The introduction and expression of transgenes in plants

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Scientists have entered a new era of agricultural biotechnology. No longer is it sufficient merely to introduce a gene into a plant. The new generation of technology requires that genes be introduced into agronomically important crops in single copy and without the integration of extraneous vector 'backbone' sequences and, perhaps, even selectable markers. The expression of transgenes must be predictable and consistent among numerous independent transformants. Recent research has more clearly defined these problems and pointed the way to their solution.

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Abbreviations

CaMV	cauliflower mosaic virus
ipt	isopentenyl pyrophosphate transferase
MAR	matrix attachment region
nptII	neomycin phosphotransferase
Ti	tumor inducing

Introduction

Despite the fact that the first transgenic plants were generated less than 15 years ago, many transgenic crops are now beginning to be widely grown, and the products of these plants are available in the marketplace. Among these first generation crops are plants that are tolerant of herbicides, plants resistant to viruses and insects, and plants that are being used as 'bioreactors' to produce pharmaceuticals and food additives. In addition, some recombinant crops have altered nutritional or storage qualities, or are being used to produce industrially important materials, such as speciality oils and plastics.

The rapid advances necessary to generate these crops have come from three areas: basic research in plant molecular biology and physiology; the development of transformation technologies; and improvements in plant tissue culture to regenerate transformed plant cells or tissues. Although breakthroughs in each of these areas were essential, I shall limit this review to recent advances in transformation and related technologies. In addition, I shall discuss recent research to understand how transgene integration patterns may affect transgene expression.

Novel plant transformation technologies

Two classes of plant transformation technologies exist: 'non-natural' or *in vitro* methods, and 'natural' methodologies. Among the *in vitro* technologies are microinjection (not commonly used), direct DNA uptake into protoplasts (with or without electroporation), and microprojectile (or particle) bombardment. 'Natural' technologies include the use of viral vectors (that will result in transient but not stable transformation) and *Agrobacterium tumefaciens* T-DNA-mediated transformation. Each of these technologies has advantages and disadvantages. The *in vitro* techniques tend to result in transformed plants containing a high copy number of often rearranged or catenated transgenes, which can result in homology-dependent suppression (transgene silencing). In addition, electroporation technology depends upon the ability to regenerate the transgenic protoplasts into whole plants, a process that is difficult or impossible for many plant species. Particle bombardment can be performed with any tissue of most species; however, the process is relatively inefficient in that few cells are stably transformed. *Agrobacterium*-mediated transformation is, for many species, relatively efficient, and a low copy number of intact, non-rearranged transgenes frequently are integrated into the plant genome. *Agrobacterium*-mediated transformation of many important crop species (such as many cereals and soybean) is at best inefficient (however, see below for recent advances in the transformation of cereals by *Agrobacterium*).

A number of scientists are interested in combining the best attributes of *Agrobacterium*-mediated transformation (high efficiency, low copy number, and intact transgenes) with particle bombardment (species-independent transformation). Two major approaches have recently been taken. Both rely upon the known mechanism of T-DNA processing in *Agrobacterium*. The T-DNA region (the region of DNA that is destined to be transferred to the plant) of the *Agrobacterium* tumor inducing (Ti) plasmid is flanked by 25 bp directly repeated (i.e. same orientation) DNA sequences called T-DNA 'borders'. These borders are acted upon by a T-DNA border-specific endonuclease encoded by the *virD1* and *virD2* genes [1]. The VirD2 protein nicks the border sequence between bases three and four and subsequently covalently binds to the 5′ end of the nicked DNA. It is then thought that a single-stranded DNA molecule (the 'T-strand') is unwound from the Ti-plasmid. T-DNA is transferred to plants as a single-stranded molecule [2,3], most likely covalently linked to VirD2 protein and perhaps coated by VirE2 protein, a non-specific single-stranded DNA-binding protein also produced by *Agrobacterium*. Work in several laboratories has shown that highly purified recombinant VirD2 protein can cleave short single-stranded oligonucleotides containing a T-DNA border [4,5]. Recent work in our laboratory has indicated that long single-stranded molecules containing a T-DNA border can also be cleaved *in vitro* by purified VirD2 protein (SB Gelvin, unpublished data). VirD2 protein–T-strands thus generated can be electroporated

into tobacco protoplasts and integrated into the plant genome. DNA blot and PCR analyses are currently being carried out to determine the integration pattern of these 'artificial T-complexes' when introduced by *in vitro* techniques.

Work from M-D Chilton's laboratory [6••,7•] used another approach to combine *Agrobacterium* and particle bombardment-mediated technologies. Two recent papers described a novel technique termed 'Agrolistic' transformation [6••,7•]. Using this approach, the authors delivered three plasmids to tobacco cells or maize protoplasts using particle bombardment or direct DNA uptake. Two plasmids contained separately the *virD1* and *virD2* genes, each under the control of the strong cauliflower mosaic virus (CaMV) 35S promoter. The third plasmid contained a target T-DNA consisting of a neomycin phosphotransferase (*nptII*) gene between T-DNA borders. The rationale for these experiments was that if VirD1 and VirD2 proteins, expressed in plants, could nick the T-DNA at the borders, the plant may integrate this T-DNA in low copy number using these borders. Sequence analysis of DNA from kanamycin-resistant tobacco cells, or paromomycin-resistant maize cells, indicated that integration of the selectable marker gene could occur in low copy number using the T-DNA borders precisely, as though these borders had been cleaved in *Agrobacterium* and the DNA transported to the plant cell. In addition, the authors detected *nptII* genes integrated as part of the plasmid DNA that was not cleaved at the T-DNA borders; thus, in some instances, the entire plasmid appeared to integrate into the genome. The authors suggested that this novel strategy could be used on any tissue that was susceptible to transformation by particle bombardment to integrate a low copy number of transgenes without integration of plasmid vector sequences.

Eliminating unwanted DNA from transgenic plants

During the early days of plant transformation, scientists were relatively oblivious of details such as the copy number or conformation of integrated transgenes, or whether selectable marker or even plasmid vector sequences, integrated into the chromosomes of transgenic plants. As the science matured, however, such 'details' became increasingly important for several reasons: regulatory agencies started to request information regarding which DNA sequences had been incorporated into plants that were being released for field trials and commercialization, and scientists became increasingly concerned about the effects of transgene conformation and copy number upon transgene expression (see below). Several recent studies have examined these problems directly.

In 1994, Martineau *et al.* [8] published a letter to the editor in which they described a curious observation: in a significant percentage of transgenic plants, T-DNA binary vector 'backbone' sequences had integrated into the plant genome. Although some evidence for the integration of 'non-traditional' T-DNA sequences had appeared in the earlier literature [9], reports of the integration of DNA sequences from outside the T-DNA borders were rare. In fact, T-DNA was operationally defined as those sequences inside the borders that were transferred to the plant. This definition of T-DNA was so pervasive that most scientists did not even bother to check for vector 'backbone' sequences in their transgenic plants. In 1995, however, Ramanathan and Veluthambi [10] reported that Ti-plasmid sequences to the left of the T-DNA left border had been integrated into plant DNA. Wenck *et al.* [11] also documented the transfer and integration of binary vector sequences beyond the left border in transgenic *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. More recently, Kononov *et al.* [12••] investigated the mechanism by which non-T-DNA vector 'backbone' sequences integrated into plant DNA during *Agrobacterium*-mediated transformation.

Kononov *et al.* [12••] built two T-DNA binary vectors containing an *nptII* selectable marker between the T-DNA borders, and a *gusA* reporter gene within the binary vector 'backbone' sequences, just outside either the left or right T-DNA border. They generated several hundred transgenic tobacco plants, selecting for kanamycin-resistance conferred by the *nptII* gene. When they assayed these plants for β-glucuronidase activity, approximately 20% of the plants showed blue staining of leaf sections using the chromogenic dye X-gluc. When the plants were assayed for the presence of the *gusA* gene using PCR, however, approximately 75% of the plants contained the unselected *gusA* gene that had been harbored by the binary vector 'backbone' sequences. DNA blot analyses indicated that these vector 'backbone' sequences could be integrated into plant DNA linked to either the T-DNA left or right border sequences, or as an independent unit that was not linked to the T-DNA. The authors determined that the transfer and integration of vector 'backbone' DNA could occur either as a consequence of T-DNA 'border skipping' (VirD2 protein failed to cleave at one of the T-DNA borders), or that VirD2 protein bound to the 5′ end of the non-T-DNA vector 'backbone' resulted in the transfer of this DNA in a manner analogous to T-DNA transfer, that is, the vector 'backbone' DNA had itself become a new T-DNA. The authors concluded that the transfer and integration of this 'non-T-DNA' region of the binary vector was part of the normal T-DNA transfer mechanism and could not be prevented. It may be possible, however, to eliminate the regeneration of plants containing this 'extraneous' DNA by placing a 'killer gene' within the binary vector 'backbone' sequences.

Although the integration of selectable marker genes into the plant genome along with 'useful' transgenes initially appeared innocuous, scientists have increasingly been concerned with their presence. Many of these genes confer antibiotic or herbicide resistance upon the host plant. The growth and development of transgenic plants, however, is

often retarded by chemicals used to select transformed cells. Some plant species are naturally tolerant of these selective antibiotics, and it is therefore difficult to distinguish transformed from non-transformed tissues. Because these resistance genes are usually expressed constitutively in the plant, the toxicological and environmental impact of their encoded proteins must be evaluated. Finally, the presence of a specific antibiotic resistance gene in a transgenic plant obviates the use of that antibiotic for recurrent transformation of the plant with additional genes. Goldsbrough *et al.* [13] previously described a transposon-based system for the elimination of marker genes from plants. More recently, Ebinuma *et al.* [14••] described a novel modification of this system. In their system, called multi-autotransformation, a chimeric *Agrobacterium* isopentenyl pyrophosphate transferase (*ipt*) gene is used as the initial selectable marker for transformation. Expression of this gene results in plants that overproduce cytokinin and, subsequently, have an extreme shooty phenotype and cannot root. The authors placed a CaMV 35S–*ipt* gene within an Ac transposable element; this 'cassette' was then itself cloned into the T-DNA region of a binary vector. Transformed plants could be visually selected because of their extreme shooty phenotype; however, should the Ac element excise from the plant genome and not reinsert at another location (which is know to occur in approximately 10% of the transposition events), the result would be a phenotypically normal plant lacking the *ipt* marker gene. The authors were able to recover such plants and show that, although the Ac element containing the *ipt* gene was lost from the plant, the remainder of the T-DNA (carrying other reporter genes) remained intact. In addition to the generation of 'marker-free' transgenic plants, the authors reported an additional advantage of their system. Plants containing multiple T-DNA copies (and thus multiple copies of the *ipt* gene) would be unlikely to lose all copies of the *ipt* gene by excision of all the Ac elements. Thus, the multi-autotransformation system selected for plants containing a single-copy T-DNA insertion.

T-DNA integration position and transgene expression

One of the major problems that has irked plant molecular biologists is the variable expression levels of transgenes in independent transgenic plants. Because there has typically been little correlation between transgene copy number and transgene expression levels, scientists have attributed the differences in transgene expression to so-called 'position effects'; that is, the position of the genome into which the T-DNA integrates contributes to the expression level of the transgenes. One hypothesis to explain this range of expression levels is that the local chromatin domain containing the transgene determines the rate of transcription of the gene. Two approaches to decreasing variability in transgene expression levels have been discussed: eliminate the 'position effect' by always integrating the transgene into the same chromosomal site, or 'buffer' the transgene from the effects of the local chromatin conformation by isolating the transgene on its own chromatin 'loop' domain.

The first approach to eliminating the position effect has been taken by the laboratory of David Ow. These scientists have used the P1 bacteriophage *cre-lox* site-specific recombination system in plants to integrate transgenes into specific chromosomal sites, or mediate the site-specific recombination between plant chromosomes [15,16]. Recent results reported by this group at the 5th International Congress of Plant Molecular Biology (September 21–27, 1997, Singapore) indicate, however, that even when a *gusA* reporter transgene is integrated into the same location of the tobacco genome, independent plants still display a wide range of β-glucuronidase expression patterns. This surprising and perhaps disappointing finding suggests that factors in addition to the position of the integrated transgene contribute to the level of transgene expression. Alternatively, some positions of the genome may be more prone to variable gene expression levels than others, although essentially the same results are seen in all five chromosomal target sites that have been examined.

An alternative approach to reducing transgene expression variability has been to flank the transgenes with matrix attachment regions (MARs) or scaffold attachment regions [17,18,19••,20]. In these experiments, the expression of a *gusA* reporter gene in numerous independent transgenic plants was assessed in the presence or absence of flanking MARs. In general, these researchers were able to demonstrate reduced variation in transgene expression levels in plants containing transgenes flanked by MARs compared to plants containing the *gusA* gene in the absence of a MAR. Although variation in reporter gene expression was still evident among independent plants containing transgenes flanked by MARs, Mlynarova *et al.* [19••] demonstrated that the same degree of variability in expression could be found in genetically identical sibling transgenic plants. This variability was attributed mostly to environmental or developmental influences upon the test plants. The authors concluded that, using the chicken lysozyme MAR in their particular constructions, they had 'approached the lower limits' in their ability to control transgene expression variability.

T-DNA integration patterns and transgene expression

It has long been known that T-DNA does not always integrate into the plant genome as a simple, unique copy event (for example, see [21]). T-DNA may integrate as multiple copies in direct or inverted repeat conformation. Other more complex patterns may also occur. It is not clear how these repeated structures are generated. Recently, De Neve *et al.* [22••] demonstrated that when two different T-DNAs are delivered to plant cells from different *Agrobacterium* strains, they frequently integrate at the same chromosomal locus. Such T-DNA multimers are joined to each other in all possible orientations,

although there appeared to be a preference for right T-DNA borders to be involved in the linkage. The authors suggested that extrachromosomal double-stranded T-DNA molecules could ligate prior to integration. Although T-DNA enters the plant cell and the nucleus as a single-stranded molecule [2,3], the presence of extrachromosomal double-stranded T-DNA molecules can be inferred from the transient expression analyses of Narasimhulu *et al.* [23•]. Alternatively, De Neve *et al.* [22••] suggested that double-stranded T-DNA molecules could ligate during the integration process. It should be noted, however, that others have suggested that T-DNA integrates into the plant genome by strand invasion of a single-stranded T-DNA molecule [24•].

Regardless of the mechanism by which T-DNA integrates into the plant genome, the presence of T-DNA repeat structures, and especially inverted repeat structures, strongly correlates with the phenomenon of transgene silencing [25••,26•,27•,28••]. Transgene silencing is the phenomenon by which the expression of a transgene is turned off. The terms 'sense suppression' or 'co-suppression' have been applied to situations in which both a transgene and a homologous resident gene are silenced. The problem of sense suppression can become particularly exasperating to scientists who attempt to over-express a product of an endogenous gene by introducing into the plant additional copies of the gene under the regulation of a strong promoter. Co-suppression has, however, been successfully used as an alternative to anti-sense technology to eliminate the expression of an endogenous gene. The ability to eliminate, or at least control, the integration pattern and copy number of T-DNA molecules (and thus reduce the possibility of sense suppression) remains an elusive goal of plant molecular biologists.

Conclusions

During the past 15 years scientists have made enormous advances in agricultural biotechnology through the creation of transgenic plants. For many agronomically important species, the question being asked is no longer "can we make a transgenic plant?"; rather, scientists are now concerned with more sophisticated problems of regulating transgene expression, establishing consistency of transgene expression, and the environmental impact of transgenic plants. Although species limitations of *Agrobacterium*-mediated gene transfer still present challenges, the recent stable transformation of rice [29,30,31•,32], maize [33••], and barley [34••] bode well for extending the host range of *Agrobacterium* to other agronomically important crops, such as wheat, sorghum, and soybean. Important problems yet to be solved include regulating the extent and consistency of transgene expression (perhaps by regulating the integration pattern of T-DNA molecules), and achieving one of the 'holy grails' of plant molecular biology: targeted gene disruption and gene replacement by homologous recombination. Although efficient homologous recombination has yet to be achieved for most plant species, recent reports of relatively efficient gene targeting in *Arabidopsis thaliana* at efficiencies up to one homologous recombination event per 750 illegitimate recombination events [35,36••] suggest that the acquisition of this important molecular tool is just beyond the horizon.

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This paper analyzes in depth the integration of T-DNA binary vector 'backbone' sequences into the genome of tobacco. The authors placed a *nos-nptII* gene into the T-DNA, and a *gusA* gene outside the T-DNA in the vector 'backbone'. Approximately 75% of the resulting kanamycin-resistant plants also contained *gusA* gene sequences. DNA blot analyses indicated that the vector 'backbone' sequences could be integrated into the tobacco genome linked to the T-DNA through either the left or right borders, or could independently integrate into tobacco DNA unlinked to the T-DNA.

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The authors transform *Nicotiana* and *Arabidopsis* cells with two *Agrobacterium* strains harboring binary T-DNA vectors containing two different selectable markers. Following selection of cells resistant to both markers, they show that approximately one half of these lines contain both T-DNA molecules linked to each other. Although all combinations of linkages were detected, linkages involving at least one T-DNA right border were prevalent. The authors suggest that T-DNA molecules, made double-stranded after the transfer of single-stranded T-strands to the nucleus, link together either extrachromosomally or during the integration process. This model differs from that of Tinland 1996 [24•] who favors integration of single-stranded T-DNA molecules into the plant chromosome by strand invasion.

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This report analyzes the very early events following T-DNA transfer from *Agrobacterium* to tobacco and maize cells. Using RT-PCR, the authors could detect the expression of a T-DNA-encoded *gusA*-intron gene within 18 hours after infection. Infection of tobacco cells with an avirulent *Agrobacterium* strain harboring a mutation in the ω domain of *virD2* still resulted in high transient levels of processed *gusA* transcript, suggesting that the ω domain of VirD2 may be involved in T-DNA integration into the plant genome. The infection of maize BMS cells with wild-type *Agrobacterium* strains resulted in only transient expression of the *gusA* transcript, suggesting that with this infection protocol, the maize cells were recalcitrant to stable T-DNA expression and/or integration.

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These authors investigated suppression of *nptII* transcript levels in transgenic tobacco. They conclude that sense suppression depends upon particular 'silencing loci' containing the *nptII* transgenes. Silencing correlates both with reduced NPTII protein, *nptII* mRNA ratios, and with extensive cytosine methylation of particular sites in the 3′ region of the transgene coding sequence.

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This paper reports the targeted disruption of the *AGL5* MADS-box gene in *Arabidopsis thaliana* by homologous recombination. One in 750 kanamycin-resistant transgenic plants that were targeted by a T-DNA binary vector containing a *nptII* gene within the *AGL5* sequence showed integration of the transgene by homologous recombination. This paper shows that relatively efficient targeted gene disruption can be effected in plants, and boosts *Arabidopsis* to the enviable position as the only higher eukaryote in which both gene disruption by homologous recombination and large-scale random mutagenesis are possible.