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Uses of Plant Gene Silencing

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Introduction

Conventional breeding methods have relied on plant genetic diversity as the source of new traits to improve our domesticated crops. Early in our history selection for characteristics affecting, e.g. ear brittleness of wild grasses (which were related to barley) allowed the first 'domesticated' cereals to be developed and grown (Dunwell, 1995). Such advances enabled humans to abandon their nomadic lifestyle in favour of settlements and farming. Domestication of cereals was the foundation for the great civilizations seen in the Middle East and continues to be the cornerstone of human civilization today.

Plant breeding techniques have generally provided increasing yields, especially over the last 50 years. This has been achieved by the introduction of new genetic traits obtained from wild relatives, by selecting for induced genetic mutations, use of fertilizers and the widespread use of pesticides to control commercially important pests and diseases. Together, these have all contributed to the 'green' revolution that has occurred since 1945. However, as genetic diversity within individual species is limited, and tolerance to many of our pesticides has been observed, new ways to improve and protect crops have been sought.

During the last decade great efforts have been directed towards understanding the genetics of all our staple crops and how to further improve them. This research has been performed, both from an academic and industrial view point, with the ultimate aim of breeding more productive crop plants. Many important crop traits have been located using restriction fragment length polymorphism analysis. Disease resistance genes and other plant genes are being cloned, and plant growth and development is better understood. Together, these advances are facilitating the development of potentially more accurate molecular breeding programmes and increase crop productivity as a result. However, such gene usage was restricted to closely related species until the development of genetic transformation methods, which have allowed the introduction of genes from any species and kingdom.

On a commercial scale, plant transformation has now produced crop varieties with new and unusual characteristics that cannot be obtained by conventional approaches alone. McElroy (1996) has reported on the industrialization of transgenic plant

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production, and this molecular plant breeding is becoming routine world-wide. New varieties produced via molecular biology are now undergoing extensive field trials and some transgenic crops have already been commercialized. In Canada during the last three years, several varieties of transgenic canola have appeared, while in America, tomatoes with a longer shelf-life have been on sale since May 1994. Biotechnology has also been used to produce insect-resistant cotton by using the *Bacillus thuringiensis* (Bt) toxin, and this new cotton variety was released to US farmers in 1996. This is thought to have benefited the environment by a substantial reduction in pesticide application on cotton and has given knock on economic benefits for the farmers (Schell, 1997).

The transfer of basic research to commercial product has identified new, and to some extent, unpredicted research opportunities. Initially, it was thought that the main obstacle to producing plants would be regeneration and transformation steps, but while this was a problem particularly for cereal crops, it has been achieved. It was also expected that the new 'transgenes' would express normally and follow Mendelian inheritance. However, various regulatory pathways are now being studied which influence the stability of transgenes in transgenic plants, and over the last decade increasing numbers of reports have appeared describing and investigating the phenomena of 'transgene silencing'.

Plant transformation techniques

There are a number of different methods which can be employed to obtain transgenic plants and these are briefly discussed below.

AGROBACTERIUM MEDIATED TRANSFORMATION

This is probably the most widely used method to obtain transformed plants. *Agrobacterium* is a gram-negative soil bacterium that causes either tumorous growths called crown galls (*A. tumefaciens*), or hairy root syndrome (*A. rhizogenes*) on a wide variety of plant species. The disease is caused by the transfer of a segment of DNA from a bacterial plasmid to wounded plant cells. This segment of DNA (T-DNA or transforming DNA) contains genes for phytohormone production (leading to gall or root production) and genes for the biosynthesis of opines, amino acid derivatives that are catabolized by the bacterium. The T-DNA is stably inserted into the hosts genome and can be transmitted to the next generation following normal Mendelian genetics (For a recent review see Sheng and Citovsky, 1996.) This natural DNA transfer system has been modified to effect transformation and transfer genes of scientific interest into the genomes of plants. Until very recently, it was thought that this biological method was restricted to broad leaf dicotyledonous plants and gymnosperms but rice, maize and barley are reported to have been transformed using this bacterium (Park *et al.*, 1996; Ishida *et al.*, 1996; Escudero *et al.*, 1996; Tingay *et al.*, 1997). Both forms of *Agrobacterium* can be used; *A. rhizogenes* has been used to transform more recalcitrant species such as *Antirrhinum majus* (Senior *et al.*, 1995), but has the disadvantage that several rounds of back-crossing may be required before normal looking transgenic plants are produced, due to the close integration of both pathogenic and the desired traits (Newbury and Senior, 1998). *A. tumefaciens* is the usual choice as it produces

transgenic plants without need to separate pathogenic characteristics from the desired trait.

Agrobacterium-mediated transformation methods were developed for whole plant explants, cultured cells and protoplasts. Transformation of explants and subsequent direct shoot regeneration has been a very successful and efficient technique for the generation of transgenic plants. It also minimizes somaclonal variation associated with regeneration from cultured cells and calli. There are many factors which can influence the efficiency of transformation such as choice of bacterial strain, phytohormones used, presence of acetosyringone, selection conditions, explant age, and tissue culture conditions. Therefore, an empirical approach to transformation methodology is required to obtain a working system.

PARTICLE BOMBARDMENT

This is the method of choice to transform monocotyledonous plant species (such as wheat, rice and barley) and over the last five years all major cereal crops have been successfully transformed using this method. The process entails DNA coated gold or tungsten particles (1 μm in diameter) being 'shot' through the cell wall and membrane into the plant cells and plants being regenerated from such transformed cells. The method was originally described by Klein *et al.* (1987) and has been extensively used for cereal transformation. Maize was one of the first plant species to be successfully transformed using this method (Gordon-Kamm *et al.*, 1990) using embryogenic suspension cultures bombarded with the *bar* gene. (For reviews see Vain *et al.*, 1995 and Christou, 1996.)

PROTOPLAST TRANSFORMATION

The methodology behind the use of protoplasts has improved significantly over the last few years. The advantage of using protoplasts over other tissues is that chimeric plants are not regenerated using these techniques. The main obstacle, however, is that it is limited to those cultivars that can be successfully regenerated from cultured protoplasts. There are a variety of ways in which protoplast have been transformed and these are outlined below.

Polyethylene (PEG) mediated plant transformation

PEG has been used to increase the porosity of protoplast membranes to allow the direct uptake of DNA. This methodology has been widely used for transformation of monocotyledons, e.g. Tall fescue transformation with the *bar* gene (Wang *et al.*, 1992), Indica rice transformation of protoplasts, again with the *bar* gene (Datta *et al.*, 1992), to produce fertile transgenic plants in both cases.

Electroporation

This method uses short electrical pulses of high field strength to enable DNA to permeate cell plasma membranes. The success of this method depends on the amplitude and duration of the electric pulses, and the medium used, as they can affect

both uptake and subsequent survival of the putative transformed protoplasts. The method has been used successfully on rice (Shimamoto *et al.*, 1989), maize (Fromm *et al.*, 1986) and oilseed rape (Guerche *et al.*, 1987) but is not widely used.

Micro-injection

It requires considerable skill to inject cells with DNA. It is widely used for animal cell transformation but has not often been used for plant cells. Few examples are available, e.g. Alfalfa (Reich *et al.*, 1986), and Oilseed rape (Neuhaus *et al.*, 1987).

Physical abrasives and laser beams

Silicon carbide fibres have been used to physically puncture cells to allow the direct uptake of DNA. Several species have been successfully transformed using this method including maize (Frame *et al.*, 1994) and tobacco (Kaeppler *et al.*, 1992). There are reports of microlaser beams being used to burn small holes through the wall of rice cells to allow the passive uptake of DNA (Weber *et al.*, 1988a, b, 1990). *Brassica napus* pollen grains have been transformed using this technique and been regenerated into whole plants (Weber *et al.*, 1990). Similarly, pollen grains from *Impatiens* have been transformed (Yamaguchi *et al.*, 1994).

IN PLANTA TRANSFORMATION – VACUUM INFILTRATION

This is a variation on *Agrobacterium* transformation. Instead of using plant parts for transformation, whole plants are used. Shoot meristems are exposed to *Agrobacterium* infection by slitting bolting shoots and inoculating the incision. Subsequent shoot production is quick for non-transformed but slow for transformed tissue. A second round of inoculation is usually performed after which new flowering shoots are allowed to set seed. On average, 5.5% of newly formed shoots produced transformed progenies (Chang *et al.*, 1994). Bechtold *et al.* (1993) reported the transformation of *Arabidopsis thaliana* by vacuum infiltration of an *Agrobacterium* suspension. Their genetic and molecular analysis suggested transformation occurred late in floral development as all transformants were hemizygous and contained different T-DNA inserts.

Gene silencing phenomena

Once transgenic plants had been produced, researchers expected that their new transgenes would act as dominant Mendelian traits. These plants were expected to be used in a variety of ways, including complementation and over-expression studies, to identify gene function or to increase yields. Early literature showed that while generally this sort of effect was seen, there were a number of noticeable deviations from expected phenotypes. Reports of such cases were infrequent during the 1980s, but since the phenomena was recognized the number of reports into gene silencing have escalated. Matzke *et al.* (1994b) undertook a survey of reported gene silencing events and found the percentage incidence ranged from 3% to 100% (zero incidence of gene silencing is also known). The exact incidence of silencing events is not known

but it is frequent enough to be a potential drawback in commercial production of transgenic plants. A report by Finnegan and McElroy (1994) stated that 30 companies working with transgenic plants had encountered problems with silencing of their introduced transgenes, while a meeting report used the title 'Treasure your exceptions' (Phillips *et al.*, 1995) when discussing the phenomena.

EARLY INDICATIONS OF [TRANS]GENE SILENCING

During the 1980s probable instances of gene silencing were reported, but presented as unusual events or non-Mendelian inheritance. Deroles and Gardner (1988a, b) reported the observation of low frequency inheritance of a kanamycin construct associated with low expression, and for some *Petunia* lines segregation ratios could not be explained by Mendelian genetics (Deroles and Gardner, 1988b). Of these, many of the parents contained high copy numbers of the construct used (Deroles and Gardener, 1988a). Jones *et al.* (1987) reported weak expression of their transgene, while Shirsat *et al.* (1989) found gene copy number did not correlate with expression level of a *legA* transgene.

Heberle-Bors *et al.* (1988) analysed seeds from 283 tobacco transgenics for embryo-lethal mutations caused by T-DNA integration. Instead of identifying recessive lethal mutations, they observed the loss of marker genes or their expression between generations. They also found transgene inactivation could be independent of other genes encoded by the T-DNA, i.e. kanamycin was inactivated whilst nopaline synthase was not. Sukhapinda *et al.* (1987) also observed suppression or loss of marker gene expression in their transgenic tomatoes.

Sinkar *et al.* (1988) used *Agrobacterium rhizogenes* to transform tobacco. This species of *Agrobacterium* causes abnormal shoot development due to transfer of hormonal genes to the plant. This usually produces plants with short internodes, altered leaf morphology, loss of apical dominance and delayed flowering. Occasionally, revertant normal looking shoots can be produced. Analysis showed reverted shoots still contained the transgene but they had been transcriptionally inactivated (Sinkar *et al.*, 1988). They suggested that silencing of T_L-DNA was age related and probably the result of 'cellular processes directed towards silencing of foreign genes in plants'. Such cases led to more detailed investigations of these 'silencing' phenomena and paved the way to the discovery of a variety of processes not previously envisaged.

TYPES OF GENE SILENCING RECOGNIZED

Gene silencing has been observed in a variety of situations: single transgene only, transgene–transgene or transgene–endogenous gene interactions. It can be due to either transcriptional or post-transcriptional mechanisms (*Table 1*).

Single copy transgene silencing has been observed in tobacco (Elmayan and Vaucheret, 1996) and *Brassica napus* (Owen, personal communication). But silencing occurs more commonly when many copies of the transgene are present in the genome (Stam *et al.*, 1997a; Matzke *et al.*, 1994a). When two or more constructs are present, silencing can occur over considerable distances, e.g. different chromosomes (*trans* silencing; Matzke *et al.*, 1989), or they can be present and silenced on the same

Table 1. Identified Gene Silencing Phenomena.

Type of silencing interaction	Transcriptional or post-transcriptional silencing	References
Single copy transgene	Transcriptional	Meyer *et al.*, 1992
	Post-transcriptional	Elmayan & Vaucheret 1996
Transgene–transgene	Transcriptional – in *cis*	Assaad *et al.*, 1993
	Transcriptional – in *trans*	Matzke *et al.*, 1989
	Post-transcriptional – in *trans*	Hobbs *et al.*, 1990, 1993; English *et al.*, 1996
	Both transcriptional and post-transcriptional	Thierry and Vaucheret, 1996
Transgene–endogenous gene	Post-transcriptional	Napoli *et al.*, 1990; van der Krol *et al.*, 1990a
Multiple endogenous genes silencing		
(a) + promoter	Post-transcriptional	Seymour *et al.*, 1993; Jones *et al.*, 1996; Stam, 1997
(b) – promoter	Post-transcriptional	
Antisense transgene–endogenous gene	Post-transcriptional	Rothstein *et al.*, 1987; Smith *et al.*, 1988; Hamilton *et al.*, 1990

stretch of DNA (*cis* silencing; Assaad *et al.*, 1993). T-DNA integrations themselves can be simple single copy inserts or very complex inserts, where transgenes are arranged as: direct repeats, inverted repeats (head to head or tail to tail), or even complex insertions where constructs are present in several different forms (Stam 1997) and they can include bacterial DNA from outside the T-DNA borders (Kononov *et al.*, 1997).

Plant gene silencing can be the result of, or influenced by:

(i) Position of T-DNA insertion into the genome
(ii) Environmental conditions
(iii) Transcriptional silencing
(iv) Post-transcriptional silencing
(v) Antisense silencing
(vi) Chromatin mediated silencing.

Some of these silencing effects are similar to those observed in non-transgenic plants and other organisms. Transcriptional silencing, for instance, is considered to be similar to paramutation (Brink, 1973; Hollick *et al.*, 1997) in plants (Matzke *et al.*, 1996; Martienssen, 1996) and to fungal *M*ethylation *I*nduced *P*remeiotically (MIP) (Rossignol and Faugeron, 1995; Irelan and Selker, 1996; Selker, 1997) and *R*epeat *I*nduced *P*oint mutation (RIP) (Singer and Selker, 1995; Irelan and Selker, 1996; Selker, 1997) phenomena. While viral-induced gene silencing appears to be a defence system of plants to inhibit viral spread (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). Antisense suppression in prokaryotes is known to be a natural control system for accessory genetic elements such as phages, plasmids and transposons (Wagner and Simons, 1994).

TRANSCRIPTIONAL GENE SILENCING

The first gene silencing observation to be examined in detail was published in 1989. Initially, two different but partially homologous transgene constructs were introduced into the same plant by a sequential transformation experiment (Matzke *et al.*, 1989), and in later experiments loci were brought together by sexual hybridization (Matzke *et al.*, 1993). The two constructs encoded either kanamycin and nopaline synthase or hygromycin and octopine synthase. Only hygromycin was controlled by the 35S CaMV promoter; the other three genes were under the control of the NOS promoter. After combining these constructs, progeny segregations were analysed. Instead of finding both hygromycin (H) and kanamycin (K) resistant phenotypes, they found kanamycin resistance was silenced when the two constructs were present in the same plant. Examination of the promoter region showed that an SstII site was methylated. This correlated with gene inactivation in the presence of the hygromycin construct, and expression when it was absent. Analysis of the progeny from such experiments indicated that loss of promoter methylation and subsequent improvement of kanamycin expression was a progressive rather than a one-step process, even if H alleles had been lost during outcrossing (Matzke and Matzke, 1991).

The extent to which K was silenced was determined by the nature of the incoming hygromycin loci. When H1 was introduced, K remained active and was not methylated; H2 caused silencing and methylation of K in both homozygous and hemizygous plants, while H3 caused partial inactivation and methylation when hemizygous but this was more complete in the homozygous condition (Matzke *et al.*, 1993). Analysis of these three loci showed; H1 to consist of a single copy transgene without methylation, H2 had four copies which were completely methylated, while H3 contained two to three partially methylated copies of the transgene (Matzke *et al.*, 1994a). Similar results have been obtained for a potent 35S silencer locus called '271' (Vaucheret, 1993, 1994). It too consists of multiple, methylated copies of the transgene and causes heritable silencing and methylation of targeted genes.

Such instances of homology-dependent transcriptional gene silencing have been compared to paramutation (Matzke *et al.*, 1996; Martienssen, 1996). This latter phenomena is thought to occur when one allele (or locus) is able to weaken the expression of a second. The change to the second allele (or locus) is inheritable and often accompanied by cytosine methylation, while the 'silencer' allele remains unchanged. Such effects are common in many plant species and have been extensively studied in *Antirrhinum majus* and maize (Martienssen, 1996). Structural analysis of maize paramutant alleles has shown them to be complex and sometimes associated with transposable elements. Martienssen (1996) suggested these elements may also play a role in gene silencing.

CO- OR SENSE SUPPRESSION

This is a particularly interesting form of gene silencing as it was so unexpected. First reports of sense suppression occurred in 1990, when two independent groups working with *Petunia* chalcone synthase (CHS) gene noticed that up to 50% of transformants had white or variegated flowers (Napoli *et al.*, 1990; van der Krol *et al.*, 1990a). Napoli *et al.* (1990) were trying to over-express the CHS gene in order to test whether the

enzyme was rate limiting to anthocyanin biosynthesis. Detailed examination of gene expression was performed and showed mRNA level of the co-suppressed line was 50-fold lower relative to the control (Napoli *et al.*, 1990). Van Blokland *et al.* (1994) used nuclear run-on experiments to analyse co-suppression further. This revealed normal levels of transcription occurred, but the CHS transcript steady-state level was greatly reduced in white and variegated tissue compared to pigmented flowers. From this data, a post-transcriptional gene silencing mechanism was thought to be operational.

Since these initial findings, co-suppression has been observed in many other plant species transformed with sense constructs which have homology to endogenous genes. Research into co-suppression mechanisms has continued to use transgenic *Petunia* plants, due to the ease of obtaining transgenics and the readily observable phenotypes. One interesting feature observed in *Petunia* plants was that co-suppression does not always occur in all side branches of a suppressed plant. *Petunias* have been obtained where fully pigmented flowers occur on one side branch, while other branches have white or variegated flowers, indicating the extent of suppression is variable. Flavell (1994) suggested these effects were somatically inherited and suppression was initiated in individual branch meristems. Various colour patterns can be obtained via CHS and dihydroflavonol reductase (DFR) co-suppression in *Petunia* flowers (van der Krol *et al.*, 1988, 1990a; Napoli *et al.*, 1990) and antisense CHS in *Eustoma* (Deroles *et al.*, 1995). Patterns are either ordered or erratic in nature and Jorgensen *et al.* (1996) have established a relationship between pattern type and transgene locus structure. For sense constructs they observed a range of phenotypic pigmentation patterns that antisense CHS constructs do not produce. Transgene organization, in particular its repetitiveness, was the primary determinant for these patterns. They also showed that the degree of co-suppression was subject to a transgene dosage effect. Interestingly, no such co-suppression-induced floral patterning has been observed in species which traditionally lack patterned varieties such as rose or *Gerbera* (Davies and Schwinn, 1997).

There is conflicting evidence for the role of transcription in initiation of co-suppression. Smith *et al.* (1990a), showed that down-regulation of expression only occurred when both transgene and endogenous genes were transcribed. While other studies have used promoterless constructs and still observed co-suppression (van Blokland *et al.*, 1994). In the latter case antisense transcripts were found, suggesting that endogenous transcription produced these transcripts. Their exact role, if any, in co-suppression was not established. Other studies have indicated a threshold level of transcript is required to initiate down regulation (Dorlhac de Borne *et al.*, 1994; Hart *et al.*, 1992). Transgenes in a homozygous state can be more effective in initiating co-suppression than hemizygous transgenes. This suggests a gene dosage effect or that a threshold needs to be surpassed before suppression occurs. An alternative explanation is that these alleles interact in some way, which results in the production of low levels of aberrant mRNAs. These are detected and are degraded along with all other correct homologous copies, switching off expression. Metzlaff *et al.* (1997) suggested a role for the 3' end of RNA in co-suppression. They observed the accumulation of aberrant poly A⁻*chs*A RNA in *Petunia* plants with active transgenes and consider this is the active inducer of co-suppression in their system. Their model suggests loss of *chs*A RNA in floral co-suppression occurs via pairing – cleavage cycles between aberrant RNA fragments and complementary 3' sequences of the full length mRNA. This is

thought to be active in the nucleus (Metzlaff *et al.*, 1997). Analysis of potato virus X carrying GUS sequences also suggested a link between gene silencing and 3' end (English *et al.*, 1996).

There are instances of co-suppression which are developmentally controlled instead. Some transgenes have been identified which are silenced after a lag period. This can occur at different stages of development (Dorlhac de Borne *et al.*, 1994; Hart *et al.*, 1992) or at a defined stage in plant development (de Carvalho *et al.*, 1992). Co-suppression can also be triggered by environmental conditions (de Carvalho Niebel *et al.*, 1995). High light intensity has been implicated in suppression of CHS in *Petunia* (van der Krol *et al.*, 1990a) and β-1,3-glucanase genes in tobacco (de Carvalho *et al.*, 1992). Cultural conditions have also been found to cause silencing. Chitinase genes in *N. sylvestris* (Hart *et al.*, 1992) and nitrate reductase in tobacco (Dorlhac de Borne *et al.*, 1994) were silenced by germination and growth conditions. The acetolactate synthase (ALS) gene in tobacco was silenced when seedlings were transplanted into the field (Brandle *et al.*, 1995).

Many examples of co-suppression are meiotically transmissible showing the silent state is stably transferred to the progeny. This is obviously important for commercial development of co-suppression technology (dubbed 'Trans-Switch'™ by DNAP).

HOMOLOGY-DEPENDENT VIRAL RESISTANCE

Plant viruses have been impossible to control directly by chemical means; only the vectors of disease have been successfully targeted by chemical sprays. With the development of transgenic plants, new ways to control viral plant pathogens have emerged. Since the concept of pathogen-derived resistance (PDR) was postulated (Sanford and Johnston, 1985), great advances in obtaining virus resistant plants have occurred, and the first commercial product, virus-resistant squash (Asgrow Co, Kalamazoo, MI, USA) became available in July 1995 (Meeusen, 1996). For an overview of PDR types and mechanisms, see Beachy (1997).

There are two basic methods for PDR: one relies on viral coat protein transgene expression for resistance, the other is a gene silencing mechanism. The latter is operational at the post-translational level and appears to be directly linked to RNA. Several papers have recently appeared discussing this phenomenon and the mechanisms by which it could operate and the reader is directed to these for more details (Lindbo *et al.*, 1993; Dougherty and Parks, 1995; Baulcombe, 1996a, b; Baulcombe and English, 1996; Angell and Baulcombe, 1997; Beachy, 1997). Viral resistance techniques are commercially important as many crop plants are susceptible to viral infections and significant yield losses can occur (strategies also exist using transgenic viruses to infect non-transgenic plants in order to produce high value therapeutic proteins and secondary metabolites; see Della-Cioppa and Grill, 1996). Plant viral resistance so far observed is a highly strain-specific phenomenon, as unrelated viruses can still infect and damage plants which are expressing resistance against another virus. It is thought that this type of resistance occurs through a homology-based inactivation system similar to co-suppression.

Covey *et al.* (1997) have identified a non-transgenic or natural gene silencing mechanism operating against cauliflower mosaic virus (CaMV). CaMV-infected *Brassica* species initially showed viral symptoms before recovery occurred. Plants

that first showed chlorosis, vein banding and necrosis quickly reverted to asymptomless phenotypes and newly emerged leaf tissue was virus free. Analysis of viral replication showed that there was a change from viral replication to a post-replicative state. The authors suggested a similarity to transgene silencing events. Analysis showed that the CaMV mini-chromosome was still highly transcribed but that the mRNA produced was degraded. They suggested that a post-transcriptional mechanism was operating. Such findings support the idea that gene silencing is a resistance mechanism against viruses causing disease, endogenous proliferating sequences such as transposable elements (Matzke *et al.*, 1996) or other invasive DNAs. Ratcliff *et al.* (1997) have also identified a natural anti-viral mechanism operating against nepovirus infection in *Nicotiana sp.* They demonstrated that recovery from nepoviral infection and gene silencing are both viral inducible and suggested both use the same mechanism to target specific RNAs. Ratcliff *et al.* (1997) proposed that plant recovery, and thus gene silencing, is initiated upon viral penetration of the meristem (viruses are usually excluded from plant meristems and surrounding areas where gametes are produced). The possible relationship between nepoviral recovery, plant meristems and gene silencing suggest the latter could be more frequent when transgenes are expressed in plant meristems. Silencing may thus occur when plants falsely identify a transgene, or its RNA product, to be viral in origin and initiate an anti-viral defence mechanism: gene silencing (Ratcliff *et al.*, 1997).

ANTISENSE SUPPRESSION

This is now a very well-established technique for manipulating gene expression in a wide variety of organisms (for a plant review see Bourque, 1995). Antisense suppression is a natural system to control gene expression in bacteria (Simons & Kleckner, 1983; Wagner and Simons, 1994) and has led to the design of strategies to control eukaryotic gene expression. The first case of antisense use in multicellular eukaryotes was in plants (Rothstein *et al.*, 1987) and, since that time, many reports using antisense technology have been published. The mechanism of antisense suppression probably results from the formation of an RNA duplex between sense and antisense transcripts. Gene suppression may result from a number of mechanisms. Interference with the normal processing of mRNA could be caused by sequestration of translation initiation signals (although this appears unlikely). Duplices could prevent transportation into the cytoplasm and inhibit its subsequent translation or the duplex could be a target for double strand RNase action. Whatever the actual cause, the result is that gene expression is prevented (Bourque, 1995; Wagner, 1996). Kumar and Carmichael (1997) have recently used mouse polyoma virus to show that antisense RNA can cause adenosine to be converted to inosines or guanosines via modification of RNA duplexes by double stranded RNA adenosine deaminase or a related enzyme. Such transcripts were retained in the nucleus and remained untranslated. Whether or not such transcript pools exist in other systems is unknown, as these modified transcripts are thought to be invisible to normal hybridization probes (Kumar and Carmichael, 1997).

One potential drawback of antisense technology is differential inhibition of expression. Many plant genes are members of extended gene families which vary in their homology to each other, timing and location of expression. This reduces the suppres-

sive effect and might allow greater endogenous expression than is wanted. Equally, it could be used to advantage by production of a range of phenotypes from which the desired type can be chosen. Elomaa *et al.* (1996) reported the effects of two different cDNAs of the *CHS* gene family, *gchs1* and *gchs2*. These were transformed into *Gerbera hybrida*, which has up to 10 members of the *CHS* super family, and the effects of the antisense constructs on expression of members of this super family was determined. They found the degree of suppression observed was dependent on the percentage of homology between the different family members. Relaxation of inhibition in some transformants was shown to start from the most distantly related gene, adding to the belief that sequence homology is a determinant of the antisense effect. Elomaa *et al.* (1995) suggested that two mechanisms may be operational in antisense mediated suppression. They observed differences in amount of antisense RNA between two family members. For anti-*gchs2* plants, a positive correlation between amount of inhibition and level of RNA was observed. But, for anti-*gchs1* a large amount of antisense RNA was associated with no inhibition of *gchs1* mRNA, when inhibition was observed neither mRNA nor antisense mRNA was detectable. To ensure efficient suppression of a family member, a gene-specific antisense transgene needs to be used (Elomaa *et al.* 1996).

There are many factors which could regulate inhibitory activity of antisense, from availability of complementary sequences, rate of binding, rate of synthesis of each strand and rates of their decay, to the mechanism that inhibits target RNA once the duplex has formed, e.g. presence of double stranded RNA-degrading enzymes (Wagner, 1996). In contrast to eukaryotic systems, prokaryotic antisense processes have been better elucidated, e.g. replication control of bacterial plasmids.

So far, plant antisense technology has been the most widely used form of gene silencing because of the simplicity in obtaining reduced expression. Antisense has been used in many ways including: to define roles for newly isolated plant genes of unknown functions (Bird *et al.*, 1991; Smith *et al.*, 1988; Hamilton *et al.*, 1990); for crop improvement (Knutzon *et al.*, 1992; Fader *et al.*, 1995) and to determine the relative effect of enzymes in metabolic processes (Heineke *et al.*, 1994; Riesmeier *et al.*, 1993, 1994). Since antisense was shown to work in plants (Rothstein *et al.*, 1987), it has been a vital component in all areas of plant molecular biology and has gained commercial uses.

Gene silencing – potential and utilization

Most research has concentrated on the 'why' aspect of gene silencing and determining mechanisms by which it could operate. Many reviews and papers are available on the subject (Flavell, 1994; Stam *et al.*, 1997b; Baulcombe and English, 1996; Baulcombe, 1996a, b; Matzke and Matzke, 1995a,b; Matzke *et al.*, 1996; Martienssen, 1996; Dougherty and Parks, 1995; Meins and Kunz, 1995; Meyer, 1995; Depicker and Montagu, 1997; Kunz *et al.*, 1996; Metzlaff *et al.*, 1997). Such phenomena are potentially very useful commercially, by controlling unwanted traits or suppressing plant activity until it is required and even redirecting metabolism from one pathway to another. This technology will only be successful if the silent phenotype is stable throughout the lifetime of the crop and maintained in following generations. If we can find reliable ways to control gene silencing, then we have a new and very powerful

technique to control plant growth, development and metabolism in ways previously impossible to achieve. Some notable successes have already been made in this area and the next section is intended to highlight some of the areas in which gene silencing has already made an impact or where gene silencing has the potential for commercial products or academic advances.

USE OF SILENCING TO IDENTIFY POTENTIAL HERBICIDE SITES OF ACTION

Development of herbicides and other pest control agents is a time-consuming and expensive business. It relies on the synthesis of a wide array of different chemical compounds from which new pest controlling agents can be discovered. This is largely an empirical process, relying on identification of compounds with some biological activity and manipulating the chemical structure in such a way as to enhance pesticidal effects. These compounds are assessed *in vivo*, in the glasshouse and finally full-scale field tests before the compounds are released for sale. Such methodology allows early identification of chemicals with the desired characteristics and these can be quickly progressed to the field for assessment alongside industry standards (Ormrod and Hawkes, 1995). This safety verification process has overtones of the procedures needed for the release of new transgenic plants around the world.

Pesticidal compounds discovered over the last 30 years have usually been single-site inhibitors (e.g. sterol biosynthesis inhibitors) rather than the older multi-site compounds (e.g. copper sulphate). Multi-site inhibitors are often more toxic to a wider range of organisms and have largely been replaced by single site types. The efficacy of these newer compounds is very good but they tend to exert a large selective pressure on the biological target for resistant mutants. If pesticide usage is not carefully controlled, these resistant mutants can arise quickly and reduce effectiveness of control agents.

One such class of single site inhibitors is the acetolactate synthase (ALS) herbicide group which encompasses a diverse range of compounds. ALS herbicides have been widely used for the past 10 years, and have provided effective control against a wide variety of weeds world-wide (Sherman *et al.*, 1996). However, there is the on-going problem of target weed resistance, as single mutations can provide enough resistance for the weed to survive and set seed. Weed resistance is a world-wide problem (Le Baron, 1991) and new approaches to identifying new control agents and maintaining efficacy against a wide range of weeds are of constant concern.

As more crop plants are now transformable, plant molecular biology techniques are increasingly applicable to agriculture. It is now possible to use gene silencing to verify which plant genes could be targets for new herbicides. Both co-suppression and antisense approaches could be used, but so far, only antisense methods have been reported (Foster and Höfgen, 1993; Höfgen *et al.*, 1995b). Instead of the empirical approach of producing compounds with little or no biological activity, a more direct or rational approach to target validation and pesticide discovery can be used. A practical definition for target validation is the suppression of enzymatic activity by 80% or less, by genetic or chemical means, which produces lethal effects (Rendina and Abell, 1994; Abell, 1996). If the genetic approach is successful in identifying a target site, it also provides a ready means to over-express the gene and obtain large amounts of the target for detailed mechanism studies, high throughput

screening and crystal structure elucidation (Rendina and Abell, 1994; Abell, 1996).

Using gene silencing to down-regulate expression, it is possible to see how important individual genes are for normal plant growth and development. Any genes found which have very deleterious or lethal effects on plant development, are candidate herbicide targets. The next step is to determine the protein structure of the gene products, which are then used to model the enzymatic reaction centre. From this information a chemical inhibitor can be designed to bind to the active site. Foster and Höfgen (1993) showed that this approach will work. They 'antisensed' three genes; the ALS gene (inhibits branched chain amino acid synthesis), threonine deaminase (an enzyme of the isoleucine biosynthesis pathway), glutamate semialdehyde aminotransferase (GSA-AT, δ-aminolevulinate a precursor of chlorophyll synthesis). They found similar phenotypes in regenerants of ALS antisense plants when plants are treated with ALS herbicides. They had reduced gene expression by 80% and this was enough for herbicidal activity. This contrasted with the results for the second target, threonine deaminase. Previous mutagenesis studies showed lack of enzymatic activity was lethal, but rescuable with addition of isoleucine (Negrutiu *et al.*, 1985). Antisense potato plants showed considerable phenotype disruption. Plants were shorter, had altered leaf / plant morphology and discoloured tubers. Antisense tobacco plants showed most inhibition in the F1 generation where plants were extremely dwarfed, had a completely different leaf morphology and lost apical dominance. However, these effects were not sufficient for good herbicidal activity. The final example used an antisense GSA-AT gene which is involved in chlorophyll biosynthesis. A number of transgenics were obtained and these showed a range of phenotypes depending on the level of gene inhibition. These phenotypes mimicked a wide variety of chlorophyll variegation patterns known from horticulture and mutation analysis (Höfgen *et al.*, 1994). Greenhouse grown plants showed leaf damage, and even death, which was dependent on the level of light and chlorophyll content. It was considered that this gene was a suitable candidate for herbicide design (Foster and Höfgen, 1993).

Mode of action studies on many pesticidal compounds have been successful in elucidating what is inhibited but rarely provide information on subsequent events leading to cellular death. Höfgen *et al.* (1995a) have used antisense of the ALS gene to further elucidate how the herbicidal effect is generated. It had been speculated by La Rossa *et al.* (1987) that toxicity of ALS herbicides was due to the build-up of 2-oxobutyrate, a precursor of acetolactate, or its transaminated product 2-aminobutyrate. Other suggestions included amino acid starvation as a cause (Schloss, 1989; Shaner and Singh, 1993). Neither reason appears to be correct. Höfgen *et al.* (1995a) found that both antisense and herbicide-treated plants showed a general increase in the free amino acid pool, which was accompanied by imbalances in their relative proportions. They considered this to indicate a general deregulation of amino acid biosynthesis, which itself might be enough to elicit retardation of plant growth, cell division and other pleiotrophic effects.

IDENTIFICATION OF HERBICIDE MODE OF ACTION

Antisense technology has been used to determine why oilseed rape and wheat are highly resistant to the herbicide quinclorac, while tomato and other vegetables are susceptible. Herbicide activity of quinclorac was thought to be caused by its auxin

activity, which induces synthesis of ethylene in sensitive plants (Grossmann and Kwiatkowski, 1993). By studying ethylene synthesis inhibitors and using antisense aminocyclopropane-1-carboxylate (ACC) synthase tomato plants, Grossmann and Schmülling (1995) showed ethylene biosynthesis was implicated in quinclorac action. Stimulation of ACC synthesis increased ethylene production and caused the accumulation of toxic levels of endogenous cyanide, produced during ACC degradation (Yang *et al.*, 1988). Cyanide causes chlorosis of growing leaves, quickly followed by wilting and shoot necrosis (Grossmann and Kwiatkowski, 1993). Antisense ACC synthase plants were highly resistant to quinclorac action. Ethylene biosynthesis inhibitors were also successful in alleviating the toxic effects of the herbicide.

Use of gene silencing for the establishment and validation of possible pesticide targets is a new and valuable tool. Down-regulation of genes leads to similar effects observed when plants are treated with herbicides. As discussed for plant herbicide design, the down-regulation of genes from plant pests and diseases would convey information on the usefulness of targeting a given gene product as a potential pesticide. Such information will allow a more rational approach to the development of new pesticides and could have an important impact on future agriculture.

CONTROL OF TOMATO FRUIT RIPENING

As a member of the family Solanacea, along with tobacco and potatoes, tomato was an early success for transformation technology. While not as widely used as tobacco as a model system, there have been major advances achieved with this species. For instance, some of the first plant resistance genes have been identified and cloned from tomato and their functions elucidated (Jones *et al.*, 1994). As an important food crop, tomato has received considerable attention from both academic and commercial organizations, to both understand fruit ripening and to modify its control. This relationship between commerce and academia has resulted in the first widely available commercial product of gene silencing technology – the long-life tomato.

One of the key components of the ripening process is a simple gas – ethylene. This gas is involved in many plant processes from leaf and flower senescence and abscission, root initiation and modification of leaf and fruit pigments to hypoxia-induced plant adaptions and interference of geotropic responses (Theologis, 1994 and refs therein). It can be induced by wounding, viral infection, chilling, and drought amongst other stimuli. Induction of senescence by ethylene causes extensive loss of fruit and vegetables world-wide. Due to its importance as a regulatory hormone of fruit ripening, the role of ethylene has been studied extensively.

Fruits are divided into two classes depending on their respiratory response during ripening. The climacteric fruits such as: tomato, pear, apple, banana, melon and mango, all undergo a sharp rise in respiration and significant changes in texture and composition. Non-climacteric fruits like orange, lemon, strawberry do not show any change in fruit composition. With the climacteric rise, there is production of ethylene. The fruit ripening process is thought to be regulated by ethylene which co-ordinates gene expression for respiratory rise, autocatalytic production of ethylene, carotenoid synthesis, chlorophyll degradation, conversion of starch to sugar and cell wall degradation (Gray *et al.*, 1992).

One approach to prevent / delay ripening is to inhibit the production of ethylene.

The biosynthetic pathway is shown in *Figure 1*. The key enzyme involved in ethylene biosynthesis (aminocyclopropane-1-carboxylate [ACC] synthase) has been cloned from tomato plants. This gene has been returned to tomato as an antisense construct in an experiment to alter ripening (Oeller *et al.*, 1991). Down-regulation of ACC synthase caused marked delaying of fruit ripening by inhibition of both isozymes of ACC synthase (LE-ACS2 and LE-ACS4). Down-regulation of LE-ACS4 mRNA by LE-ACS2 antisense RNA was attributed to an identical stretch of 180 base pairs near to the amino terminus (Rottmann *et al.*, 1991). Antisense fruits did not: show a climacteric rise, turn red, soften or develop an aroma (Oeller *et al.*, 1991). This antisense effect can be reversed by treatment with ethylene or propylene for six days. Exogenously applied ethylene induces the normal rise in climacteric respiration, full colour, texture and taste development (Oeller *et al.*, 1991). Ethylene has also been inhibited using antisense RNA to ACC oxidase (Tom 13) (Hamilton *et al.*, 1990). Confirmation that Tom 13 did encode ACC oxidase was obtained when yeast transformed with a full length cDNA could convert ACC to ethylene (Hamilton *et al.*, 1991; Spanu *et al.*, 1991). This is an example of how gene silencing can be used to determine the function of an unknown gene. As for ACC synthase, antisensed fruit could be stored for several weeks at room temperature without over-ripening or shrivelling. Partial restoration of ripening to wild-type levels was obtained by exogenously applied ethylene (Picton *et al.*, 1993). Suppression of ethylene by antisense also delayed the onset of leaf senescence in tomato (Picton *et al.*, 1993). Analysis showed a delay of 10–14 days in the ACC oxidase antisensed plants compared to controls (John *et al.*, 1995). Control of leaf senescence has the potential to increase yields from crop plants.

Many genes have now been cloned from both ethylene biosynthesis and fruit ripening, opening up the possibilities of understanding the complexity of ripening and to improve the quality characteristics of tomatoes for consumption. Some of the genes which have been isolated and manipulated are shown in *Table 2*.

The first gene to be manipulated was polygalacturonase, an enzyme thought to control cell wall degradation during ripening. An antisense construct containing this gene was made to down-regulate its expression (Smith *et al.*, 1988). A major decrease in the levels of PG activity was observed, but initially no differences in fruit firmness was found between transgenic and control plants (Smith *et al.*, 1988). However, further work showed minor differences to be present between control and transgenics at later stages of ripening (Grierson and Schuch, 1993). The transgenic tomatoes were more resistant to mechanical damage and cracking (Schuch *et al.*, 1991) and the low level PG phenotype surprisingly increased pathogen resistance to *Rhizopus stolonifer* and *Geotrichum candidum* (Kramer *et al.*, 1992). Tomato juice made from these fruits was shown to have a higher viscosity than the controls. These characteristics are commercially significant as they allow tomato fruit to be ripened more fully on the vine before harvest, reduce handling and storage losses which would usually occur and improve the quality characteristics conventionally achieved by hot-break processing (Schuch *et al.*, 1991).

A second gene involved in cell wall metabolism is pectin esterase (PE). This is responsible for de-esterification of galacturonic acid residues in high molecular weight pectin. Activity has been detected in a variety of plant fruits such as avocado, banana, pear and tomato. There are three endogenous PE genes in tomato and two of

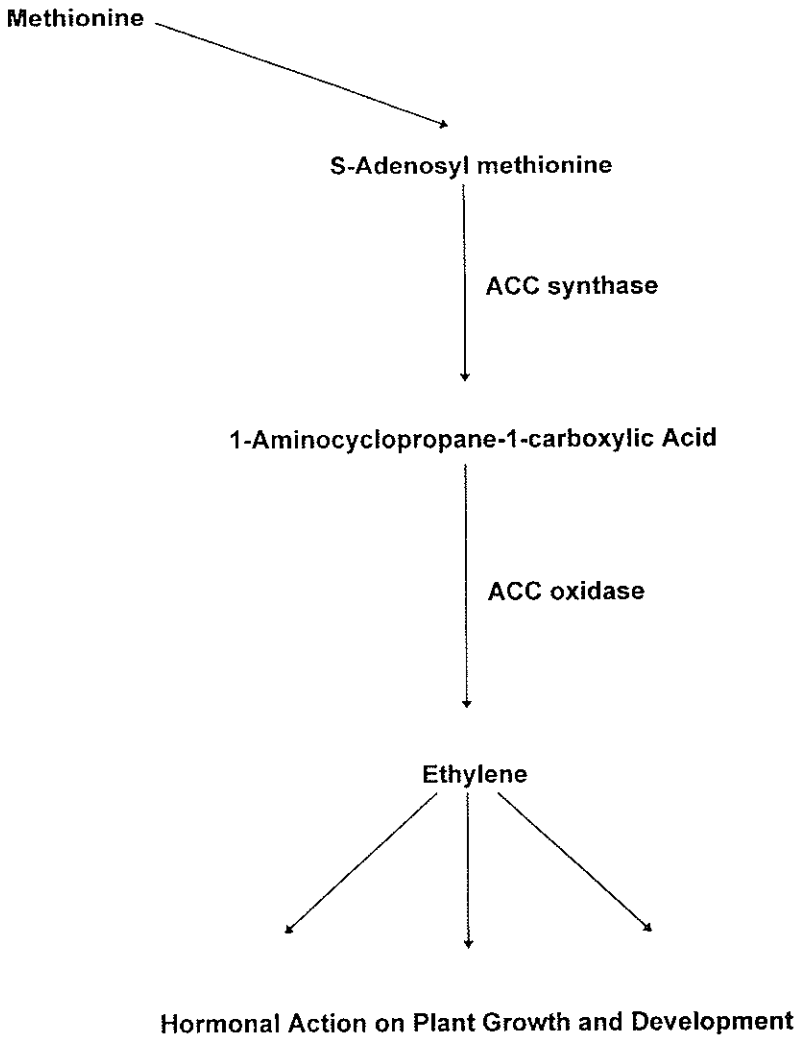

Figure 1. Ethylene Biosynthetic Pathway.

these are expressed in fruit. Hall *et al.* (1993) used an antisense approach to determine the role of this enzyme in fruit ripening. Although 93% inhibition of activity was obtained, no major differences in either fruit development or ripening were detected. Pectin remained heavily esterified at all stages of fruit maturation, confirming the gene's role in pectin de-esterification. Although no differences in either ripening or storage capabilities were observed, processing properties of the antisense tomatoes were improved as serum viscosity of fruit was increased. This was attributed to the different methylation levels of soluble pectin in tomato. This viscosity gave a glossy appearance to the tomato paste (Hall *et al.*, 1993; Grierson and Schuch, 1994). Combining both PE and PG down-regulated genes into one plant produced plants showing characteristics of both and increased soluble solid levels.

Table 2. Examples of identified and modified genes from ethylene biosynthesis and fruit ripening

CDNA	Gene identity	Antisense inhibition	Co-suppression
TOM6	Polygalacturonase	Smith *et al.*, 1988, 1990a	Smith *et al.*, 1990b
F1	Polygalacturonase	Sheehy *et al.*, 1988	
PG16	Polygalacturonase		
PE1	Pectinesterase	Hall *et al.*, 1993	
PET1	Pectinesterase	Tieman *et al.*, 1992	Tieman *et al.*, 1992
TOM13	ACC oxidase	Hamilton *et al.*, 1990 Picton *et al.*, 1993	
tACC2 tACC4	ACC synthase	Oeller *et al.*, 1991	
E8	Unknown	Penarrubia *et al.*, 1992	
TOM5	Phytoene synthase	Bird *et al.*, 1991	Fray and Grierson, 1993

Additionally, combining these genes dissipated the 'granular taste' associated with PG alone (Schuch, personal communication).

The reported research and new technology has resulted in commercial products. Calgene has used an antisense polygalacturonase gene (PG), while Zeneca used a truncated sense PG to effect longer shelf-life and different processing characteristics (Kramer *et al.*, 1993; Smith *et al.*, 1990a). Both companies have been selling genetically modified tomatoes for the last few years, either as the fresh product, 'FlavrSavr ™' (Calgene) or as tomato paste (Zeneca). DNA Plant Technology Corporation have taken a slightly different approach, by using a full length LE-ACS2 coding region to sense suppress ACC synthase and hence ethylene production (Howie *et al.*, 1996). These workers reported that just 5% of their primary transgenic plants had 90% or more of their ethylene production suppressed. From these sense-suppressed lines, they have shown sense ripening arrested fruit can be stored for 30 days prior to ethylene ripening, and these fruits have at least a 14-day life after ripening. This, coupled with much lower losses during production, makes the new tomato commercially viable.

This same technology has been applied to Cantaloupe melons by Ayub*et al.* (1996). This fruit was chosen owing to its good eating character but poor storage. By antisensing the melon ACC oxidase gene, they successfully inhibited gene expression and reduced ethylene production to under 1% of the controls. Transgenic fruit remained attached to the plant, and normal pigment production in the flesh was observed but the rind remained green. Storage of transgenic and control fruit for 10 days at 25°C, produced no change to the transgenic fruit, whereas the controls were shrivelled and yellow, with fungal infections starting to appear. Application of ethylene restored the yellow rind to the transgenic fruit.

Many other fruits and vegetables could be altered similarly to prolong the useful life of food. This would be particularly useful in hot climates where fresh fruit and vegetables rot quickly.

TRANSGENIC TREES

Forestry is an important area of agriculture. We rely on a plentiful source of trees for food, building, furniture and paper amongst others. As for other plant species, the aim

of tree biotechnology is to improve the quality and / or the characteristics of trees for human use. Prevention of disease, pest control, modification of lignin and cellulose are all targets for alteration (Schuch, 1991). The biotechnology of trees has lagged behind other areas of plant biology. However, with recent advances in the transformation and regeneration protocols of both hardwood and conifers, new opportunities are now arising. Most success in transformation of trees has occurred with poplar and larch tree species which are susceptible to *Agrobacterium*, although particle bombardment has also been used. Transformation techniques have allowed the introduction of new characteristics such as herbicide tolerance (glyphosate, Riemenschneider and Haissig, 1991; chlorosulfuron, Brasilero *et al.*, 1992 and glufosinate ammonia, De Block, 1990), and pest resistance (*Bacillus thuringiensis* delta endotoxin (BT) gene, McCown *et al.*, 1991) into trees. Stability of introduced transgenes (*rolC* gene from *A. rhizogenes*) has recently been examined and, as for other plant species, transgene inactivation via methylation can occur (Fladung, 1996). Trees are long-lived organisms, and reach reproductive maturity over a period of years. Introduction of transgenes into trees, requires them to be stably maintained over the long lifetime of the plant. Any unwanted transgene silencing could cause considerable losses to the industry.

Lignin modification

Lignin is an important component of wood, making up 15–35% of the dry weight. In order to manufacture wood pulp and paper, lignin has to be removed. This process is costly both in terms of inputs required and in pollution produced via the process. In the US it is estimated that 20 Mt of lignin is removed from wood fibres each year, and for each tonne 800 kg of sodium hydroxide and 300 kg of sodium sulphide are needed during the process. Lignins also limit the digestibility of forage crops by cattle. However, lignin reduction would only be advantageous for non-ruminants as bloat can otherwise occur (Chesson, 1997). Any reduction in lignin content of trees will give cost benefits both to industry itself and to the environment by reduced pollution.

Characteristics of lignin in conifers differs from the hardwoods because they contain structurally condensed guaiacyl lignin rather than guaiacyl-syringyl lignin. Gymnosperm lignins have a 5-aromatic position available for carbon–carbon bonds. These are very strong bonds making them resistant to the pulping depolymerization process. Guaiacyl lignin thus costs more to process than guaiacyl-syringyl lignin, which does not have these bonds. Conifer trees are still preferred for the pulping process, as they produce longer and better quality cellulose fibres which are suited to many manufacturing processes. A second desirable change to lignin is the substitution of $-OCH_3$ to $-OH$ groups in angiosperm lignin. This would increase lignin solubilization and result in decreased amounts of sulphur-containing compounds released into the atmosphere during processing (Boudet *et al.*, 1995).

The metabolic pathway leading to lignins is relatively well known (Boudet *et al.*, 1995), although recent evidence has indicated two methoxylation pathways for lignin biosynthesis exist. The first uses free acids (caffeic acid and 5-hydroxyferulic acid) as substrates, while the alternative route uses Co A esters of hydroxycinnamic acids as substrates (Ye *et al.*, 1994). There are three types of monolignols that can be formed in plants. These are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Once formed these are transferred to the cell wall where polymerisation occurs forming

condensed lignin. Down-regulation of specific enzymes of the pathway to condensed lignin would result in considerable reduction in pulping costs. To this end, various suppression experiments have been performed using different genes of the biosynthetic pathway.

Some of the genes involved in the lignin biosynthetic pathway have been cloned, opening up the opportunity for genetic manipulations. The phenylalanine ammonia-lyase (PAL) enzyme was one of the first genes isolated and was used in antisense experiments. Reduced lignin content was obtained in transgenic tobacco but it also caused pleiotrophic effects such as altered leaf shape and texture, altered flower morphology and pigmentation (Elkind *et al.*, 1990). Such early enzymes of the pathway are unsuitable for manipulations due to these effects.

O-methyltransferase (OMT) genes are a target for manipulation as reduction in activity should improve lignin solubilization and require less polluting chemicals in paper manufacture. This idea has already been tested by several groups. Ni *et al.* (1994), introduced a heterologous antisense OMT fragment into tobacco and obtained significant reductions in lignin content without alteration of monomer composition. Atanassova *et al.* (1995) successfully altered tobacco lignin using both sense and antisense OMT constructs. For the full-length sense construct, only 1 out of 20 transformants showed significant co-suppression, with just 3% of control activity. Most other transformants over expressed OMT activity. Antisense constructs were more successful in down-regulating OMT expression (15 plants obtained), with the most suppressed line exhibiting just 3% of control activity. Even though considerable inhibition of activity was demonstrated, total amount of lignin was unaffected contrasting with the result of Ni *et al.* (1994). Atanassova *et al.* (1995), showed composition of lignin obtained from OMT down regulated plants was significantly different from controls. The syringyl : guaiacyl ratio was altered owing to a decrease in syringyl unit abundance and a new monomer, 5–OH guaiacyl unit, appeared. To obtain such changes, OMT activity had to be reduced by 80% or more. Similar changes have also been observed in transgenic poplar trees (van Doorsselaere *et al.*, 1995), with an additional change to xylem colour. This changed from the control white / yellow to pale rose. This has been compared to the brown midrib mutants of maize and sorghum which are known to have altered lignin composition and increased digestibility (Grand *et al.*, 1985; Pillonel *et al.*, 1991). Caffeoyl CoA O-methyltransferase, has also been suggested as a target to alter the monomeric composition of lignin. This enzyme may be involved in an alternative pathway to G units, which could produce lignins with different monomer compositions (Boudet and Grima-Pettenati, 1996).

There are two enzymes considered to be lignin biosynthetic pathway specific, Cinnamoyl-CoA reductase (CCR) and cinnamoyl alcohol dehydrogenase (CAD). CCR has been down-regulated by antisensing the gene and this caused an orange-brown colour in the xylem area. It is thought that this indicated significant changes in composition of lignin had occurred. CAD was first targeted by Halpin *et al.* (1994) who used an antisense CAD tobacco construct to down-regulate expression to as little as 7% (line 50). The plants obtained had low CAD activity and the xylem was red-brown. Analysis indicated that there was an increase in coniferyl and sinapyl aldehydes in line 50. CAD down-regulation had thus produced a novel lignin. The pulping characteristics of the CAD down-regulated tobacco and poplar plants have been

investigated and antisense plants had better pulping characteristics than their controls. The pulp yield was increased along with decreased chemical inputs (Boudet and Grima-Pettenati, 1996).

Ralph *et al.* (1997) have shown a loblolly pine mutant produced a novel lignin monomer, dihydroconiferyl alcohol. This accounted for up to 30% of the units in the mutant. They also observed increased levels of aldehydes including the production of two new methoxybenzaldehydes. The wood of this mutant pine was red-brown and was likened to that of the brown midrib mutants. The changes observed resulted from a CAD deficiency. For homozygous mutant pines carrying the *cad-n1* allele, CAD activity was 1% or less compared to wild-type. Such results indicate the plasticity of lignin biosynthesis and show large changes to composition do not necessarily disrupt lignin functions (Ralph*et al.*, 1997). Results obtained from both mutant and transgenic studies indicate lignin manipulation can be achieved through the use of gene silencing.

Fruit tree modification

Genetic transformation of fruit trees has been achieved (James *et al.*, 1989). Apple trees have been shown to reliable maintain transgene stability throughout the long juvenile stage (James, 1993). Transgenic apple trees have now been shown to display stable expression of kanamycin, both in fruit flesh and in subsequent generations (James *et al.*, 1996). Apples are climacteric fruits and as such their ripening is controlled by ethylene. These trees can therefore be modified to prolong the survival of their fruits using an antisense approach against ethylene production, in the same manner as for tomatoes (see earlier). ACC synthase and ACC oxidase have been cloned from apples (Dong*et al.*, 1991, 1992) and these have been introduced back into apple trees (James, 1993). Another ACC synthase has recently been identified from ripening McIntosh apples (MdACS-2) and has been transformed back into Royal Gala and McIntosh apple plants in another attempt to down-regulate ethylene and hence fruit ripening (Hrazdina *et al.*, 1997). No reports of success have been published. As for forestry trees, genetic modification will introduce a wide variety of genes to control pests and diseases.

OIL MODIFICATION

This area of research has been at the forefront of transformation technology because of the potential for valuable new and novel products it encompasses. Since the first report of novel oil production in the early 1990s (Knutzon *et al.*, 1992), there have been many successes in manipulations of oil composition, some of which have now entered full scale agricultural production (Kridl and Shewmaker, 1996). Higher plants are thought to produce over 200 different fatty acids, but the majority of plants used for oil production only accumulate about ten of these to any extent. The majority of these differ only in chain length and level of unsaturation, but these can be converted to a range of useful products such as coatings and paints, adhesives, surfactants and lubricants. Natural sources of fatty acids are a mixture and these need to be converted to a common derivative or separated into individual fatty acids before they can be processed. By using molecular biology to increase purity of natural fatty acids, some of this processing could be reduced. The other aim is to increase the variety of fatty

acids available from field crops, reducing the need for chemical conversion or use of non-renewable raw materials.

Alterations to lipid composition

Oilseed rape (*Brassica napus*) is an obvious choice for molecular plant breeding. In our laboratory it is readily transformable and manipulated at both molecular and tissue culture levels. Since the early 1970s, oilseed rape has become a common agricultural crop in the UK and its oil has wide commercial application. Fatty acid composition is known to be amenable to manipulation by conventional breeding methods (Lühs and Friedt, 1993). Reduction in erucic acid (C22:1) content and glucosinolates by conventional plant breeding has produced the canola varieties extensively grown in the USA and Canada. Considerable molecular breeding has already occurred and new canola lines have entered commercial agriculture, e.g. high laurate canola (Voelker *et al.*, 1992). Most of this molecular breeding has been by incorporating new genes into *Brassica* to produce novel oils. High laurate canola was produced using a lauroyl-ACP thioesterase gene from the Californian bay tree (Voelker *et al.*, 1992). However, there are opportunities for gene silencing in oil modification. Knutzon *et al.* (1992) successfully increased the stearate (C18:0) levels of mature seeds of both *B. napus* and *B. rapa,* using an antisense seed specific stearoylacyl carrier protein desaturase gene construct. This enzyme catalyses the first desaturation reaction in fatty acid biosynthesis. By down-regulating this enzyme, seed oil contained up to 25 times more stearate acid than controls and, by using a seed specific promoter, they maintained the normal membrane fatty acid composition in the rest of the plant. By increasing 18:0 levels in oilseeds, a cocoa butter-like fat is produced. A similar fat is normally produced by cocoa itself, or by the hydrogenation of vegetable oil. Using oilseed to make this fat reduces the dependency on other resources, there are health benefits as no *trans* fatty acids are produced and it may have novel properties, as stearate is not incorporated into the *sn*-2 position of the triacyl glycerides. Kinney (1994) suggested that further increases in saturated oil content could be achieved by hybridisation of down regulated Δ^9 desaturase phenotype with over-expressed thioesterase phenotype, but such attempts have not so far been reported.

Seed companies are also interested in reducing the level of 18:2 and 18:3 fatty acids, which will improve both oil stability to oxidation and its taste characteristics. Various mutants are known with lower levels of these fatty acids such as IMC01 mutant of canola, and the A5 mutant of soybean (Kinney, 1994 and refs therein). Both canola and soybean have also been genetically modified to produce lower levels of 18:3. This has been achieved using both antisense and sense suppression techniques to inhibit the Δ^{15} desaturase (Fader *et al.*, 1995). These new sources of modified fatty acids have uses for frying and in salad oils. Canola and soybean have been genetically modified so that they produce high levels of 18:1 fatty acids (Fader *et al.*, 1995). This has resulted from the down regulation of oleate Δ^{12} desaturase (*fad2*) by both antisense and sense suppression, preventing the insertion of the second double bond. This type of oil again has uses for frying, as salad oil and also as high stability spray oils (Kridl and Shewmaker, 1996). Broglie *et al.* (1997) reported that gene silencing of *fad2* in soybean, had remained stable through multiple generations and at many test sites. Seeds from these transgenic plants contained less than 5% polyunsaturated fatty acids

and greater than 80% monounsaturated acid. As expected, this had greatly improved the oxidative stability of the oil.

Cotton is the world's second largest oilseed crop but, due to the presence of a toxic sesquiterpene gossypol and derivatives, use of cottonseed meal and oil is somewhat limited. Wilkins and Loguercio (1997) are attempting to produce gossypol-free germplasm by down regulating the HMG CoA reductase (HMGR) in pigment glands of developing embryos using antisense RNA.

Production of polymers in transgenic plants

One of the most exciting possibilities in manipulating oil biosynthesis pathway is the production of polyhydroxybutric acid (PHB). PHB is a biodegradable polymer which has a growing commercial market. It is presently produced by fermentation of bacteria. This is an expensive process and causes the end-product to be more expensive than other non-renewable plastics. If this polymer could be made by plants, production cost will be greatly reduced and this will allow greater use of a renewable, environmentally friendly plastic. There are other forms of biopolymers that can be produced in plants and bacteria, e.g. protein-based polymers, that do not rely on silencing; see Daniell and Guda, 1997.

Production of PHB is dependent on the main building block for fatty acid synthesis, acetyl CoA. Sequestration of acetyl CoA into PHB synthesis will reduce overall fatty acid levels in seeds. As both antisense and sense suppression produce a range of phenotypes, it should be possible to obtain a level of suppression of carboxylase (the enzyme responsible for acetyl CoA incorporation into lipid), which allows for normal plant growth and development, but which reduces flux into storage oil. The acetyl CoA could then be utilized predominantly for PHB synthesis. To enable this objective, three new bacterial genes and one suppressing gene construct need to be incorporated into oilseed rape. So far, PHB synthesis enzymes have been expressed in *Arabidopsis thaliana* and a level of 14% dry weight PHB has been attained in leaves (Nawrath *et al.*, 1994), while 1–4% (seed dry weight) PHB production has now been achieved in leucoplasts of both soybean and canola (Slater *et al.*, 1997). Slabas *et al.* (1997) have indicated that they have used antisense expression to suppress the plasticidal ACCase and fatty acid synthetase using 35S CaMV and ACP promoters, but little information is currently available on the resultant phenotypes. At present, much more work is required before we can farm PHB bioplastics.

MOLECULAR FLOWER BREEDING – NOVEL TRAITS

The cut flower industry is another area in which gene silencing may enhance the commercial end product. Over the last few years a greater understanding of flowering has emerged. New genes controlling floral organ identity have been discovered (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994), the biochemistry and genetics of flower colour production has been elucidated (Forkmann, 1991, 1993), and progress has been made into how flowering architecture is controlled (PERIANTHIA gene from *Arabidopsis*, Running and Meyerowitz, 1996; CENTRORADIALIS gene from *Antirrhinum majus*, Bradley *et al.*, 1996; CYCLOIDEA gene from *A. majus*, Luo *et al.*, 1996; CLAVATA1 gene from Arabidopsis, Clark *et al.*, 1997), how plants

switch from vegetative to floral forms (analysis of LEAFY activity in *Arabidopsis* and Aspen, Weigel and Nilsson, 1995) and how Myb transcription factors regulate gene expression (Martin and Paz-Ares, 1997). With the availability of all these genes and appropriate transformation technologies, we can potentially manipulate flowers and inflorescences by either, gene expression, or silencing techniques.

Flower colour manipulations

The obvious first choice for genetic manipulation was flower colour as so much is known about how colour is produced, there are a range of genes already available for manipulation (Davies and Schwinn 1997) and because of the added premium that unusual flower colour can command in the market-place. Many flowers have a limited colour range, for instance, blue-flowering varieties are missing from carnations, chrysanthemums and roses. Genetic changes to flower colour are non-disruptive to plants themselves and provide an easy assay for success. It is expected that genetic manipulation of biosynthetic pathways will help to broaden the colour spectrum available. Other workers have shown how to alter pigmentation patterns (Mooney *et al.*, 1995), or flower colour intensity and lustre (Noda *et al.*, 1994). Other characteristics could also be targeted for manipulation, e.g. petal senescence, plant shape and fragrance to enhance the commercial appeal of such plants. The main cut flower industry plants and whether they are transformable are shown in *Table 3.*

Flowers have been at the forefront of gene silencing research as they readily show any altered phenotype, even if these are only subtle changes. Co-suppression was discovered by flower colour change (Napoli *et al.*. 1990; van der Krol *et al.*, 1990a). A purple *Petunia* line was transformed with a chalcone synthase transgene, and a proportion of the progeny were unexpectedly white. Analysis showed the transgene to be present and that it, and the endogenous CHS gene, had both been silenced. Early research showed that transgenes can also be influenced by their environment, as hot dry conditions induced gene silencing of a *Petunia* line containing an A1 gene from maize (Meyer*et al.*, 1992).*Petunia* plants have continued to be used for gene silencing research both in Europe and America providing a greater understanding of the phenomena.

Use of sense and antisense techniques to manipulate flowers

The biosynthetic pathway for flavonoid production is well characterized with genes being cloned from a variety of sources (Holton and Cornish, 1995; Forkmann, 1991). This has allowed comparison of homologous (*Petunia*) and heterologous (chrysanthemum) sequences for their ability to suppress *Petunia* chalcone synthase and dihydroflavonol reductase genes. The heterologous genes were 70% identical to the endogenous *Petunia* sequences. Each was driven by the 35S promoter and had the nopaline synthase 3' terminator sequence. For the *Petunia* sequence, 16% of transformants showed suppression of CHS, and 10% for DFR. The chrysanthemum sequences did not suppress expression of either gene. Gutterson (1995) suggested that, for efficient sense suppression, the host gene is the favoured gene source. Gutterson (1995) went on to show that sense suppression in *Petunia* could be achieved with as little as 225 bp of sequence. There was a penalty incurred, however, while suppression

Table 3. The main plant species of the cut flower industry : transformation and regeneration

Plant	Transformable?	Method used	Plants regenerated?	References
Rose	Yes	At	Yes	Gutterson 1995
		Ar	Yes	van der Salm *et al.*, 1997
Carnation	Yes	At	Yes	Lu *et al.*, 1991
Tulip*	Yes	PB	Yes	Wilmink *et al.*, 1994
		Ar	No	Wilmink *et al.*, 1992
		PB (pt)	No	Tanaka *et al.*, 1995
Chrysanthemum	Yes	At	Yes	Ledger *et al.*, 1991
Freesia*	TE	PB (pt)	No	Tanaka *et al.*, 1995
Gerbera	Yes	At	Yes	Elomaa *et al.*, 1993
Lily*	TE	PB (pt)	No	Nishihara *et al.*, 1993
		E (pt)	No	Miyoshi *et al.*, 1995
		PB (t)	No	Tsuchiya *et al.*, 1996
		At	No	Langeveld *et al.*, 1994
Iris*	No	–	–	–
Daffodil*	No	–	–	–
Gypsophila	No	–	–	–

* = Monocot spp.
At = *Agrobacterium tumefaciens* mediated transformation.
Ar = *Agrobacterium rhizogenes* mediated transformation.
PB = Particle bombardment transformation.
(pt) = Pollen, transient gene expression.
E = Electroporation transformation.
TE = Transient expression.

could be seen, the proportion of plants with high suppression fell when the sequence size was reduced to below 550 bp.

CHS sense suppression has been used to either produce white or pale-coloured flowers for *Petunia*, chrysanthemum, carnation and rose. The extent of suppression varied between the species. For chrysanthemum, completely white flowers were obtained by both sense and antisense constructs (Courtney-Gutterson *et al.*, 1994), although the frequency of such plants was low for both construct types (3/133 sense and 3/83 antisense). For carnations and roses, only pale-coloured flowers were obtained as CHS suppression was incomplete (Gutterson, 1995). To address transgene stability, 200 chrysanthemum cuttings were prepared for each of three field sites. No differences were found for the original cultivar (moneymaker) or for control regenerants. Cuttings from transgenic white flowered plants generally remained white; however, four plants produced pink colouration. This was thought to be environmentally induced as cuttings from pink flowering branches subsequently gave white flowers. At the two other test sites a higher proportion of transgenics gave pink flowers (up to 12%). Overall, sense-suppressed plant cuttings gave higher proportion of pink flowered offspring than antisense plants. The transformation process had also affected both plant height and time to flowering, but neither change was considered commercially important (Courtney-Gutterson *et al.*, 1994). Suppression of CHS can have other effects on flowers. Variegated *Petunia* flowers have been produced which are similar to horticultural varieties such as picotee type, Cossack Dancer, stars and highly disordered patterns (Jorgensen, 1995; van Blokland *et al.*, 1994). If these pattern types can be stabilized, new novel variegated varieties could become commercially available.

Antisense technology was first used to modify flower colour in 1988. Van der Krol *et al.* (1988) used antisense technology to down-regulate *Petunia* CHS expression. Fully white transformants were obtained along with other variable phenotypes having sectored pigmentation patterns. This unexpected effect was influenced by plant hormones and light intensity. The natural *Petunia chs* mutant, Red Star, also shows these effects (van der Krol *et al.*, 1990b).

By targeting different genes, it is possible to use antisense to increase the colour intensity of flowers. Holton *et al.* (1993) antisensed a flavonol synthase (FLS) gene involved in flavonol biosynthesis. Introduction of this gene into *Petunia* produced 4 out of 12 transformants having redder flowers, thought to be caused by alteration to copigmentation. Flavonol synthesis was markedly reduced compared with controls. This same construct produced flowers with increase levels of anthocyanins in an heterologous tobacco system (Holton *et al.*, 1993). Antisense modification of flavonoid pathway can affect copigmentation and might be a way to increase anthocyanin levels of weakly pigmented flowers. Targeting other modification steps of anthocyanin biosynthesis have also resulted in altered flower colours. Brugliera *et al.* (1994) antisensed anthocyanidin-3-glucoside rhamnosyltransferase and 7 out of 12 transformants had uniformly pink flowers in most cases, with some having purple and red sectors instead of the control flowers purple pigmentation.

Fertility control

Flavonoids are probably best known for the blue, red and purple colouration they provide in flowers, fruits and leaves. However, this is not their only function as they also play important roles in many other areas. These include: defence, signalling compounds in reproduction, pathogenesis, symbiosis and allelopathy (Shirley, 1996). Therefore, any modification of such genes could have consequences for the well being of the plant and its ability to reproduce. Recent evidence has implicated CHS repression by mutation, antisense or sense constructs in reduced plant fertility (Coe *et al.*, 1981; van der Meer *et al.*, 1992; Franken *et al.*, 1991). Van der Meer *et al.* (1992) showed CHS antisense construct caused male sterility in *Petunia*. This was associated with tapetum expression of the construct, which suppressed normal flavonoid production during male gametogenesis and infertile white pollen was produced. While these findings pose possible problems, any method which could control fertility, might offer the prospect of a new transgenic hybrid seed process. Such a system is under investigation by Bovy *et al.* (1997). They have confirmed that flavonols are required for *Petunia hybrida* self-fertilization and that antisensing the *CHS-a* gene prevented this. They are currently investigating how to restore self-fertility to the F1 generation. Their approach is to cross in a maize *Ic* gene, which can up-regulate several flavonoid biosynthesis genes. By doing this, they have partially restored flavonoid production and fertility.

Long-life cut flowers

Once cut, flowers deteriorate rapidly, and a short vase life is an obstacle to flower marketing. It is the onset of petal senescence that is the determining factor in the vase life of flowers. To address this problem, carnation flower senescence has been studied extensively. It is widely known that vase life can be extended by addition of silver ions

or cytokinins, which inhibit ethylene biosynthesis or action (Mol *et al.*, 1995). Ethylene production, ACC oxidase and two ACC synthase gene's expression have recently been examined (ten Have and Woltering, 1997). Using cut flowers of the variety White Sim, they found that ACC oxidase was expressed in all floral organs, whereas the two ACC synthase genes (CARACC3 and CARAS1) showed tissue-specific expression. Savin *et al.* (1995) created an antisense ACC oxidase construct and transformed carnation varieties Scania and White Sim. One transgenic from each variety showed increased cut flower life. These were shown to produce 10% of the normal control levels of ethylene, and had low levels of mRNA at the time when inrolling of control flowers occurred. Addition of exogenously applied ethylene could at least partially reverse the inhibition. Carnation plant varieties with similar modifications have been field tested in Australia over the past few years.

Gene silencing and metabolic pathway engineering

Manipulation of metabolic pathways is the ultimate aim of many transgenic plant programmes. Redirecting carbon flux from one pathway to another will facilitate the production of high value commodities, reduce reliance on non-renewable resources and give higher quality food products. However, there are still many problems to be overcome before these objectives become reality.

CAN MULTIPLE GENES BE DOWN-REGULATED AT THE SAME TIME?

In some instances it might be essential to knock out several pathways at once or sequentially as part of an overall strategy in manipulating metabolism. So far the author has only concentrated on manipulation of single genes to alter metabolism, but it is conceivable that two, three or more genes may be required to be silenced in order to attain a new product or pathway. Can this be achieved? The evidence so far is encouraging. It has been shown that chimeric gene constructs encoding two different genes can successfully inhibit the expression of corresponding endogenous genes. In the first study of this kind, Seymour *et al.* (1993) suppressed endogenous PE and PG genes using a chimeric sense PE-PG construct driven by 35S CaMV promoter. They showed the construct was expressed in leaves at the level of mRNA. Wild-type fruit express PE and PG during ripening, but transgenic plants carrying a chimeric copy of PE-PG showed significant reduction in expression along with the transgene. They also found this construct was inherited following Mendelian principles. Stam (1997) showed CHS, dihydroflavonol reductase A (DFRA) and flavonol synthase (FLS) endogenous genes could be targeted and silenced by promoterless chimeric constructs. DFRA was fused to either CHS or FLS and in transformants showing silencing, the endogenous expression of both genes was shown to be co-ordinately suppressed. Detailed analysis of the silencing T-DNA loci, showed they consisted of two or more T-DNAs. The strongest suppression occurred where T-DNAs had integrated as inverted repeats (Stam, 1997). The amount of repetitive sequence and arrangement of transgenes in a single locus appears to be the essential component of the silencing rather than chromosomal position of T-DNA or presence of multiple unlinked endogenous homologues (Stam, 1997). The level of transcript also appears to be of low importance for gene silencing in this case. Jones *et al.* (1996) used a single

gene construct encoding PG and PE to silence the same two non-homologous endogenous genes. They compared their construct against plants produced by hybridizing the two antisense construct together. They found that the chimeric transgene acted in both sense and antisense orientations similar to transgenes encoding the single gene. The suppression observed was co-ordinated even though the two genes are located on different chromosomes. This was considered incompatible with ectopic pairing, unless co-ordinated transient interactions can occur. This technique, when fully developed, will allow the manipulation of commercially important multiple gene traits which cannot be altered at present.

WILL 'STACKING' TRANSGENES CAUSE PROBLEMS?

As more genes controlling different biochemical pathways are identified, metabolic engineering becomes possible. If more than one construct is required, there is the possibility that transgenes could interact and gene silencing be encountered. As already discussed, transgene silencing can occur in a number of different ways. The silencing of transgenes seems linked to the copy number of the transgene in a given plant. Multiple copies at the same genetic locus can exert extensive effects on transgenes introduced by a second transformation event (Matzke *et al.*, 1989) or by crossing two different transgenes together (Matzke *et al.*, 1993). The effects of such interactions can be felt even after the transgenes have been separated again and can take several generations to fully reactivate. The amount of homology between the two different transgene constructs is considered important as this seems to be sensed by the cell and silenced accordingly. The common factor in gene silencing phenomena appears to be the presence of more than one transgene at the silencer locus (Hobbs *et al.*, 1990; Linn *et al.*, 1990; Mittelsten Scheid *et al.*, 1991; Assaad *et al.*, 1993; Matzke *et al.*, 1994; Vaucheret, 1993, 1994; Cluster *et al.*, 1996; Jorgensen *et al.*, 1996; Stam, 1997; Stam *et al.*, 1997b).

However, there is evidence showing transgenes can be successfully stacked in the same plant. Work by Boulter *et al.* (1990) showed that different transgenes can be successfully crossed together. They found an additive effect of combining the two constructs together over each individually, enhancing pest resistance. Similar work by Zhu *et al.* (1994), showed fungal resistance of tobacco was enhanced against *Cercospora nicotianae* by using a combination of two different transgenes compared to either transgene alone. The authors' own work on *Brassica napus* has used single copy transgenes which had regions of homology to each other, in both coding and promoter regions. When hybridized together, the reporter genes used operated effectively, even though four copies of the 35S Cauliflower mosaic virus promoter were present in a single plant (Senior *et al.*, unpublished observations). This observation further illustrates that the make-up of genetic loci is important in the silencing of transgenes rather than the DNA sequence alone.

By selecting transgenic plants with single copy inserts and reducing sequence similarity to known host genes to below 70%, it should be possible to 'stack' a range of constructs together to improve or even make new biochemical pathways in plants. With the possible exception of highly expressing transgenes causing silencing (Elmayan and Vaucheret, 1996), single copy transgenic plants should be used in stacking hybridisations.

Another way to build a pathway is to use BIBAC vectors (binary bacterial artificial chromosomes) which can house 150 kb of genetic material (10 or so genes) sufficient for a new pathway (Hamilton *et al.*, 1996). With such large stretches of DNA, new controlling factors can be manipulated to enhance expression. Such large stretches of DNA might eliminate site-dependent gene expression, which will be essential in building multigene pathways. No evidence has so far been published showing whether these vectors express the encoded DNA, but, as the secondary structure can be manipulated by altering the sequence of genes and inserting matrix attachment regions (MAR) and other control elements its possible that gene expression could actually be enhanced.

One final way to produce commercially viable plant products is to infect non-transgenic plants with transgenic viruses (tobacco mosaic virus). Large quantities of product can be obtained within two weeks, alleviating the need to produce stable transgenic plants and any possible risk associated with them. This approach has been successfully used in the US by Biosource Technologies CA (Della-Cioppa and Grill, 1996). Kumagai *et al.* (1995) reported the modification of key isoprenoid intermediates by up to 50-fold with genetic sequences expressed in either sense or antisense orientation. This is the first demonstration that antisense transcripts generated in the cytoplasm can down-regulate the expression of endogenous genes. Such manipulations allow the production of secondary products in amounts normally lethal if produced by 'conventional' transformation techniques. As the technology develops, multiple genes in both sense and antisense orientations will be incorporated into viral vectors allowing construction of novel pathways in plants to divert carbon flow into the desired commercial product (Della-Cioppa and Grill, 1996).

Conclusions

Gene silencing was initially an unexpected hurdle to producing stable transgenic plants with the desired new trait. It has cost-implications for industry trying to obtain efficient transformation systems and increased the time required to obtain stable transgenic plants. However, it is now clear that gene silencing offers an exciting opportunity to produce new and interesting plant products, allowing industry a valuable opportunity to recoup its investment in plant biotechnology (Senior and Dale, 1996). With the advent of transformation protocols for monocotyledonous species, silencing technology is already expanding into this area, e.g. antisense of allergenic components of rice (Tada *et al.*, 1996) and as cereals form a large part of the human diet more examples will quickly follow.

It is interesting to note that the first products of plant biotechnology have arisen from gene silencing using either antisense or sense suppression techniques. The large chemical / biotechnology companies have invested the resources to develop products from initially obscure findings, which have led to the FlavrSavr™ tomato, for instance. Examination of USDA field releases shows how important gene silencing is becoming. Many unwanted traits have been removed by down regulation of the relevant gene by silencing techniques. Thus new plant varieties have been created that have resistance to viral diseases (e.g. potatoes), longer shelf-life (e.g. tomatoes), extended cut flower life (e.g. carnations) and modification of storage compounds (e.g. potatoes and oilseed species). The opportunities seem endless. However, only

those products that fulfil a real need should be developed and brought to market. Ultimately, the approval of such genetically modified organisms relies on consumer acceptance, which is at least partially dependent on how the new product and technology is presented. So far, the acceptance of genetically modified tomato should be an example of how perceived need is translated into a marketable product. Other transgenic products, whether from silencing technology, or introduction of new traits, need to fulfil these criteria to be accepted over non-transgenic alternatives (Senior and Dale, 1996). Overall, plant gene silencing phenomena have now entered a new commercial age from which valuable products should ensure their continued success.

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