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Review

Gene stacking in transgenic plants – the challenge for 21st century plant biotechnology

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Summary

One of the major technical hurdles impeding the advance of plant genetic engineering and biotechnology is the fact that the expression or manipulation of multiple genes in plants is still difficult to achieve. Although a small proportion of commercial genetically modified (GM) crops present 'stacked' or 'pyramided' traits, only a handful of products have been developed by introducing three or more novel genes. On the research front, a variety of conventional and more novel methods have been employed to introduce multiple genes into plants, but all techniques suffer from certain drawbacks. In this review, the potential and problems of these various techniques and strategies are discussed, and the prospects for improving these technologies in the future are presented.

Introduction

Transgenic crops have the potential to promote revolutionary change in agriculture, industry, nutrition and even medicine. By manipulating plant genomes, crops can be engineered to provide enhanced nutritional value and to be resistant to biotic and abiotic stresses, plant raw materials can be better adapted to the requirements of industry, and 'green factories' can be used to produce a host of novel products, including pharmaceuticals, in an environmentally benign and sustainable manner. Although much has been written about the staggering potential of genetically modified (GM) crops, achievements to date have been relatively modest, as the technology itself is still in its infancy. This gap between the hyperbole and the reality of the relatively crude first-generation 'input trait' GM crops, perceived by many to offer benefits only to seed companies and farmers, has prompted several commentators and pressure groups to deem the technology a failure. Progress towards second-generation 'output trait' products with nutritional, environmental or other benefits that consumers can appreciate directly has been slow, and will continue to be so until the bottleneck of developing technologies for the co-ordinated manipulation of multiple genes or traits has been removed. This bottleneck is perhaps not widely appreciated, but is amply evidenced by the huge body of literature describing the manipulation or expression of single useful genes in plants and the comparative paucity of publications dealing with the manipulation of multiple genes. Yet, the advantages of 'stacking' or 'pyramiding' existing GM traits in crops are obvious, and offer the potential for providing durable multi-toxin resistance to particular pests or for engineering multiple resistance to different types of pathogen, perhaps in crops that are also herbicide tolerant. Similarly, the potential for sophisticated metabolic engineering in plants is enormous, and could lead to the development of plants able to grow in inhospitable environments, which might provide healthier foodstuffs or improved raw materials. However, most metabolic processes that are targets for manipulation depend on the interaction between numerous genes, and flux through biochemical pathways is often co-ordinated with that of competing pathways; therefore, effective metabolic engineering will only be achieved by controlling multiple genes on the same, or interconnected, pathways. For example, although three carotenoid biosynthesis genes have been successfully engineered into 'Golden rice' to allow it to produce provitamin A (Ye *et al*., 2000), efficient provitamin A absorption may require the resorbable iron content to be enhanced, potentially requiring the introduction of another three genes. Similarly, it is estimated that the production of reasonable levels of a really durable biodegradable plastic [a copolymer of polyhydroxybutyrate (PHB) and polyhydroxyvalerate] in crops would require the introduction of four to six genes and

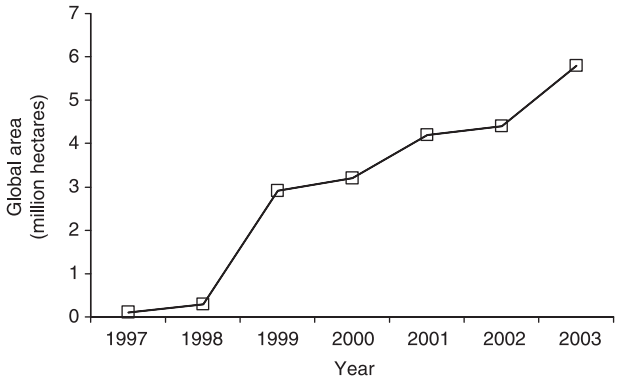

Figure 1 Global area of stacked trait transgenic crops. Redrawn from James (2003).

the manipulation of several metabolic pathways (Slater *et al*., 1999).

Only 8% of commercially grown GM crops in 2003 contained 'stacked' traits (James, 2003) conferred by the expression of two or more 'effect' genes (an 'effect' gene is one intended to effect some useful change in the final plant product, as opposed to a selectable marker gene which may only be used for the initial selection of transformed material). Nevertheless, high growth rates in the global area planted with stacked trait crops is expected in future years following a significant and consistent increase over the past 5 years (Figure 1). The current predominant stacked trait crops are insect-resistant and herbicide-tolerant maize and cotton. These products often contain only two introduced genes, as the 'effect' gene conferring herbicide tolerance can also be used as the selectable marker for transformed plant material. Indeed, only a handful of GM crops with three or more stacked genes have yet gained regulatory approval (Table 1).

Despite such slow progress, over the past few years significant advances in multigene manipulation have been made using a variety of conventional and novel techniques that, despite imperfections, provide a framework for future improvements. This article reviews the various strategies that have been employed, commenting on their specific advantages and problems, in order to provide some perspective on what has already been achieved and on the scale of the challenge facing plant biotechnology.

Iterative strategies

Two or more transgenes can be sequentially introduced into a plant by conventional iterative procedures, e.g. a plant containing one transgene is crossed with individuals harbouring other transgenes or, alternatively, is re-transformed with new genes. These techniques have been used, at the research level

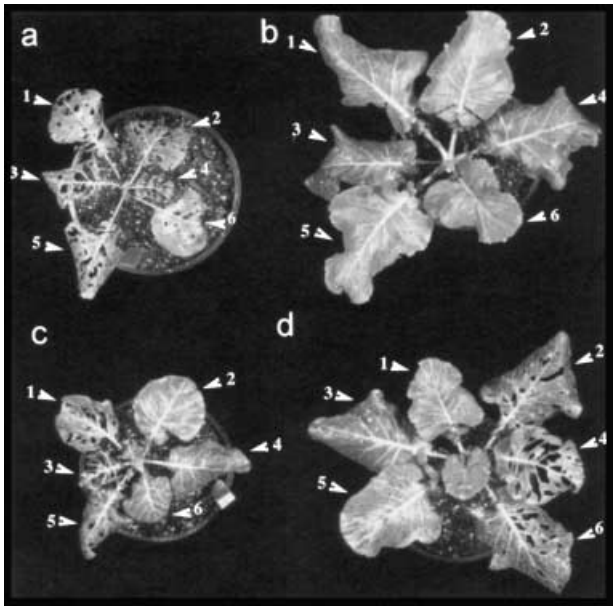

Figure 2 Bioassay of intact *cry1Ac*, *cry1C*, *cry1Ac* + *cry1C* (produced by crossing) and untransformed broccoli plants for control of late second instar Cry1A^R and Cry1C^R diamondback moth larvae. Each odd-numbered leaf was infested with five Cry1A^R larvae and each even-numbered leaf with five Cry1C^R larvae. Larvae were allowed to feed for 5 days. (a) Green Comet control; (b) *cry1Ac* + *cry1C* hybrid (Q23 × H1); (c) *cry1Ac* plant (Q23); (d) *cry1C* plant (H1). Reproduced from Cao *et al*. (2002) (figure 3), with kind permission of Springer Science and Business Media.

at least, to combine or reinforce existing transgenic traits. For example, crossing plants expressing different *Bacillus thuringiensis* (Bt) toxins can provide an effective way of delaying the emergence of Bt-resistant pests, as recently illustrated in broccoli where pyramided *cry1Ac* and *cry1C* Bt genes controlled diamondback moths resistant to either single protein (Cao *et al*., 2002; see Figure 2), and significantly delayed the evolution of resistant insects (Zhao *et al*., 2003). Similarly, disease- and pest-resistant rice has been developed by crossing plants expressing the *Xa21* gene (resistance to bacterial blight) with plants expressing both a Bt fusion gene and a chitinase gene (resistance to yellow stem borer and tolerance to sheath blight, respectively) (Datta *et al*., 2002). Iterative rounds of cross-fertilization have also been used to introduce new biochemical pathways into plants. Two genes for a bacterial organic mercury detoxification pathway (mercuric reductase, *merA*, and organomercurial lyase, *merB*) were combined by crossing in *Arabidopsis*, and plants expressing both genes were able to grow on 50-fold higher methylmercury concentrations than wild-type plants (Bizily *et al*., 2000). In early work aimed at producing the biodegradable polymer PHB in plants, a three-gene biosynthetic pathway from the bacterium *Alcaligenes eutrophus* was reconstituted in *Arabidopsis* by serial crossing of plants harbouring single *Alcaligenes* genes

Table 1 Global status of approved genetically modified (GM) crops with three or more stacked genes

Trait	Crop	Genes	Method	Company	Events
Herbicide tolerance + fertility restored	Canola	*bar; barnase or barstar; neo*	*Agrobacterium*/ Linked genes, 1 T-DNA	Aventis Crop Science	MS1, RF1; MS1, RF2; PHY14; PHY35; PHY36
Herbicide tolerance + fertility restored	Chicory	*bar; barnase; neo*	*Agrobacterium*/ Linked genes, 1 T-DNA	Bejo Zaden BV	RM3-3; RM3-4; RM3-6
Multiple virus resistance	Squash	*neo; CP-CMV; CP-ZYMV; CP-WMV2*	*Agrobacterium*/ Linked genes, 1 T-DNA	Asgrow (USA); Seminis Vegetable Inc. (Canada)	CZW-3
Multiple virus resistance	Squash	*neo*; CP-ZYMV; CP-WMV2*	*Agrobacterium*/ Linked genes, 1 T-DNA	Upjohn (USA); Seminis Vegetable Inc. (Canada)	ZW20
Modified colour + herbicide tolerant†	Carnation	*surB†; dfr; hfl*	*Agrobacterium*/ Linked genes, 1 T-DNA	Florigene Pty Ltd.	4, 11, 15, 16
Modified colour + herbicide tolerant†	Carnation	*surB†; dfr; bp40*	*Agrobacterium*/ Linked genes, 1 T-DNA	Florigene Pty Ltd.	959A; 988A; 1226A; 1351A; 1363A; 1400A
Insect resistant + herbicide tolerant	Cotton	*bxn; cry1Ac; neo*	*Agrobacterium*/ Linked genes, 1 T-DNA	Calgene Inc.	31807/31808
Insect + virus resistant	Potato	*cry3A; PLRVrep; neo or EPSPS*	*Agrobacterium*/ Linked genes, 1 T-DNA	Monsanto	RBMT21-129; RBMT21-350; RBMT22-082
Insect resistant + herbicide tolerant	Maize	*bar; cry1Ac; pinII‡*	Biolistics/3 plasmid co-transformation	Dekalb Genetics Corporation	DBT418
Insect resistant + herbicide tolerant	Maize	*cry1Ab; EPSPS; gox*	Biolistics/2 plasmid co-transformation	Monsanto	MON802; MON809; MON810; MON832§

Information was taken from the Agbios database (http://www.agbios.com/dbase.php) and is as accurate as possible, although records are not always explicit on the detailed method of stacking. In some cases, particularly when biolistic transformation is used, extra genes (e.g. for bacterial selection) may also be transferred to the plant but are not expressed. Regulatory approval does not necessarily indicate that the product is in commercial production. The reader is referred to the Agbios database for detailed information on the regulatory approval status for each genetically modified crop in different countries.

Bar, phosphinothricin *N*-acetyltransferase; *neo*, neomycin phosphotransferase II; *EPSPS*, 5-enolpyruvylshikimate-3-phosphate synthase; *goxv247*, glyphosate oxidoreductase; *CP-CMV,* coat protein cucumber mosaic cucumovirus; *CP-ZYMV*, coat protein zucchini yellow mosaic potyvirus; *CP-WMV2*, coat protein watermelon mosaic potyvirus; *surB*, acetolactate synthase; *dfr*, dihydroflavonol reductase; *hfl*, flavonoid 3′,5′-hydroxylase; *ACC*, 1-amino-cyclopropane-1-carboxylic acid synthase; *bp40*, flavonoid 3′,5′-hydroxylase; *bxn*, nitrilase; *cry*, various Cry delta-endotoxins; *PLRVrep*, replicase and helicase open reading frames of potato leafroll luteovirus; *pinII*, protease inhibitor; *gox*, glyphosate oxidoreductase.

*nptII was included within the T-DNA, but only plants lacking it were selected for commercialization.

†*surB* herbicide tolerance was used as a selectable marker only, not as a commercial trait.

‡Incorporation of *pinII* was incomplete as a result of DNA rearrangements.

§cry1Ab was present on the plasmid but not incorporated in MON832.

(Nawrath *et al*., 1994). The bacterial genes had been modified by the addition of sequences for pea chloroplast transit peptides, and plants expressing all three bacterial genes accumulated PHB granules in plastids at up to 14% of plant dry weight. Even more ambitiously, four genes encoding different immunoglobulin polypeptides were combined in tobacco by sequential cross-fertilizations in order to enable the production of a functional secretory IgA (SigA) antibody in plants (Ma *et al*., 1995). Loss-of-function transgenes (e.g. co-suppressing or antisense genes) have been combined by crossing in order to manipulate processes such as lignin biosynthesis and fruit ripening (Chabannes *et al*., 2001; Pinçon *et al*., 2001a; Abbott *et al*., 2002; Powell *et al*., 2003).

Re-transformation has also been proven to be a viable research strategy. Flower colour has been modified in forsythia by inducing anthocyanin synthesis through sequential transformation with the genes for dihydroflavonol 4-reductase from *Antirrhinum majus* (*AmDFR*) and anthocyanidin synthase from *Matthiola incana* (*MiANS*). The double transformants displayed a novel bronze-orange petal colour caused by the

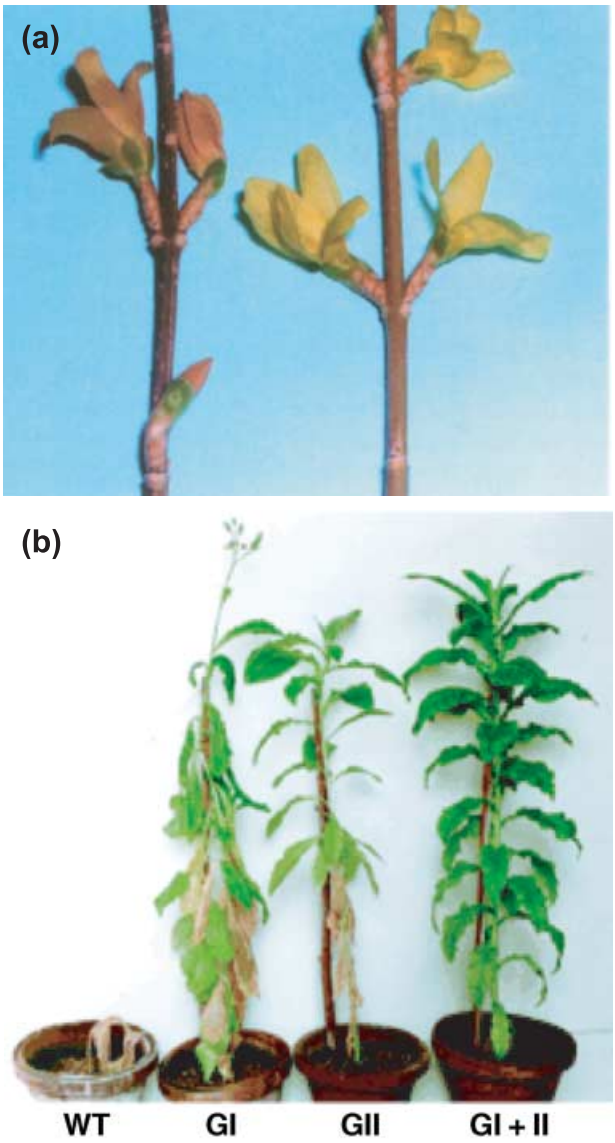

Figure 3 Re-transformation as a method of expressing multiple genes related to flower colour or salinity tolerance. (a) Phenotype of forsythia wild-type (WT) (right) and altered flower colour in *AmDFR* + *MiANS* transformants (left). (b) Relative salt tolerance of WT and glyoxalase over-expressing transgenic T_1 generation tobacco plants (GI, gly I; GII, gly II; GI + II, double transgenics) grown in the continued presence of 200 mM NaCl for 98 days. Reproduced from Rosati *et al*. (2003) (figure 7c), with kind permission of Springer Science and Business Media, and from Singla-Pareek *et al*. (2003) (figure 4b) (copyright 2003 National Academy of Sciences, USA).

de novo accumulation of cyanidin-derived anthocyanins over the carotenoid yellow background of the wild-type (Rosati *et al*., 2003; see Figure 3). Similarly, the introduction of a two-gene glyoxalase pathway into tobacco led to enhanced salinity tolerance, with the double transgenics responding better under salinity stress than plants harbouring either single transgene (Singla-Pareek *et al*., 2003). In potato, antisense inhibition of three starch synthase genes was achieved by re-transforming plants, already altered in amylopectin synthesis as a result of the suppression of SSII and SSII, with an antisense gene for granule-bound starch synthase (Jobling *et al*., 2002). This resulted in the production of an extremely freeze–thaw-stable starch that was amylose free with short-chain amylopectin. This starch could have significant environmental and consumer benefits in the food industry if it replaced current technology where freeze–thaw-stable starch is produced by chemical modification. Recently, three genes involved in the synthesis of long-chain polyunsaturated fatty acids were sequentially introduced into *Arabidopsis* in order to illustrate the potential of transgenic plants for producing the health-promoting omega-3 and omega-6 fatty acids normally obtained from fish oils (Qi *et al*., 2004). Re-transformation has also been used in trees to manipulate two genes involved in lignin biosynthesis by introducing an antisense gene for caffeate/5-hydroxyferulate *O*-methyltransferase into plants already containing an antisense cinnamyl alcohol dehydrogenase gene (Lapierre *et al*., 1999). Manipulation of lignin in this way is a target for reducing the environmental impact of papermaking.

Limitations of iterative procedures

Despite these and other success stories, iterative procedures for combining transgenes have several significant limitations. Principal among these is the fact that transgenes introduced by these procedures are not linked and will be sited at different random loci in the plant's genome. This means that they can segregate apart again in subsequent generations and that larger progeny populations will need to be maintained and screened in order to identify those in which all transgenes are retained. In the commercial context of a continuous crop breeding programme, where yield improvements have to be maintained, each unlinked transgenic locus to be introduced would double the size of the breeding population, and the incorporation of a three-gene trait, such as PHB synthesis, would potentially require an eightfold increase in plant numbers (Hitz, 1999). This increased breeding effort limits the number of independent loci that can realistically be incorporated and incurs a cost that will be reflected in higher seed prices or reduced yield. Even at the laboratory scale, cross-fertilization or re-transformation strategies are slow and labour intensive as multiple transformations must be performed and each transgenic population has to be individually characterized to identify plants expressing the transgene(s) at an appropriate level. Typically, transgenes are combined by crossing homozygote parents, and the production of homozygote breeding stock alone can take two to three generations. Thus, it may take four to six generations to

combine three or four transgenes in one plant (Halpin *et al*., 2001). Moreover, obtaining plants homozygous for all transgenes may be virtually impossible as a result of the complexity of the segregation of the independent transgenes. On the other hand, these problems are not an issue for species that are normally propagated vegetatively and, in this situation, the strategy of combining multiple transgenes by re-transformation can have considerable advantages, allowing the effects of each added gene to be assessed sequentially.

A second problem associated with the re-transformation strategy is that it requires a variety of selectable marker genes to be available so that a different one can be used with each sequential transformation. Although the range of potentially useful selectable markers is constantly increasing, GM crops with an accumulation of such genes would encounter significant hurdles to regulatory approval and public acceptance. This is a problem for many of the transgene stacking approaches discussed here, but is particularly relevant to the re-transformation of vegetatively propagated crops, where the possibility of eliminating selectable markers via outcrossing during subsequent breeding is not an option. Recently, several systems have been developed that enable the removal of selectable marker genes, which may help to overcome this limitation.

Selectable marker removal systems

Many selectable marker gene removal systems are slow, multistep procedures that may involve sexual crossing and are therefore not applicable to vegetatively propagated plant species (for a full review of marker gene removal systems, see Hohn *et al*., 2001 or Hare and Chua, 2002). Only a couple of single-step marker gene removal systems have so far been developed. The MAT vector system (for multi-autotransformation) works in the absence of sexual crossing and uses transposon- or, more recently, recombinase-based excision to enable the production of marker-free transgenic plants, facilitating repeated transformations (Ebinuma *et al*., 1997; Sugita *et al*., 2000a,b). An *Agrobacterium* isopentenyltransferase (*ipt*) gene is inserted between two recombination sequences from the yeast site-specific recombination system R/RS. The *ipt* gene provides a positive and visual selectable marker for transformation by catalysing cytokinin synthesis and inducing a 'shooty' phenotype on hormone-free medium. After selection, subsequent excision via the R/RS system produces marker-free transgenic plants with normal phenotype, allowing *ipt* and MAT to be used again for another round of transformation. Recent improvements to the method have increased its efficiency and have allowed its applicability to species that do not regenerate through cytokinin-dependent organogenesis but rather via embryogenesis (Endo *et al*., 2002a,b). A second promising marker excision system, termed CLX (for Cre/*loxP* DNA excision system), uses chemical-regulated expression of the Cre recombinase to excise the marker gene (together with all other 'used' components, such as the recombinase itself), which is placed between two *loxP* sites (Zuo *et al*., 2001). The CLX system is claimed to be tightly controlled, with DNA excision being induced at high efficiency – all 19 transgenic lines examined had undergone some level of DNA excision although some were chimeric. Although these marker excision technologies are, as yet, too immature and inefficient for routine use in a commercial context (König, 2003), the prospects for their further development and refinement are very good.

Recently, strategies have been devised that allow for transformation without the use of selectable markers by, for example, employing a virulent *Agrobacterium* strain and screening for transformed cells/shoots by polymerase chain reaction (PCR) (de Vetten *et al*., 2003). Using this approach, 1%–5% of harvested potato shoots were found to be transformed, and shoots with single T-DNA insertions and free of backbone-vector DNA sequences could be identified. This approach may prove to be effective for the generation of marker-free transgenic plants in species for which reasonably efficient transformation systems exist, such as potato and cassava. However, costs may become prohibitive as a result of the relatively low transformation frequency and the need to screen large populations of plants in order to identify lines suitable for commercialization (Rommens *et al*., 2004). A new transformation system may help to overcome this limitation and allow the more efficient generation of marker-free transgenic plants. This system allows for easier identification of transgenics by screening for temporary marker gene expression (using a short selection phase) whilst selecting against marker gene integration (using the negative selectable marker gene cytosine deaminase) (Rommens *et al*., 2004). When two DNAs, one encoding a fragment of the polyphenol oxidase (PPO) gene and the other encoding the positive:negative selection cassette, were co-introduced into potato, 6.1% of plantlets had integrated the PPO transgene, and temporarily expressed the selection cassette before eliminating it. Moreover, by using a plant-derived repeat sequence (P-DNA) to replace the *Agrobacterium* T-DNA on the 'effect' transgene to be stably integrated, modified plants containing only native DNA could be produced. Further development and more widespread use of this strategy will determine to what extent it can be adapted to other crop species and whether efficiency can be further improved.

Co-transformation with multiple transgenes

Co-transformation has been proven to be one of the most promising approaches taken to date for the introduction of multiple genes into plants. This strategy is quick and can be used in a variety of species and with both direct (e.g. biolistics) and indirect (*Agrobacterium*-mediated) transformation methods. The methodology is simple, even when using *Agrobacterium* – multiple transgenes, either harboured within different T-DNAs in a single *Agrobacterium* strain or harboured separately within different strains, are merely inoculated together on to plant tissues. A major advantage of co-transformation is that the co-introduced transgenes tend to co-integrate at the same chromosomal position in a high proportion of transgenics. This ensures that 'effect' genes are unlikely to segregate apart in subsequent generations. Conversely, linkage with the selectable marker gene may not be desirable, and many groups have explored the value of unlinked insertions to allow the subsequent generation of marker-free transgenic plants by segregating out the marker gene (e.g. see Komari *et al*., 1996). Several studies have examined how transformation conditions might be manipulated to promote co-transformation and/or co-integration. For example, one tobacco study using two T-DNAs recorded co-transformation frequencies as high as 100%, with 40%–50% of lines showing the capacity for segregational separation (McCormac *et al*., 2001). The strategy therefore has obvious advantages as a one-step procedure for the introduction of multiple 'effect' genes into plants. Alternatively, as a marker removal system, co-transformation is considered to be one of the more viable current methodologies, but potentially a labour-intensive one as a result of the increased numbers of transformation events that have to be handled in order to find lines suitable for marker gene elimination (König, 2003).

Several examples of deregulated products have used biolistic co-transformation as a means of stacking herbicide tolerance and insect resistance in maize (see Table 1). However, the most spectacular examples of the co-transformation strategy come from projects that are as yet at the research stage, such as the engineering of 'Golden rice'. Using *Agrobacterium*-mediated co-transformation, two T-DNAs, each harbouring two genes (one bacterial gene, two daffodil genes and a selectable marker gene), were introduced into rice, enabling the endosperm to express a carotenoid biosynthetic pathway and produce β-carotene (provitamin A) (Ye *et al*., 2000). The production of this vitamin A precursor in rice endosperm could potentially help to alleviate vitamin A deficiency in certain regions of the world where rice is a staple. Subsequent work has introduced the 'Golden rice' trait into commercially important *indica* and *japonica* rice varieties that are amenable to deregulation (Datta *et al*., 2003; Hoa *et al*., 2003; see Figure 4). Co-transformation, this time via particle bombardment, has also been used to simultaneously introduce three insecticidal genes (the Bt genes *cry1Ac* and *cry2A*, and the snowdrop lectin gene *gna*) into *indica* rice (Maqbool *et al*., 2001). Transgenic plants containing all three genes showed significant levels of protection against three of the most important insect pests of rice: rice leaf folder (*Cnaphalocrocis medinalis*), yellow stemborer (*Scirpophaga incertulas*) and brown planthopper (*Nilaparvata lugens*). In *Arabidopsis*, six genes (including two selectable marker genes), co-transformed on two T-DNAs, enabled the production of a biodegradable plastic copolymer in leaf plastids (Slater *et al*., 1999). In order to produce the copolymer, the availability of metabolic precursors had to be increased by redirecting intermediates (acetyl-CoA and threonine) from fatty acid and amino acid biosynthesis. Nevertheless, the yields of copolymer were very low, and further metabolic engineering will be necessary if sufficient levels of a durable copolymer are to be produced to allow commercial exploitation.

Figure 4 Co-transformation of multiple genes for carotenoid biosynthesis to produce 'Golden rice'. Polished grains from transgenic indica rice cultivar IR64 showing the white endosperm of wild-type plants (left) and yellow endosperm of transgenic plants (right). Reproduced from Datta *et al*. (2003) (figure 5a), with kind permission of Blackwell Publishing.

Co-transformation has also been used for sophisticated metabolic engineering of several plant pathways in which the simultaneous introduction of both protein-expressing and gene-suppressing transgenes has been necessary. On the lignin biosynthetic pathway, for example, suppression of certain genes can reduce the lignin content, whilst the over-expression or suppression of other genes alters lignin composition (see Baucher *et al*., 2003 or Halpin, 2004 for a review). Both types of change can potentially facilitate the ease of wood pulping for paper production. A recent study combined both strategies, using *Agrobacterium*-mediated co-transformation of aspen

to introduce two transgenes, one designed to over-express the ferulate 5-hydroxylase gene and the other to suppress the 4-coumarate-CoA ligase gene (Li *et al*., 2003). The resulting transgenic trees were concurrently improved in several wood quality traits and illustrated the potential of multigene manipulations for future tree improvement (Halpin and Boerjan, 2003). The flavonoid branch of the phenylpropanoid pathway has also been similarly manipulated, by co-transformation via particle bombardment, to increase the isoflavone levels in soybean (Yu *et al*., 2003). By combining the expression of a chimeric gene consisting of a fusion of the maize C1 and R transcription factor genes (which activate the isoflavone branch of the pathway) with co-suppression of flavanone 3-hydroxylase (to block the competing anthocyanin pathway), higher levels of isoflavones were produced. These examples illustrate the feasibility and benefits of complex manipulations of multiple genes for sophisticated metabolic engineering in plants. Given that co-transformation has been shown to be effective in introducing at least 14 different genes into plants (Chen *et al*., 1998), the stage is set for further development and implementation of this technique. No serious disadvantages of the co-transformation strategy have been identified thus far, although some reports have indicated that transgenes introduced in this way tend to integrate as high copy number repeats, especially when using biolistic transformation methods (for example, see Hadi *et al*., 1996; Maqbool and Christou, 1999). One recent *Agrobacterium*-mediated co-transformation experiment in *Arabidopsis* found that most inserts were very complex loci consisting of multiple tandem or inverted T-DNA repeats that often also included the complete binary vector sequence (Stuitje *et al*., 2003). Such complex integration patterns could both increase the potential for problems with transgene silencing in subsequent generations and retard or prevent the regulatory approval of crops transformed in this way. Nevertheless, the number of deregulated products developed using this technology attests to the feasibility of finding transgenics with stable expression of multiple co-transformed genes and relatively simple integration patterns, at least when small numbers of 'effect' genes are involved. Further work may help to define the experimental parameters that might maximize the co-integration of single copies of multiple transgenes. Alternatively, DNA excision technologies, similar to those used for marker gene removal, could be employed. By flanking transgenes with recombination sites in inverted orientations, loci with complex integration patterns could be resolved to a single gene copy in wheat, via recombination between the outermost recombination sites (Srivastava *et al*., 1999).

Another area in which further research could prove to be valuable is in the delineation of methods for the co-ordination of the expression of multiple co-transformed genes. Although there is little information in the literature to draw upon, some reports have indicated large variations in the level of expression of different co-transformed genes. For example, when three genes necessary for PHB synthesis were co-infiltrated into *Arabidopsis*, 84% of plants did not produce PHB (Poirier *et al*., 2000), presumably because of negligible or low expression of one or more gene(s). Indeed, only 9% of transgenics both incorporated the transgenes at a single locus and synthesized detectable amounts of the plastic. Where the expression of different co-transformed transgenes has been looked at directly, the proportion of plants expressing all transgenes, not surprisingly, seems to decrease with increasing transgene number. Whereas two transgenes co-transformed into potato were both expressed in 75%–80% of plants (Romano *et al*., 2003), four genes co-bombarded into rice were all expressed in only 50% of plants (Tang *et al*., 1999), and only 9% of rice lines co-expressed seven or eight genes when nine different plasmids/genes were co-introduced (Wu *et al*., 2002). In experiments in which the relative expression levels of different co-transformed genes have been investigated in a semiquantitative fashion, a large variation in the level of expression of different transgenes has been noted (Maqbool and Christou, 1999). In all cases described, many of the plants co-expressing multiple introduced genes contained multiple integration sites and/or several copies of one or more of the transgenes, and only a very small proportion of plants exhibited the dual benefits of co-expression together with simple integration patterns.

Linked effect genes

For the purposes of this discussion, the term 'linked effect genes' describes two or more 'effect' genes, each with its own promoter and terminator, that are positioned contiguously on DNA that will transfer as a single entity into a plant (i.e. on a single T-DNA for *Agrobacterium*-mediated transformation). Some commentators consider this a special case of co-transformation. Linking 'effect' genes in this way is a simple and obvious strategy that has been used with great success to stack traits in many GM crops that have gained regulatory approval (see Table 1). Although, in these examples, a relatively small number of genes (typically three) have been used, ambitious research projects have successfully used larger linked-gene cassettes. For example, as many as four or five genes (three or four genes related to PHB synthesis, plus a selectable marker gene) have been linked within

one T-DNA and introduced into *Arabidopsis* (Bohmert *et al*., 2000, 2002; see Figure 5) or oilseed rape (Slater *et al*., 1999). The most obvious practical problem associated with the linked 'effect' genes strategy is that the size of the DNA to be introduced and the possibilities for the engineering of convenient, unique restriction sites to aid construct assembly rapidly become limiting when attempting to link three or more genes, each with an individual promoter, in conventional vectors for plant transformation. A very successful strategy when four or more genes need to be introduced is therefore to use several T-DNA cassettes of moderate size (two or three linked 'effect' genes) in combination with the co-transformation of the different T-DNAs. Using this strategy, three genes for PHB biosynthesis, plus a selectable marker gene, were introduced on two T-DNAs into *Arabidopsis* and 16% of the resulting plants produced PHB (Poirier *et al*., 2000). Similarly, 'Golden rice' was produced by the co-transformation of two T-DNAs, each containing two linked genes (Ye *et al*., 2000).

Several groups have endeavoured to develop new vector systems to simplify the assembly of multigene cassettes and enable greater numbers of 'effect' genes to be directly linked. Two similar independently-derived systems comprise plant transformation vectors that have been engineered to contain an array of octanucleotide restriction sites and/or homing endonuclease cleavage sites together with unidirectional 'auxiliary' or 'shuttle' vectors with combinations of the same rare endonuclease sites (Goderis *et al*., 2002; Thomson *et al*., 2002). By constructing individual gene cassettes in shuttle vectors and then transferring these to the plant transformation vectors to assemble an artificial gene cluster, six-gene clusters were successfully introduced into soybean and tobacco plants (Thomson *et al*., 2002) or *Arabidopsis* plants (Goderis *et al*., 2002). Another system, also using homing endonuclease sites together with the Cre/*loxP* recombination system, sequentially delivers multiple genes from donor vectors (via multiple rounds of site-specific recombination) to an artificial chromosome-based acceptor vector competent for plant transformation (Lin *et al*., 2003). Constructs containing as many as 10 foreign DNA fragments were created using this system and the *Agrobacterium*-mediated transfer of six linked resistance genes was demonstrated in rice (Lin *et al*., 2003).

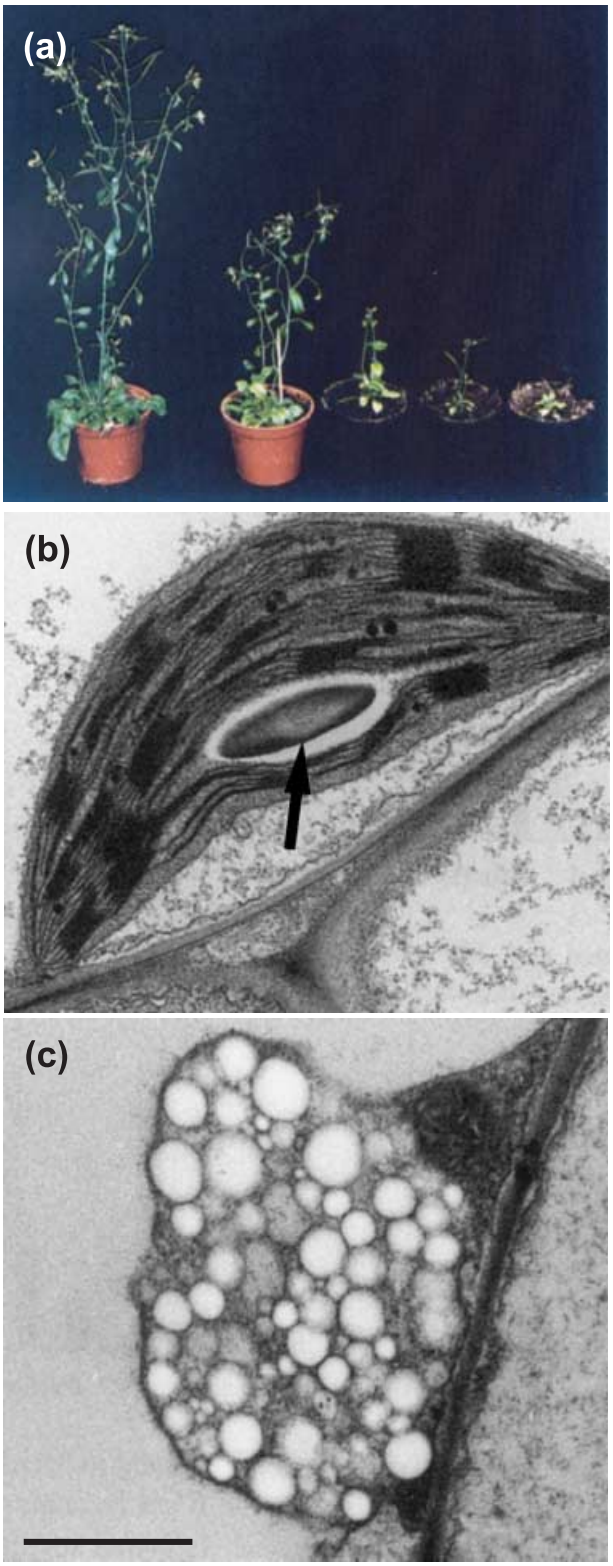

Figure 5 Linked transgenes used to introduce a pathway for polyhydroxybutyrate (PHB) synthesis into *Arabidopsis*. (a) Phenotype of transgenic *Arabidopsis* lines with increasing PHB content (left to right). (b) Transmission electron micrograph of a chloroplast from a mesophyll cell of a mature wild-type leaf (left) showing starch granule (arrow) and (c) a chloroplast of a transgenic leaf showing agglomerates of electron-translucent PHB granules. Bar = 1 μm. Reproduced from Bohmert *et al*. (2000) (figures 2(top) and 3a,b), with kind permission of Springer Science and Business Media.

Co-ordinating the expression of introduced genes

A limitation of all the approaches described so far is that they cannot ensure that different 'effect' genes will be co-ordinately expressed at similar levels, even when the 'effect' genes are physically linked. Whilst a degree of co-ordinated expression of linked genes has been reported in some cases (e.g. Gidoni *et al*., 1988; van Engelen *et al*., 1994), many studies have indicated that linked genes can be expressed at very different levels (e.g. Jones *et al*., 1987; Peach and Velten, 1991), even if the same or similar promoters are used. The phenomenon, that many researchers will be familiar with, of plants that grow on selective medium (i.e. express the selectable marker gene) and that harbour but do not express a linked 'effect' gene at any significant level, attests to this general lack of co-ordinated expression between two genes transferred together on a single piece of DNA. Clearly, transgenes that are not linked and that reside at independent genomic loci have even less chance of being co-ordinately expressed, partly because host sequences flanking each site of integration can have different influences on transgene expression ('position' effects). One recent study has suggested that flanking linked reporter genes with matrix-associated regions (MARs) results in the highly co-ordinated expression of the two genes in tobacco (Mlynárová *et al*., 2002). These MAR sequences attach active chromatin to the proteinaceous nuclear matrix and may create an artificial chromatin 'loop' wherein genes are co-ordinately regulated. MARs have also been proposed to be useful for reducing the expression variability between independent primary transformants (e.g. see Mlynárová *et al*., 1996; Petersen *et al*., 2002), although some studies have had no success with the strategy (e.g. see Liu and Tabe, 1998; De Bolle *et al*., 2003).

Other factors that may affect transgene expression are the number of transgenic loci, the number of insertions at a given locus and the stability of each locus. Multiple copies of either coding or regulatory sequences within transgenes are known to promote the activation of gene silencing mechanisms. Silencing of transgenes previously showing stable expression can also be triggered '*de novo*' when a new transgene is added by crossing or re-transformation if, for example, the same promoter has been used in both transgenes in an effort to promote co-ordinated expression. A couple of studies have tried to overcome this problem by developing synthetic promoters that are bidirectional (Xie *et al*., 2001; Li *et al*., 2004) or functionally equivalent (Bhullar *et al*., 2003), thereby enabling the expression of two or more genes at similar levels without triggering homology-based silencing. Other work has shown that the incorporation of MARS elements into transgenes may, in some cases, protect against transgene silencing and improve the stability of expression over a number of generations (Ulker *et al*., 1999; Vain *et al*., 1999; Mlynárová *et al*., 2003), although MARS cannot protect transgene expression from strong silencing loci that act in *trans* (Vaucheret *et al*., 1998). For most applications, the identification of individuals with simple, single-locus transgene integration patterns is a useful way to reduce the probability of transgene silencing.

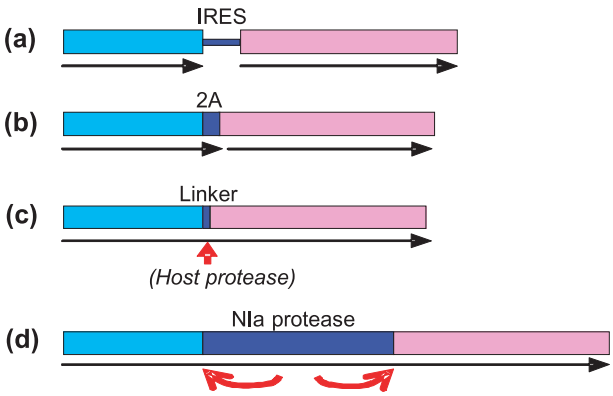

Figure 6 Strategies for expressing multiple proteins from a single promoter. Two or more proteins (blue box, pink box) can be encoded behind a single promoter and separated by: (a) an internal ribosome entry site (IRES), (b) foot-and-mouth disease virus (FMDV) 2A, (c) a linker sequence that is a substrate for a host cell proteinase or (d) the NIa protease. The products of the translation of each construct are indicated beneath by black arrows. Protease cleavage sites are indicated by red arrows.

Polycistronic transgenes

One way of overcoming the difficulties of co-ordinating the expression of different transgenes without duplicating regulatory sequences is to express several 'effect' genes from a single promoter as a single transcriptional unit. There are currently several alternative single-transgene systems for doing this (see Figure 6), although none has yet been widely applied in plants.

Chimeric polycistronic constructs that incorporate internal ribosome entry sites (IRESs) from different viruses have been tested and shown to function in plant systems (Urwin *et al*., 2000, 2002; Dorokhov *et al*., 2002; Jaag *et al*., 2003). IRESs are specific sequences, usually of several hundred nucleotides, that can directly recruit ribosomes to internal positions within mRNAs and initiate translation in a cap-independent manner. The IRES of crucifer-infecting tobamovirus (crTMV), for example, promotes the translation of a second cistron at 21%–31% of the levels of the first cistron, when a bicistronic

construct is expressed in transgenic plants (Dorokhov *et al*., 2002). Thus, although both cistrons are co-ordinately regulated, they are expressed at different levels. In some studies, IRES-mediated expression has also been shown to be significantly lower than normal cap-dependent expression in certain plant tissues (Urwin *et al*., 2002). The moderate inefficiency of IRESs is the main limitation to their more widespread use in plant biotechnology. Even if efficiency could be improved, their relatively large size could potentially initiate transgene silencing if the same element was used more than once in a polycistronic construct. Nevertheless, IRESs are being increasingly used in gene therapy applications in animal systems and wider use in plant systems may follow.

A special but hugely important opportunity for using polycistronic constructs in plants is presented by the development of technologies for engineering the plastid genome (reviewed by Daniell and Dhingra, 2002; Bock and Khan, 2004). A big advantage of plastid transformation is that entire bacterial operons can be easily introduced into, and expressed in, chloroplasts, where their regulatory signals, including internal initiation sequences, will be recognized. An entire, three-enzyme pathway for the production of PHB has been expressed in tobacco chloroplasts from a bacterial-type operon (Lössl *et al*., 2003). Similarly, the *Bacillus thuringiensis* cry2Aa2 operon encoding the cry2Aa2 insecticidal protein and two other proteins (including a putative chaperonin that facilitates folding of Cry2Aa2) was introduced into the chloroplast genome (De Cosa *et al*., 2001). This resulted in the highest level of foreign protein production yet achieved in transgenic plants, representing 45.3% of the total soluble protein in mature leaves and illustrating the lack of gene silencing in this system. Insects that are normally difficult to control were killed after consuming transgenic leaves. Thus, chloroplast expression has the very significant advantages of enabling the high-level expression of multiple recombinant proteins in plants whilst reducing the risk of transgene escape via pollen (since paternal plastid inheritance is rare). The system will, however, only be useful for crops that can be efficiently transformed in this way and for certain types of protein in which post-translational modification or targeting to other subcellular locations is not necessary.

Polyprotein expression systems

Several research groups have investigated the idea of co-ordinating the expression of multiple proteins by expressing them initially as polyproteins. In the simplest of these systems, different protein sequences are connected in a single open reading frame via short linker sequences that are substrates for a host cell proteinase. After translation, the polyprotein is cleaved into its constituent protein units during its passage through the plant endomembrane system. This strategy has been used to express a translational fusion of two different proteinase inhibitors joined by a protease-susceptible linker sequence in *Arabidopsis* (Urwin *et al*., 1998). The same strategy was independently used to construct a polyprotein comprising a leader peptide and two different antimicrobial proteins fused via a linker peptide originating from a natural polyprotein produced by seeds of *Impatiens balsamina* (François *et al*. 2002a,b). When expressed in *Arabidopsis*, the chimeric polyprotein was cleaved post-translationally and the individual, active, antimicrobial peptides were secreted into the extracellular space. In both of the preceding examples, the identity of the proteinases responsible for cleavage of the chimeric polyproteins is unknown, as is their exact location within the endomembrane system. The dependence of the approach on the temporal and spatial coincidence between the expression of the polyprotein and the expression of the endogenous plant proteinase is a limitation, making it useful only for certain applications.

A refinement of the polyprotein strategy overcomes these limitations by encoding a protease within the polyprotein itself. Several groups have constructed chimeric polyproteins that incorporate the NIa protease from various plant potyviruses which can cleave at specific heptapeptide sequences either in *cis* or in *trans* (Marcos and Beachy, 1994; von Bodman *et al*., 1995; Dasgupta *et al*., 1998). In these polyproteins, two 'effect' genes are separated by the coding sequence for the 48 kDa NIa proteinase, which is flanked on both sides by its requisite cleavage sites. When expressed in cell-free systems or transgenic tobacco, the viral proteinase cleaves within its recognition sequence, dissociating the polyprotein and releasing the 'effect' proteins. Using this strategy, a short, two-step, biochemical pathway for mannityl opine production was introduced into tobacco cells (von Bodman *et al*., 1995). Similarly, a polyprotein incorporating coat proteins of tobacco mosaic tobamovirus and soybean mosaic potyvirus was expressed in tobacco to yield plants resistant to multiple viruses (Marcos and Beachy, 1997). Although cleavage of these polyproteins appeared to be efficient, a number of limitations of the system have been recognized. Expression levels are generally low and influenced by the position of each gene within the polyprotein, possibly as a result of mistargeting of polyprotein to the nucleus due to a nuclear localization signal within the NIa protease, although this can be removed without impairing function (Ceriani *et al*., 1998). Furthermore, targeting of component proteins to different subcellular compartments may be complicated by the fact that translocation of the

polyprotein can occur more rapidly than polyprotein cleavage, resulting in the accumulation of partially processed products in specific compartments (Dasgupta *et al*., 1998).

A novel polyprotein strategy, which ensures co-translational polyprotein 'cleavage', incorporates the 20 amino acid 2A peptide of foot-and-mouth disease virus (or similar peptides from other viruses). This peptide can mediate polyprotein 'cleavage' by a unique non-proteolytic mechanism thought to involve an intra-ribosomal 'skip' during translation, such that a peptide bond is not formed between amino acids 19 and 20 of 2A, yet translation continues (Donnelly *et al*., 2001). Incorporation of the 2A peptide as a single open reading frame between two protein coding sequences therefore results in the translation of two polypeptides: (i) the first protein incorporating a C-terminal extension of 19 amino acids of 2A; and (ii) the second protein including a single N-terminal proline from 2A. Chimeric polyproteins incorporating 2A have been widely tested in eukaryotic systems, including mammalian, human, insect, yeast and fungal cells (Roosien *et al*., 1990; Ryan and Drew, 1994; Suzuki *et al*., 2000; Varnavski *et al*., 2000; de Felipe *et al*., 2003; François *et al*., 2004), and several reports have described their successful use in plants (Halpin *et al*., 1999; Ma and Mitra, 2002; El Amrani *et al*., 2004; François *et al*., 2004). The small size of 2A is seen as a particular advantage, as it facilitates the incorporation of multiple proteins within a single polyprotein construct. In animal systems, this strategy has been used to express several multimeric proteins, including the recent spectacular expression in the mouse of a single 2A-linked polyprotein encoding all four CD3 subunits, which restored T-cell development in CD3-deficient animals (Szymczak *et al*., 2004). Encoding proteins within 2A-polyproteins is entirely compatible with the potential need to target individual proteins to different subcellular compartments within plants either co- or post-translationally (El Amrani *et al*., 2004). A potential disadvantage of the system is the impact that the C-terminal 2A extension might have on the activity or targeting of proteins to which it remains attached after polyprotein translation. In practice, and despite the fairly widespread use of 2A with disparate proteins by independent research groups, this has not proved to be a problem. However, even if only from the perspective of simplifying the regulatory process for products generated using 2A-polyproteins, a fully removable 2A would be preferable. Initial attempts to design such a removable 2A linker have been partially successful (C. Halpin, unpublished data 2003; François *et al*., 2004), and further work in this area may promote the wider application of 2A-polyproteins to the expression of multiple useful proteins in plants.

Chimeric transgenes for multiple gene suppression

Single transgenes can also be used to simultaneously suppress multiple genes. A number of studies have demonstrated the value of transforming plants with single chimeric transgenes containing fused sequences (or partial sequences) of several target genes under the control of a single promoter. Although it is possible that chimeric transgenes might produce active fusion proteins, in most cases they seem to target homologous plant genes for silencing, probably via co-suppression or post-transcriptional gene silencing. The strategy has been used to co-ordinately suppress two cell wall hydrolases, polygalacturonase and pectinesterase, in tomato, thus altering fruit ripening (Seymour *et al*., 1993), and the effect is stably inherited over at least two generations (Simons and Tucker, 1999). A single chimeric transgene derived from two distinct viruses was also successful in conferring multivirus resistance to transgenic *Nicotiana benthamiana* plants (Jan *et al*., 2000). Our own work has shown that three (or possibly more) lignin biosynthetic genes can be co-ordinately suppressed in tobacco by a chimeric transgene composed of partial sense-orientated sequences of the three target genes (Abbott *et al*., 2002). The plants produced showed significant changes to lignin content, structure and composition, and exhibited more consistent levels of gene suppression than plants produced conventionally by crossing parents with different antisense transgenes. Indeed, the orientation of the suppressing sequences within chimeric transgenes does not appear to be important, as sense or antisense sequences have been shown to work equally well (Jones *et al*., 1998). Chimeric antisense constructs have been used in potato to co-ordinate the suppression of two starch biosynthetic genes (Edwards *et al*., 1999) and to manipulate two lignin biosynthetic genes in tobacco (Pinçon *et al*., 2001b). Moreover, our preliminary results suggest that the strategy can be combined with the use of RNAi-inducing constructs to effect very efficient co-ordinated suppression of multiple genes (A. Barakate and C. Halpin, unpublished data 2004). Chimeric gene-suppressing constructs therefore appear to offer great potential for the rapid and co-ordinated down-regulation of multiple genes on diverse biochemical pathways, and the technique deserves to be more widely adopted.

Conclusions and perspectives

A number of conventional and more novel techniques already exist for the stacking of genes and/or traits in transgenic plants. Many of these methods are tried and tested and some have already been used in the commercial production of GM

crops. No single method is, as yet, ideal, and individual methods may suit some purposes more than others. One of the most effective current approaches appears to be a combination of the co-transformation and linked transgene strategies, such that different DNA molecules, each harbouring several linked genes, are transformed together into plants. Re-transformation of a GM plant with additional transgenes is not a particularly attractive method for crops that are sexually propagated, but may be an option for vegetatively propagated species. Any strategy, such as re-transformation, which results in the stacking of multiple selectable marker genes in crops will probably need to incorporate techniques for marker gene removal if products are to gain regulatory approval. For multiple gene suppression, chimeric transgenes containing fused sequences of several target genes under the control of a single promoter offer very significant advantages, although the method has not yet been widely employed. Similar, novel, single-transgene methods have also been developed for multiple gene expression in the form of self-cleaving polyproteins. These techniques are a valuable addition to the more conventional techniques currently available in the 'toolkit' for multigene manipulation in plants, and deserve to be more widely tested and refined. Single-transgene methods also have the advantage of co-ordinating expression from a single promoter and avoiding multiple marker gene stacking in GM crops.

The post-genomic era offers unrivalled opportunities for the complex genetic manipulation of plants towards useful ends. Our increasing understanding of metabolic pathways and identification of the genes involved provide the basic tools for producing hardier crops that could resist disease and thrive in adverse environmental conditions, whilst having enhanced nutritive value and health-promoting properties. Plant raw materials, such as fibres, oils and starch, could be improved to allow more cost-effective and environmentally benign processing by industry, and entirely new industrial and therapeutic products could be produced in crops in a sustainable manner. In order to realize these opportunities, we will need to refine and supplement the existing 'toolkit' for co-ordinated multigene manipulation in plants to provide more durable and cleaner transgenic technologies that can simplify the route to regulatory approval and can reassure the public about the safety and stability of GM products. Technical advance in these areas is the major challenge facing 21st century plant biotechnology, but is also its ultimate opportunity for demonstrating the unprecedented contribution that GM crops could make to the sustainable and environmentally friendly production of foods, raw materials and novel products world-wide.

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