutation of the satellite RNAs to pathogenic forms (Palukaitis and Roossinck 1996) limit the use of this approach as a protection strategy.

4.1.2 Virus Resistance from Coat Protein Genes

Coat protein-mediated protection is accomplished through the expression of a stably transformed cDNA gene derived from a virus coat protein RNA (Powell-Abel et al. 1986). Using this method, transgenic crops (notably squash and papaya) resistant to viruses which threatened to devastate their industries have been extensively tested and grown (Fitch et al. 1992; Fuchs and Gonsalves 1995; Tricoli et al. 1995; Pang et al. 2000; Ferreira et al. 2002). The recombinant coat protein most commonly works by interfering with the uncoating of the viral genome during the virus replication cycle, thus preventing infection (Beachy 1997). However, it was observed that a high degree of virus resistance did not necessarily correlate with a high accumulation of the coat protein viral transgene mRNA and that transformation with modified viral transgenes encoding non-translatable RNAs also conferred resistance, which suggests that in some circumstances resistance is mediated not at the protein level but at the RNA level (Baulcombe 1996). In this case, transcription from the virus-derived transgene triggers post-transcriptional gene silencing of both the transgene RNA and homologous RNA transcripts from the potential pathogen. Both transcripts are degraded, suppressing the accumulation of viral RNA and thus preventing infection. This is termed homology-dependent resistance (Baulcombe 1996).

There are now several reports of populations of pepper transformed with a virus coat protein gene under the control of the 35S promoter. In the first of these, a CMV coat protein gene was integrated into the genome of a sweet

pepper variety and five putative transformants were recovered (Zhu et al. 1996). DNA gel blot analysis confirmed that three lines showed a strong band hybridising to a CMV coat protein probe, and two lines exhibited accumulation of immunodetectable CMV coat protein. However, further characterisation of the lines was not reported.

A similar study with two chilli pepper varieties produced 12 primary transformants containing a TMV coat protein gene, some of which were shown to accumulate TMV coat protein mRNA (Lee et al. 2004). Self-pollinated T_0 plants were used to produce 408 segregating T_1 plants, which were mechanically inoculated with TMV in glasshouse tests. Of these, 28 plants were resistant to TMV infection, as assessed by a lack of mosaic development or hypersensitive response.

A vector designed to independently co-express two coat proteins (those of CMV and ToMV) was used to transform the hot pepper variety Golden Tower (Shin et al. 2002a). Out of 12 putative transformants, seven showed the presence of both coat protein genes by DNA and RNA gel blot analysis. In the T_1 generation, a high accumulation of immunodetectable ToMV coat protein in leaves was correlated with a reduced number or size of lesions after inoculation with ToMV. Similarly, a high accumulation of CMV coat protein mRNA was correlated with reduced CMV propagation relative to control non-transformed plants. A high accumulation of ToMV coat protein mRNA also caused reduced viral propagation after inoculation of leaves with PMMV, which has a related but diverged coat protein amino acid sequence.

In the most extensive transgenic study to date, Cai et al. (2003) used *Agrobacterium* co-cultivation of hypocotyls to create a population of 49 kanamycin-resistant primary transformants of a chilli pepper cultivar containing a T-DNA designed to independently co-express coat proteins of CMV and TMV. Of these 49 T_0 plants, PCR tests found that 22 contained at least one of the coat protein genes and 11 were identified as containing both. From these, T_1 plants were produced by self-pollination and homozygous T_1 plants were identified by examination of the segregation of kanamycin resistance in the T_2 generation. Inheritance of the T-DNA into the T_3 generation was confirmed in one homozygous line, as well as the accumulation of CMV and TMV mRNA and protein. In glasshouse tests, T_1 plants from six independent T_0 lines expressing both coat protein genes were successively challenged with CMV and TMV; and plants from four of these lines showed resistance to virus infection. Resistance to visible viral infection was correlated with low rates of virus multiplication, as assayed on a local lesion host. Three field studies were also carried out, using homozygous progeny of the best line exposed to normal field mixed infections. Two small-scale trials with T_3 progeny found that transgenic plants showed delayed appearance and reduced severity of symptoms relative to controls. A large-scale planting (10,000 plants) of T_4 progeny showed similar results. However, in all the field trials, the percentage of transgenic plants exhibiting symptoms, although these were milder, was eventually almost as high as in control plants. This was explained as possibly due to infection by pathogens

other than CMV or TMV occurring under field conditions, such as TEV and PVY, which produce symptoms similar to those of CMV. Nevertheless, vegetative growth was stronger in the transgenic lines, with greater fruit weight and fruit number per plant, and fruit quality attributes were similar to those of controls. This study shows the potential for developing pepper lines exhibiting coat protein-mediated virus resistance which is robust enough to produce measurable improvements in fruit yield and quality under field conditions.

4.1.3 Virus and Broad Spectrum Resistance from Transcription Factors

In other species, virus resistance has also been achieved through transgenic interference with virus movement and replicase proteins and by gene silencing (Baulcombe 1996; Beachy 1997). To date, these have not been attempted in pepper, but two studies have used the over-expression of transcription factor genes to up-regulate the expression of downstream genes that form part of the natural plant response to infection.

Constitutive over-expression of the tobacco *Tsi1* gene (*Tobacco stress-induced gene 1*) in tobacco induced the expression of several pathogenesis-related (PR) protein genes under normal conditions and improved the plants' tolerance to salt stress and pathogens (Park et al. 2001). Over-expression of the tobacco *Tsi1* gene in transgenic hot pepper plants was performed with a *35S::Tsi1::nos* construct and 15 primary transformants were generated (Shin et al. 2002b). RNA gel blot analysis of a selection of these T_0 plants showed constitutive expression of the *Tsi1* transgene and eight PR protein genes to varying levels. Pathogen challenge of plants of the T_1 generation showed reduced multiplication of PMMV and CMV virus, reduced incidence and severity of infection by the oomycete pathogen *Phytophthora capsici* (responsible for late blight disease) and slightly reduced accumulation of the bacterial pathogen *Xanthomonas campestris* (bacterial spot disease). Increased resistance to unrelated virus, bacterial and oomycete pathogens shows the promise of using regulatory transcription factor genes as a defence strategy.

Similarly, *PPI1* (*pepper-PMMV interaction 1*, a bZIP transcription factor gene isolated from *Capsicum chinense*) was over-expressed in hot pepper under the control of the 35S promoter (Lee et al. 2004). Six primary transformants positive for the *35S::PPI1* transgene were identified by PCR; and four of these were confirmed by DNA gel blot analysis to contain a single transgene copy of *PPI1* in addition to the endogenous gene. However, no data on the expression of PR genes or the pathogen resistance of the transgenic lines were presented.

Although data so far are limited (particularly with regard to yield penalties), the over-expression of transcription factor genes to trigger the expression of the numerous plant defence genes which together provide systemic acquired resistance may be the route to developing plants with long-lasting, broad spectrum resistance to a variety of unrelated pathogens.

4.2 Insect Resistance

The expression of synthetic genes encoding the active part of the protein *δ*-endotoxins of various subspecies of *Bacillus thuringiensis* (*Bt*) has been shown to protect crops from foliage damage and loss caused by herbivorous insects (Koziel et al. 1993). Excellent protection has been observed against insect pests; and there are now millions of hectares under cultivation of corn, cotton and potato plants expressing different insecticidal *Bt* toxin genes and consequently with enhanced resistance to European corn borer, bollworm and Colorado potato beetle, respectively (Frutos et al. 1999). A major pest of pepper farming in Korea is oriental tobacco budworm and a report (in Korean) of the production of transgenic *Bt* toxin-expressing pepper plants has appeared (Kim et al. 2002).

A plant-optimised version of the *cry1Ac* gene was produced and ligated into plasmids under the control of the constitutive 35S promoter, or the ribulose-1,5-bisphosphate carboxylase small-subunit (*rbcS*) promoter from rice to give tissue-specific expression in green organs. Two populations of transgenic hot peppers (19 plants each with *35S::cry1Ac* and *rbcS::cry1Ac* constructs) were examined by protein gel blot for the accumulation of cry1Ac protein; and three and seven positives, respectively, were identified. Preliminary laboratory data indicated that damage to fruit due to feeding by insect larvae was reduced. Although further characterisation and field trials are necessary, the success of *Bt* toxin-mediated protection of other crops against insect pests gives no reason to doubt that this mechanism would also be effective in pepper.

4.3 Growth Habit

MADS-box genes encode a large eukaroyote-wide family of transcription factors and include most of the developmental control genes that regulate apical meristem development in plants (Theissen et al. 2000). The expression of various MADS-domain floral organ identity genes controls the differentiatiation of sepals, petals, stamens and carpels (Theissen et al. 2000). A member of this gene family from rice, *OsMADS1*, plays an important role in floral meristem determination, since a mutant defective in this gene develops multiple florets per spikelet instead of the usual one (Jeon et al. 2000a). When *OsMADS1* was constitutively over-expressed in tobacco using the strong 35S promoter, development was dramatically altered and short, bushy, early-flowering plants with reduced apical dominance resulted (Chung et al. 1994). However, if over-expression was controlled by the weaker *nos* promoter, transgenic rice plants were only slightly dwarfed and flowered a few days earlier without pleiotropic effects (Jeon et al. 2000b), suggesting that limited expression of *OsMADS1* could be used to modify plant growth habit.

Transformation of hot pepper with a *35S::OsMADS1::T7-T* construct resulted in two lines shown by DNA gel blot to possess a transgene insertion in the

genome (Kim et al. 2001). One line (line 2) possessed insertions at two loci, and the other (line 5) at a single locus. Both lines accumulated *OsMADS1* mRNA in leaves, with much higher accumulation in line 2. Plant height was reduced in both lines, but less effectively in line 5, and only in line 2 was internode length reduced and flowering time shortened slightly. Studies with a greater number of primary transformant lines and using weaker promoters may be able to identify pepper plants with improved stature or flowering-time phenotypes.

4.4 Fruit Quality

In many fruit species, particularly berry fruit such as strawberry and raspberry, excessive softening is the main factor limiting post-harvest life. Ripening-related softening is thought to be due mainly to a disassembly of the various polysaccharide components of the cell wall (Brummell and Harpster 2001). Pepper fruit do soften somewhat during ripening but the loss of firmness is moderate, partly because (unlike wild accessions) the fruit of cultivated pepper varieties lack accumulation of polygalacturonase mRNA (Rao and Paran 2003), and pectin depolymerisation is almost undetectable during ripening and over-ripening (Harpster et al. 2002a).

In tomato, ripening-related accumulation of endo-1,4-β-glucanase (EGase) activity was suggested to be a major determinant of fruit softening (Lashbrook et al. 1994). However, in tomato at least three ripening-related EGase genes have been detected (Brummell and Harpster 2001) and suppression of one gene alone did not affect fruit firmness (Lashbrook et al. 1998; Brummell et al. 1999). In pepper, a single ripening-related EGase gene, *CaCel1*, is apparently responsible for all EGase activity in the fruit (Harpster et al. 2002a). The protocol of Engler et al. (1993) was used to create the largest population of pepper primary transformants to date, transformed with a truncated version of the *CaCel1* cDNA driven by the 35S promoter to trigger sense suppression of the endogenous *CaCel1* gene. Out of 57 primary transformants, only two lines exibited strong post-transcriptional gene silencing of *CaCel1* and undetectable accumulation of CaCel1 protein and EGase activity (Harpster et al. 2002a). This work confirmed that *CaCel1* alone is responsible for ripening-related EGase activity in pepper, but showed that the lack of CaCel1 activity did not prevent or reduce hemicellulose degradation during ripening. Furthermore, over-expression of functional CaCel1 protein in tomato did not increase hemicellulose degradation or softening in tomato fruit (Harpster et al. 2002b). The role of EGase activity in fruit softening remains obscure.

Perhaps the most commercially significant fruit quality issue for pepper, that of post-harvest water loss (Kissinger et al. 2005), has not so far been the subject of transgenic intervention.

4.5 Transient Transformation Using Viral Vectors

Virus-induced gene silencing (VIGS) exploits the natural defence system of the plant against viruses, an RNA-based mechanism designed to silence virus RNA accumulation. Transgenes derived from the host's genes are incorporated into modified viral vectors which, as they spread systemically after infection, additionally trigger silencing of the corresponding host mRNAs. This allows a much more rapid analysis of gene function than through creating stably transformed transgenic lines (Lu et al. 2003). VIGS has been limited in the past to *Nicotiana benthamiana* and a few other host species whose infecting viruses have been characterised, but additional viral vectors are being developed. Transgenes consisting of portions of pepper cDNAs coding for the carotenoid biosynthetic enzyme phytoene desaturase (*PDS*) and *rbcS* were cloned into a version of a viral vector based on tobacco rattle virus (TRV) and used to infect chilli pepper plants (Chung et al. 2004). Almost 100% of the plants exhibited the expected phenotypes of photobleaching and chlorosis, respectively, showing that TRV induces high frequency VIGS in pepper plants. VIGS may be a very useful tool for examining gene function in pepper, circumventing the low frequencies of stable genetic transformation.

Viral vectors can also be used for the over-expression of novel recombinant proteins in plants (Pogue et al. 2002). A vector based on ToMV was used to transiently express a protein consisting of GFP fused to peptides of amyloid-β, a protein thought to be involved in Alzheimer's disease (Szabo et al. 2004). When green pepper plants were used as the production host, accumulation of the recombinant protein in leaves was sufficient for use in oral vaccination experiments with mice.

5 Pepper Cell Cultures for Capsaicin Production

Capsaicinoids are the alkaloids responsible for the burning sensation experienced when eating hot chilli peppers. Capsaicinoids are synthesised from a condensation between an aromatic skeleton derived from phenylalanine (via the phenylpropanoid pathway) and a branched fatty acid moiety derived from valine (Ochoa-Alejo and Ramirez-Malagon 2001). The most abundant capsaicinoids are capsaicin and dihydrocapsaicin, which also contribute most to the pungency. In addition to its widespread use as a spice, capsaicin is also used therapeutically as a stimulant, analgesic or for anti-inflammatory applications (http://www.ars-grin.gov/duke/ethnobot.html). However, capsaicin has also been reported to inhibit the growth of, or cause apoptosis in, certain types of cancer cell lines in vitro (Surh 2002).

In the plant, capsaicinoids accumulate in the placenta of maturing chilli pepper fruit. Cultured plant cells and tissues derived from hot pepper fruit have been used in an attempt to produce high quantities of capsaicin for

the pharmaceutical and food industries (for a review, see Ochoa-Alejo and Ramirez-Malagon 2001), usually using an immobilised cell culture system with capsaicin being secreted into the medium (Lindsey and Yeoman 1984). The technology has been improved by the use of placental tissue as the starting material (Sudhakar Johnson et al. 1990), using lines resistant to an analogue of phenylalanine (Ochoa-Alejo and Salgado-Garciglia 1992), treating with elicitors (Sudhakar Johnson et al. 1991) and feeding intermediates of the biosynthetic pathway (Sudhakar Johnson and Ravishankar 1998; Nuñez-Palenius and Ochoa-Alejo 2005). Yields of capsaicin several-fold higher than that of fruit have been reported (Sudhakar Johnson and Ravishankar 1998).

6 Future Prospects

Pepper remains one of the species most recalcitrant to genetic transformation. Although there are now reports of studies generating significant numbers of primary transformants (Harpster et al. 2002a; Cai et al. 2003), contradictory findings that successful transformation should proceed only without a callus phase (Engler et al. 1993) or only with one (Lee et al. 2004) emphasise the problems caused by genotype-specific procedures and the lack of repeatability and consistency of the methods used in different laboratories. The difficulties inherent in pepper transformation necessitate using the most efficient technologies available. For example, the use of inverted repeats in transgene constructs for gene silencing has resulted in a huge improvement in the frequency of post-transcriptional gene silencing (Wesley et al. 2001). In this way, transgenic population size can be kept to a minimum and only a few successful transformation events may be sufficient to generate the required phenotype. VIGS may be used as an alternative to genetic transformation in studies of gene function in pepper, or for the expression of novel, pharmaceutically important proteins. The use of small populations combined with efficient technologies should allow the genetic engineering of pathways involved in the biosynthesis of nutritionally desirable pigments, capsaicinoids and vitamins in pepper fruit.

Virus resistance is still the most desired target for pepper biotechnology and attempts so far have been relatively successful. Improvements could be made by increasing the broadness of the resistance, such as by constructs designed to express three virus coat protein genes, as used in melon (Fuchs et al. 1997), or by linking multiple virus-specific cDNA sequences together (Jan et al. 2000). The expression of peptides which interfere with nucleocapsid proteins, involved in almost all steps in the virus life cycle, gave resistance to four different tospoviruses in *Nicotiana benthamiana* (Rudolph et al. 2003) and will be a useful technology for crop plants. Furthermore, the use of breeding to combine transgenic with natural resistance in a single plant has been used successfully in tomato to create broad-spectrum virus resistance (Gubba et al. 2002). However, the expression of transcription factors to up-regulate natural

plant resistance to infection may be necessary to give broader resistance to unrelated pathogens, provided that pleiotropic effects and yield penalties can be minimised.

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V.2 Onion, Garlic and Related Species

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1 Introduction

The genus *Allium*, belonging to the family Liliaceae, comprises about 700 species, including both economically important vegetables and wild species. The plant is generally adapted to temperate environments with low or erratic supplies of water and can be found across a wide range of latitudes and altitudes in Europe, Asia, North America and Africa. Various *Allium* species produce an underground storage bulb in the first year of growth and flower in the following year. Many species, but not garlic, also produce seeds. While *A. cepa* (bulb onion), *A. sativum* (garlic) and *A. tuberosum* (Chinese chives) are commercially important, other *Allium* species are important locally (Table 1).

Many members of *Allium* are used as foods. They can be consumed fresh, but are also used in food processing when dehydrated. Garlic and onion have also

Table 1. Economically important *Allium* spp.

Species	Subdivision	Horticultural name
A. sativum		Garlic
A. schoenoprasum		Chives
A. tuberosum		Chinese chives
A. ampeloprasum porrum		Leek
	Aegyptiacum	Kurrat
		Elephant garlic
		Pearlzwiebel
A. cepa	*Cepa*	Bulb onion
	Ascalonicum	Shallot
	Aggregatium	Potato onion
		Ever-ready onion
	Proliferum	Tree onion
A. triquetrum		Onion weed
A. chinense		Rakkyo
A. ursinum		Ramsons
A. fistulosum		Welsh onion

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been used as medicinal agents for thousands of years and have been shown to possess antimicrobial, antithrombotic, antitumor, hypolipidemic, antiarthritic and hypoglycemic properties. These chemopreventive activities are thought to be related to their high content of organosulfur compounds (Fig. 1 Ali et al. 2000; Harris et al. 2001; Knowles and Milner 2001; Griffiths et al. 2002; Banerjee et al. 2003; Thomson and Ali 2003).

Breeding plays an important role for *Allium* improvement. Interspecific hybridizations between wild and cultivated species have generated new genotypes possessing biochemical and genetic properties of both parental plants. For the past two decades, the establishment of efficient genetic transformation systems has been shown to be important for improvement of a wide range of crop species. However, the development of genetic transformation systems for *Allium* spp has been slow and difficult. The integration and expression of introduced genes in plants with large genomes such as *Allium* spp have not been studied extensively. Recently, *Agrobacterium*-mediated transformation has been used to express valuable genes in onion, shallot and garlic. This technology may lead to accelerated progress in *Allium* biotechnology, especially for garlic, which is propagated vegetatively.

2 Onion (*Allium cepa* L.)

Onion is one of the oldest cultivated plants, with its cultivation dating back to before 5,000 BC. The plant probably originated from central Asia, but is no longer known in the wild. Onions are grown throughout the temperate and subtropical regions of the world (Woodward 1996). The species shows a great diversity in form, varying in color, shape, dry matter content and pungency. This diversity is reflected in the success of the species in adapting to a wide range of environments (Griffiths et al. 2002). Onions are diploid ($2n = 2x = 16$), with a nuclear genome of 1.6×10^9 base pairs per 1C, which is approximately equal to that of hexaploid wheat and about 34 and 6 times larger than that of rice and maize, respectively (Arumuganathan and Earle 1991).

Global onion production has increased by at least 50% over the past 10 years. In 2003, the world production of onions was approx. 52.5×10^6 t (FAO 2004), rendering it the second most important horticultural crop after tomato. The main onion producers are China (800,713 ha, 17.5×10^6 t), India (530,000 ha, 5×10^6 t), the Russian Federation (116,870 ha, 1.6×10^6 t) and Pakistan (104,000 ha, 1.4×10^6 t).

Bulb onion produces a large bulb in the first year of growth and flowers after vernalization. Onions are attacked by a number of pests, diseases and viruses during growth and storage. The control of onion pests and diseases constitutes one of the most important goals of onion biotechnology. Onion is dehydrated for food processing and is consumed fresh in the form of green salad material. It has also been used for thousands of years as a medicinal agent.

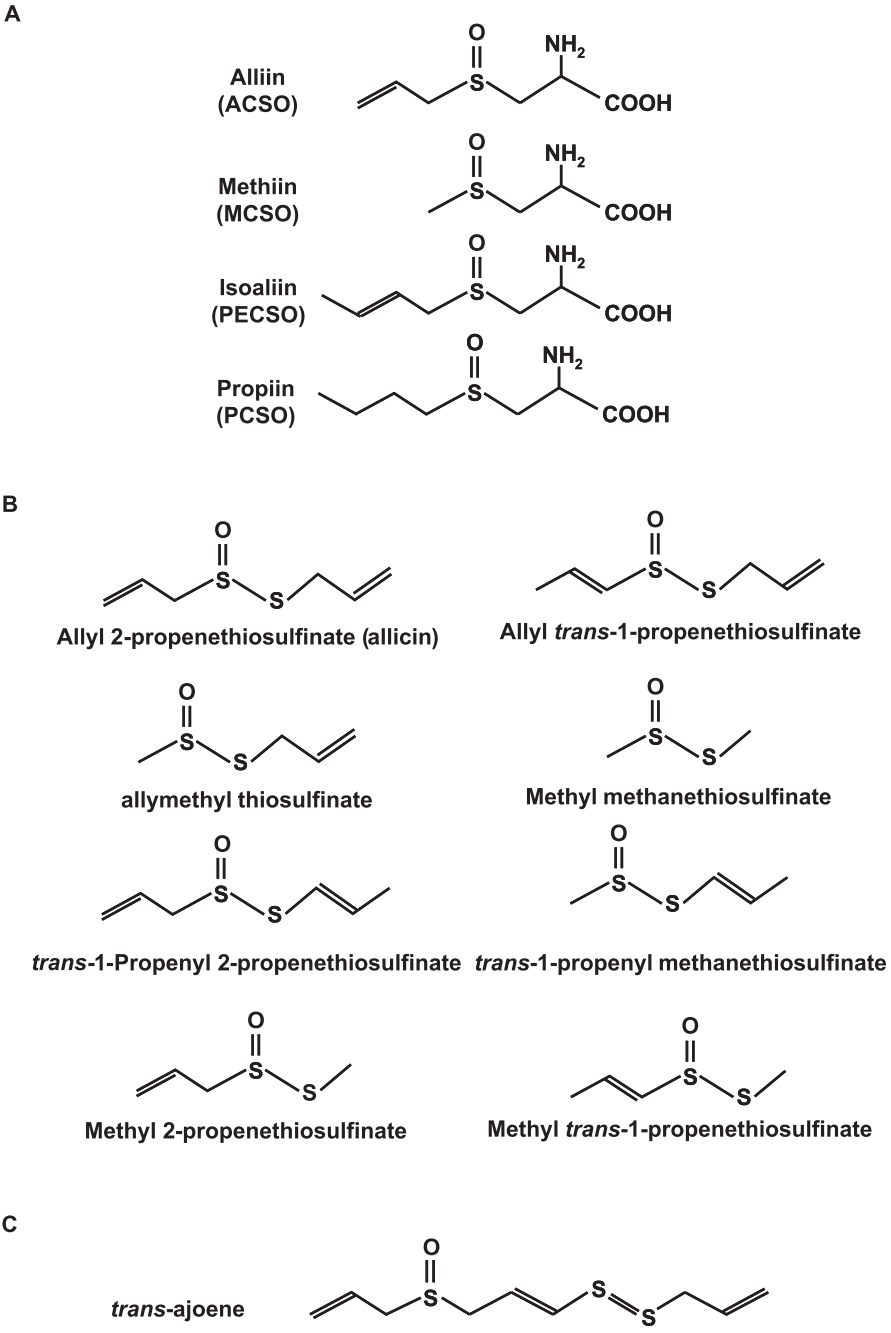


Fig. 1. Chemical structures of organosulfur compounds in *Allium* spp. Flavor precursors (**A**), thiosulfinates (**B**) and *trans*-ajoene (**C**)

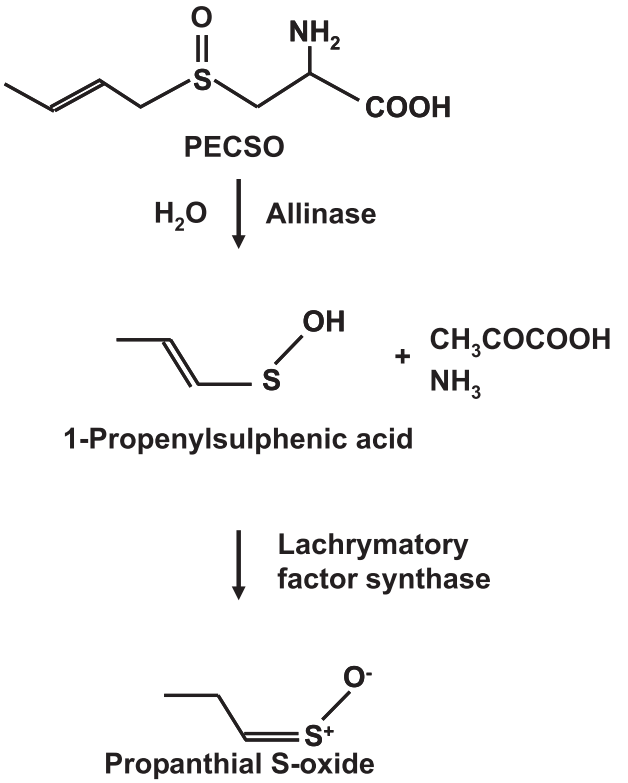


Fig. 2. The chemical reactions in lachrymatory factor synthesis (Imai et al. 2002)

Onions contain two chemical groups that have been reported to provide health benefits, namely the flavonoids and the alk(en)yl cysteine sulfoxides (ACSOs; Fig. 1). In plants, flavonoids have various functions in protection against UV radiation, plant–microbe interactions and fertility (Jones et al. 2004). The two subgroups of flavonoids, anthocyanins and flavonols, are also present in onions. The ACSOs are the flavor precursors that generate the characteristic odor and taste of onion after cleavage by the enzymes alliinase and lachrymatory-factor synthase (Fig. 2; Griffiths et al. 2002).

2.1 Breeding and Genetic Transformation of Onion

Breeding programs are used widely to improve onion quality and productivity. An important aspect of onion breeding programs involves the development of new varieties, with traits such as resistance to major pests and diseases, and variations in bulb shape, color and firmness. Most of the countries that produce onions have developed cultivars that are appropriate for their environment and the demands of consumers. It is very important that breeding pro-

grams take into account the diverse onion cultivars developed in each country. The breeding of new varieties, containing higher concentrations of medicinal or health-beneficial compounds, is on the horizon with the development of analytical methodology and molecular genetics.

Most of the commercially grown onion cultivars on the market are F_1 hybrids. Onion breeding is a slow process. The development of an onion inbred line may require 8–10 years, depending on the complexity of the traits under selection. Using doubled-haploid techniques, truly homozygous lines can be obtained from the gametes of F_1 hybrids, thereby shortening by two or three generations the time required to develop new varieties. The approach requires the methods for both haploid plant production and chromosome doubling (Alan et al. 2003). Haploids can be produced by anther culture and interspecific hybridization techniques. Doubled haploids after chromosomes doubling can then be produced following the application of colchicine. Doubled haploids are useful for studies of quantitative inheritance. These techniques can also shorten the time required for inbred line development.

Genetic markers are useful indirect selection tools that are closely linked to economically important traits. Isozyme and random amplified polymorphic DNA (RAPD) markers have been utilized to classify and categorize the genetic diversity of onion cultivars. Havey et al. (2001) reported the development of a low-density genetic map of onion comprising primarily of restriction fragment length polymorphisms (RFLPs), while Song et al. (2004) developed the microsatellite markers in bunching onion (*A. fistulosum* L.). The results of these molecular genetic studies will be useful for breeding programs to generate cultivars with economically important traits.

Interspecific hybridization amongst wild and cultivated species of the genus *Allium* has been performed to improve the biochemical and genetic properties of these species. Cross-breeding has generated interspecific hybrids between *A. cepa* and *A. kermesinum* (Storsberg et al. 2004) and between *A. roylei* and *A. cepa* (Alan et al. 2003).

Such interspecific hybrids may serve as useful tools in the improvement of *Allium* species. As mentioned earlier, the development of an inbred onion line may require many years and the selection of an inbred line containing only desirable properties may not be straightforward. A genetic transformation system to introduce genes that confer agronomically useful traits could be a solution to these shortcomings encountered in conventional breeding techniques.

Several researchers have investigated the proliferation of calli from different explants and plant regeneration from calli with the goal of developing an efficient transformation protocol for onion. Results indicate that cultures generally grow better in suspension because *Allium* callus grows very slowly on semi-solid medium (Zhang et al. 2004). Although suspension cells that are capable of regeneration have been reported in *A. cepa* (Hansen et al. 1995; Mukhopadhyay et al. 2005), *A. fistulosum* (Kim and Soh 1996) and an interspecific hybrid (*A. fistulosum* × *A. cepa*; Song and Pefflet 1994), the regeneration capacity

is generally low. Klein et al. (1987) developed a high-velocity microprojectile method that can be used to transform onion epidermal tissue. Dommisse et al. (1990) demonstrated that onion is a host for *Agrobacterium*, as evidenced by tumorigenic responses and opine production in these tumors. Eady et al. (1996) used both particle bombardment and *Agrobacterium*-mediated DNA delivery systems to transform onion cultures. Recently, successful transformation of onion and shallot, mediated by *Agrobacterium*, has been reported (Zheng et al. 2001, 2005; Eady et al. 2003a, b). *Agrobacterium* and biolistic transformation of onion using non-antibiotic selection marker phosphomannose isomerase have also been reported (Aswath et al. 2006). Confirmation of the stable expression and inheritance of the transgenes are the crucial steps in transgenic plant production. Eady et al. (2003b) demonstrated Mendelian inheritance of both green florescent protein (GFP) expression and phosphinothricin tolerance in transgenic onions. The level and range of reporter gene expression were consistent with those observed in other transgenic plant species. The authors also showed that there were no obvious detrimental phenotypic effects caused by transgene insertion or somaclonal variation arising due to tissue culture for the majority of the plants. Recent progress in *Agrobacterium*-mediated transformation techniques for onion is expected to facilitate the development of onion biotechnology.

2.2 Biotechnology of Onion

An important aspect of onion biotechnology involves the development of new onion varieties with traits such as red-colored bulbs, altered levels of secondary sulfur compounds, a mild and sweet flavor, the ability to be brought to market early, resistance to weed control methods and disease/pest/virus resistance. Griffiths et al. (2002) highlighted an important point that onion acts as a delivery system for nutrients. Onions, which are eaten by almost all consumers and ethnic groups, are found in a wide range of processed foods, particularly in fast foods. As such, onion is a good candidate for the delivery of beneficial mineral nutrients such as selenium, making it a potential "functional food". In the near future, breeding programs and genetic transformation systems could allow the production of numerous specially modified onion cultivars.

Traditionally, pungent onions with white/yellow flesh and yellow to brown skins have been used for cooking. However, the demand for red/purple colored onions is increasing in many parts of the world. There are four conventional classifications for bulb colors, namely white, yellow, brown and red. The bulb color of onions is an important trait and homogeneous coloration of commercial red onion cultivars is one of the major goals of onion breeding programs. The heterogeneous red onions are often commercial varieties. Flavonoids, one of the major classes of plant secondary metabolites, are thought to be the main pigments that determine bulb color. Anthocyanins are responsible for a red/purple color in some varieties. Flavonols such as quercetin and its deriva-

tives are responsible for the yellow flesh and brown skins of many other varieties (Griffiths et al. 2002). Anthocyanins absorb parts of the visible spectrum to produce red, orange, or blue colors, depending on their specific structural modifications. The actual colors are further dependent on pH, the presence of metals and interactions with other colorless flavonoids. The four main anthocyanins of the red onion cultivars Ruby, Southport Red Globe, Kurenai, Red Baron, Comred, Tropea, Mambo, Red Jumbo, Red Bone and Red Granex were identified as 3-(6″-malonylglucoside), 3-(3″-glucosyl-6″-malonylglucoside), 3-(3″-glucosylglucoside) and the 3-glucoside of cyanidin. In addition, some minor anthocyanin pigments have been detected in these cultivars: 3-(3″,6″-dimalonylglucoside), 3-(3″-malonylglucoside), the 3,5-diglucoside of cyanidin and the 3-glucoside, 3,5-diglucoside and 3-malonylglucoside of peonidin (Donner et al. 1997; Fossen et al. 2003).

Early studies on the inheritance on bulb colors revealed a complex pattern resulting from the interaction of five loci. The first locus, *I*, which is incompletely dominant over *i*, is responsible for a color-inhibiting factor. A second factor, controlled by locus *C*, which exhibits complete dominance over *c*, is also required for the coloration of onion bulbs. A third factor, *G*, produces golden-yellow bulbs when present in either the homozygous or heterozygous state, whereas homozygous recessive *gg* bulbs are chartreuse in color. The final two loci, *L* and *R*, act in a complementary manner and result in the production of a red pigment (Davis and El-Shafie 1967). These five loci are closely related to the genes involved in anthocyanin biosynthesis, because the pigments of the different colored onions are considered to be flavonoid compounds (Davis and El-Shafie 1967). Recently, a new locus (*P*) was identified, which leads to a pink color in red onions (Kim et al. 2004). The *P* locus is likely to be the gene encoding anthocyanidin synthase (ANS), one of the late genes in the anthocyanin pathway. Co-segregation of the *ANS* allele with the pink phenotype in segregating populations strongly suggests that the *P* locus is identical to the *ANS* gene. Kim et al. (2005) developed a co-dominant, PCR-based marker for allelic selection of the pink trait in onions, based on the insertion mutation in the promoter. This information could be used to design breeding strategies to remove the recessive allele or to deliberately utilize this trait for the development of novel pink onion cultivars (Kim et al. 2004). The anthocyanin synthesis pathway has been extensively studied; and almost all of the genes encoding the enzymes comprising the pathway have been characterized in multiple species. Mutations in regulatory genes that result in color changes have also been characterized in many species (Yamazaki et al. 2003). The generation of red/pink onion by genetic modification may be complicated, because six or more genes related to the six loci must be identified. The relationships between these genes and the composition of flavonoid anthocyanins must also be explained. However, previous studies, together with a genetic transformation system, could make it easier to produce red/pink onion cultivars.

Interest is growing in onions of low pungency, or "sweet" onions, which are attractive for fresh uncooked use. When *Allium* tissues are chopped, over 50

sulfur-containing compounds are produced. The compounds include thiosulfinates, thiosulfonates, mono-, di-, tri-sulfides and the lachrymatory or tear factor, propanthial *S*-oxide. These compounds are presumed to be produced spontaneously following the action of the enzyme alliinase, which cleaves flavor precursors. Some flavor precursors found in *Allium* spp are *S*-methyl cysteine sulfoxide (MCSO, methiin; present in most *Allium* spp and some Brassicaceae), *S*-allyl cysteine sulfoxide (ACSO, alliin; characteristic of garlic), *S*-*trans*-prop-1-enyl cysteine sulfoxide (PECSO, isoalliin; characteristic of onion) and *S*-propyl cysteine sulfoxide (PCSO, propiin; in onion and related species; Jones et al. 2004; Fig. 1A). In garlic, ACSO is the major cysteine sulfoxide and is responsible for the characteristic flavor of this plant. However, in onion, PECSO is the most abundant cysteine sulfoxide, often representing more than 80% of these compounds, and is responsible for the majority of the flavor chemistry. Previous studies have shown that alliinase from any source is the only enzyme required to produce lachrymatory factor (propanthial *S*-oxide) from PECSO in onion. The reactions from the intermediate sulfenic acid to propanthial *S*-oxide and thiosulfinate are presumed to be spontaneous because sulfenic acid is very unstable and has never been isolated. Imai et al. (2002) reported that lachrymatory factor is not formed as a byproduct of this reaction in onion but is, instead, specifically synthesized by a previously undiscovered enzyme, lachrymatory factor synthase (Fig. 2). The downregulation of alliinase could lead to a non-lachrymatory onion, but such an onion might have compromised flavor and nutritional value. However, it may be possible to develop a non-lachrymatory onion that still retains its characteristic flavor and high nutritional value, by downregulating the activity of this synthase using antisense or RNA interference (RNAi) technology (Imai et al. 2002).

Although onions that are sweet and less pungent are increasing in popularity, the more pungent varieties still dominate the onion market, because they impart more flavor to cooked dishes, are easier to handle and have longer storage and shelf lives. In 1994, the first genetically modified food was approved to go to market by the United States FDA. The Flavr Savr tomato had been generated by the biotechnology company, Calgene, using antisense technology, which resulted in altered ripening characteristics. In Flavr Savr, the expression of the gene for the polygalacturonase enzyme, which is responsible for the breakdown of pectin, a building block in cell walls, had been suppressed. Flavr Savr tomatoes can ripen on the vine longer, while maintaining firmer skin, thus producing a better-flavored tomato on supermarket shelves. A similar technology could be applied to onions. The genes for other enzymes responsible for the softening of the cell wall, such as cellulases, galactanases and pectin methylesterases, could also be targeted. In addition, the relatively simple secondary metabolite pathway that leads to the production of onion flavor compounds could be manipulated to produce onions with precise flavor characteristics.

Weed control is a major problem in the production of field vegetables, especially where herbicides are not used. Effective weed control is often more

difficult for onion than for many other crops because it grows relatively slowly and does not form the dense leaf canopy needed to suppress weed development. Additionally, the crop can be exposed to both warm- and cool-season weed species. Managing weeds is critical for successful onion production. Herbicide resistance genes, such as the *bar* gene and the *EPSPS* gene, which encode resistance to phosphinothricin and glyphosate, respectively, are being used to produce herbicide-resistant crops, despite some public concern. These genes may be useful in the development of new cultivars of onion, given that onion crops suffer severely from weed competition. Monsanto produces herbicide-tolerant crops such as Roundup Ready corn and Roundup Ready soybeans. A similar strategy could be applied to the production of herbicide-tolerant onions. Eady et al. (2003a) developed transgenic herbicide-resistant onion plants, derived from commercially important genotypes; and they demonstrated the effectiveness of the transgenes in the primary transformants (T_0 plants) with respect to imparting resistance to field application rates of commercial formulations of phosphinothricin and glyphosate.

Onions are attacked by a number of pests, diseases and viruses. Maggots and thrips are responsible for most of the losses attributed to insects. Maggot infestations are common in wet spring seasons; and thrip infections are more severe in dry seasons. Field diseases include smut, downy mildew, pink root, smudge, leaf blight and several basal rots. Some of the storage diseases that affect onions are common field rots, botrytis neck rot and bacterial soft rot. Onion also suffers from a damaging mosaic disease caused by a virus. Protection of the onion crop against onion pests and diseases is a crucial issue. The use of chemical protection agents is difficult, owing to the underground growth habit of the bulb and the wax cover on the leaf. A potentially efficient and environmentally friendly approach toward onion protection would be the production of pest- and disease-resistant onion varieties, by extensive inter-mating and screening campaigns. Some *A. fistulosum* (Welsh onion) lines are resistant or moderately resistant to *Stemphylium* leaf blight (causal agent *S. vesicarium*). *A. roylei* is a non-bulbing wild relative of common onion, with several desirable traits, including resistance to *Botrytis* leaf blight and downy mildew. Such pest- and disease-resistant lines could be used as breeding materials and the progeny used to develop pest- and disease-resistant onion lines. A more active strategy for developing such lines would be a genetic transformation system. Transgenic onion lines resistant to pests, diseases, or viruses could be produced by the introduction of genes encoding such proteins as members of the cystatin and thyropin gene families or antimicrobial proteins.

3 Garlic (*Allium sativum* L.)

Garlic ranks second in importance to onion among the *Allium* species. One possible ancestor of this plant is *A. longicuspis*, a native of the mountainous re-

gions of central Asia. The earliest indication of the use of garlic is in clay models in Egyptian cemeteries, dated to as early as 3,750 BC (Woodward 1996). The global production of garlic in 2003 was about 14×10^6 t, grown on 1 142 000 ha (FAO 2004). China has become the major area of garlic cultivation, producing 10×10^6 t of garlic on 632,000 ha in 2003. In 2002, the worldwide trade of garlic exceeded 1×10^6 t, worth U.S. $ 620×10^6.

Garlic cloves are used in cooking all over the world. Garlic combines well with an enormous range of foods, adding its own flavor as well as enhancing the flavors of the foods with which it is mixed. The flavor of garlic varies, depending on the cultivar and the climate in which it was grown. The best garlic comes from temperate areas with hot and dry summers (Woodward 1996). Garlic has long been used medicinally as a home remedy for protective and curative purposes. The reputation of garlic as an effective remedy for various diseases extends back to the Egyptian medical text, *Codex Ebers*, which dates from about 1,550 BC (Woodward 1996). Garlic is reported to exert antimicrobial (Harris et al. 2001), anticancer (Thomson and Ali 2003), antioxidant (Banerjee et al. 2003) and cardiovascular lipid-lowering activities (Banerjee and Maulik 2002).

The main constituents of garlic that have the most medicinal value are sulfur-containing compounds (Fig. 1B), including allicin (Ankri and Mirelman 1999). Allicin is an oxygenated sulfur compound that is produced from its stable precursor, alliin, by the enzyme alliinase (or alliin-lyase) when the bulb is cut or bruised (Van Damme et al. 1992; Rabinkov et al. 1994). The wide-spectrum antimicrobial effects of allicin are attributed to its interaction with the thiol groups of certain thiol-containing enzymes in the microorganisms (Rabinkov et al. 1998). The amounts of alliin (or allicin) in garlic vary considerably among cultivars, ranging over 2–8 mg g^{-1} fresh weight. Alliinase is present in unusually high amounts in garlic cloves, comprising at least 10% of the total protein (10 mg g^{-1} fresh weight). An isolated garlic alliinase gene encodes a 55-kDa glycoprotein, consisting of 448 amino acid residues (Van Damme et al. 1992; Rabinkov et al. 1994).

Raw garlic cloves are processed and supplied as dietary supplements, including dehydrated garlic powder, garlic oil and aged garlic extract (AGE). Garlic preparations exert antioxidant activity by scavenging reactive oxygen species (ROS) that may cause numerous disease processes. AGE enhances the activities of cellular antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, and increases the glutathione content of cells (Borek 2001). Variations in the processing procedures yield quite different garlic preparations. Highly unstable thiosulfates, such as allicin, are quickly transformed into a variety of organosulfur components (Amagase et al. 2001).

Ajoene (Fig. 1C) is a condensation product of allicin formed by application of heat, having the advantage of a greater chemical stability than allicin (Hassan 2004). In addition to antithrombosis, antimicrobial and cholesterol-lowering effects, ajoene appears to stimulate peroxide production to induce apoptosis in human leukemic cells, implying a novel promising role of this

compound for the treatment of acute myeloid leukaemia (AML) patients (Hassan 2004).

3.1 Molecular Genetics of Garlic

Garlic is a perennial monocotyledon that is usually grown as an annual. Garlic has been classified into two forms, based on a botanical feature: the softneck and hardneck garlics (Woodward 1996). Softneck garlics produce no flower stems or flowers; the leaves simply die back when the bulb is mature. In contrast, hardneck, or top-setting garlics produce a smooth, round, solid flower stem in early summer. The flowers develop at the top or can be embedded in the stem, but are rarely open and usually wither as buds without producing seeds. If seeds are produced, they are black and are contained in a small capsule, but are infertile. In general, garlic is propagated vegetatively via the clove and bulblet.

More than 300 cultivars and varieties of garlic, varying in color, size, pungency and flavor, are grown worldwide. Most areas have their own distinct cultivars that match the local climatic conditions and the demands of the market; and cultivars are often named after their place of origin. In general, the softneck cultivars are stronger and more pungent and the top-setting cultivars are usually milder, sweeter and more nutty in flavor (Woodward 1996).

Different garlic cultivars are distinguished according to their morphological, physiological and ecological characteristics. However, such characteristics may differ under different environmental conditions. DNA-related traits and methods have been used to differentiate the genetic backgrounds of garlic cultivars. Isozyme and RAPD markers have been utilized to classify and categorize the genetic diversity of garlic cultivars (Klass 1998). Molecular genetic studies have revealed that bulb-related traits or geographical origins have little predictive value in the genetic relationships of different accessions.

Garlic is a diploid ($2n = 16$), with two pairs of satellite chromosomes (McCollum 1976). It has a large genome of approximately 1.58×10^{10} base pairs, much larger than that of *Arabidopsis* (1.2×10^{8} base pairs) or rice (4.3×10^{8} base pairs). Garlic partial bacterial artificial chromosome (BAC) libraries have been constructed for molecular and cytogenetic studies (Lee et al. 2003).

3.2 Genetic Transformation of Garlic

Because cross-fertilization of garlic is not practical, the breeding of this plant relies on simple selection and multiplication of spontaneous or induced mutants. However, success in the production of germinable garlic seeds was reported by Pooler and Simon (1994). In this study, removing the vegetative top-sets from the inflorescence and cutting the inflorescences from the underground bulbs led to the production of a number of germinable garlic seeds. Establishing sexual reproduction in garlic may provide access to genetic variations for

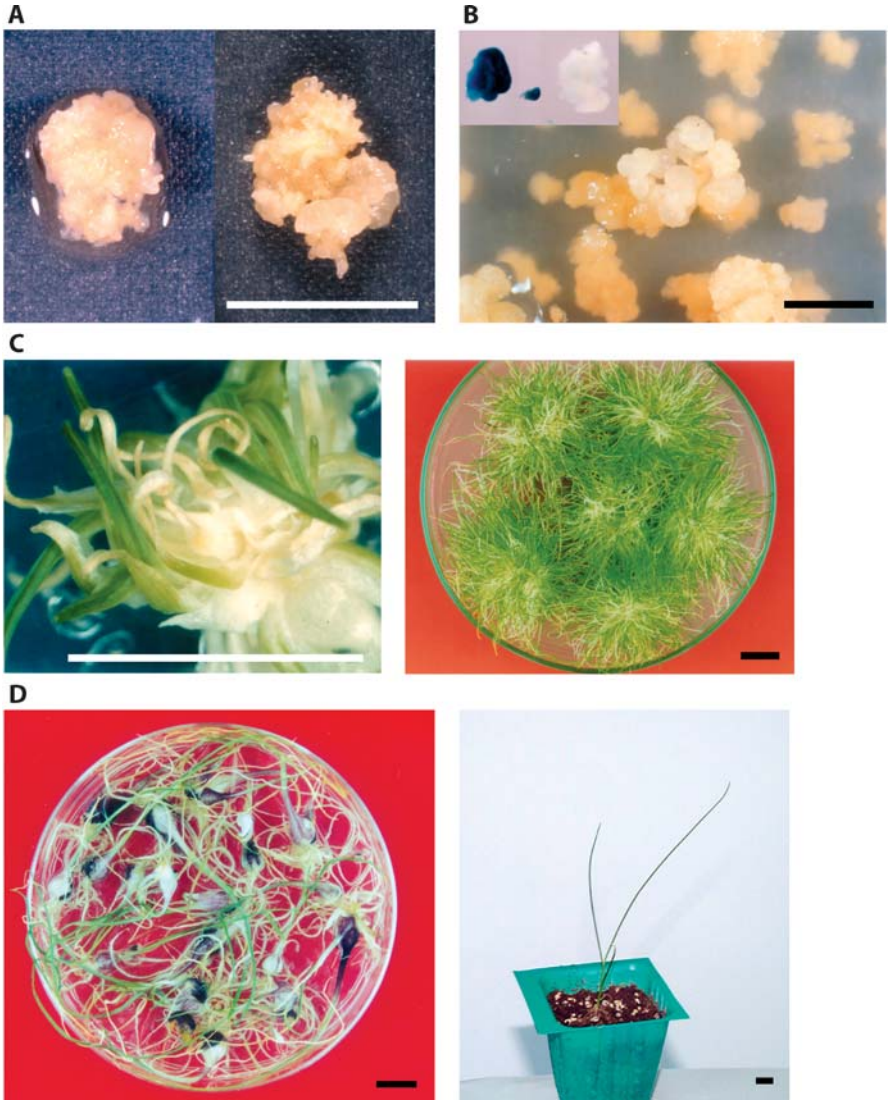
breeding programs, but the reliable generation of new recombinants through this technique remains to be demonstrated. Recently, an interspecific hybrid was produced between leek and garlic (Yanagino et al. 2003). This hybrid is able to be propagated vegetatively by the planting of cloves, suggesting the possible, direct use of interspecific hybrids as new crops.

Following the demonstration of the biolistic delivery of foreign genes into onion epidermal tissue (Klein et al. 1987; Eady et al. 1996), transient expression was described for the *uidA* (*gus*) reporter gene in garlic, using this technique (Barandiaran et al. 1998; Robledo-Paz et al. 2004). The technique was adapted to the generation of herbicide-resistant transgenic garlic plants (Park et al. 2002). In addition, the *Agrobacterium*-mediated method of garlic transformation and regeneration was reported (Kondo et al. 2000: Eady et al. 2005). Recently, this method was also employed for the production of transgenic garlic lines resistant to beet armyworm, using the *cry1Ca* resistance gene from *Bacillus thuringiensis* (Zheng et al. 2004).

In order to produce transgenic plants efficiently, target tissues must be used appropriate for both biolistic transformation and regeneration. Callus has often been used for transformation of monocotyledon because it may remain for an extended period in the early-log growth phase and, thus, continuously maintain active mitotic cells. Since callus tissues actively synthesize cell walls, membranes and other organelles in dividing cells, foreign genes can easily be introduced into the cells and integrated into the genomes without serious disruption of tissues. Barandiaran et al. (1998) detected a high endogenous nuclease activity that might degrade exogenous DNA in garlic tissues. It was shown that treatment of garlic tissue with aurintricarboxylic acid, an inhibitor of endogenous nuclease, significantly increased the transformation efficiency (Baradiaran et al. 1998; Sawahel 2002). An effective biolistic transformation procedure was established recently, as illustrated by the generation of transgenic plants resistant to chlorsulfuron, a sulfonylurea

▶ **Fig. 3.** Transgenic garlic. **A** Preparation and transformation of garlic calli. Calli induced from apical meristems of garlic cloves. Most of the newly induced calli are covered with viscous, gummy material (*left*). Calli exhibiting brittle, crispy surfaces without the viscous material (*right*) are selected repeatedly in subcultures and used for gene transformation. **B** Embedding cultures for garlic callus selection. Transformed garlic calli are embedded and grown in selective medium containing 50 $mg\,l^{-1}$ hygromycin B for at least 2 months after bombardment. The inset shows histochemical staining of transformed garlic calli for GUS activity. Hygromycin-B-resistant calli, selected for over 2 months (*left*), are compared with non-transformed calli (*right*). The calli were stained with X-Gluc for 24 h and destained in 95% ethanol. **C** Regeneration of transgenic garlic shoots. *Left* Regenerated shoots. The selected calli were allowed to regenerate on regeneration medium for 6 months. *Right* Propagated multishoots. The selected shoots were propagated for 4 months on multishoot medium containing 20 $mg l^{-1}$ hygromycin B. **D** Transgenic garlic plants. *Left* Bulb induction. Bulbs and roots were induced from regenerated hygromycin-B-resistant shoots by culture, for 4 months, in induction medium lacking hygromycin B. *Right* A transgenic garlic plant. Bulbs were germinated in soil and grown to maturity. *Size bar* in each panel = 1 cm. From Park et al. (2002), with the permission of Kluwer, Dordrecht

herbicide (Park et al. 2002). In this study, callus subcultured from the apical meristems of garlic cloves were repeatedly selected over six months until the calli exhibited brittle, non-mucilaginous surfaces, which were found to result in increased transformation efficiencies (Fig. 3A). Herbicide resistance was conferred by transforming garlic callus with a mutant *Arabidopsis* gene encoding a chlorsulfuron-insensitive acetolactate synthase (ALS; Haughn and Somerville 1986). Garlic calli were bombarded twice with tungsten particles coated with a construct containing the *ALS* gene, the cauliflower mosaic virus 35S promoter, the β-glucuronidase (GUS) reporter gene and the hygromycin



phosphotransferase (HPT) selectable marker gene. Transformed calli were selected efficiently by embedding them in semi-solid agar medium containing hygromycin B (Fig. 3B) and the transformed calli were grown to adult plants (Fig. 3C,D). The multishoot (Fig. 3C) propagation technique employed in this experiment for efficient reproduction of the progeny could be widely utilized for the commercial mass production of seed bulbs. The regenerated adult plants survived in the presence of 3 mg l^{-1} chlorsulfuron, demonstrating that their ALS protein was insensitive to the herbicide.

For successful gene transformation, the establishment of an efficient procedure for the regeneration of garlic callus into whole plants is a critical prerequisite. However, for garlic, the rate of multiplication and the number of plantlets regenerated per explant are too low to be practical. Garlic shoot regeneration from callus has been reported using several explant sources, including root tips, shoot tips, leaf tissue and basal plates. Advances have been made toward the regeneration of tissue-cultured garlic. The newly developed in vitro garlic shoot regeneration protocol has the potential to be applied to the production of transgenic garlic, in conjunction with *Agrobacterium*-mediated (Kondo et al. 2000) and biolistic (Park et al. 2002) gene transformation procedures. These transformation protocols are expected to be applicable to the generation of transgenic garlic varieties that possess various beneficial traits. Like all field crops, garlic plants are challenged by a variety of pathogens and weed competitors. Thus, breeding new varieties that possess agronomically beneficial traits, such as resistance to pathogens or herbicides, would increase the yield and quality of the garlic crop.

Because garlic plants are propagated through vegetative reproduction, they are readily infected by a variety of viruses; and these viruses constitute one of the most serious problems in garlic farming. The leaves of garlic plants infected by viruses exhibit mosaic or streak symptoms and eventually produce bulbs and cloves of reduced size. Even if virus-free seed garlic plants could be generated by laboratory techniques, such as tissue culture, they would be infected with viruses again when planted in the field, because garlic-infecting viruses are nearly endemic. Garlic viruses include carla-, poty-, allexi- and potex-viruses. Viral infection leads to yellow mosaic or streak symptoms on leaves and results in a smaller bulb size. Chronic and persistent infection through vegetative propagation might occur, owing to the discreet symptoms and slow multiplication of these viruses. When attempting to control these viral diseases, the first essential steps are to establish the identity of the viruses responsible and to determine their molecular characteristics. Two main sap-transmissible filamentous garlic viruses have been described, according to a previously classification, based on their morphological and cytopathological properties, namely garlic latent virus (GLV or garlic carlavirus) and garlic mosaic virus (GMV or garlic potyvirus). Song et al. (2002) determined the complete GLV genome sequence. The presence of GLV in virus-infected garlic plants has also been confirmed by immunoelectron microscopy, using an anti-GLV coat protein (CP) polyclonal antibody. An unassigned garlic virus,

garlic virus X (GVX), which has features of potexviruses and carlaviruses, has also been reported (Song et al. 1997) and the complete genome sequence of GVX has been determined (Song et al. 1998). GVX contains a single-stranded positive-sense RNA of about 8000 nucleotides and has a flexuous rod shape with a length of 700–800 nm. Sumi et al. (1999) determined the complete genome sequences of garlic viruses A and C, which have structures similar to the GVX genome. These viruses have been assigned to the genus *Allexivirus* by the International Committee on the Taxonomy of Viruses. This new type of virus appears to be responsible for the mosaic symptoms observed in garlic plants from Korea, Japan and Europe. Various filamentous viruses associated with mosaic symptoms in garlic have been reported and are often present in complex mixtures.

It has been shown that transformation of a ribosome inactivating protein (RIP) gene into plants can lead to enhanced viral resistance (Lodge et al. 1993; Moon et al. 1997), an approach that offers the possibility of developing plants resistant to a broad spectrum of viruses through the expression of a single gene. Thus, an attempt to generate virus-free garlic is in progress, by transforming a RIP gene (unpublished results).

The alliin present in uninjured garlic cloves is colorless and odorless. Upon bruising, cutting, or crushing of the cloves, alliinase is released from the vacuoles of the cells and catalyzes the breakdown of alliin into numerous constituents, including allicin. Allicin has a characteristic odor, but breaks down into diallyl disulfide and ajoene when heat is applied. Although allicin is an important compound for the medicinal properties of garlic, there is some demand for odorless or weak-odored garlic varieties. Transgenic garlic plants, transformed with an antisense *ALS* gene or genes encoding allicin-degrading enzymes, could be generated in the near future.

4 Future Perspectives

Concerns related to the health issues associated with food quality and safety are widespread. The chemopreventive activities of *Allium* spp are thought to be related to the organosulfur compounds they contain. Extensive research has been conducted recently to investigate the beneficial and medicinal properties of garlic and onions. In particular, the use of these agents in the treatment and prevention of cardiovascular disease and cancer constitutes an area of considerable interest and investigative effort. Although the question of how these compounds result in chemoprevention has not yet been fully answered, several modes of action have been proposed (Knowles and Milner 2001; Griffiths et al. 2002; Thomson and Ali 2003). These proposed modes of action were summarized by Izzo et al. (2004) as: (1) effects on drug metabolism, (2) antioxidant activity, (3) inhibition of tumor growth, (4) induction of apoptosis and (5) effective stimulation of the immune response. At the present time, the com-

ponent(s) of the *Allium* spp that are essential for delivering health benefits are unknown. Detailed chemical profiles of onion bulb compositions are needed to determine the *Allium* compounds that offer enhanced health benefits. Identifying the component(s) that provide health benefits and understanding the biosynthetic pathway of such component(s) could lead to the development of onion as a "functional food".

The future direction in *Allium* biotechnology must be related to the demands of consumer and producer. An important aspect is the development of new varieties with traits such as: (1) resistance to major pests/diseases/viruses, (2) variety in bulb shape, color and firmness, (3) improved profiles of secondary sulfur compounds, (4) mild, sweet (low pungency) flavor and (5) improved shelf life (analogous to the Flavr Savr tomato). Successful transformation of *Allium* spp will allow biochemical and molecular biological studies of *Allium* genes and enzymes, which synthesize and catabolize the cellular constituents, with medicinal value. The large genome sizes of *Allium* spp may impede the use of genomics in the study of secondary metabolism and developmental processes. The accumulation of such information, however, could aid in the development of new *Allium* varieties. In the near future, breeding programs and a genetic transformation system could make possible the production of specially modified onion cultivars. In addition, transgenic *Allium* varieties could be used as bioreactors to produce the biochemical compounds that are therapeutically useful. Production of more diverse *Allium* genotypes could lead to the rapid diversification of the *Allium* market.

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V.3 Mint

F. Jullien[1]

1 Introduction

1.1 Systematics of Mints and the Economic Importance of Their Oils

The genus *Mentha* belongs to the family Lamiaceae and is distributed across all five continents. According to Harley and Brighton (1977), *Mentha* can be subdivided into five sections, namely *Audibertia*, *Eriodontes*, *Pulegium*, *Preslia* and *Mentha*. The section *Mentha* comprises most of the species that have significant commercial benefit. Species in this section are widespread in Asia, Europe and Africa and include *M. suaveolens*, *M. longifolia*, *M. aquatica* and *M. arvensis*, plus many inter- and intra-specific hybrids with a complex taxonomy. Three mechanisms can explain why hybridisation is important: (1) interspecific hybrids occur with great frequency both in wild populations and in cultivation whenever two or more species come into breeding contact, (2) vegetative multiplication by a vigorous rhizome system allows the settlement of hybrids within wild populations and (3) polyploidy restores hybrid fertility and leads to phenotypic changes. Historically, mint systematic has been based on morphological criteria, e. g. inflorescence morphology, calyx or corolla pilosity (Linneaus 1767; Malinvaud 1880; Harley 1972; Lebeau 1974). Over time, plants with similar phenotypes have been described and many synonymous names have been given to the same species, with the consequence of making mint nomenclature very confusing. Cytogenetic studies have proved of particular value to unify mint taxonomy and to characterise hybrids and polyploids (Ruttle 1931; Morton 1956; Sharma and Bhattacharyya 1959; Harley 1967, 1972; Singh and Sharma 1986). Chemical polymorphism of mint oils has also been a valuable marker for mint taxonomy (Lawrence 1978, 1980), both at the inter- and infra-specific levels; and several chemotypes have been characterised (Kokkini 1991). More recently, RAPD- or AFLP-based molecular markers have been developed to evaluate genetic distances and genetic diversity in cultivated mints (Fenwick and Ward 2001), or to assess phyletic relationships within the section *Mentha* (Khanuja et al. 2000; Gobert et al. 2002; Shasany et al. 2005). Analysis based on a combination of the above techniques suggested that peppermint and spearmint may be derived from the hybridisation of several known species (Fig. 1). Additionally, DNA poly-

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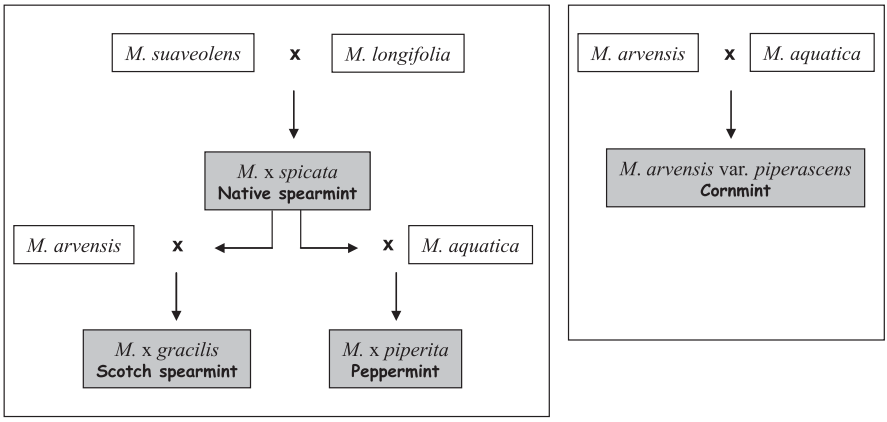

Fig. 1. Breeding scheme between wild species. Cultivated hybrids are in *shaded boxes*

morphism analysis indicates the lack of genetic diversity within these hybrid species.

Mints are mostly cultivated for their oil, which is used as an additive by the food, cosmetic and pharmaceutical industries. Mint essential oil production (excluding cornmint) is the world's second-largest essential oil crop behind citrus, with a cumulative output of approximately 6,000 t year^{-1}, including 2,000 t year^{-1} for scotch and native spearmint and twice this amount for peppermint. The United States is the main producer of peppermint and spearmint oils. Most of the production is located in Indiana, Michigan, Oregon, Washington and California. Nevertheless, the production of spearmint oil has been increased recently in Australia, Canada and Argentina in response to increasing demand. Cornmint is the poor man's mint oil. It is the main source for natural (−)-menthol, which may represent up to 80% of the mature oil content. The low added value of cornmint oil leads to its mass production (approx. 10,000 t year^{-1}) in countries with low labour cost, such as China, India and Brazil. The earliest written records of the usage of mints can be dated back from the times of the Pharaohs in ancient Egypt, where leaves or leaf extracts were used as perfume and for culinary flavouring. During Roman times, mint was additionally incorporated into tooth pastes. Knowledge of the medicinal properties of mints is also very ancient (Banthorpe 1996). Fresh or dried leaves and essential oils of peppermint, spearmint and cornmint have long been used as syrups in confection and infusions for their antiseptic and anaesthetic properties. The antiseptic properties of mint oils are supported by many recent studies that demonstrate their capacity to inhibit the growth of several pathogenic bacteria (Imai et al. 2001), including some that are involved in respiratory tract diseases (Inouye et al. 2001). An understanding of the mechanism of inhibition of bacterial growth is still partial and may derive from a combination of: (1) alteration of lipid membrane integrity and cell perme-

ability, (2) modification of enzyme activities and more especially those related to energy flow and (3) destruction or inactivation of nucleic acids (Kim et al. 1995).

Peppermint oil also acts as an antispasmodic agent. Experiments using in vitro guinea-pig ileum showed that ingestion of peppermint oil from watery infusions induced relaxation of gastric walls. The antispasmodic action was suggested to derive from a rise in intracellular cAMP levels (Lis-Balchin and Hart 1999). Peppermint oil is also very efficient in vivo to reduce a hyperperistaltic state in the stomach of patients during endoscopy treatment. This is probably related to the ability of the oil to inhibit smooth muscle contraction and leads to the suggestion that mint oils could be used as a replacement to current synthetic chemical drugs to lower the incidence of side-effects (Asao et al. 2003; Micklefield et al. 2003).

Mint oils possess psychoactive action and modify behaviour. Injection of peppermint oil into mice leads to an increase in ambulatory activity. Most of the chemical constituents of the oil display biological activity, except pinanes and limonene (Umezu et al. 2001). The influence of essential oil on human attention is not clear (Limberger et al. 2001). Nevertheless, it has been shown that fragrance perception of essential oils is affected by mental fatigue or physical activity and that positive or negative perception of fragrance is accompanied by modifications of brain activity (Sugawara et al. 1999; Satoh et al. 2003).

Antifungal activities of peppermint oil have been reported on several pathogenic fungi (Ezzat 2001). Menthol was found to be the most active ingredient of peppermint essential oil, while menthone alone was found to be ineffective (Edris and Farrag 2003). These observations are in agreement with the hypothesis that the release of volatiles emitted by the vegetative parts of mint may act as a defence mechanism against microbial pathogens. The spectrum of protection may be wider and probably includes herbivores and insect pests, since mint oils can act as phagodeterrents and have both repellent and larvicidal activity against several mosquito species (Ansari et al. 2000; Barnard 1999). In addition to their insecticidal properties, some mint essential oil constituents, like menthone, exhibit a genotoxic activity (Franzios et al. 1997).

1.2 Biosynthesis of Monoterpenes in Mint

Mint oils are composed mainly of monoterpenes that are secondary metabolites composed of a C10 hydrocarbon skeleton. Their biological roles are to repel herbivores, insects and microbial pathogens, as already mentioned above, while attracting pollinators or acting in tritrophic relations (Pichersky and Gershenzon 2002). The metabolism of monoterpenes can be subdivided into four general areas, including origin of the acyclic precursor, isopentenylpyrophosphate (IPP), cyclisation reactions and secondary transformations of cyclic compounds and catabolism.

For a long time, it has been assumed that IPP was synthesised in the cytosol from acetyl-CoA via the mevalonic acid pathway (MVA). Ten years ago, the group of Rohmer et al. (1993) discovered an alternative pathway in eubacteria. In this metabolic path, pyruvate and glyceraldehyde 3-phosphate (GA-3-P) are the first precursors (Rohmer et al. 1996). In a thiamin-dependent transketolase-type reaction, a C2 unit derived from pyruvate (hydroxyethylthiamine) is transferred to GA-3-P, whereby 1-deoxy-D-xylulose-5-P is formed (DOXP). This step is catalysed by the enzyme DOXP-synthase, or DXS. In a second step, DOXP is transformed into 2C-methyl-D-erythritol-4-phosphate (MEP), involving an intramolecular rearrangement and reduction step catalysed by enzyme DOXP-reducto-isomerase (DXR). MEP has been chosen to name this pathway and this compound, after several reactions, generates IPP as the end-product. The unveiling of the existence of the MEP pathway in plastids of plants was achieved a few years later (Arigoni et al. 1997; Lichtenthaler et al. 1997), explaining why some earlier labelling experiments were not in agreement with the MVA pathway. The involvement of MEP pathway in monoterpene synthesis of mint has been demonstrated using cell cultures fed with ^{13}C-glucose (Eisenreich et al. 1997; Fowler et al. 1999). Over the following two years, all steps of the MEP pathway were elucidated with impressive rapidity; and this subject has been largely reviewed (Lichtenthaler 1999; Eisenreich et al. 2001; Rodriguez-Concepcion and Boronat 2002). Thus, plants are able to produce IPP in the cytosol and plastids via the MVA and MEP pathways, respectively (McCaskill and Croteau 1999a; Fig. 2). The presence of these two biosynthetic routes agrees with the hypothesised origin of plastids as endosymbionts and the presence of the MEP pathway in bacteria (Lichtenthaler et al. 1997). More recently, evidence has emerged that a dichotomy of terpenoid biosynthesis exists in plants, leading to the production of sesquiterpenes (C15) and triterpenes (C30) in the cytosol and isoprene (C5), monoterpenes (C10), diterpenes (C20) and carotenoids (C40) in plastids. Although a compartmentation of these two pathways is admitted, exchanges of IPP and GPP between plastids and cytosol have been observed during labelling experiments (Adam et al. 1999; Schuhr et al. 2003). At the end of both pathways, IPP is first isomerised into dimethyl-allylpyrophosphate (DMAPP), although both isomers are directly produced by the MEP pathway, therefore making the role of IPP isomerase non-essential (Hahn et al. 1999; Lange et al. 2001). The presence of both IPP and DMAPP facilitates their condensation "head to tail" and the synthesis of geranylpyrophosphate (GPP), the genuine precursor of most monoterpenes. Further steps of condensation of isoprene units to generate the precursors of mono-, sesqui- and diterpenes are catalysed by a family of enzymes named prenyltransferases (Croteau 1987; Alonso and Croteau 1993).

Monoterpene synthases (cyclases) catalyse the formation of acyclic or cyclic monoterpenes from GPP. These enzymes are of major interest, since they construct the basic carbon skeletons and thus define structural subfamilies of monoterpenes like *p*-menthane or pinanes in mints (Fig. 3). Some cyclisation reactions are highly stereo-specific and lead to the synthesis of only one given

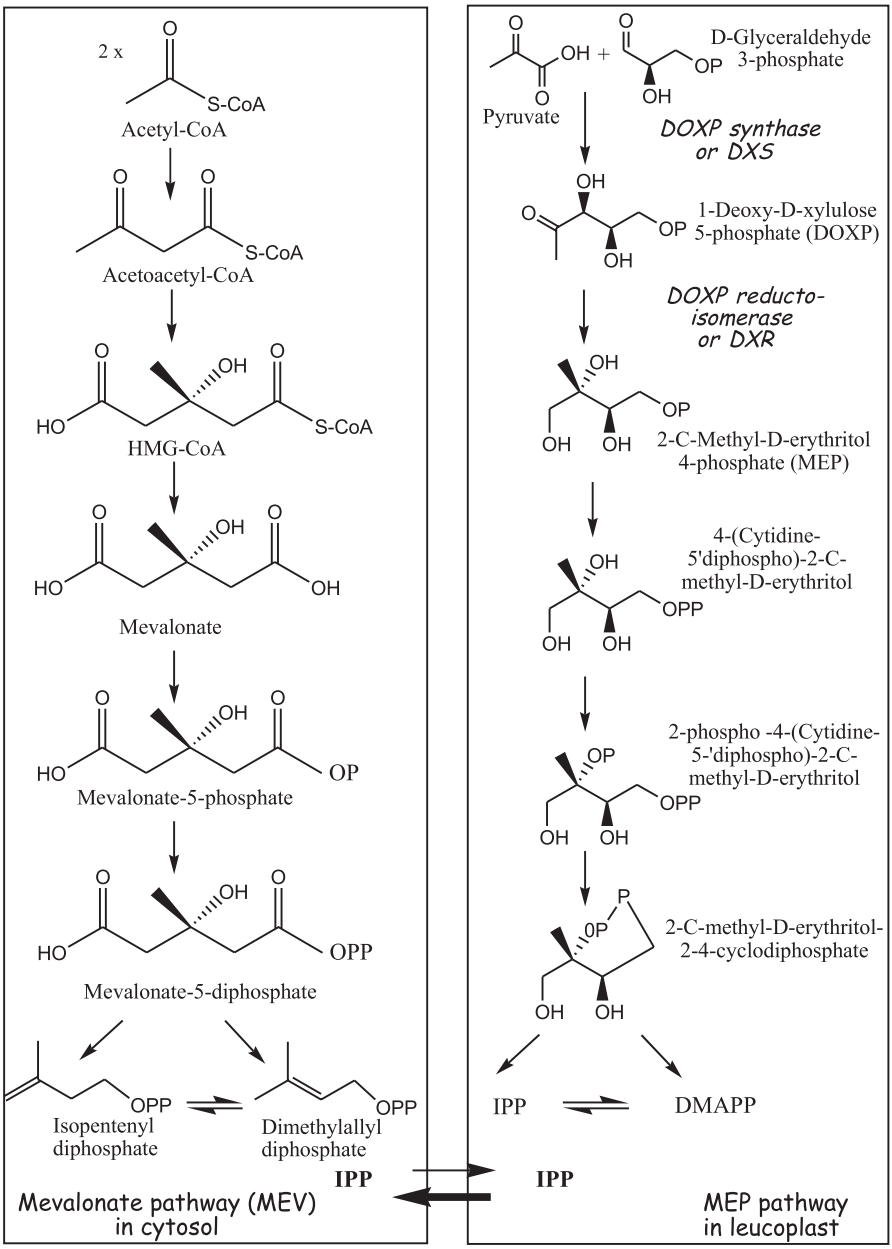

Fig. 2. Biosynthesis of IPP and DMAPP via the MEV and the MEP pathways (Mahmoud and Croteau 2001). *IPP* Isopentenyl diphosphate, *DMAPP* dimethylallyldiphosphate, *MEV* mevalonate, *MEP* 2-C-methyl-D-erythritol-4-phosphate

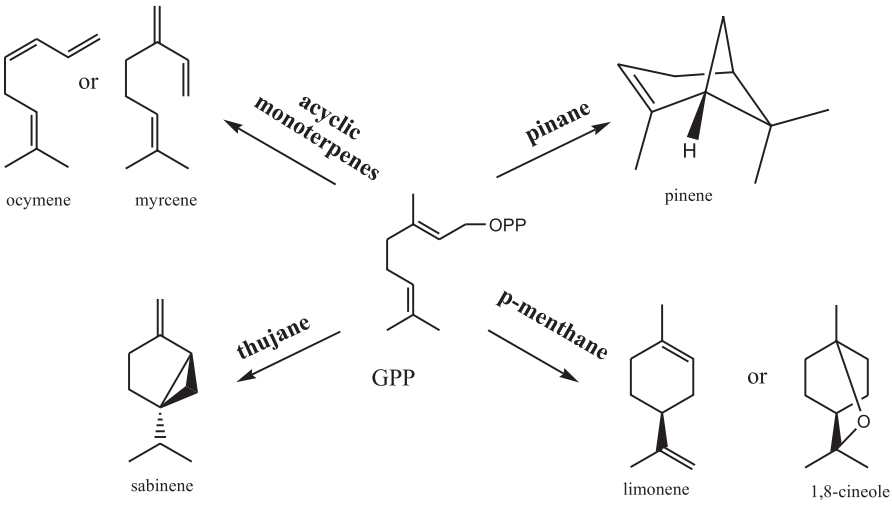

Fig. 3. Chemical classes of monoterpenes in peppermint derived from different cyclisation processes (Wise and Croteau 1999)

enantiomeric form. For example, two distinct limonene synthases have been characterised, one leading to (+)-limonene in caraway (Bouwmeester et al. 1998) and one responsible for the formation of (−)-limonene in mints (Colby et al. 1993). Nevertheless, other synthases catalyse the co-synthesis of several products as exemplified by the activity of (−)-limonene synthase of spearmint, which leads to the production of limonene (94%) and myrcene and pinenes (6%) (Rajaonarivony et al. 1992).

Even though several minor monoterpenes of mint oils like pinenes or myrcene do not undergo any modifications after cyclisation of GPP, most, like carvone in spearmint or menthol in peppermint, require secondary modifications via oxidation, reduction and isomerisation reactions. The diversity of such modifications explains why plants secrete such a large array of monoterpenes. In the *p*-menthane pathway, for example (Wise and Croteau 1999), limonene is hydroxylated in C3 or C6 depending of the mint species to respectively yield (−)-*trans*-isopiperitenol or (−)-*trans*-carveol (Fig. 4). These limonene-hydroxylases are characterised by a high degree of substrate specificity and regio-selectivity (Karp et al. 1990). Enzymes of monoterpenes metabolism that add further modification to C3- or C6-hydroxylation products are present in both peppermint and spearmint, so that a spearmint mutant where hydroxylation of limonene was artificially switched from C6 to C3 was shown to produce menthol (Croteau et al. 1991).

Metabolic turnover of monoterpenes has been observed in mints (Croteau 1991). Catabolic processes were seen to increase during the latest stages of plant development and were maximum after flowering where a loss of up to 40% of the total oil content has been observed over the course of a few

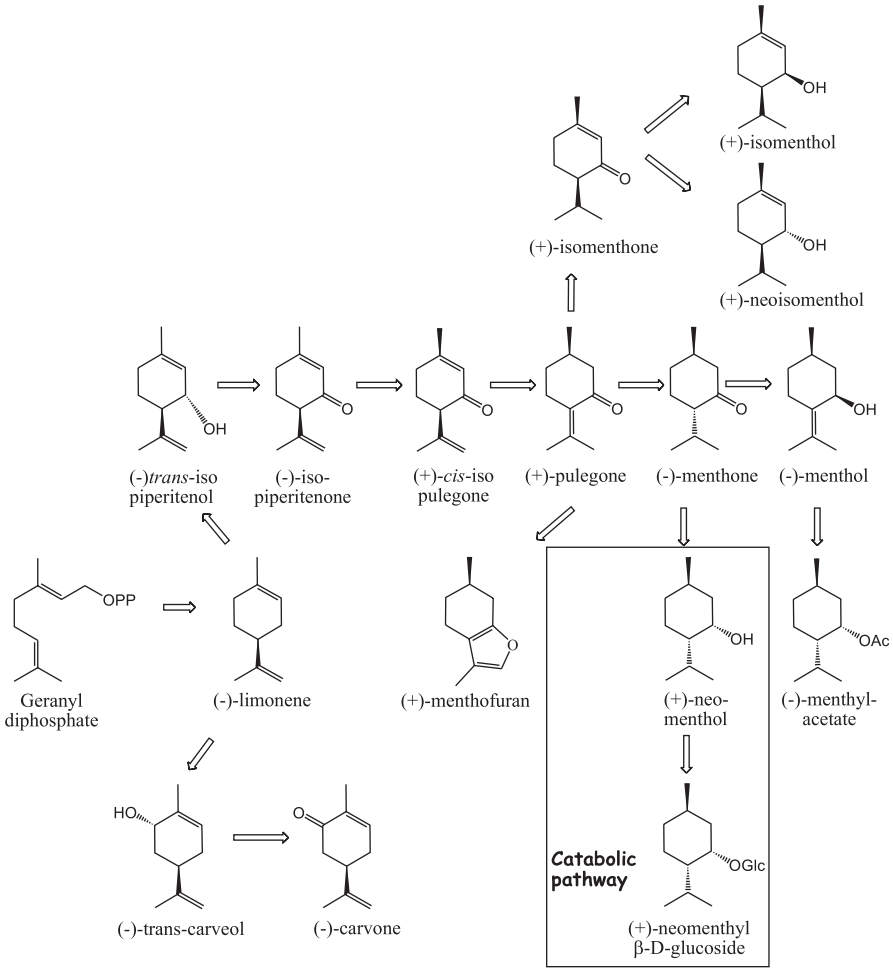

Fig. 4. Monoterpene biosynthesis in peppermint and spearmint (Large 1999b). Catabolic pathway is *framed*

weeks (Croteau 1987). Many field studies have been performed to optimise the harvest date with regards to yield and quality of the oil (Clark and Menary 1979; Perrin and Colson 1991; Rohloff et al. 2005). Radioactive labelling experiments on mature plants have revealed that menthone can be either reduced to menthol and menthyl acetate in epidermal glands or transformed into neomenthol in mesophyll cells, where it is further glycosylated (Croteau and Winters 1982). Neomenthol-β-D-glucoside is subsequently transported to rhizomes and catabolised by hydrolysis of the glucoside to the aglycone which undergoes oxidation to (−)-menthone and ring cleavage to yield the corresponding lactone (Croteau et al. 1984). Finally, the lactone is metabolised and transformed into

several unidentified non-volatile polar and non-polar products. This breakdown of monoterpenes allows the recycling of carbon and energy from foliar terpenes to supply the needs of the developing rhizomes (Croteau and Sood 1985).

1.3 Regulation of Monoterpene Biosynthesis in Mints

Biosynthetic pathways of mint monoterpenes are well known, but very little information is available on the underlying regulatory mechanisms. Monoterpene biosynthesis is restricted to specific tissues, where individual metabolic pathways are differentially regulated both by environmental factors and by the stage of plant development. All three aspects of the regulation of monoterpene biosynthesis (tissue specific expression, relationship with plant development, influence of biotic/abiotic factors) will be covered in this review.

Monoterpene biosynthesis is located in specialised epidermic structures named glandular trichomes. Two types of secretory structures, capitate and peltate trichomes, have been described in mint (Fahn 1979; Turner et al. 2000a). Capitate trichomes have a basal cell surmounted successively by a stalk cell and a secretory cell. Peltate trichomes, likewise, consist of single basal and stalk cells but, in contrast to the capitate structures, they possess eight radially distributed secretory cells (Fig. 5). Analysis of enzymatic activities from separated trichomes and histochemical studies of enzymes of the carvone pathway confirmed that peltate trichomes are the principal site of monoterpene biosynthesis (Gershenzon et al. 1989). In addition, intracellular compartmentation of the different biochemical steps inside glandular cells has been demonstrated. 4-(S)-Limonene synthase, which catalyses the first committed step

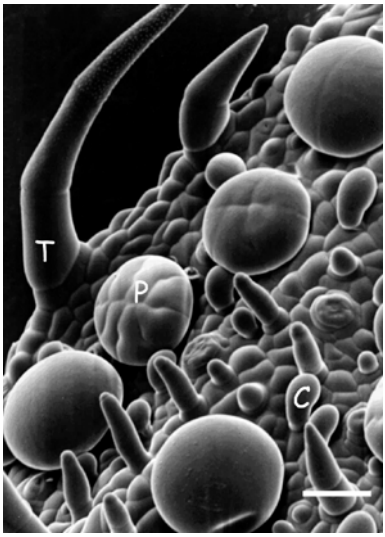

Fig. 5. Scanning electron micrograph of *M.* × *piperita* leaf trichomes. *C* Unicellular capitate secretory trichome, *P* peltate pluricellular trichomes formed of height secretory cells, *T* non-secretory trichome, *bar* 50 µm. Picture by courtesy of Prof. M. Colson

of the *p*-menthane pathway, is only present in leucoplasts of secretory cells (Turner et al. 1999), whereas enzymes involved in subsequent metabolic steps like hydroxylations accumulate in microsomal fractions (Lupien et al. 1999). While maturing, secretory peltate trichome cells undergo remarkable ultrastructural transformations which involve the differentiation of an abundant smooth endoplasmic reticulum (SER) into glandular disc-cells and the formation of a large sub-cuticlar secretion storage space on the outside surface of the gland cells (Turner et al. 2000b). These observations suggest the existence of a secretion route for monoterpenes from leucoplasts to the plasma membrane via SER. Recently, immunocytochemical localisations of several enzymes involved in monoterpene pathway suggested the involvement at least of four compartments, respectively leucoplasts, SER, mitochondria and cytoplasm, in the secretion of monoterpene (Turner and Croteau 2004).

Several studies of the distribution of peltate glandular trichomes on developing leaves of peppermint have been conducted in order to describe the time-course of epidermis differentiation (Maffei et al. 1989; Colson et al. 1993; Turner et al. 2000b). It was shown that new peltate glands continue to form until leaf expansion ceases and that regions of active gland initiation are unevenly distributed. Nevertheless, initiation of new glands was observed mostly in the basal immature part of the leaf and decreased towards the age. Moreover, the density of secretory glands was found to be unequal on leaves and subject to modifications depending on leaf expansion and position. Turner et al. (2000b) characterised several developmental stages of peltate trichomes and observed that the number of glands at the secretory stage increases as a function of leaf development. The chemical composition of monoterpene exudates of ageing leaves of peppermint has been studied (Duriyaprapan and Britten 1982; Maffei et al. 1989; Brun et al. 1991; Rohloff 1999) in order to assess the relationship between monoterpene biosynthesis and gland initiation and development. These experiments revealed that leaves sampled from the top to the base of plants were gradually enriched in menthol and cineole and depleted in menthone and limonene. The analysis of terpene secretion by individual peltate trichomes further indicated that these dynamic changes in monoterpene content commence at the distal end of the leaf and progressively move towards its base (Voirin and Bayet 1996). The rate of monoterpene biosynthesis, as determined by $^{14}CO_2$ incorporation, closely paralleled that of monoterpene accumulation and appeared to be directly related to monoterpene concentrations in peppermint leaves (Gershenzon et al. 2000). The loss of monoterpene by catabolic turnover or release through the cuticle was found to be low during leaf development. In addition, head space extracts had a different monoterpene profile than those made from glandular trichomes, suggesting that these volatilised products may derive from a separate secretory pathway. Since the rate of biosynthesis is the limiting step in monoterpene accumulation, interest has focused on the characterisation of gene expression and enzymatic activities of enzymes involved in the *p*-menthane pathway. All enzymes responsible for the transformation of limonene to menthone displayed very similar and co-ordinated expression

profiles, with a maximum of activity in 15-day-old leaves. Only menthone reductase, which catalyses the reduction of menthone to menthol, was expressed with 5 days of delay. The time-course of steady-state mRNA levels of four genes giving rise to *p*-menthane products showed the same pattern of expression as their corresponding enzymes, therefore suggesting that the developmental regulation of monoterpene biosynthesis in peppermint oil glands resides at the level of gene expression (McConkey et al. 2000).

Environmental factors such as day/night temperatures and light intensity are known to affect peppermint oil composition (Burbott and Loomis 1967; Clark and Menary 1980). These factors act mostly on the primary metabolism and consequently affect terpenoid synthesis. Light quality and day length are known to act on plant morphogenesis. Cultures of peppermint under additional blue or UV light displayed photomorphogenetic changes (stem elongation, leaf area reduction) and a modification of essential oil yield and composition (Maffei and Scannerini 1999; Maffei et al. 1999). Although it is hypothesised that light quality could lead to a modification of the expression of genes involved in the *p*-menthane pathway, no direct evidence has yet been published. X-ray irradiation experiments have been performed on dormant rhizomes of mint to introduce genetic mutations (Kak and Kaul 1981) and generate new cultivars with either improved essential oil characteristics or enhanced resistance against *Verticillium* wilt. Some peppermint clones have been selected for reduced sensitivity. However, information about the underlying molecular mechanisms was lacking (Johnson and Cummings 2000). More interestingly, a spearmint mutant exhibiting a monoterpene content similar to peppermint has been described (Croteau et al. 1991). In this mutant, gamma irradiation induced a dramatic alteration in oil composition resulting, in part, from an increased ability to hydroxylate limonene on C3 instead of C6 as in wild spearmint. Early expectations were that a mutation in the gene encoding for 6-limonene-hydroxylase would lead to a modification of the catalytic activity of the corresponding enzyme. Domain swapping and reciprocal site-directed mutagenesis experiments on C3 and C6 limonene hydroxylase enzymes supported this hypothesis and demonstrated that a single amino acid substitution converts the regiochemistry of the enzyme from a C6- to a C3-hydroxylase (Schalk and Croteau 2000). Nevertheless, a comparison of the cDNA sequences of mutant and wild spearmint limonene hydroxylase suggested that a mutation had not occurred within the structural gene for limonene hydroxylase and that a regulatory site controlling the expression of C3 or C6-limonene-hydroxylase gene had been affected (Bertea et al. 2003). The infection of mint by microorganisms is also known to affect both yield and composition of essential oils. Colonisation of mint roots by endophytic fungi induced a growth response characterised by a greater dry biomass, a sustained lowering of (+)-menthofuran production and an increase in (+)-menthol accumulation (Mucciarelli et al. 2003). However, infection with pathogenic fungi, such as *Puccinia menthae*, induced defoliation and a resulting loss of oil production (Edwards et al. 1999).

2 In Vitro Studies

2.1 Cell Culture and Monoterpene Biotransformation

The use of callus and cell suspensions in the micropropagation of mint was reviewed by Banthorpe (1996) and Cellarova (1992). This review therefore discusses only major advances and provides an update in this field of research. Mint cell cultures have been performed with the aim of producing monoterpenes either directly by the cell culture, or via the addition of precursor molecules. These studies have highlighted the existence of factors regulating the capacity of cultured cells to synthesize and accumulate monoterpenes.

Callus and cell suspensions of mint were first induced by Lin and Staba (1961), but the production of monoterpenes was absent or very low from such undifferentiated cell cultures (Suga et al. 1980; Lee et al. 1997). This led to the assumption that monoterpene production and accumulation were strictly associated with the differentiation of glandular trichome hairs (Wiermann 1981). This idea was supported by several observations. First, meristematic tissues of several mint species producing adventitious buds were able to synthesize monoterpene compounds (Bricout and Paupardin 1975; Hirata et al. 1990; Kawabe et al. 1993). Second, the monoterpenes produced by these clusters of buds were in lower concentrations, but similar to those synthesised by the mother plant (Bricout et al. 1978a). Third, microscopic studies revealed the presence of peltate glands on primary leaves surrounding buds (Medou et al. 1997). Nevertheless, the low production of monoterpene by undifferentiated cell suspensions may also be the result of a feedback regulation mechanism linked to the toxicity of terpene compounds. In one case where undifferentiated calli produced monoterpenes, histological studies revealed that giant cells were the site of terpene accumulation (Kireeva et al. 1978). This sequestration of monoterpenes controlled their toxicity and allowed the synthesis of monoterpenes by undifferentiated cells. The phytotoxicity of monoterpenes was confirmed by biotransformation studies where the glycosylation of exogenous monoterpenes by cell suspension as a detoxification mechanism was observed (Galun et al. 1983). Biotransformation studies have also suggested that several enzymes involved in the *p*-menthane pathway are potentially active in undifferentiated cells, so that cell suspensions of mint have the potential to transform (+) pulegone and (−) menthone into (+) isomenthone and (+) neomenthol, respectively (Aviv and Galun 1978; Aviv et al. 1983). Feeding suspension cells with monoterpenes like isopiperitenone led to the production of several hydroxylated isopiperitenone as a consequence of the expression of hydroxylases which produce different stereoisomers to those found in planta. Hydroxylation was followed most of the time by glycosylation and only glycosylated compounds were found within cells, while hydroxylated isopiperitenone remained in the medium (Park et al. 1997; Park and Kim 1998). The hydroxylase activity of cell suspension on exogenous monoterpenes seems to be common and has been observed with several compounds, like isomenthone,

isopiperitenone and carvone (Kim et al. 2002). The conversion of menthyl acetate or neomenthyl acetate into menthol or menthone has been observed in cell suspensions of *M. canadiensis* and *M.* × *piperita* (Werrmann and Knorr 1993). These biotransformations represent the reverse reactions to those observed in oil glands, where menthol is acetylated, and those in catabolic pathways, where neomenthol is acetylated. Because no acetate was found in the medium, it is supposed that this reaction is catalysed by an acetyltransferase working in an opposite direction to that in planta.

Many stress factors such as irradiation, colchicine and chitosan can regulate monoterpene biosynthesis. Bricout et al. (1978b) noted that tissues of peppermint cultured with colchicine exhibited a 3-fold increase in the synthesis of essential oil. The main effect of this chemical treatment was to increase the number of neoformed secretory glands which are known to undergo several endoreplication cycles during their differentiation (Berta et al. 1993). More intriguing is the action of elicitors on the yield of monoterpenes in peppermint cultured cells. Several elicitors, such as yeast extract (Lee et al. 1997), pectinase or chitosan (Ha et al. 1997; Chang et al. 1998), were able to enhance monoterpene production in plant cell cultures. These results are in agreement with the hypothesised role of monoterpenes as plant defence molecules. Son et al. (1998) confirmed this observation using jasmonic acid (JA). This compound was able to increase the biotransformation of isopiperitenone into hydroxyl-isopiperitenone via the action of a cytochrome P450 oxidase, whose mRNA was shown to accumulate in parallel. Although the molecular pathway by which JA regulates monoterpene synthesis is unclear, this compound may act more or less directly on the expression of the P450 oxidase involved in the *p*-menthane pathway. This hypothesis is in agreement with the observed ability of methyl jasmonate to induce terpene biosynthesis and terpene synthase gene expression in Norway spruce (Fäldt et al. 2003; Martin et al. 2003).

2.2 Plant Regeneration and Somaclonal Variation

Callus initiation for the establishment of cell suspensions was performed initially on leaf explants. In these preliminary experiments, organogenetic callus was produced (see Sect. 2.1). Plant regeneration has been carried out on different vegetative or sexual tissues of several mint species (Table 1). Early attempts were conducted on meristematic explants, such as axillary buds, embryos or the base of leaves, which are known to retain a high cell division activity (Repcakova et al. 1986; Kukreja et al. 1991; Caissard et al. 1996). The critical ingredients needed to induce adventitious buds formation in both systems were the cytokinins benzylaminopurine (BAP) or kinetin (KIN), applied in a range of concentrations from 0.1 $mg\,l^{-1}$ to 2.0 $mg\,l^{-1}$ (Repcakova et al. 1986; Rech and Pires 1986). Van Eck and Kitto (1990) showed that callus could be produced from non-meristematic parts such as petals, hypocotyls or cotyledons. An auxin [either naphthaleneacetic acid (NAA) or 2,4-D] was essential to ini-

Table 1. Explants used for plant regeneration in several *Mentha* species. *BL* Bases of leaves, *AB* axillary buds, *E* embryos, *S* seedlings, *FP* floral parts, *LD* leaf disks, *IN* internode

Species	Explants	Reference
M.× piperita	BL	Repcakova et al. (1986)
M. arvensis, *M. pulegium*, *M.× piperita*, *M.× spicata*	AB	Rech and Pires (1986)
M.× piperita, *M.× spicata*	E, S, FP	Van Eck and Kitto (1990)
M. arvensis	AB	Kukreja et al. (1991)
M.× citrata, *M.× piperita,*	LD	Van Eck and Kitto (1992)
M.× piperita	BL	Caissard et al. (1996)
M. arvensis	IN	Shasany et al. (1998)
M.× piperita, *M.× spicata*	LD, AB, IN	Faure et al. (1998)
M.× spicata	BL	Li et al. (1999)
M. arvensis	LD	Phatak and Heble (2002)

tiate callus production, but shoot differentiation could only be obtained from embryos of peppermint. In further experiments, non-meristematic explants like leaf disks (Van Eck and Kitto 1992) or internodes (Shasany et al. 1998) were used as explants capable of shoot regeneration. Both cases required the presence of auxin and a high concentration of cytokinin (ranging from 5 mg l^{-1} to 25 mg l^{-1}). Primary cultures obtained from leaf disk explants needed to be kept in the dark to avoid the callus becoming necrotic. The regenerative capacity of calli correlated positively with the age of the leaf disk, the youngest being optimal. For all of these reasons, plant regeneration from leaf disks remained difficult and bound to a low efficiency. We improved the efficiency of this system in our laboratory by adding mannitol to the medium as a plasmolytic agent and by using a stronger cytokinin, thidiazuron (TDZ). Under those conditions, leaf disks proved to be a suitable source of explant, with a percentage of regeneration of 78% for peppermint and 49% for spearmint (Faure et al. 1998). Further experiments have been conducted to evaluate the efficacy of this regeneration protocol on additional species (Diemer 2000). However, different species and cultivars did not respond in the same fashion and differed in their potentials for callus initiation and shoot differentiation (Table 2). Amongst cultivated mints, *M. arvensis* exhibited the highest regeneration potential (Kukreja et al. 1991; Phatak and Heble 2002).

Plant regeneration is a powerful tool for mass propagation which can also be used to produce desirable somaclonal variants. Such phenotypic variations result from either genetic variability already existing in the explant or meiotically stable epigenetic DNA modifications induced during culture (Matzke and Matzke 1996; Duncan 1997). Somaclonal variation has been used for the genetic improvement of cornmint (Kukreja et al. 1991). More than 280 plants were regenerated by adventitious bud formation and transferred to the field. Agronomic traits of these plants were compared with those of micropropagated control plants. The mean phenotypic character altered in the variants was plant height (greater or smaller than controls). Clones with higher herb yield also

Table 2. Regeneration of several mint genotypes from leaf disks in vitro. Regeneration data followed by two different letters in a column are significatively different ($\alpha = 0.05$), with a χ^2 comparison followed by a contrast test. For each species, data are calculated from one to seven independent experiments

Species	Cultivar	Number of explants	Regeneration (%)	
			6 weeks	10 weeks
M. × *piperita*	'38'	500	10.6a	52.8a
	'19'	445	7.4a	23.4b
	'Todd's'	290	35.9b	78.3c
	'Kubanskaya'	100	0.0c	0.0d
M. × *spicata*		280	49.3d	63.9e
M. aquatica		269	0.0c	26.8b
M. × *citrata*		310	40.6bd	65.2e
M. arvensis		350	72.6e	84.9c
M. suaveolens		60	0.0c	0.0d
M. longifolia		190	0.0c	0.5d

exhibited an increase in oil production, but the ratio of oil yield over biomass did not differ from control plants. In some cases, a modification in the composition of the oil was observed with an increase in menthol content. Recently, Dhawan et al. (2003) screened more than 2000 somaclones for their ability to grow on a selection medium containing menthol. After several rounds of selection, they selected only five clones with an increased production of menthol per gram of leaf biomass. This last report underlined how the induction and selection of somaclonal variation remains an empirical technique based on the screening of large samples.

2.3 Protoplast Isolation and Somatic Hybridization

This field of research has been poorly investigated and only a few successful studies have been reported. Plant regeneration from leaf-derived protoplasts of peppermint was achieved first by Sato et al. (1993). While isolation was easily performed from in vitro grown leaves incubated with a combination of pectinase and cellulase, several factors proved to be critical to obtain sustainable cell division. Then, NAA (1.0 mg l^{-1}) and BAP (0.4 mg l^{-1}) were added to B5 salts media (more suitable than MS medium; Murashige and Skoog 1962; Gamborg et al. 1968) to induce callogenesis and the production of cell colonies. Darkness was also important to avoid cell necrosis and an osmolarity of 0.2 M mannitol was needed to obtain firm green callus. Light and a high concentration of cytokinin (BAP at 1 mg l^{-1} or 5 mg l^{-1}) favoured shoot differentiation, but the frequency remained low (8% of calli). Several synthetic cytokinins have been used to induce caulogenesis, like TDZ (Chaput et al. 1996) or 4-phenyl urea (Sato et al. 1994). In all cases, the frequency of regenerating calli remained lower than 10% and was mainly cultivar-dependent (Jullien et al. 1998). In conclusion,

plant regeneration from protoplasts of mint is still time-consuming, exhibits a low efficiency and can be obtained only with some cultivars of peppermint, orange mint, spearmint and gingermint (*Mentha gracilis* cv. *variegata*).

Because peppermint is almost sterile, its hybridisation with another species is very unlikely. Protoplast fusion is, therefore, an attractive alternative strategy for the production of new, improved mint hybrids. Somatic hybrids have been reported between peppermint and gingermint (Sato et al. 1996) and between peppermint and spearmint (Krasnyanski et al. 1998). In both experiments, somatic hybrids were selected on the basis of leaf shape and inflorescence types that were intermediate with those of their parents. RAPD and Southern blot analyses confirmed the hybrid origin of the selected clones. Nevertheless, physiological features, such as oil composition or *Verticillium* resistance, remained similar to one of the two parents. Only one selected hybrid of peppermint and gingermint accumulated equal proportions of the monoterpenes produced by the two parents: menthol and linalool. The limited genetic modification observed in these somatic hybrids may result from the polyploidy nature of the parents in these experiments (spearmint: tetraploid; gingermint and peppermint: hexaploid).

3 Transformation and Genetic Bioengineering

3.1 Transformation Protocols for Mint Species

Early transformation experiments aimed at: (1) developing axenic differentiated culture for in vitro biosynthesis of monoterpenes and (2) establishing a general transformation protocol using reporter genes for further genetic manipulation with genes of agronomic interest, e. g. genes involved in monoterpene synthesis, and pathogen and herbicide resistances. These first attempts at mint transformation are summarized in Table 3.

Spencer et al. (1990) obtained axenic crown galls of *M. citrata* with several wild strains of *Agrobacterium tumefaciens*. These calli could be maintained by regular subculture but did not synthesize monoterpenes. Co-culture of stems with T37 and C58 strains of *Agrobacterium* produced galls that subsequently differentiated into shooty teratomas possessing oil glands. This shoot differentiation did not require the addition of growth regulators in the culture medium and was induced by tDNA insertion and expression of chromosomic bacterial genes coding for auxin and cytokinin synthesis. Bacterial strains mutated in genes leading to auxin (*aux1*, *aux2*) or cytokinin biosynthesis (*ipt*: isopentenyl transferase) were unable to induce shoot organogenesis. Successful transformation was confirmed by the accumulation of nopaline and by Southern blot analysis of the tDNA gene *ipt*. Monoterpene secretions by these organogenic tissues contained linalool and linalyl-acetate as major constituents. In further experiments, Spencer et al. (1993a, b) reported the successful transformation of

Table 3. Summary of transformation experiments on different mint species with wild-type strains of *Agrobacterium tumefaciens* or with disarmed strains carrying reporter genes

Species	*Agrobacterium* strain	Plasmid	Explant used	Transformation events	Reference
M. × *citrata*	T37, C58	Nopaline pTi	Stem	Shooty teratomas	Spencer et al. (1990)
M. × *piperita*, *M.* × *citrata*	T37	Nopaline pTi p35S *ipt*	Stem	Shooty teratomas	Spencer et al. (1993a, b)
M. × *piperita*, *M.* × *spicata*, *M.* × *gracilis*	A281	pTi Bo542	Leaf disks, petioles	Axenic callus	Berry et al. (1996)
M. × *piperita*	C58 MP90/GI, GV 2260/GI	p35S GUS-INT	Leaves, petioles	Transient GUS expression	Caissard et al. (1996)
M. × *piperita*	C58 MP90/GI	p35S GUS-INT	Leaf disks	Stable integration	Diemer et al. (1998, 1999)
M. arvensis	GV 2260/GI	p35S GUS-INT		GUS and NPTII genes	
M. × *spicata*	AGL1/BGI EHA105/MOG	pB+GIN pMOG410			
M.× *piperita*	EHA105	pBISN1, pOC (from pBI121)	Leaves with petioles	Stable integration, GUS and NPTII genes	Niu et al. (1998, 2000)

Mentha × *piperita*. Caulogenesis of axenic galls was later improved with the use of a disarmed T37 strain of *Agrobacterium* carrying a binary vector with the *ipt* coding sequence under the control of the powerful 35S promoter, in agreement with the higher concentration of exogenous cytokinin needed to induce shoot organogenesis during plant regeneration. Monoterpene production by shooty teratomas matched the composition of the essential oil of the parent plants and the two major constituents were menthofuran and menthol. Subsequently, Berry et al. (1996) tried to transform several mint species with the hypervirulent *Agrobacterium* strain A281. Even though they could obtain transformed calli, they failed to induce shoot differentiation on media containing 10 mg l^{-1} BAP, which may reflect the low capacity of this particular cv. for plant regeneration. Because wild strains of *Agrobacterium* modify the plant hormonal balance in an uncontrolled manner, we conducted experiments with disarmed pTi *A. tumefaciens* strains bearing a binary vector with reporter genes coding for β-glucuronidase (GUS) and neomycin phosphotransferase (NPTII) and we reported the first transient expression of GUS in peppermint leaves transformed with disarmed *A. tumefaciens* strains and biolistics using pBI221.1 (Caissard et al. 1996). This experiment demonstrated that leaves could regenerate shoots and that leaf tissues can be used for genetic engineering. Improvements in the regeneration and transformation protocols led us (Diemer et al. 1998; Niu et al.

1998) to create transgenic peppermint via co-cultivation with *A. tumefaciens*. Two critical factors were identified in our experiments: the *Agrobacterium* strains and the co-culture period. Thus, by using a 5-day co-cultivation with the strain EHA105/MOG, 45% of leaf disks produced transformed calli on selection medium containing 50 mg l^{-1} kanamycin. These calli underwent shoot organogenesis and transformed plants were rooted on medium containing 150 mg l^{-1} kanamycin. On this selection medium, and with an improved regeneration rate, the transformation efficiency could reach up to 10%, with a low rate escape or chimaeric plant formation. This transformation protocol has been used on spearmint and cornmint (Diemer et al. 1999). Niu et al. (1998) reported similar results using the same strain EHA105, but with another binary plasmid (pBISN1). The explants used for co-cultivation were leaf bases with attached petiole. Transformed plants were selected on medium with 15 mg l^{-1} kanamycin. Under these conditions, a transformation efficiency of 1% was reported. Transient GUS expression obtained by biolistics did not yield stable transformants with this technique. Niu et al. (2000) further improved the efficiency of their transformation system from 1% to 20% by omitting coconut water from the co-cultivation medium, by adding a feeder layer of tobacco cells onto the medium and by increasing co-cultivation time and selection pressure (50 mg l^{-1} vs. 15 mg l^{-1} kanamycin). Reliable peppermint transformation protocols are, therefore, now available for genetic improvement of this essential oil crop.

3.2 Alteration of Terpene Biosynthesis in Transgenic Mint

Mint is probably the plant where the metabolic pathway leading to monoterpene synthesis has been most studied (Wise and Croteau 1999; Croteau and Davis 2005). All enzymatic steps are well characterized and a cDNA library has been constructed from isolated secretory glands (Lange et al. 2000). A comparison of predicted DNA sequences of purified enzymes with an EST database led to the cloning of several genes involved in monoterpene biosynthesis by Lange and co-workers (2000; Table 4). In contrast, knowledge of the regulatory mechanisms underlying monoterpene production is still poor. Progress in mint transformation and the availability of genes involved in terpene biosynthesis have opened new avenues to investigate the regulation of monoterpene synthesis and attempt to improve mint oil production.

4S-Limonene synthase (*ls*; Colby et al. 1993) is the first gene to be overexpressed in transgenic peppermint (Krasnyanski et al. 1999; Diemer et al. 2001; Mahmoud et al. 2004). This enzyme carries the first committed step of the *p*-menthane pathway from GPP and may act as a control point for monoterpene production. Krasnyanski et al. (1999) obtained transformed peppermint using both co-cultivation of *Agrobacterium* with peppermint internodes and direct gene transfer into protoplasts. Both transformation approaches were successful, but analysis of oil constitution did not reveal differences between

Table 4. Summary of genes associated with biosynthesis of terpenes isolated from different mint species

Gene	Function	Accession Number	Plant	Reference
DXP synthase	Transketolase	AF019383	*M.* × *piperita*	Lange et al. (1998)
DXR	Reductoisomerase	AF116825	*M.* × *piperita*	Lange and Croteau (1999a)
Isopentenylphosphate kinase	Kinase	AF179283	*M.* × *piperita*	Lange and Croteau (1999c)
GPP synthase	Prenyltransferase	AF182827 AF182828	*M.* × *piperita*	Burke et al. (1999)
4-S Limonene synthase	Monoterpene synthase	L13459	*M.* × *spicata*	Colby et al. (1993)
		No data	*M.* × *piperita*	Lange et al. (2000)
		175323	*M. longifolia*	Crock and Croteau (unpublished data)
Linalool synthase	Monoterpene synthase	AY083653	*M.* × *citrata*	Crowell et al. (2002)
β-Farnesene synthase	Sesquiterpene synthase	AF024615	*M.* × *piperita*	Crock et al. (1997)
Muroladiene synthase	Sesquiterpene synthase	AJ786641	*M.* × *piperita*	Prosser et al. (2006)
Limonene-3-hydroxylase	Hydroxylase	AF124816	*M.* × *piperita*	Lupien et al. (1999)
		AY622319	*M.* × *spicata* L.	Lücker et al. (2004b)
		AY281027	'*Crispa*' *M.* × *gracilis*	Bertea et al. (2003)
Limonene-6-hydroxylase	Hydroxylase	AF124815	*M.* × *spicata*	Lupien et al. (1999)
		AY281025	*M.* × *gracilis*	Bertea et al. (2003)
Menthofuran synthase	Pulegone hydroxylase	AW255974	*M.* × *piperita*	Bertea et al. (2001)
Pulegone reductase	Reductase	AY300163	*M.* × *piperita*	Ringer et al. (2003)
Isopiperitenone reductase	Reductase	AY300162		
Menthone reductases	Reductase	AY288137 AY288138	*M.* × *piperita*	Davis et al. (2005)
Isopiperitenol(−)-carveol dehydrogenase	Dehydrogenase	AY641428	*M.* × *piperita*	Ringer et al. (2005)

transgenic and control plants. Diemer et al. (2001) analysed oil yield and composition of transgenic peppermint and cornmint over-expressing the *ls* gene. Plants accumulating higher concentrations of total monoterpenes were obtained, but neither limonene accumulation nor the activity of 4S-limonene

synthase increased. Recently, Mahmoud et al. (2004) provided a more detailed study on *ls* ectopic expression in transgenic peppermint. Even though the *ls* transgene was constitutively expressed in leaves under the CaMV 35S promoter, the activity of the corresponding enzyme was not significantly enhanced in the glandular trichomes and no effect was observed on oil yield or composition.

The conversion of DXP to methylerythritol phosphate by 1-deoxyxylulose-5-phosphate reductoisomerase (*dxr*) is a committed step for the production of IPP and may, therefore, represent another potential control point of monoterpene metabolism. Over-expression of *dxr* (Lange and Croteau 1999a) in transformed peppermint (Mahmoud and Croteau 2001) led to an increased accumulation of oil by up to nearly 50%. The oil composition was, nevertheless, unaffected. In addition, the co-suppression of *dxr* activity lowered oil content and impaired chlorophyll biosynthesis. These results suggest that DXR regulates terpene synthesis in plants and controls the flux of precursor molecules of monoterpene biosynthesis towards the oil glands.

(+)-Menthofuran is an undesirable monoterpenoid component of peppermint oil which derives from (+)-pulegone. A cDNA encoding cytochrome P450 (+)-menthofuran synthase (*mfs*) was isolated recently from peppermint (Bertea et al. 2001). Transformation of peppermint plants with an antisense construct of *mfs* led to a significant reduction in mentofuran accumulation (Mahmoud and Croteau 2001). In subsequent experiments, plants over-expressing *mfs* were also analysed for changes in menthofuran, pulegone and menthone content (Mahmoud and Croteau 2003). Plants transformed with sense *mfs* exhibited an increased concentration of menthofuran and pulegone, whereas menthone content was lowered when compared to the control. In contrast, transgenic plants with an antisense construct of *mfs* produced less menthofuran and pulegone and more menthone. Analysis of the expression of *mfs* and pulegone reductase (*pr*) (catalyses the conversion of pulegone to menthone) in transformed plants over-expressing or co-suppressing *mfs* revealed that menthofuran yield is regulated at the transcriptional level and that *pr* and *mfs* gene expression patterns are inversely correlated. This result explains why a decrease in menthofuran accumulation is always accompanied by an increase in pulegone content because of its reduced conversion into menthone. Feeding experiments with menthofuran on untransformed plants showed that this small molecule is responsible for the decrease in pulegone reductase activity (Mahmoud and Croteau 2003).

The regioselectivity of the hydroxylation of limonene is a critical feature of mint synthesis since it determines the type of aromas that can be produced. Peppermint and cornmint express limonene-3-hydroxylase, which catalyses the conversion of limonene into *trans*-isopiperitenol that is ultimately converted into menthol as an end-product. A hydroxylation on C6 is carried out by spearmint as the first step for carvone synthesis (Fig. 4). Consequently, alterations in the limonene hydroxylation pattern by genetic engineering are attractive approaches to produce plants with a modified oil profile. Unfor-

tunately, essential oil composition and yield were not markedly affected in transgenic plants that constitutively expressed limonene-3-hydroxylase (*3lh*) in leaves. In agreement, no change in the level of transcript for *3lh* was found in isolated glands, as already observed during limonene synthase over-expression experiments. Nevertheless, high limonene content was found in peppermint plants in which *3lh* had been silenced by co-suppression. Interestingly, the overall essential oil yield was unaffected in this later construct, therefore suggesting that the point of control of metabolic flux intensity may reside upstream of this hydroxylation step (Mahmoud et al. 2004).

In conclusion, transformation experiments with different genes of monoterpene biosynthesis led to the production of novel plants with improved oil quality (lower menthofuran content) or yield. Moreover, some information on the regulation of monoterpene synthesis has been obtained through these experiments.

3.3 Prospects for Transgenic Mints with Improved Monoterpene Production

Molecular engineering of isoprenoid production in plants has been reviewed recently (McCaskill and Croteau 1998, 1999b; Haudenschild and Croteau 1998; Chappell 2002; Mahmoud and Croteau 2002) and some reviews have placed special emphasis on the bioengineering of mint essential oil (Lange and Croteau 1999b; Veronese et al. 2001; Wildung and Croteau 2005). Through the experience gained in early attempts (Sect. 3.2), improvement of terpene production in mints can be envisaged through three different approaches: (1) manipulating plant metabolism, (2) improving resistance to herbicide or pathogens and (3) increasing or altering sites of synthesis.

Manipulation of mint metabolism has been successful by over-expressing or silencing genes involved in monoterpene biosynthesis (Sect. 3.2). This approach will be extended in the near future by the concomitant expression of several transgenes in the same plant. Three different synthases have been introduced into separate tobacco plants and subsequently combined into one plant by sexual crossing. This led to the accumulation of an increased and altered blend of monoterpenoids (Lücker et al. 2004b). Moreover, these transgenic plants which were able to produce limonene, terpinene and pinene have, in a second step, been further transformed with a limonene-3-hydroxylase gene leading to the production of (+)-*trans*-isopiperitenol (Lücker et al. 2004a). Similar experiments could have been carried out on transformable and fertile spearmint or cornmint. Another strategy is the heterologous expression of synthases absent in mint in order to produce novel compounds. This idea has already been investigated by expressing linalool synthase in carnation (Lavy et al. 2002) and petunia (Lücker et al. 2001). In carnation, linalool and linalool oxide were produced whereas, in petunia, linalool concentrations remained low and a glycosidic form, *S*-linalyl-β-D-glucopyranoside, accumulated. Both approaches revealed that it was possible to express heterologous synthases

in transformed plants to alter the blend of monoterpenes. A finer tuning of monoterpene blend quality may even be obtained thanks to the recent characterization of several domains in monoterpene synthases and their role in the overall enzyme activity and specificity (El Tamer et al. 2003; Katoh et al. 2004). The main problem in these transgenic constructs is that they are based on the use of the constitutive CaMV-35S promoter. Over-expression of a synthase may result in a redirection of the flux of the isoprenoid precursors IPP and DMAPP towards GPP and thus lower the formation of FPP and GGPP. Depletion of some of these isoprenoids can have a dramatic effect on the overall plant phenotype, as observed during the over-expression of a phytoene synthase in tomato which competed with gibberellin synthesis and resulted in a dwarf genotype (Fray et al. 1995). In contrast, transformation of *Brassica napus* with the same gene under the control of a tissue-specific promoter led to the specific accumulation of carotenoids in seeds and did not affect the growth pattern of the transgenic plants (Shewmaker et al. 1999). In future, trichome-specific promoters should allow the specific expression of terpene metabolism genes in glandular trichomes. One such promoter was characterized recently (Wang et al. 2002) and is attached to a gene involved in the hydroxylation of a diterpene in tobacco trichomes (Wang et al. 2001).

Weed control is a serious concern in the field production of mint because the successive arrival of annual and perennial herbs affects both yield and oil quality. Eradication of weeds cannot be managed by mechanical processes in a field-grown crop and most farmers use herbicides to control weeds in mint. Currently, most registered herbicides have a limited weed spectrum and the use of increased quantities of these chemicals has led to the emergence of herbicide-resistant weed species, the pollution of soil and water and the need to develop mint cultivars that are resistant to a broad spectrum of environmentally more acceptable herbicides (Veronese et al. 2001). Recently, transgenic peppermint expressing the *bar* gene (generates a phosphinothricin acetyltransferase) was obtained (Li et al. 2001). These transgenic lines are resistant to as much as four times the normal concentration of bialaphos (Liberty; Bayer, CropScience). Such plants will facilitate the development of post-emergent herbicide control strategies with reduced environmental impact and which do not lead to the accumulation of residues. In this respect, bialaphos can be equally metabolised by microbes and transgenic plants. During a two-year survey, the combined expression of the *bar* gene and bialaphos treatment caused no alteration in oil production or quality.

Diseases limit mint production, with mint rust (*Puccinia menthae*) and *Verticillium* wilt (*Verticillium dahliae* Kleb) being most severe pathogens (Veronese et al. 2001). To date, only the physiology of the interaction of mint with *P. menthae* has been characterised and information is not available about the molecular mechanisms controlling disease resistance. In contrast, research on *Verticillium* wilt is more advanced and several antifungal proteins like osmotin (Abad et al. 1996), inhibiting in vitro pathogen growth, have been reported. Veronese et al. (2001) expressed an osmotin gene isolated from to-

bacco in transformed peppermint. This led to an increased tolerance towards *Verticillium* infection, suggesting that resistant plants could be obtained during the co-expression of multiple antifungal proteins. Recently, a dominant locus controlling *Verticillium* disease resistance was identified in *Arabidopsis* (Veronese et al. 2003). Further studies on this model plant system may soon provide new candidate genes to engineer mint for *Verticillium* resistance.

A radically different approach to improve monoterpene yield is to increase the density of secretory glands on leaves. This approach will be possible when a basic knowledge becomes available about the transcription factors which coordinate development of secretory trichomes. At present, trichome development of *Arabidopsis* provides a model to analyse processes regulated to the distribution and the differentiation of unicellular non-secretory trichomes on the leaf epidermis. As trichomes are not essential for the growth of *Arabidopsis* plants, many mutants defective in trichome development have been isolated and more than 20 genes involved in hair development have been identified (Marks 1997), including regulatory genes involved in trichome initiation (Szymanski et al. 2000; Schiefelbein 2003; Pesch and Hülskamp 2004). GL1, GL3 and TTG respectively code for members of the R2R3 MYB class of transcriptional regulators, a R-like bHLH and a TTG protein interacting with GL3 and possessing a WD40 repeat which may function as a protein–protein interaction domain. These genes act as positive regulators of trichome formation, as suggested by the over-expression of both GL3 and GL1 which trigger excess trichome formation (Payne et al. 2000). In contrast to these factors, CPC and TRY are MYB transcriptional factors and act as negative regulators of trichome formation, since the ectopic expression of either CPC or TRY led to a reduction in trichome density (Schellmann et al. 2002). Trichome formation is probably regulated by additional factors like growth regulators. Gibberellic acid has been shown to act as a positive regulator of GL1 and trichome formation (Perazza et al. 1998). Trichome formation is a complex process which is still not fully understood, but over-expression of transcription regulators like AtMYB23 (Kirik et al. 2001), or both GL3 and GL1, leads to the development of ectopic trichomes. Moreover, the expression in tobacco of a MYB-related transcription factor that controls the development of conical cells in *Antirrhinum* petals leads to the over-production of multicellular secretory trichomes on leaves and floral organs (Glover et al. 1998). This result suggests that differentiation of non-secretory and secretory trichomes may share some common elements like endoreduplication (Berta et al. 1993; Vespa et al. 2004). Recently, a tomato mutant insensitive to jasmonic acid (jai1) was characterized (Li et al. 2004). This mutant was also affected in the development of glandular trichomes and showed a decrease in glandular trichome density on leaf, sepals and green fruit. Genetic complementation experiments showed that jai1 is the tomato homologue of CORONATINE-INSENSITIVE 1 of *Arabidopsis* and that this gene promotes multiple processes, including resistance to herbivory, seed maturation and trichome development.

4 Concluding Remarks

Mint essential oil is produced mainly in the United States, where growers recently encountered several difficulties, leading to a decline in the value of this crop and a decrease in the production of both peppermint and spearmint (USDA, unpublished data). Irrigation has been cut back in some counties as a consequence of regional drought. *Verticillium* infestations have occurred on vast areas of land and, because spores remain viable for several years in the soil, farmers had to move their mint cultures away from their stills. Finally, adulteration of foreign oil, such as cornmint into peppermint products, has depressed the pricing of peppermint oil. In contrast, the market of mint oil increases by 5% every year and new countries, like India, have begun to produce peppermint oil of good quality with a competitive price (Shahi et al. 1999; Dwivedi et al. 2004). In this economic context, genetic improvement of mint is needed to engineer mint plants to produce large amounts of a high quality essential oil.

Over-production or qualitative modifications in mint essential oil have already been performed. In contrast, engineering of terpene metabolism in non-aromatic model plants like *Arabidopsis*, tobacco or petunia has been difficult. Several theories have been proposed to account for the limited success in the metabolic engineering of terpene metabolism, including substrate limitation, stringent metabolic feedback regulation or absence of suitable compartments (subcuticular chamber of gland) to sequester toxic monoterpenes (Chappell 2004). New approaches will require knowledge on additional genes like transcription factors which up-regulate entire metabolic pathways (Gantet and Memelink 2002; Broun 2004), or enhance trichome formation (Pesch and Hülskamp 2004). However, a prerequisite for such studies will be the development of a genetic and molecular model aromatic plant. This model plant should be easily genetically manipulated and grown. Moreover, it should yield a mutant collection to allow the discovery of genes involved in isoprenoid metabolism or trichome development. Characterisation of elicitor molecules, like jasmonic acid, which increase metabolic flux of monoterpenes in Norway spruce (Martin et al. 2003), could allow a functional genomics approach towards the understanding of secondary metabolism (Goossens et al. 2003).

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