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Superfluous Transgene Integration in Plants

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ABSTRACT: Recent reports suggest the transfer of superfluous DNA sequences to plant genomes during transformation processes. This review investigates the evidence from the published literature for the prevalence of this phenomenon and highlights methods to limit or prevent DNA transfer and subsequent potentially detrimental evolutionary consequences.

Evidence for superfluous foreign DNA transfer using both *Agrobacterium*-mediated transformation and direct DNA transfer methods such as microprojectile bombardment and PEG-mediated transformation of protoplasts is reported. In the case of *Agrobacterium*-mediated transformation, the lack of information on the integration of sequences from outside of the T-DNA borders has been due to the general belief by researchers that T-DNA processing is precise. This assumption was based on analysis of T-DNA in tumors and as a result the majority of T-DNA integration events have been identified exclusively using DNA probes, which are homologous only to DNA from within the T-DNA borders. Where direct gene transfer protocols are employed, any part of the transforming plasmid and indeed accompanying carrier DNA may become integrated into the plant genome. The main body of evidence proving that superfluous vector DNA sequences are present in plant genomes transformed using direct transfer methods is confined to the identification of plasmid concatamers integrated into plant genomes.

The limited amount of recorded evidence pertaining to superfluous vector DNA integration in transgenic plants and transformed tissues makes it impossible to draw definitive conclusions as to the factors involved in promoting this phenomenon. However, there are methods available for removing superfluous sequences from transgenic plants. These have been developed for the removal of selectable marker genes, whose presence in transgenic plants has been a source of much controversy, but can equally be applied to other DNA sequences. Suggestions have been made in the review that might limit or prevent the integration of superfluous vector sequences during transformation procedures; however, these are not proven and further research is required.

KEY WORDS: superfluous DNA, plant transformation, GMOs, *Agrobacterium tumefaciens*, microprojectile bombardment, agrolistics.

I. INTRODUCTION

Rapid progress in gene manipulation and the desirability of improving agricultural productivity, both to decrease inputs of pesticides and fertilizers as well as to benefit quality, have fueled the development of genetically modified plants. This technology allows plants with specific qualities to be developed in a much shorter period of time than when using conventional plant breeding. It also makes possible the introduction of characteristics that cannot be achieved through plant breeding alone. Nevertheless, it is recognized that the technology could pose potential risks to the environment and human health. The introduction of foreign genes into plant genomes and the expression of foreign proteins in plants carries concerns for the evolution of the plant species itself and for other living organisms that come into contact with it. In addition, any novel proteins expressed in a plant have the potential to produce new interactions between themselves and other proteins. In instances where transgenic plants are destined for consumption, there is the risk of

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toxic or allergenic responses to be considered (Nordlee et al., 1996). The release of genetically modified organisms (GMOs) into the environment therefore is controlled in the U.K. by domestic and European legislation. All applications to release GMOs into the environment must identify any superfluous DNA integration events and evaluate the possible consequences of these.

The first published report of *in vitro* single-cell plant transformation appeared in the literature in 1979 (Marton et al., 1979). Further reports of plant genomes transformed with foreign DNA began in the early 1980s (Barton et al., 1983; Herrera-Estrella et al., 1983). These first experiments utilized the ability of a soil bacterium, *Agrobacterium tumefaciens*, to transfer a specific region of DNA on its Ti plasmid into plant cells where subsequently it becomes incorporated into the plant's own genome. The potential this discovery had for rapid agronomic improvement was realized and has served to fuel the enormous amount of research and development in plant genetic engineering.

Although *Agrobacterium*-mediated transformation has been hugely successful for a vast number of plant species, initially this was confined to dicotyledonous plants and precluded the exploitation of this method for engineering many of the world's most important crops. As a result, a number of other plant transformation methods have been developed with varying degrees of success. The two most widely practiced and successful plant transformation techniques are *Agrobacterium*-mediated transformation and microprojectile bombardment. These techniques mediate the transfer of foreign DNA to plant genomes in completely different ways. Further research in transformation technology has brought together what are thought to be the best features of both these systems to produce a new "hybrid" transformation technique coined agrolistic transformation (Hansen and Chilton, 1996). *Agrobacterium*-mediated transformation has now been used successfully for monocot, yeast (Bundock et al., 1995; Bundock and Hooykaas, 1996), and fungi (De Groot et al., 1998) transformation.

A. Cloning Vectors

1. Vectors for Direct Gene Transfer Protocols

A gene of interest is usually inserted into a plasmid vector to make an expression cassette. This vector includes promoters and terminators for the gene such that when it is integrated into the plant genome it can be expressed under the control of the plant's own transcription and translation machinery. The vector carries origin of replication (ori) sequences that allow the plasmid to replicate in bacteria facilitating the production of large quantities of plasmid. Usually a bacterial antibiotic resistance gene cassette is included in the plasmid. This enables only bacteria containing the required plasmid to be selected during the engineering process. A second selectable marker is also added to the plasmid that facilitates the selection of transformed plant cells over and above untransformed cells subsequent to the transformation event. Usually these genes code for herbicide or antibiotic resistance and are part of an expression cassette that also includes a promoter and terminator. A multiple cloning site in the plasmid provides a number of restriction sites to facilitate cloning of the necessary components, and a variety of other sequences are typically included to aid the cloning process. For example, pUC vectors (approximately 2700 bp in length) contain a region of β-galactosidase sequence into which the polylinker has been inserted. When a sequence has inserted into the polylinker it disrupts β-galactosidase; on appropriate selection plates bacterial colonies containing a plasmid with a disrupted β-galactosidase gene appear white and those that lack an inserted DNA fragment appear blue. In this way, the selection of bacteria containing plasmids that carry the required cloned sequence can be performed visually.

2. Vectors for Agrobacterium-Mediated Transformation

Vectors used in *Agrobacterium*-mediated transformation are a little different to those used in direct gene transfer. Unlike pUC vectors, they carry

broad host range replicators that facilitate replication in *Agrobacterium* species and a T-DNA region bordered by right and left repeats that facilitate the transfer of specific DNA sequences. They are generally larger; Bin 19 a typical and widely used binary vector for *Agrobacterium*-mediated transformation is 11, 777 bp in length. For many years Bin 19 was used extensively, even though the only available data for the plasmid was a limited restriction map. In 1995, Frisch et al. published the complete sequence of Bin 19 revealing exactly which sequences the plasmid was made from. They found a number of suboptimal functional elements and that approximately half the plasmid consisted of superfluous sequences. Within the T-DNA region of the plasmid, 800 bp of superfluous sequence lies between the multiple cloning site and the right border. Included in this region are *gene III* sequences from M13 phage, orthinine cyclodeaminase from *Agrobacterium* plasmid C58 (Fray et al., 1994), and the 3′ region from *lacI*. Further superfluous sequences include 5′ *lacI* and M13 *ori* DNA. The *nptII* gene present inside the right border contains a point mutation that has been shown to cause a fourfold reduction in enzyme activity (Yenofsky et al., 1990). The non-T-DNA region of the plasmid contains a broad host-range origin of replication, *ori V*, which allows the plasmid to replicate in both *E. coli* and *Agrobacterium*. It also contains an *nptIII* gene that facilitates selection in both bacteria and a *trfA* locus that codes for two proteins involved in promoting the replication of the plasmid. A number of sequences are present within the non-T-DNA region that appear to provide no function in this plasmid. These include a *tetA* gene that has been inactivated through the insertion of the T-DNA, *traF*, *is1* , a 379-bp fragment with homology to the origin of replication in pUC vectors (pMB1) and a nonfunctional fragment of gene, *klaC*, which is normally part of the *kilA* locus that when unregulated is lethal to the host cell. There are also several pieces of sequence, the origin of which have not been identified.

B. DNA Integration

When introducing foreign genes into plant genomes using direct DNA transfer methods, the complete vector in which the genes have been cloned is usually presented to plant cells as a supercoiled circular plasmid but sometimes as linearized plasmid DNA. Generally, the whole plasmid is taken into the cell, and any part of this foreign DNA can become integrated into the plant genome. *Agrobacterium*-mediated transformation differs in this respect, processing of the plasmid occurs within the bacterium and DNA from between two T-DNA borders is transferred to the plant cell.

The integration of vector sequences other than those required for the improved phenotype of resulting transgenic plants could produce unforeseen or unwanted changes in the biology of these plants if released into the environment. In theory, it would appear that direct gene transfer protocols are more likely to produce transgenic plants containing superfluous sequences than *Agrobacterium*-mediated transformation. However, large portions of superfluous vector sequence present in transformants created using *Agrobacterium*-mediated transformation have been reported and these are discussed.

C. Superfluous DNA Integration

The presence of selectable marker genes in transgenic plants is an area of much concern and discussion (Bryant and Leather, 1992a; Gressel, 1992; Bryant and Leather, 1992b; Goldsbrough, 1992; Malik and Saroha, 1999). Antibiotic- and herbicide-resistance genes in transgenic plants have been seen by some as unacceptable and potentially harmful because of their expression and the theoretical potential for these genes to transfer to other organisms. The *nptII* gene, conferring resistance to the antibiotic kanamycin, is widely used in the generation of transgenic plants and has been shown by Flavell et al. (1992) and Fuchs et al. (1993) not to compromise the efficacy of oral administration of kanamycin in humans. They also report the probability of its transfer to another organism as being extremely low, and even if this were to occur the impact would be minimal. Other selectable and screenable marker genes have not been so studied extensively. Their transfer to gut microorganisms or to other plants

through pollination could potentially compromise the use of antibiotics and herbicides in controlling the spread of bacteria and weeds. Although plant-selectable marker genes have received a lot of attention, other sequences included in vectors used for plant transformation have been largely overlooked. As mentioned above, these sequences can also become integrated into the plant genome, but what are the consequences of this?

The risks with gene transfer of this nature may relate directly to possible expression of superfluous genes in the plant, resulting in the production of protein products from these sequences that may cause allergenic reactions or interactions with other proteins in an unusual way once the plant material has been consumed. A gene not normally expressed in a plant could be induced to do so if it became integrated into the plant genome downstream of a plant promoter. Integration of any foreign DNA sequences could knock out genes that are important in the detoxification of certain chemicals, the greater the number of integration events within a genome the greater the chance of this occurring. This type of effect is particularly important with respect to the safety of a food product, its quality, and its equivalent nutritional value.

There are concerns that DNA sequences not normally present in foods could transfer to gut microorganisms and subsequently to pathogenic microorganisms, which might ultimately compromise the clinical treatment of infections. The presence in plant genomes of broad host range sequences involved in the transfer and replication of plasmids in bacteria could help to maintain such sequences in a bacterial population if the DNA was ever transferred. This could be particularly detrimental if an antibiotic resistance gene was also present. It is particularly important to remember that sometimes only a fragment of a full-length gene is necessary to produce an active enzyme or protein product. The expression of antibiotic resistance markers in bacterial cells could be avoided by the use of introns to interrupt the gene sequence (Vancanneyt et al., 1990). Where an intron is present in a gene, it must be spliced out before expression of the protein can occur; eukaryotes are able to do this, but prokaryotes are not. This idea has been used as a tool by researchers to eliminate the question of reporter gene activity being the result of bacterial expression rather than expression in the plant cell (Ohta et al., 1990; VanWordragen et al., 1992; Shabnam et al., 1996; Pavingerova et al., 1997). Superfluous sequences from nuclear genomes could also pose risks to the environment through the potential to transfer to other plants via pollination.

The introduction of a gene or piece of DNA into a plant genome may also have unpredictable effects on secondary metabolism. In instances where an enzyme is introduced to produce a single new chemical, that chemical might act as a substrate for other enzymes resulting in novel compounds or changes in the levels of different compounds. The introduced enzyme itself could result in the production of more than one compound in a particular plant because of its ability to be active on compounds not normally found in the organism from which it originated. Insertion of the foreign DNA into the genome could itself affect secondary metabolism through the disruption of a gene involved in secondary metabolism (Firn and Jones, 1999). An idea of the numbers of compounds and associations involved in secondary metabolism can be gained from two examples taken from terpenoid metabolism. In *Mentha gracilis* (spearmint), a radiation-induced mutant was shown to produce a new enzyme product plus other new compounds unexpectedly (Croteau et al., 1991). Two enzymes from *Abies grandis* (grand fir) are able to produce multiple products from just one substrate, one enzyme can produce 52 compounds and another enzyme 34 (Steele et al., 1998).

During the preparation and purification of vector DNA, it is not inconceivable that co-purification of contaminating bacterial DNA occurs from contaminated cultures. In all scenarios, the chance of a detrimental effect occurring is increased by the presence of multiple copies of superfluous sequences present in transgenic plant genomes.

II. *AGROBACTERIUM*-MEDIATED PLANT TRANSFORMATION

Agrobacterium tumefaciens is a phytopathogen that has evolved a parasitic mechanism al-

lowing it an ecological niche in which to flourish. The mechanism by which it is able to achieve this involves the transfer of certain genes from itself to the plant nuclear genome. Once these genes are incorporated into the plant genome, they are capable of being expressed using the plant's own transcriptional and translational machinery that results in the formation of tumors on the host plant. These tumors subsequently produce specific amino acid derivatives called opines that *Agrobacterium* is able to utilize as a carbon and nitrogen source. Virtually no other soil microorganism is capable of catabolizing opines, and thus *Agrobacterium* has evolved to occupy a niche in which to thrive.

A. *Agrobacterium* Plasmids

Within *Agrobacterium*, the DNA to be transferred (T-DNA) is located on a large tumor-inducing plasmid (Ti plasmid) between two imperfect 25-bp direct repeat sequences known as the left and right borders. The Ti plasmid also contains a 35-kbp virulence (vir) region that includes genes *virA*, *virB*, *virC*, v*irD*, *virE*, *virF, virG, virH,* up to *virR* (Zambryski, 1992; Sheng and Citovsky, 1996; Zhu et al., 2000). A number of the corresponding Vir proteins are involved in producing a copy of the T-DNA and transporting it from the bacterium to the plant cell in response to chemicals released from the wounded plant, some of the *vir* gene functions are, as yet, unknown. Also involved in the transfer process are a number of virulence genes (*chv*) (Zupan and Zambryski, 1997) located on the *Agrobacterium* chromosome that mediate chemotaxis and attachment of the bacterium to the plant cell wall. This exchange of genetic information from bacterium to plant cell is the only known natural transfer of DNA across kingdoms (Ream, 1989) and has provided an elegant tool for the manipulation of plant genomes.

Wild-type Ti plasmids themselves were not useful to molecular biologists because tumorigenesis that occurs in transformed tissue as a result of the expression of oncogenes present on the plasmids makes regeneration of plantlets from the transformed tissue very difficult. The solution was to create a disarmed Ti plasmid, where all the oncogenes were removed. Zambryski et al. (1983) achieved this simply by replacing, with pBR322 sequences, all of the DNA (apart from the *nos* gene) from between the T-DNA borders of pTiC58. The resulting plasmid was named pGV3850. Further improvements came with the inclusion of selectable markers within the T-DNA that allowed transformed cells to be distinguished from untransformed cells (Bevan et al., 1983). These plasmids were still not ideal as vehicles for gene delivery. Their very large size made standard procedures for manipulation arduous, and unique restriction sites within the T-DNA, in which the gene of interest could be subcloned, were difficult to find. The solution was the creation of intermediate vectors (Matzke and Chilton, 1981) based on the standard pBR322 vector for *Escherichia coli* (*E. coli*) (Sutcliffe, 1979; Backman and Boyer, 1983; Peden, 1983). This vector is small and contains an array of unique restriction sites (multiple cloning site) useful for inserting genes of interest. The intermediate vector is transferred from *E. coli* to *Agrobacterium* by conjugation; however, the vector itself is conjugation deficient, and this function is provided by a helper plasmid such as pRK2013 (Ditta et al., 1980). Once the intermediate vector has been transferred to *Agrobacterium*, homologous recombination occurs between itself and *Agrobacterium's* resident disarmed Ti-plasmid (for example, pGV3850). The result is a new larger plasmid known as a cointegrate disarmed Ti-plasmid. Cointegrate plasmids have been used extensively and are still used today; however, they do result in a very large plasmid.

B. Binary Vectors

One of the remarkable features of DNA transfer from *Agrobacterium* to the plant cell is that none of the virulence genes themselves is required to be transferred. This means they can be placed on a plasmid, separate from the T-DNA, to function *in-trans* to facilitate the transformation process. The segment of DNA to be mobilized is placed between the two cis-acting sequences, the right and left borders, on a smaller plasmid capable of ma-

nipulation in *E. coli*. An example of such a plasmid is Bin 19 (Frisch et al., 1995). Once cloning is complete the plasmid is transferred, either conjugatively using a helper plasmid or via direct methods, to an *Agrobacterium* strain containing a plasmid (pAL4404) harboring the *vir* genes. Cocultivation of the *Agrobacterium* strain with appropriate plant cells will induce the *vir* genes on pAL4404 to mediate, *in trans*, the transfer of T-DNA from the binary vector to the plant genome. This system is known as the binary vector system (Hoekema et al., 1983; Bevan, 1984), and it is used extensively for *Agrobacterium*-mediated transformation.

It is important at this point to mention a further binary vector system discovered by Buchanan-Wollaston et al. (1987). A plasmid such as pAL4404 carrying the *vir* genes is able to mobilize plasmids of incompatibility class Q introduced into the *Agrobacterium* strain, just as described for the binary vector system. The difference however, is that incQ plasmids contain no right or left borders; instead they contain a bacterial *ori*T and *mob* genes encoding mobilization proteins that mediate relaxosome formation, which is similar to border nicking by the Vir D proteins. This type of system has not been adopted by many researchers, because incQ plasmids are more difficult to handle than binary vectors such as Bin 19. The whole plasmid is usually transferred in multiple complete or truncated copies (Bravo-Angel et al., 1999).

C. *Agrobacterium* Rhizogenes

Although the most widely used *Agrobacterium* species for plant transformation is *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes,* the causative agent of hairy root disease in dicotyledonous plants, has also been used to introduce foreign genes into plant genomes (Porter, 1991). It carries an Ri plasmid that is analogous to the Ti plasmid of *Agrobacterium tumefaciens*. In an experiment using a disarmed Ti plasmid and an Ri plasmid simultaneously to infect both *Arabidopsis* and carrot cells, the induction of hairy root disease served as the only selectable marker of transformation (Van Sluys et al., 1987). *Agrobacterium rhizogenes* has not been used extensively for plant transformation because the pRi T-DNA carries onc-genes that result in plants with an abnormal phenotype. However, pRi vectors are used to make hairy root cultures for the production of secondary plant metabolites.

D. T-DNA Transfer

1. Mechanism of T-DNA Transfer

One of the main factors contributing to the preferred use, by molecular biologists, of *Agrobacterium tumefaciens* as a tool for transformation of plant cells is the widely accepted view that transfer of DNA from *Agrobacterium* to the plant genome is discrete, with a high proportion of integration events containing a single or low T-DNA copy number (Hiei et al., 1994; Marton and Browse, 1991). Despite these reports, some groups provide evidence to the contrary. Jorgensen et al. (1987) evaluated the structure of integrated T-DNA sequences transferred to tomato plants using *Agrobacterium.* Seven from eleven transgenic plants exhibited multiple copies of T-DNA organized predominantly as inverted repeats. In this report, they suggest that the prevalence of inverted repeats may be a feature unique to the *Agrobacterium* vector C58 and its derivatives. A recent report of wheat transformation mediated by *Agrobacterium* found that from 26 transformants, 35% contained a single copy of the transgene, 50% contained two or three copies, and 15% carried four or five copies (Cheng et al., 1997). Although more than half of the transformants in this experiment carried more than one copy of the transgene, the maximum number of transgene copies was five, which in general is low compared to transformants created using direct DNA transfer methods. Unusually, this group also performed a comparable transformation experiment. Using similar constructs, the same cultivar and regeneration and selection procedure but microprojectile bombardment rather than A*grobacterium*-mediated transformation, they found that the percentage of transformants carrying single transgene copies fell to 17% (13 plants from a total of 77). Unfortunately, the results of this experiment are not shown; further comparison of the integration events would have been valuable.

Sarmento et al. (1992) using *Agrobacterium* to transform pickling cucumber found that 3 plants, from 21 kanamycin-resistant plants, contained approximately 10 copies of the *npt II* gene. A very interesting set of experiments by Grevelding et al. (1993), designed specifically for comparison purposes, revealed different patterns of T-DNA integration dependent on the type of method/explant used to transform *Arabidopsis* using *Agrobacterium*. Southern blot analysis showed that 89% of transgenic plants derived from leaf disc transformations contained multiple T-DNA insertions, whereas 64% of transgenic plants derived from a root transformation method contained single T-DNA insertions. In contrast, *Arabidopsis* leaf transformation has also been shown to produce transformants containing a high proportion of single copy transgene insertions. Experiments performed by Van der Graaff and Hooykaas (1996) show that 68% of transformants contained just one T-DNA insert.

When examining transformation data, it is important not to be misled by confusing wording. Valvekens et al. (1988) reported confirmation of low copy number T-DNA insertion using evidence of segregation patterns that suggested the presence of one or two functional transgene copies. Transgene segregation in progeny often reports the number of functional transgene copies, but it is important to remember that the number of functional copies is not always a reflection of the actual number of transgene copies present. Transgene silencing is a well-documented phenomenon and can occur for a variety of reasons (Meyer, 1996). Consequently, transgenic plants frequently contain functional and nonfunctional copies of the same transgene.

It is widely reported that T-DNA alone from binary and cointegrative vectors is transferred from *Agrobacterium* to the plant cell causing only minor deletions at the ends of the T-DNA and within the plant genome during integration (Tinland, 1996). However, there are a handful of publications that challenge this understanding (Martineau et al., 1994; Ramanathan and Veluthambi, 1995; Van der Graaff et al., 1996; Cluster et al., 1996; Wenck et al., 1997; Kononov et al., 1997). Joos et al. (1983b) were able to show tumors produced in a plant after placing the tumor-inducing genes outside of the T-DNA borders in the Ti plasmid pGV3850. Other evidence to suggest that plasmid sequences other than from between the T-DNA borders may be capable of being transferred and incorporated into plant genomes during *Agrobacterium*-mediated transformation came from work by Stachel et al. (1987) and Veluthambi et al. (1988). Both these groups found DNA sequences from either side of the T-DNA borders on T-DNA strands formed within *Agrobacterium*.

Further anomalies during the transfer and integration of T-DNA have been reported. It is widely understood that T-DNA transfer is a polar process, initiating from the right border continuing through the T-DNA until the left border. The T-DNA is excized because VirD1 and VirD2 proteins together have site-specific endonuclease activity that cleave between the third and fourth base pair of the bottom strand of the borders (Wang et al., 1987a; Scheiffele et al., 1995). There is actually no difference between the right and left border sequences; the right border is more active than the left, however, because of the presence of 'overdrive' enhancer sequences close to the right border (Van Haaren et al., 1987a). The overdrive sequence is an enhancer that means that it is able to exert its effect over large distances. Therefore, it is possible that the overdrive sequence could stimulate initiation of transfer from the left border (Ramanathan and Veluthambi, 1995; Van der Graaff et al., 1996). This effect may be mediated by factors such as the size and topology of the intervening DNA sequence. The type of plasmid used and gene of interest inserted in the T-DNA region could therefore be important in mediating 'normal' T-DNA transfer. There are many unpublished accounts, where using the same binary vectors and methods of transformation different genes have produced widely varying frequencies of plants stably transformed with the gene of interest. The T-DNA size and topology theory could be an explanation for this anomaly. In many studies, the right border has been shown to be essential for tumorigenesis (Joos et al., 1983b; Hepburn and White, 1985; Ooms, et al., 1982; Peralta and Ream, 1985a; Shaw et al., 1984; Wang et al., 1984) and thus transfer of the T-DNA, whereas absence of the left border has had no

effect on virulence (Joos et al., 1983a; Peralta and Ream, 1985b). Two of these groups (Shaw et al., 1984; Wang et al., 1984) also showed that inversion of the right border in the Ti plasmid dramatically reduces tumorigenicity. This is probably explained by the results of Miranda et al. (1992), who were able to show that when the right border in a Ti plasmid was inverted, rather than the T-DNA being transferred, vector sequence adjacent to the T-DNA was transferred. The Ti plasmids are very large and it is likely that, in a large percentage of cases, transfer is broken off before reaching the T-DNA region, resulting in the genes responsible for tumorigenesis not being transferred to the plant and hence reduced tumorigenicity. Other groups, however, have shown that in the absence of a right border, the left border in fact becomes the point of initiation for transfer (Van Haaren et al., 1987b; Wang et al., 1987b). Despite these observations, it seems that for many years little research was carried out to establish to what extent non-T-DNA sequences were being transferred to the plant genome during *Agrobacterium*-mediated transformation.

2. Prevalence of Vector Backbone Transfer

In a letter to the editor of *Plant Cell* in 1994, Martineau et al. outlined the results of analysis of several hundred crop plants independently transformed with a binary vector (McBride and Summerfelt, 1990). DNA from the Ti plasmid outside of the T-DNA borders was found to be present in plant genomes of 20 to 30% of the plants. Investigation of further plants transformed using a different binary vector (An et al., 1985) also resulted in 20% of plants containing binary plasmid sequences from outside the T-DNA borders. Unfortunately, this paper does not specify whether the transformants were created using the same *Agrobacterium* transformation method or whether a number of methods was employed. However, it appears that this publication sparked a revival in analyzing T-DNA transfer more extensively.

Ramanathan and Veluthambi in 1995 developed a vector specifically for easy identification of non-T-DNA transfer from *Agrobacterium* to the tobacco genome during co-cultivation of leaf discs with *Agrobacterium*. Using an *nptII* expression cassette located outside of the left border in pTiA6, they were able to recover kanamycin-resistant calli, which could only be the result of transfer of non-T-DNA. The calculated frequency of non-T-DNA transfer in these experiments was 1.25%. Southern blotting techniques were able to establish that DNA transfer had been initiated at or near to the left border moving away from the T-DNA. Each of the kanamycin-resistant calli contained no typical T-DNA sequences, suggesting that the DNA was broken off at some point in the transfer process before reaching the right border. These results are interesting not only because they illustrate transfer of non-T-DNA sequences from a Ti plasmid to a plant genome, but they also contradict previous models of T-DNA transfer (Zambryski, 1988; Peralta et al., 1986; Caplan et al., 1985), which suggest initiation of transfer occurs from the right border extending through the T-DNA until it reaches the left border.

In the following year a molecular analysis of the structural organization of T-DNA from a population of Petunia plants exhibiting co-suppression revealed seven plants from a total of 47 containing large fragments of binary vector attached to the T-DNA (Cluster et al., 1996). During that year, further evidence of non-T-DNA transfer was reported by Van der Graaff et al. (1996). From 29 T-DNA inserts isolated from *Arabidopsis thaliana* plants, four contained a complete right border attached to which was further binary vector sequence. Further analysis of two of these proved that in fact the complete binary vector was present. The evidence from the molecular analysis of these integration events suggests that instead of transfer beginning at the right border it had originated from the left border and continued around the whole plasmid until reaching the left border once again. The frequency of this integration event calculates to 14%; however, the frequency of transfer initiating from the left border and incorporating vector sequence is likely to be higher. The method of selection in these experiments did not identify events arising from transfer initiated from the left border and continuing only to the right bor-

der sequence as was detected in the experiments of Ramanathan and Veluthambi (1995).

Kononov et al. in 1997 reported on experiments carried out to investigate the nature of non-T-DNA transfer. They created two interesting binary vectors in which a selectable marker was present within the T-DNA and a mas2′-gusA expression cassette carried outside of the T-DNA borders, either adjacent to the left (pBSG-1) or right border (pBSG-2). From 202 kanamycin-resistant plants, 75% contained the gusA gene and 20% tested positive for GUS activity. From evidence that T-DNA transfer is a polar process (Zambryski, 1988; Peralta et al., 1986; Caplan et al., 1985), beginning at the right border and continuing until the left border, if any 'read through' was to occur one would expect that plants transformed using pBSG-1 would have a higher probability of carrying the gusA gene than those transformed with pBSG-2. This in fact was not the case, no major differences could be found between either plasmid in their ability to transfer the gusA gene. This experiment also encompassed an analysis of the strain of *Agrobacterium tumefaciens* used in the co-cultivation with tobacco leaf discs. Three strains commonly used for plant transformation were examined: LBA4404 (an octopine-type), GV3101 (a nopaline-type), and EHA105 (an agropine-type supervirulent strain). The two latter strains are regarded as being more virulent than the octopine-type strain LBA4404 (Hood et al., 1993); however, on some hosts octopine strains can be more virulent. From the three *Agrobacterium* strains used, there was no discernible difference between the frequency of non-T-DNA sequences transferred. This group report vector 'backbone' sequences attached to both left and right borders; they also report vector 'backbone' sequences integrated into the plant genome that are unlinked to the T-DNA region. Because selection of transgenic plants was based on the presence of the *nptII* gene present within the T-DNA borders, T-DNA and vector 'backbone' must have integrated into the genome at independent locations. This suggests that the real frequency of 'backbone' integration must be higher than recorded because excluded are 'backbone' integration events that have occurred without simultaneous integration of T-DNA at any location.

3. Factors Involved in Vector Backbone Transfer

The widely varying frequencies of vector 'backbone' transfer reported may be indicative of differences in transformation method, plant species/cultivar, *Agrobacterium* strain, or plasmid construction. The research by Kononov et al. (1997) indicated that there was no difference in the ability of three strains of *Agrobacterium* to transfer binary vector sequence to the tobacco genome under the experimental conditions employed. This result may reflect the correct association between *Agrobacterium* and 'backbone' transfer, but it must not be assumed from such a small study that this is the norm.

A series of experiments performed by Wenck et al. (1997) was carried out to analyse the frequency of vector 'backbone' transfer under different transforming conditions. Co-cultivation of *Agrobacterium* LBA4404 with *Nicotiana plumbaginifolia* protoplasts produced five transformants, three of which contained sequences from the transforming vector other than those from within the T-DNA. *Arabidopsis* root transformation using *Agrobacterium* GV3101 exhibited a frequency of 33% of transformants containing vector 'backbone' sequences. Of particular interest are the results from vacuum infiltration experiments. From 63 transformants, 62% contained DNA from outside the traditionally defined T-DNA region. This group also identified concatamers of the complete binary vector integrated into the plant genome, suggesting that transfer may not have stopped at the right border even after one complete circuit of the vector. The results cannot be used in direct comparison because a variety of vectors and *Agrobacterium* strains were used. However, the results do support the findings of other groups already discussed that not only the defined T-DNA region is transferred to the plant genome during *Agrobacterium*-mediated transformation.

III. DIRECT GENE TRANSFER

Although *Agrobacterium*-mediated transformation has proven very successful for many plant

species, a number of agronomically important crop species has been recalcitrant to transformation using *Agrobacterium*. For this reason, direct DNA transfer methods were developed that dispensed with the restrictive elements of *Agrobacterium*-mediated transformation such as genotype and host cell specificity. Particle bombardment was a further improvement to this transformation technology. It provided the advantages of direct gene transfer but also circumvented difficult tissue culture stages, such as protoplast regeneration, which is unavoidable when producing whole transgenic plants using electroporation and PEG-mediated transformation techniques.

A. Microprojectile Bombardment

The pioneers of this technology were John Sanford and colleagues who first described particle bombardment in 1987 (Sanford et al., 1987). The technology uses gold or tungsten particles coated in DNA that are propelled at high speed toward the plant tissue required to be transformed. Some particles penetrate the plant cell walls and introduce foreign DNA into intact cells.

In 1987 Klein et al. demonstrated transient CAT (chloramphenicol acetyl transferase) activity in epidermal onion cells 3 days after delivering the DNA to cells using a modified bullet gun. Shortly after, in 1988, Christou et al. were the first to report stable transformation events resulting from microprojectile bombardment. Using an electrical discharge gun, they were able to deliver viable DNA into immature soybean embryos and generate stably transformed callus material from isolated protoplasts.

Expression of foreign DNA introduced to plant cells by microprojectile bombardment can occur in two ways. It is most commonly expressed transiently either in the nucleus or other plant cell compartments for a limited period only, 2 to 3 days on average, before being degraded. Alternatively, the foreign DNA may become integrated into the nuclear or chloroplast genome where it is maintained as part of the plant's own DNA and may or may not be expressed. If the foreign DNA is passed faithfully to subsequent progeny, the DNA may be regarded as being stably integrated. DNA-coated particles that penetrate the nuclear membrane are 45 times more likely to be expressed transiently than particles that reach the cytosol and more than 900 times more likely to exhibit transient expression than DNA-coated particles in the vacuole (Yamashita et al., 1991). When delivered by a direct transfer method, DNA expression in the cytosol is more likely to occur if it has been deposited close to the time that the nuclear membrane disappears at mitosis (Bower and Birch, 1990). In the case of wheat cell suspensions, as the cells move into stationary phase, transient expression frequencies fall dramatically (Vasil et al., 1991). The efficiency of transformation using microprojectile bombardment has been shown to be influenced by the stage of the cell cycle (Iida et al., 1991). It has also been suggested that cell cycle stage influences the complexity of the DNA integration pattern in stably transformed plants (Kartzke et al., 1990).

1. Vectors for Direct Gene Transfer

Vectors used for direct gene transfer experiments are in general significantly smaller than those used for *Agrobacterium*-mediated transformation. *Agrobacterium* vectors are naturally large because they carry numerous genes required for the process of tumor formation and a large broad host range replicon for maintenence in *Agrobacterium*. In contrast, small high copy number plasmids that are easy to manipulate are usually chosen as vectors for direct gene transfer. These small vectors also carry sequences that are not necessary for their function, but they are generally limited and only make up small areas between essential genes. The salient features of direct transfer vectors are (1) a bacterial selectable marker cassette (usually antibiotic resistance), which allows for selection of the plasmid when using bacteria in the initial cloning steps; (2) a bacterial origin of replication that facilitates the replication of the plasmid in multiplying bacterial populations; (3) a selectable marker gene (often herbicide or antibiotic resistance) linked to a promoter and terminator that function once in the plant cell and allows for the selection of transformed cells as opposed to

nontransformed cells, and, finally, (4) a multiple cloning site situated between a plant promoter and termination signal into which the gene of interest can be inserted and expressed once inside the plant cell.

It is usual in particle bombardment to coat the gold or tungsten particles in supercoiled plasmid DNA. However, there are reports that linearized plasmid DNA can sometimes increase the frequency of transformation (Sautter et al., 1991). In contrast to *Agrobacterium*-mediated transformation, vectors used for microprojectile bombardment do not undergo specific processing at sequences like T-DNA borders. Therefore, any DNA sequence within the plasmid has an equal chance of being incorporated into the plant genome. In any transformation event, using a direct method, the following can occur, (1) the whole plasmid will be integrated into the plant genome at a single locus, or (2) the plasmid will fragment resulting in parts of the plasmid becoming integrated into the genome possibly at different locations. In reality the pattern of integration is often more complex and is discussed below.

2. Integration of Transforming DNA

There is a vast quantity of literature reporting the pattern of integration of marker genes and the gene of interest, in plant genomes, following microprojectile bombardment to the extent that it is widely accepted that integration events can frequently be complex and multiple in nature. During the period from 1987, when Sanford et al. (1987) first described the technique of particle bombardment, until December 1995, approximately 200 papers were published documenting plant transformation events using microprojectile bombardment. These have been listed (defining the plasmid construct used, the resulting type of expression, the type of tissue/explant used, and the plant in which the transformation was performed) in a bibliographic search by Luthra et al. (1997). The search highlights the vast array of plasmids and explants used to achieve transient and stable expression events all using the same transformation principle, microprojectile bombardment, but subtly different approaches.

3. Integration Patterns of Selectable Marker Genes and Genes of Interest

The insertion patterns of the genes of interest and selectable marker genes have been extensively reported, but as yet few definite conclusions have been made as to what the controlling factors are in the pattern of integration. These questions are difficult to answer because there is no standard transformation method used and extremely limited data from experiments specifically designed to make these types of direct comparisons.

Integration patterns of selectable marker genes and genes of interest are generally documented in reports of plant transformation. In the case of microprojectile bombardment, transgenes are found to be frequently present as full-length copies or occasionally truncated in a wide variety of rearranged forms and in multiple copies. Most experiments are not comprehensive in their analysis of transgene integration, often reporting only frequency of transformation events, copy number of transgenes present in the plant genome, and levels of expression of the transgenes. Less frequently, characteristics such as the orientation of transgenes, co-transformation frequency, or single vs. multiple loci integration are documented.

a. Copy Number

The majority of reports show the transgene copy number ranging between one and twenty (Cooley et al., 1995; Yao et al., 1997; Van der Mass et al., 1994; Klein et al., 1989; Register et al., 1994), but in some cases the copy number has been reported to be far greater (Christou et al., 1989). It is possible for very large amounts of DNA to be inserted into the plant genome following microprojectile bombardment. An interesting experiment carried out by Hadi et al. in 1996 used microprojectile bombardment to introduce 12 different plasmids into cells of a soybean embryonic suspension culture. After selection for hygromycin resistance, carried on one of the plasmids, 26 clones were found to be resistant and most contained all the co-transforming plasmids. The copy number of plasmids was highly variable between

clones, but within each individual clone the copy number for each plasmid was roughly equal, suggesting no bias for specific plasmids. Some clones contained just 0 to 3 copies of each plasmid, others contained as many as 10 to 15 copies of each. This experiment proved that up to 600 kb of DNA can be introduced to the plant genome at once using microprojectile bombardment.

Multiple copies of transgenes at the same locus are often found to have short sections of plant genomic DNA interspersed between them (Cooley et al., 1995). In eukaryotes, replication of DNA occurs at a number of replication forks within the same proximity. It has been suggested that ligation of transgenes to plant DNA at these replication forks would result in the integration of multiple transgenes separated by small amounts of plant genomic DNA.

The prevalence of multiple copy transgene integration is likely to be due, in part, to variable amounts of DNA entering the plant cell nucleus. It may be possible to reduce integrated transgene copy number, particularly in systems with high transformation frequencies, by reducing the amount of DNA used in the bombardment. This is supported by two pieces of experimental evidence: (1) increased amounts of DNA used to bombard plant tissue can result in unstable transgene expression (Drummond et al., 1991), and (2) unstable expression of an introduced transgene is frequently the result of one of a variety of silencing phenomena caused by the presence of multiple copies of the integrated transgene (Meyer, 1996). The ability of *Agrobacterium* to mediate transformation events that predominantly result in the integration of a single copy of a transgene is the result of an evolutionary process that has produced a near optimal mechanism.

b. Integration Site

Multiple copies of transgenes can be found integrated at the same or tightly linked loci, as described above (Becker et al., 1994; Christou et al., 1989; Register et al., 1994). Kohli et al. (1998) reports that transgene integration performed in rice using direct DNA transfer resulted in an integration pattern suggestive of a two-phase integration mechanism. In the first instance, intact or partial transforming DNA molecules become spliced together and integrated, resulting in rearrangements without interspersing plant genomic DNA. These integration sites then act as hot spots for further integration of transforming molecules that result in foreign DNA integrating largely at the same genomic location with the first preintegration event, and the second integration event being separated by plant genomic DNA. Integration of intact transgene copies at a single locus with additional rearrangements, further copies and truncated transgene fragments interspersed with plant genomic DNA is supported by transformations, and co-transformations performed using microprojectile bombardment of oat tissues (Pawlowski and Somers, 1998). This work reported all 13 transgenic oat lines showing cosegregation of transgenes and the presence of interspersing plant DNA in all cases. It is suggested that there must be a predominating integration mechanism. It was estimated that the transgene loci must have been between 35 to 280 kb.

Less frequently, but of significance, co-transforming molecules can be found to be integrated at unlinked sites. This is a useful phenomenon because it lends itself to the removal of selectable marker genes. Whether linked or unlinked, the site of integration is thought to be random, although there is no conclusive evidence to confirm this. Lindsey et al. (1993) report that T-DNA integration events mediated by *Agrobacterium* disrupt transcriptional units in more than 90% of cases, and within these transcribable regions integration site is random (Koncz et al., 1989; Mandal et al., 1993).

c. Rearrangement of Transgenes

Molecular analysis of transgenic genomic DNA, using techniques such as Southern blotting and PCR, have established that DNA introduced into plant genomes using microprojectile bombardment frequently undergoes extensive rearrangement. In fact, the majority of microprojectile bombardment experiments that used Southern analysis of transgene integration events report

some degree of transgene rearrangement (Brar et al., 1994; Cooley et al., 1995; Vasil et al., 1992; Weeks et al., 1993). Typical rearrangements observed include deletion from the ends of linearized transforming constructs, probably due to endogenous nuclease activity, direct repeats, inverted repeats, deletion, and ligation of fragments and concatamerization.

It has been shown that the integration patterns of transgenes usually remain unchanged through repeated subculturing of tissues (Christou et al., 1992; Gordon-Kamm et al., 1990). This suggests that rearrangements occur prior to integration rather than after the event. In support of this theory are a number of co-transformation experiments that report linkage of different co-transformed plasmids (Gordon-Kamm et al., 1990; Spencer et al., 1992). This phenomenon has also been reported in co-transformation experiments using *Agrobacterium* (De Neve et al., 1997).

d. Concatamerization

Linearized copies of transforming plasmid DNA linked together in either a head-to-head or head-to-tail configuration and integrated at a single genomic locus is a type of rearrangement known as concatamerization. It has been observed most dramatically by Hadi et al. (1996) but also in a more modest fashion by Finer and McMullen (1990) and as a result of other direct DNA transfer methods by Hain et al. (1985), Kartzke et al. (1990), and Riggs and Bates (1986).

The majority of complex Southern blot analyses are never accurately explained. The most comprehensive method of analyzing integration events would be to DNA sequence, but this is not a routine procedure. We have been unable to find a report documenting what would be extremely valuable information in understanding the mechanism of transgene integration. A further valuable analysis of transgene integration that could be performed is the use of plasmid DNA, cut with a restriction endonuclease that does not cleave within the plasmid itself, to probe transgenic genomic DNA (Kartzke et al., 1990). Such analyses can help to establish the number of transgene integration sites and the complete size of the integrated DNA.

4. Integration of Superfluous DNA Sequences

It seems that in the course of producing transgenic plants, researchers have not routinely assessed the extent of superfluous vector sequences integrated into plant genomes as a result of microprojectile bombardment. The vast majority of published manuscripts reporting plant transformation events are those where the intention was not to release transgenic plants into the environment. Basic research accounts for a high percentage of experiments using microprojectile bombardment to study processes such as transient gene expression, promoter analysis, plant development, biosynthetic and metabolic pathways, and the mechanism of viral infection. Therefore, in many cases it has been regarded as unnecessary to carry out molecular analysis using vector DNA other than the gene of interest and the selectable marker. The emphasis of research has been to identify and analyze integration events of the gene of interest and the selectable marker gene, with the intention of understanding the way in which expression of these genes is regulated.

As mentioned previously, in theory any sequence within a plasmid that is used as a vector in a transformation experiment employing a direct transfer protocol has a chance equal to that of any other sequence in the plasmid of being transferred and incorporated into the plant genome. Therefore, where the selectable marker gene and gene of interest are equal in size to the remaining DNA making up the completed vector, the total amount of superfluous vector sequence transferred to plant cells during a transformation experiment is equal to the amount of selectable marker and gene of interest transferred.

Microprojectile bombardment is known to produce transgenic plants with rearranged and multiple copies of the required transgene. Therefore, a certain proportion of transformed cells are also likely to contain rearranged and/or multiple copies of remaining superfluous vector sequence. Based on knowledge of vector construction one therefore surmizes that numerous transformed plant cells are likely to contain multiple copies of antibiotic-resistance genes and/or their regulatory sequences and bacterial origins of replication. It

is also highly likely that other vector sequences will be present in the genomes of transformed cells, possibly in multiple copies. These insertions, as is evident for selectable marker genes and genes of interest, may be located at the same locus as the required genes or at a single unlinked locus or multiple unlinked loci.

The paragraphs above have theorized about the possible integration patterns of unwanted vector sequences in transformed plant cells. In the case of transgenic plants, the prevalence and pattern of integration of such sequences may be skewed because these plants are not representative of all transformed cells. They have been selected for the presence of the selectable marker gene. Co-transformation frequencies for the selectable marker gene and gene of interest are generally reported to be high, ranging from 10 to 100% (Kuehnle and Sugii, 1992; Hagio et al., 1991; Gordon-Kamm et al., 1990); however, the majority of experiments report frequencies well above 50%. This suggests that superfluous vector sequences may be integrated into the plant genome at similar frequencies. However, the position of a sequence in relation to the selectable marker gene may influence the likelihood of it being integrated along with the selectable marker. It may be that superfluous plasmid sequences in transgenic plants are present in significantly greater rearranged forms than the selectable marker gene. Register et al. (1994) discovered that during transformation of embryogenic maize suspension cells, transgene cassettes that did not undergo selection during callus growth were more likely to be present in a rearranged form than transgenes whose expression had been selected for.

It has been suggested that plasmids larger than 10 kb may be subject to greater fragmentation during microprojectile bombardment (Birch and Bower, 1994). With respect to the integration of superfluous vector sequences, fragmentation might mean that the potential to select plants without superfluous DNA sequences may be higher. At the same time, such a strategy may also serve to reduce the number of transformants containing an intact selectable marker and gene of interest. Conversely, larger plasmids may simply mean greater portions of superfluous DNA present in transgenic plants.

Reports of plasmid concatamers present in the genome of transformed tissues (Hadi et al., 1996; Finer and McMullen, 1990) give the best evidence to date of unwanted plasmid DNA integrated into transgenic plants as a result of microprojectile bombardment. In Hadi's experiments, several independent transformation events contained over 10 copies of each of 12 transforming plasmids and many contained approximately 120 intact plasmids integrated into the soybean genome. Each plasmid was between 4 to 5 kb in mass. The mass of DNA integrated into soybean cells therefore was in the region of 600 kb, a large proportion of this, inevitably, sequence that would normally be unrequired in the development of an improved plant trait.

Notably, the only direct evidence for superfluous DNA integration has occurred because radioactive probes used to obtain information regarding the required integration of transgenes in Southern blots has highlighted hybridizing fragments that are equal to or are multiples of the mass of the complete introduced plasmid. In instances where unexpected fragments hybridize to radioactive probes, rearrangement of transgenes must have occurred and in many cases, are likely to contain superfluous plasmid sequences. However, it is usual for no further analysis to be carried out, and the complete nature of the event is never established. Consequently, the integration of superfluous DNA sequence is never confirmed. There are no reports of superfluous plasmid sequence being used as a probe to assess the extent to which integration of these sequences has occurred.

B. Silicon Carbide Whisker-Mediated Transformation

Silicon carbide whisker-mediated transformation is a relatively new method used to transfer foreign DNA into plant cells. Silicon carbide crystals, having an average diameter of 0.6 μm and length of between 10 to 80 μm, are mixed in liquid medium, usually with a vortex, in the presence of DNA and plant cells. The crystals appear to physically pierce the cell wall (Songstad et al., 1995) and thus facilitate entry of DNA into the plant cell.

The first report of DNA delivery into plant cells using silicon carbide fibers was by Kaeppler et al. in 1990, and 2 years later they reported stable transformation of tobacco and maize cells (Kaeppler et al., 1992). In 1994, Frame et al. presented the first report of transgenic plant production using this technology in maize and later Dalton et al. (1998), using silicon carbide whiskers, reported the creation of transgenic grasses.

Initial development of the technique centered on maize, probably due (1) to the relatively late development of transgenic maize plants capable of producing transgenic seed (Fromm et al., 1990; Gordon-Kamm et al., 1990), and (2) to the economic potential for improvement of such a widely grown crop species. There are still only a handful of papers reporting transformation events using this method, but there are several reasons that make this technology worth investigating: (1) it requires no elaborate equipment such as a helium gun or electroporation apparatus, which means that it is inexpensive and accessible; (2) the procedure is simple, which allows large numbers of transformation experiments to be processed in a short period of time; and (3) although simple there are a wide variety of variables that lend themselves to the optimization of the technique for different cell types. Further to this, Thompson et al. (1995) report the ability to transform cells using very low amounts of DNA; 0.1 μg of input DNA was found to achieve a transformation efficiency of 50% when compared with that achieved using 25 μg of DNA. Using silicon carbide whiskers as opposed to other direct transformation methods, it is therefore possible to have better control over the amount of DNA that can potentially enter a cell during transformation and thus possibly reduce the total amount of DNA that becomes integrated into the plant genome.

Although the number of reported silicon carbide transformation events is low, the evidence on copy number integration to date appears to be similar to that observed for other direct transformation methods. However, because this is a small sample it is possible that these results are not representative of the norm. Frame et al. (1994) observed the majority of maize transformants containing 1 to 5 copies of the *bar* gene, although some contained more than 10 copies. Stable transformants of tobacco and maize generated by Kaeppler et al. (1992) contained 1 to 20 copies of intact and rearranged *bar* and GUS genes. Dalton et al. (1998) has reported 1 to 6 transgene copies present in transformants of *Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea,* and *Agrostis stolonifera*. The authors suggest the prevalence of transgene rearrangement and number of transgenes present in each transformant is lower than reported for other transformation systems.

C. Protoplasts, Polyethylene Glycol, and Electroporation

1. Protoplast Transformation Using PEG and Electroporation

Protoplasts are plant cells that have had their cell wall removed usually by enzymatic degradation. Removal of the cell wall eliminates the main barrier to DNA uptake and generates single cells from large pieces of tissue. The first reports of DNA delivery into plant protoplasts (Draper et al., 1982; Krens et al., 1982) were carried out using polyethylene glycol (PEG), which causes permeabilization of the plasma membrane allowing the passage of macromolecules into the cell. The first transgenic plants to be produced using this method were tobacco by Pazkowski et al. (1984) and since this time a wide range of dicotyledonous and monocotyledonous plants have been transformed using PEG-mediated protoplast transformation. A further method for transforming plant protoplasts uses electroporation in which the protoplasts are subjected to electrical pulses that again render the plasma membrane permeable to macromolecules. Potrykus (1991) suggests that these techniques lead predominantly to the stable integration of single copy transgenes (Potrykus et al., 1985). This suggests that the integration of superfluous vector sequences may also be limited.

However, there are many studies that provide evidence to the contrary. Hain et al. (1985) and Riggs and Bates (1986) both encountered concatamerization of transforming plasmids in the genomes of tobacco protoplasts subsequent to

transforming conditions using electroporation (Riggs and Bates, 1986) and a chemical-based protocol (Hain et al., 1985). Czernilofsky et al. (1986), using the same chemical-based tobacco protoplast transformation system, discovered 10 to 25 copies of integrated donor DNA linked together in transformed cells. They also discovered significant structural changes to integrated transgenes. Christou and Swain (1990), using electroporation of soybean protoplasts, generated stably transformed cell lines that frequently contained rearranged and intact copies of transgenes. A more recent protoplast transformation experiment using PEG to create transgenic plants of *Linum usitatissimum* and *L. suffruticosum* produced plants that contained 1 to 2 copies of a *NPTII-GUS* cassette, the second copy being integrated at a separate locus (Ling and Binding, 1997).

The wide range of results experienced by different groups with different systems may be due to the cell cycle stage of the protoplast. Kartzke et al. (1990) reported significant differences in the pattern of transgene integration according to the cell cycle stage of tobacco protoplasts at the point of transformation. Nonsynchronized protoplasts produced predominantly nonrearranged single copy transgenes in contrast to M phase protoplasts that gave rise to transgenic plants containing more copies of the transforming plasmid usually at separate loci and S phase protoplasts that resulted in high copy numbers and frequent rearrangements.

Several experiments using protoplast transformation have shown that irradiation of protoplasts shortly prior to or after the addition of DNA during direct transformation procedures increases the frequency of transformation (Koehler et al., 1989; Koehler et al., 1990; Gharti-Chhetri et al., 1990). Gharti-Chhetri's experiments showed a 4- to 10-fold increase in the transformation rate when transformed diploid protoplasts were treated with UV light. It also served to increase the number of integration sites in the genome. A similar increase in transformation rate was observed by Koehler et al. (1990). Using protoplasts of *Petunia hybrida* and *Brassica nigra,* they demonstrated an increase in transformation rate of 6- to 7-fold after X-ray treatment. It is likely that increased transformation rates are a result of an increased number of nicks in the genomic DNA caused by the irradiation. If integration is partly mediated by DNA repair mechanisms, it is probable that they are induced during X-ray and UV irradiation and subsequently will contribute to raising the rate of transformation.

Protoplast systems have produced transgenic plants from species such as rice (Donn et al., 1990) and wheat (Vasil et al., 1990) that previously had been recalcitrant to *Agrobacterium*-mediated transformation. However, embryogenic cell culture systems for the production of protoplasts are frequently very difficult to establish and maintain, plus the regeneration of plants from protoplasts is often unreliable. In most cases, transformation protocols using protoplasts are technically difficult to perform and are by no means routine. Electroporation as opposed to PEG-mediated transformation has improved the simplicity of the technique and the reproducibility of high-frequency DNA delivery. However, the limitations of protoplasts have resulted in their use primarily as transient expression systems rather than for production of transgenic plants.

2. Electroporation of Intact Tissues

Electroporation has also been used for the transformation of intact plant tissues, this strategy being developed to circumvent the problems associated with protoplasts. Tobacco pollen was the first tissue to be transformed successfully (Abdul-Baki et al., 1990; Matthews et al., 1990); however, since these papers further reports have been few in number and generally describe transient expression only.

3. Carrier DNA

One of the biggest differences between protoplast transformation methods and other direct transformation methods is the inclusion of carrier DNA in the transformation mix, usually calf thymus DNA. It serves to increase the frequency of transformation (Paszkowski et al., 1984; Potrykus et al., 1985; Saul and Potrykus, 1990), but at the

same time it has been shown to increase the prevalence of transgene rearrangements (Saul and Potrykus, 1990). Carrier DNA is usually prepared to approximately 500 bp in length. It is taken up by the protoplasts along with plasmid DNA, and in theory has as much chance of becoming incorporated into the genome as the plasmid. Evidence suggests that it can promote transgene rearrangement, probably becoming complexed with the transgenes and integrates into the genome at the same locus. However, the DNA could equally become integrated at different loci, and as these sequences are not normally probed in Southern blots they would never be detected. One group, however, in the 1980s, published results from plant protoplast transformations using polyethylene glycol. They showed that nonselected calf thymus DNA was co-transformed along with other pTi sequences, and that integration had occurred at both linked and unlinked sites (Peerbolte et al., 1985).

D. Agrolistic Transformation

Although microprojectile bombardment has been revolutionary in the development of successful transformation for plant species recalcitrant to *Agrobacterium*-mediated transformation, the frequency of complex transgene integration events is usually high, which often results in the reduction or absence of transgene expression. Conversely, it is widely accepted that *Agrobacterium*-mediated transformation generally results in a greater proportion of less complex, single transgene integration events. The prevalence of this problem has fueled continued research to improve *Agrobacterium*-mediated transformation of monocotyledonous plant species. The most recent published advance in this area is 'Agrolistic' transformation (Hansen and Chilton, 1996; Hansen et al., 1997), which is striving to combine advantages of microprojectile bombardment and *Agrobacterium*-mediated transformation to produce a high frequency of transgenic plants that lack the superfluous vector sequence and contain just a single copy of the required transgene.

The strategy involves a co-transformation approach to deliver a gene of interest and selectable marker along with plasmids carrying *Agrobacterium* virulence genes *vir*D1 and *vir*D2 using microprojectile bombardment. The *vir*D genes during conventional *Agrobacterium*-mediated transformation function in the bacterium to release the single-stranded T-DNA before it is transported to the plant cell. Hansen and Chilton (1996) were able to show that these genes are functional *in planta* and are capable of mediating T-DNA-type insertion events from plasmids that have been delivered to plant cells using microprojectile bombardment. In a second published report, Hansen et al. (1997) achieved VirD1- and VirD2-mediated integration events at a frequency of 20 to 35% of the total number of integration events. The addition of a *vir*E2 gene to the co-transformation mixture reduced the number of degraded transgene integrations, particularly from the 3′ end. VirE2 binds in a cooperative manner to single-stranded DNA (Citovsky et al., 1997), and *in vitro* experiments have shown that it is capable of protecting bound DNA from nuclease activity (Citovsky et al., 1989).

The work by Hansen and Chilton (1996) and Hansen et al. (1997) addresses superfluous vector DNA integration during microprojectile bombardment. Vir-mediated events achieved a selectable marker copy number of 1 to 2, which was less than the number of copies reported for non-vir-mediated integration events. However, the technique requires considerable development. Southern blot analysis provided evidence of vir-mediated integration events that were not always of the expected size, suggesting that some form of rearrangement had occurred and the possible integration of vector DNA from the *vir* gene co-transforming plasmids was not considered in any detail.

IV. CHLOROPLASTS AND FOREIGN DNA INTEGRATION

It must be remembered that, in addition to the nuclear genome, a plant cell also contains chloroplast and mitochondrial genomes that can also be subject to integration of foreign DNA during plant transformation. These genomes therefore could carry superfluous vector sequences. It is unlikely that chloroplast transformation occurs via *Agrobacterium*-mediated

transformation because of the membrane surrounding the chloroplast, separating the DNA from the contents of the cell. However, microprojectile bombardment has been used to achieve stable chloroplast transformation (Daniell et al., 1998). Promoters designed for expression in nuclear genomes are likely to be less active in the chloroplast genome. However, it is possible that a gene integrated downstream of a chloroplast promoter could be expressed and produce a protein product.

Chloroplast transformation carries a number of advantages over nuclear transformation. A plant cell can contain up to 50,000 copies of the chloroplast genome (Bendich, 1987). Stable transformation of a foreign gene in the plastid genome under selective conditions can result in tremendous amplification of the transgene that has been shown to result in very high levels of transgene expression (McBride et al., 1995). Integration of transgenes into the nuclear genome occurs at random loci, and there are several reports of transgene integration mediated by homologous recombination (Schaefer and Zyrd, 1997), but so far this has proven difficult and by no means routine. In contrast, however, the integration of foreign genes into the chloroplast genome is achieved routinely using homologous recombination strategies (Daniell et al., 1998; McBride et al., 1995; Carrer and Maliga, 1995). Controlling the site of integration in this way can reduce the variation in transgene expression experienced when using random site integration protocols. It may also help to prevent the integration of superfluous vector sequences into the chloroplast genome. Two-thirds of higher plants transmit their chloroplast DNA maternally rather than through pollen dispersal. Therefore, it may be possible to prevent the transmission of transgenes to other plants using stable chloroplast transformation rather than stable nuclear transformation. These advantages might allow chloroplast transformation the potential to become a more widely practiced transformation technique.

V. DETECTION OF SUPERFLUOUS DNA SEQUENCES

A number of methods are available for verifying the nature of transgene integration events. Although the tools required for comprehensive analysis of transformed tissues are available, the majority of reports using these techniques fail to fully characterize the pattern of transgene integration events in their experiments. A combination of Southern hybridization, PCR, and DNA sequencing analysis combined with Mendelian inheritance studies are sufficient to establish the presence or absence of superfluous DNA sequences and the pattern of required transgene integration in transgenic plants provided they are used in the correct manner.

Southern hybridization is generally the technique of choice for the verification of transgene integration in plant genomes. However, because of the ease with which PCR samples can be processed, this method is becoming more popular despite being more limited in the amount of information it produces.

A. Southern Hybridization

With careful planning, a Southern blot can produce a lot of information about the pattern of transgene integration in a plant genome. Total genomic DNA from the putative transformant is digested with a restriction enzyme and run on an agarose gel to separate the DNA fragments. The DNA is blotted onto a membrane and probed with a radioactively labeled piece of DNA. If the same sequence of radioactive DNA used to probe the membrane is present among the genomic DNA, it will bind to its homologous sequence on the membrane and produce a radioactive signal that is visualized on photographic film. Two factors that determine the amount of information obtained from such an experiment are (1) the choice of restriction enzyme used to digest the genomic DNA, and (2) the DNA sequence used as a radioactive probe. In most cases, Southern blots are performed by cleaving the genomic DNA with an enzyme that cuts within the T-DNA. For tissues transformed using direct gene transfer, enzyme cleavage is usual within the transforming marker gene or gene of interest. The radioactive probe employed is usually a DNA fragment from within the T-DNA or the

selectable marker/gene of interest cassette. Where the detection of superfluous DNA sequences is required, a different strategy must be employed. A radioactive probe corresponding to DNA sequence from outside of the T-DNA borders or outside of the selectable marker gene of interest cassette will identify the presence of these sequences. If the genomic DNA is cleaved using an enzyme that is known not to recognize any sequence present within the probe itself, this will help to identify the number of independent integration events that have occurred. In instances where rearrangements have occurred it can be very difficult to establish whether bands on a Southern blot correspond to independent or rearranged integration events. To distinguish unambiguously the nature of the integration events the corresponding fragments can be cloned and sequenced to establish the exact nature of any rearrangement and define the number of plant-vector DNA junctions. Alternatively, sexual crossing can be performed to determine the number of independent transgenic loci.

A hybridization signal on a Southern blot does not necessarily indicate that the sequence in question is integrated into the plant genome. The addition of uncut DNA on a Southern blot can further substantiate a positive hybridization signal on a Southern blot as being a true integration event. The uncut genomic DNA is of high molecular weight, and if the probe binds to this DNA it is very unlikely that it could be binding to plasmid DNA, which is normally a lot smaller.

An assessment of transgene copy number is often performed when analyzing putative transgenic plants. The usual way of calculating this is by mixing known amounts of probe DNA (having been calculated to be equivalent, for example, to one, two, or ten copies of the sequence in the genome of the transformed plant) with genomic DNA from an equivalent untransformed plant. The relative hybridizing signals from the putative samples are compared with the known samples, and in this way the copy number is estimated. A second method is sometimes employed that involves probing the genomic DNA with an endogenous gene having a known copy number (Sanders et al., 1992).

B. PCR

PCR is a useful technique for rapidly screening large numbers of putative transformants. Specific sequences can be identified by using primers that are homologous to the ends of that sequence. Although widely used, PCR has severe limitations in the amount of information it can provide concerning integration patterns. A positive result shows only that sequences homologous to the primers were present in the sample in close enough proximity to produce a product. In instances where extensive rearrangements have occurred the sequence in question may be present, but the primers bind too far apart or in the wrong orientation, resulting in no PCR product. Actual integration into the plant genome is difficult to prove using PCR alone. A positive PCR result in conjunction with the correct controls merely concludes that the sequence is present in the sample, and this could result from contamination, presence of *Agrobacterium,* or nonintegrated sequence. Quantitative PCR (Coen, 1994) can be used to establish the copy number of specific sequences, but it is technically difficult and Southern hybridization remains the preferred method of analysis.

C. DNA Sequencing

The advantage of sequencing cloned insertions is that it provides detailed knowledge of the inserted vector sequences and plant-vector DNA junctions. A positive signal from a Southern blot indicates that either the entire probe sequence or only part of it is present within the genomic DNA. A combination of different-sized probes can help to deduce exactly which pieces of DNA are present, but the most comprehensive analysis is achieved using DNA sequencing. In instances where foreign DNA has integrated into a gene sequence, sequencing of the junctions can reveal the identity of the disrupted endogenous gene (Hansen and Chilton, 1996). This type of infor-

mation could be useful for further analysis in determining the consequences of transformation. Vector-plant DNA junctions can be cloned using particular PCR techniques, an example of which is described by Hansen et al. (1997). Alternatively, the whole integrated vector fragment plus plant genomic sequence can be cloned using plasmid rescue. Genomic DNA is digested with a restriction endonuclease and run on an agarose gel; DNA fragments of the appropriate size are excized and cloned into a suitable vector. Bacteria are transformed with the plasmid DNAs and colonies screened for the correct insert using colony filter hybridization (Hansen and Chilton, 1996) or by selection of a marker gene present in the T-DNA (Van der Graaff and Hooykaas, 1996). When the latter method is employed, the entire foreign DNA is present and therefore the entire insert can be sequenced.

D. Inheritance of Foreign DNA

By studying the inheritance of foreign DNA sequences, it is possible to determine whether specific sequences are integrated at linked or unlinked loci. Two copies of vector sequence that are tightly linked will always be transmitted to progeny as a single unit, that is, some progeny will get both copies of the sequence, and some will get none. Sequences that are integrated at independent loci will be found in some progeny to be present in the absence of the second copy.

VI. REMOVAL OF SUPERFLUOUS GENE SEQUENCES

In instances where the same selectable marker is to be used in a further round of transformation using a previously transformed plant, it is necessary to remove the selectable marker gene from the transgenic plant before commencing with the second round of transformation. Molecular biologists are now able to use genetic engineering to create transgenic plants with more than one desirable trait. At present, there are a limited number of genes available for use as selectable markers in plants, which means from a practical perspective that in many cases it may be necessary to remove selectable marker genes from transgenic plants before embarking on further rounds of transformation. The removal of selectable marker genes may also be necessary where there are concerns over safety. Extensive studies have been carried out which confirm the safety of the neomycin phosphotransferase (*nptII*) gene present in transgenic plants (Calgene, 1990 and 1991; Flavell et al., 1992; Fuchs et al., 1993). If any other genes used as selectable markers for plant transformation are found to compromise the safety of transgenic products, then this may provide further reason to remove superfluous foreign DNA sequences from the genomes of transgenic plants to facilitate approval for deliberate release into the environment.

There are a number of transformation strategies developed recently that enable selective elimination of transgene sequences from the transgenic plant.

A. Co-Transformation

Co-transformation is the delivery of two different transforming DNA constructs into a single plant cell. This type of strategy allows a selectable marker gene to be introduced separate from the gene of interest. Integration of both DNAs into the plant genome must occur, and they must be at sites sufficiently unlinked to facilitate removal of the selectable marker by conventional crossing. Co-transformation can be performed with either (1) *Agrobacterium* using a single strain or with a mixture of two strains carrying different T-DNAs, or (2) by a direct gene transfer method such as microprojectile bombardment or PEG-mediated transformation. Some groups have found co-transformation a good method for introducing two DNAs at unlinked sites; others, however, have found that the incidence of integration at linked sites is high.

One of the first published co-transformation experiments performed by McKnight et al. (1987) used two *Agrobacterium* strains, one carrying the *nptII* gene and the other a nopaline synthase gene. Eleven tobacco plants tested positive for NPTII and from these, three also tested positive for

nopaline synthase at unlinked sites. De Block and Debrouwer in 1991 co-transformed *Brassica napus* hypocotyls using the *Agrobacterium* mixture method and a nopaline-type *Agrobacterium* strain: C58. They recovered 18 co-transformed plants in which 14 proved to have linked T-DNAs. In a more recent study (De Neve et al., 1997) designed to evaluate the nature of T-DNA integration events using co-transformation as a tool, it was found that co-transformed T-DNAs were frequently integrated at the same genomic location. In these experiments, co-transformation was carried out using co-cultivation of an *Agrobacterium* strain, C58C1Rif^R (a cured derivative of the nopaline *Agrobacterium tumefaciens* strain C58), with tobacco protoplasts and roots and leaf discs of *Arabidopsis*. The percentage of linked co-transformation events established by molecular analysis of the *Arabidopsis* transformants was 45%, and the results suggested that the frequency of linked integration events was independent of transformation method or plant species.

In contrast to these results, Komari et al. (1996) showed that linked loci were not favored by either a single-strain or mixture method of co-transformation when using a derivative of an octopine *Agrobacterium* strain, LBA4404. They were able to report a frequency of 47% for co-transformation of tobacco and rice using a 'super-binary' vector carrying two separate T-DNAs on the same plasmid. More than half of the progeny produced from co-transformants carried the nonselectable marker but not the selectable marker. The frequency of co-transformation was found to be very low in instances where a mixture of two *Agrobacterium* strains carrying different T-DNAs were used to transform either tobacco or rice.

There are numerous papers reporting a wide variety of frequencies of co-transformation, but many of these do not report the frequency of plants that received unlinked T-DNAs at unlinked sites. The reason for this is probably because the researchers were merely assessing the ability to receive different DNA constructs, because it is often simpler to engineer genes into different plasmids rather than into the same plasmid. Using electroporation of protoplasts (a direct gene transfer method) Christou and Swain (1990) were able to obtain co-transformation frequencies, using unlinked genes, of 18 to 27% in soybean with various combinations of marker genes. No experiments were carried out to determine whether these genes were present in the genome at unlinked sites. The frequency of co-transformation using linked genes was quite low in this study at 50%. Aragao et al. (1996) using microprojectile bombardment of *Phaseolus vulgaris* L. (bean) produced co-transformation frequencies of between 40 to 50% for unlinked genes and 100% for linked genes. Microprojectile bombardment of barley explants by Wan and Lemaux (1994) produced co-transformation frequencies of 84% with linked genes and 85% with unlinked genes. They suggest that the high frequency of unlinked co-transformation could be due to the use of a 1:2 molar ratio of selected:nonselected genes. Often, co-transformation frequencies are established by phenotypic expression of markers rather than by molecular analysis. In these instances it is important to remember that gene silencing may have occurred, leading to an underestimation of the frequency of co-transformation.

Differences in the frequency of unlinked and linked integration events may be influenced by the method of plant transformation, the transformation vector, the *Agrobacterium* strain, or the plant species used. In plants where the frequency of transformation is very low, it is unlikely that co-transformation would be viable. However, the research to date suggests that co-transformation as a tool for the development of transgenic plants lacking selectable marker genes could be viable under some circumstances. Where different T-DNAs are transferred to the plant genome using a single plasmid, the frequency of unlinked integration events will be influenced by the efficiency of T-DNA processing. This highlights the need for a plant transformation method with a high frequency of successful transformation where co-transformation is desired.

B. Site-Specific Recombination

In 1989, Cregg and Madden demonstrated the removal of a selectable marker gene that had been introduced into the yeast *Pichia pastoris* using a site-specific recombinase (FLP) and its substrate,

an asymmetric inverted repeat sequence (FRT) from *Saccharomyces cerevisiae*. Lyznik et al. (1996) have now demonstrated the successful use of this system in maize to remove an introduced *nptII* gene. Furthermore, molecular analysis of the plant genome at the FRT sites proved that precise rearrangement of genomic DNA sequences had occurred.

Since the first demonstration of selectable marker gene removal using the FLP/FRT system, a number of other strategies have been developed. All of the systems require the selectable marker gene to be introduced into the plant genome flanked by two specific DNA sequences. A second round of transformation is performed to introduce a gene coding for a recombinase enzyme that specifically catalyzes recombination between the two specific DNA sequences at either side of the selectable marker gene. The recombinase gene can work effectively either by transient expression or by integration into the plant genome, in which case it must be removed through crossing.

The most attractive system for plant use is the bacteriophage P1 Cre/*lox* system (Dale and Ow, 1991). In Dale and Ow's experiments, a hygromycin phosphotransferase (*hpt*) gene flanked by 34 bp direct *lox*P sequences was linked to the firefly luciferase (*luc*) gene and introduced into tobacco via *Agrobacterium*-mediated transformation. Transgenic plants expressing both the *hpt* and *luc* genes were transformed again using *Agrobacterium*-mediated transformation to introduce a Cre recombinase (*cre*) gene linked to a second selectable marker, neomycin phosphotransferase (*npt*). Plants expressing the Cre recombinase precisely excized the *hpt* gene leaving the *luc* gene intact. The plants were self-pollinated and in those which had received the *cre-npt* DNA cassette not linked to the *luc* gene, the T-DNAs segregated to yield progeny containing the *luc* gene but not the *cre-npt* cassette.

A third site-specific recombination system for plants is the pSR1 system of *Zygosaccharomyces rouxii*. Demonstrated in tobacco protoplasts (Onouchi et al., 1991), recombinase encoded by the R gene of *Zygosaccharomyces rouxii* catalyzed site-specific recombination of two specific DNA sites (RSs) to remove an intervening DNA sequence from a GUS gene introduced into the plant genome. The result was precise excision of the sequence and the restoration of GUS activity. Finally, a fourth system of this nature, the Gin recombinase system of phage Mu, has shown to be successful in *Arabidopsis* and tobacco protoplasts (Maeser and Kahmann, 1991). Site-specific recombination was achieved using a mutant but not wild-type Gin recombinase introduced both transiently and stably integrated into the plant genome.

C. Transposition

One of the most well-studied transposition systems is the Ac-Ds system in maize (Ferdoroff et al., 1983). The Ac element encodes a transposase that is capable of activating *in trans* the excision and relocation, within the maize genome, of Ds sequence elements. This phenomenon has been adapted to create a modified Ds element, where virtually any DNA sequence bound by specific DNA repeat sequences can be moved about the plant genome in the presence of the Ac transposase.

Goldsbrough et al. (1993) utilized this system as a method for repositioning transgenes in the plant genome in order to create a selection of transgene expression levels without having to perform many individual transformations. They also used this system to remove an *nptII* gene that had been introduced into the plant genome linked to a GUS reporter gene cassette. In Goldsbrough's experiments, the T-DNA region was constructed such that it contained the GUS gene flanked by the Ds sequences linked to a transposase gene and selectable marker gene. It was introduced into the tomato genome using *Agrobacterium*-mediated transformation. Where transposition of the DNA region flanked by the Ds elements had occurred to regions of the genome sufficiently distant from the remaining T-DNA, self or outcrossing of these plants produced progeny that contained either the GUS gene or the selectable marker plus transposase. In this way, the selectable marker gene and transposase had been removed from the transgenic plant, leaving the transgene of interest intact.

Alternatively, the removal of a selectable marker could be facilitated by flanking the marker with Ds elements rather than the gene of interest. By carrying out the experiment in this way, the selectable marker may also be lost automatically whenever the Ds element fails to reintegrate into the plant genome. In tomato, lack of reinsertion or reinsertion into sister chromatids that subsequently become lost by somatic segregation occurs in 10% of excision events. The advantage of this is that it allows marker gene removal to be achieved in plant species that propagate only vegetatively and not sexually. The gene encoding transposase does not need to be introduced as part of the T-DNA along with the gene of interest and selectable marker. It could be introduced in a second transformation event or by crossing with a plant containing the transposase. It is important to note that with most transposable elements, once transposition has occurred it is usual for whole or partial copies of the border repeats to be left at any location in the genome that has been occupied by the mobile element.

All the mechanisms described above are not confined to the removal of selectable marker genes alone. They can be applied to the removal of virtually any DNA sequence. For some of the systems, there are likely to be restrictions on the size of DNA, which can be manipulated successfully in this way. However, the range of plasmids in use today is unlikely to cause any problems.

VII. SELECTION PRESSURE

A number of reports suggest that the degree of selection pressure applied to putative transformants and/or level of transgene expression can affect the proportions of transformants recovered containing single and multiple copy transgenes. In instances where selection pressure is high, it is more likely that a transformant with numerous copies of a functional selectable marker gene, rather than a single copy, will produce enough enzyme to detoxify the normally toxic agent. A recent paper by Dalton et al. (1995) confirmed that, in their experiments, application of high concentrations of hygromycin in the selection procedure resulted in a prevalence of transformants with multiple copies of the transgene. In contrast, low concentrations of hygromycin produced transformants containing two or fewer inserted copies of the transgene. Therefore, it seems logical that when a single integrated copy of a transgene is the requirement in a transgenic plant, the best policy to adopt is a degree of selection that is as low as possible allowing only a few or no escapes.

Commonly used promoters for driving selectable marker gene expression in plants are the cauliflower mosaic virus (CaMV) 35S and nopaline synthase (NOS) promoters. Both of these promoters have been shown to be developmentally regulated and differentially active in plant parts used as explants for transformation (An et al., 1988; Benfrey et al., 1989). NOS promoter activity was studied at various developmental stages in different plant structures of tobacco (An et al., 1988). Promoter activity was found to be high in the lower parts of the plant (roots) and decreased gradually moving up the plant (leaves). These results suggest that if the NOS promoter is used to drive selectable marker gene expression, where, for example, leaf is the chosen explant, a percentage of the transformants particularly single copy transgene insertions may be selected against. This might explain the results of Grevelding et al. (1993) discussed earlier in this review. The majority of *Arabidopsis* transformants derived from root explants contained single copy transgene insertions, whereas 89% of transformants produced from leaf-disc explants carried multiple T-DNA insertions. If promoter activity is high in transformed cells derived from root explants, the amount of selectable marker enzyme produced from a single copy of a transgene is likely to be sufficient to confer resistance to the selection. If the activity of the promoter is low because it is being expressed in transformed tissue derived from leaf explants, a single copy of the selectable marker is not likely to be enough to confer resistance to the selection. Transformants from leaf-disc explants therefore would be more likely to contain multiple copies of functional transgenes. The same theory applies to the *nptII* gene in the vector Bin 19. It contains a point mutation that reduces the activity of the enzyme fourfold. It is possible when using this plasmid that the reduction in

activity could result in a predominance of transformants selected that contain multiple copies of the selectable marker gene and thus, possibly, multiple copies of other superfluous DNA sequences.

VIII. HOMOLOGOUS RECOMBINATION

Foreign DNA integration into plant cells, using any of the variety of methods for plant transformation, is usually achieved by a form of illegitimate recombination (Gheysen et al., 1991; Mayerhofer et al., 1991). Recombination of only a few identical nucleotides, or none in the case of end-joining, occurs between the donor and recipient DNA (in the case of *Agrobacterium*-mediated DNA transfer recombination occurs at or near the T-DNA border sequences), resulting in the DNA becoming integrated into nonhomologous genomic sequences. In some lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*, foreign DNA predominantly becomes integrated not by illegitimate recombination but by homologous recombination (Hinnen et al., 1978). Homologous recombination is also used in animal cells for site-directed mutagenesis, which is commonly known as gene targeting (Capecchi, 1989).

Homologous recombination, as its name suggests, uses foreign DNA that contains large regions of homology to replace homologous endogenous genomic DNA. This type of foreign DNA integration is useful because there is more control over where in the genome the foreign DNA is inserted. It can be used to replace specific genes; a gene can be replaced with a mutant nonfunctional form to study the function of the gene. A mutant gene in the genome can be replaced with a functional gene, or a functional gene encoding an enzyme could be replaced with a gene whose sequence has been altered, resulting in increased enzyme activity.

Homologous recombination has the potential to give the molecular biologist greater control over foreign DNA sequences integrating into the plant genome during transformation experiments. By increasing the efficiency of homologous recombination and eliminating or significantly reducing the amount of illegitimate recombination between foreign DNA and plant genomic DNA, it may be possible to prevent superfluous vector sequences becoming incorporated into plant genomes during plant transformation. Until recently, reports of homologous recombination events in plants have been limited, and the frequency of homologous recombination transformation events as opposed to illegitimate events has been in the region of one targeted integration in 10,000 to 300,000 transformants (Offringa et al., 1990; Paszkowski et al., 1988; Risseeuw et al., 1995; Risseeuw et al., 1997). Two recent papers report homologous recombination integration events in the moss *Physcomitrella patens*. Schaefer and Zyrd (1997) targeted three single-copy genomic loci in *Physcomitrella patens* with DNA containing homologous sequences. The transformation frequency proved to be 10-fold higher using the targeted vectors and the efficiency of targeting was found to be above 90%. However, further development of this technique is required to prevent multiple copies of the gene being incorporated into the genome. In the same year, Kempin et al. (1997) reported one knockout for an endogenous *Arabidopsis* gene, AGL5. Using vacuum infiltration, they obtained one targeted event from a total of 750 transformants. Although this is very encouraging, it remains to be seen whether this can be repeated. It has been suggested that the vacuum infiltration process may have played a vital role in the success of this experiment by mediating the transformation in meristematic or meiotic tissues that were particularly active in homologous recombination (Puchta, 1998).

A similar alternative to homologous recombination is that of gene targeting by site-specific recombination. This has been carried out utilizing the Cre-lox system discussed earlier in the context of marker gene removal. DNA has been shown to be integrated specifically at lox sites previously placed in the plant genome (Albert et al., 1995; Vergunst and Hooykaas, 1998; Vergunst et al., 1998). A possible disadvantage of such an approach is that the system itself requires the integration of DNA sequences other than the gene of interest.

IX. CHEMICAL CONTROL OF GENE EXPRESSION

In instances where superfluous genes cannot be removed, elimination or reduction in the amount

of protein produced might be achieved with the use of inducible promoters to drive expression of genes in a temporally and spatially regulated manner. Some of the chemicals used for this type of expression include tetracycline, dexamethasone, copper, and salicylic acid (Gatz, 1996 and 1997). Not all systems are appropriate for field-scale regulation, but some, for example, may be suitable for inducible expression to facilitate the selection of transformed cells from untransformed cells.

Özcan et al. (1993) showed that a wound-inducible promoter, AoPR1 from *Asparagus officinalis,* was expressed at high levels at wound sites during tobacco leaf disc transformation, but at very low levels in leaves, roots, and seeds of the mature plant. When used to drive the expression of the selectable marker, *nptII*, a large number of tobacco and potato transformants were obtained. The resulting transgenic plants contained low levels of the protein product, NPTII, whereas control plants that had used CaMV35S to drive expression contained much higher levels of NPTII.

Cadddick et al. (1997) have described a method for inducible gene expression in plants based on the *alc* regulator of the fungus *Aspergillus nidulans*. In *A. nidulans*, the *alcA* gene codes for alcohol dehydrogenase I, which is regulated by the transcription factor AlcR. By using the *alcA* promoter and *alcR* gene to promote expression of a transgene, it is possible to switch expression on and off with the application of ethanol to the tissue. In preliminary experiments with tobacco the *suc-2* gene, when expressed constitutively using the CaMV 35S promoter, had deleterious effects on plant health. In contrast, however, using the inducible system, plants were able to develop normally. One advantage of this system is the low phytotoxicity of the inducer, ethanol.

More recently, a new and possibly more acceptable selection mechanism for transgenic sugar beet cells has been developed (Joersbo et al., 1998 and 1999). The system uses the phosphomannose isomerase (PMI) gene from *Escerichia coli* and mannose that becomes converted to mannose-6-phosphate by endogenous hexokinase. Transgenic cells are able to convert mannose-6-phosphate to fructose-6-phosphate, but non-transgenic cells accumulate mannose-6-phosphate that causes the inhibition of growth that facilitates the selection of transformed cells.

X. PLANT PROCESSES AND DNA INTEGRATION

It is known that integration of T-DNA into the plant genome is regulated to some degree by plant processes (Sonti et al., 1995; Nam et al., 1997). UV and γ radiation can cause breaks and deletions in genomic DNA. In normal cells these breaks are repaired, but a mutant unable to repair these breaks would become hypersensitive and eventually die. Sonti et al. (1995) have discovered two *Arabidopsis* mutants, originally isolated as hypersensitive to irradiation (one UV hypersensitive and the second γ hypersensitive) that show impaired ability to produce stable transformants compared with nonmutant controls. Transient expression assays using a T-DNA-encoded GUS gene resulted in no significant difference in GUS activity between the two mutants and nonmutant parental lines. These results suggest direct evidence for plant control in the integration of T-DNA into the *Arabidopsis* genome and that genomic integration may involve a DNA repair mechanism. However, later work using these two mutants (Nam et al., 1998) found that the γ-hypersensitive mutant, *rad5*, showed a significant reduction in the efficiency of both transient and stable transformation. This suggests that it is blocked at a step in the transformation process before T-DNA integration. The UV-hypersensitive mutant *uvh1* was found to be as susceptible to *Agrobacterium tumefaciens*, as its nonmutant equivalent, in an *in vitro* root inoculation and an *in vivo* flower bolt inoculation assay.

Ecotypes of *Arabidopsis* show a high degree of variation in their susceptibility to crown gall disease. A number of ecotypes are recalcitrant to tumorigenesis, several due to reduced binding of *Agrobacterium* to root explants and one, UE-1, in which recalcitrance is mediated at a late stage in the transfer of T-DNA. With this particular ecotype, transient expression from a T-DNA-encoded GUS gene is efficient, but the ecotype is not susceptible to crown gall disease, stable GUS

expression, or transformation to kanamycin resistance. When compared with ecotype Aa-0, which displays high susceptibility to tumorigenesis, Aa-0 was found to contain five times more integrated foreign DNA than UE-1. This suggests that the deficiency resides in the T-DNA integration process (Nam et al., 1997). This finding is consistent with research on maize that suggests that the difficulty in transforming this particular plant may reside in its poor ability to integrate T-DNA (Narasimhulu et al., 1996). Narasimhulu's group found that when comparing transient expression in tobacco and maize cells, using DNA delivered from two different *Agrobacterium* strains, initially there is no significant difference. T-DNA transfer, nuclear targeting, conversion of single-stranded T-DNA to double-stranded DNA, and transcription all appear to function similarly. However, GUS transcripts in the maize cells had disappeared 36 hours after infection whereas in tobacco cells they were present for at least 3 days, and in some cases more than 7 days depending on the *Agrobacterium* strain used for the infection. This suggests that expression of transgenes introduced into maize, using *Agrobacterium*, is highly transient, and that the difficulty in stably transforming maize using *Agrobacterium* may be due either to rapid degradation of T-DNA molecules or because of a block in T-DNA integration itself. Interestingly, UE-1 *Arabidopsis* ecotype was found to be 2 to 5 times more sensitive to γ radiation than ecotype Aa-0.

A further series of *Arabidopsis* mutants resistant to transformation by *Agrobacterium tumefacieins* have been identified. These mutants, known as *rat* mutants, appear to be blocked either at an early step in the transformation process or at a step subsequent to the translocation of T-DNA into the nucleus (Nam et al., 1999). The mutant *rat5* is resistant to *Agrobacterium* root transformation. Generated by insertional mutagenesis, it contains two copies of T-DNA integrated into the 3′ untranslated region of a histone H2A gene, upstream of the polyadenylation signal sequence and is deficient in T-DNA integration (Mysore et al., 2000). Overexpression of RAT5 in wild-type plants or transient expression from the incoming T-DNA in the *rat5* mutant has shown to increase the efficiency of *Agrobacterium*-mediated transformation

The importance of the hosts recombination machinery in T-DNA integration has also been illustrated by Bundock et al., 1995 and 1996. In these experiments, T-DNA exhibiting homology to the yeast genome was shown to integrate into the yeast genome via homologous recombination. In the absence of homology, integration was shown to occur via end-joining as in plants.

The evidence above, along with that of Koehler et al. (1990), indicates that plant processes are involved in the integration of *Agrobacterium* T-DNA and foreign DNA transferred by direct methods into plant genomes. It is likely that plant species, particularly model plant systems such as tobacco and *Arabidopsis,* may benefit from the use of engineered or mutant plant lines for improved transformation characteristics.

XI. SUMMARY AND CONCLUSIONS

There is evidence for the integration of superfluous vector DNA from *Agrobacterium* vectors and direct transfer vectors into plant genomes during transformation. This phenomenon has been occurring largely unchecked among researchers, because of the common practice to verify the nature of foreign DNA integration using DNA probes that are homologous only to sequences found within the T-DNA borders or to selectable marker genes and/or genes of interest. There are a limited number of publications illustrating this phenomenon, particularly for *Agrobacterium*-mediated superfluous DNA integration.

A. *Agrobacterium*-Mediated Transformation

From the literature available at present, it is impossible to draw any conclusions as to what the controlling factors are in the transfer and integration of non-T-DNA vector sequences. To date, the objective of experiments to specifically analyze this phenomenon has not been to make direct comparisons in order to establish the reasons for transfer, but merely to prove that it does occur.

With respect to producing transgenic plants for release into the environment, the widely varying frequencies of transfer reported under different transformation conditions, particularly transformation method (Wenck et al., 1997), suggest that the frequency of superfluous DNA transfer might be controlled.

It is clear that there is still a significant amount of research to be carried out in order to fully understand the processes by which DNA is transferred from plasmids within *Agrobacterium* to the plant cell. However, until an understanding of the processes can be achieved through new research, it may be possible to identify plant transformation conditions that have lower frequencies of superfluous DNA integration by reanalyzing the vast quantity of transgenic seed held by most molecular plant laboratories.

Thousands of transgenic lines for a huge range of plants, created via *Agrobacterium*-mediated transformation, exist. Southern and PCR analysis of these plants could provide enough data for a statistical survey to establish whether transformation method, such as vacuum infiltration or co-cultivation of roots or leaf discs, influences the frequency of superfluous plasmid DNA being transferred to the plant genome. Although vacuum infiltration is virtually confined to use with *Arabidopsis thaliana* at present, the elimination of tissue culture has made it the preferred method of transformation for this important model plant. As Wenck et al. (1997) have reported from one experiment a frequency of 62% of vacuum-infiltrated transformed plants carrying superfluous DNA sequences, it is of particular concern that this method is becoming so widespread.

Although there is a vast resource of previously transformed plants, these were not prepared in a manner suitable for understanding the factors controlling superfluous DNA integration. Existing or newly designed vectors to aid selection of anomalies could be employed in new transformation experiments designed to allow the effect of a single factor to be analyzed. Well-constructed vectors would aid detailed molecular analysis, using PCR, Southern blotting, and DNA sequencing.

It is possible that engineered or modified *Agrobacterium* strains could provide enhanced T-DNA processing, which would facilitate precise T-DNA transfer. The evidence of the presence of binary vector concatamers in transformed plants by Wenck et al. (1997) and other groups who report read-through of border sequences suggests that endonuclease activity is inefficient. This could be due to low amounts of VirD1 and VirD2 proteins and/or the sequence context of the borders (Wang et al., 1987b). It is interesting that 50% of transgenic plants, created in an experiment where the *virD2* gene was mutated, contained concatamers of vector DNA (Tinland et al., 1995). It may be the case that in some binary vector systems VirD2 is limiting. It is the helper plasmid in these systems that carries the virulence functions, and this is present as 1 to 2 copies per cell (Rogowsky et al., 1990; Sheikholeslam et al., 1979), the binary vector, however, has been shown to be present at 3 to 5 copies per cell (Hajdukiewicz et al., 1994) or even greater (Gallie et al., 1985).

It is interesting that in a review on transformation technology, Christou (1996) lists two unique problems associated with *Agrobacterium*-mediated transformation that he suggests must be addressed before the release of any genetically engineered plant into the environment. One of these problems relates to the presence of vector sequences, from outside of the T-DNA borders, in the genomes of plants transformed using *Agrobacterium*. It is true that the mechanics of this phenomenon are confined only to *Agrobacterium*-mediated transformation. However, the consequence of the phenomenon, superfluous vector sequence present in the genomes of transgenic plants, constitutes the problem and is not unique to *Agrobacterium*-mediated transformation. It must be stressed that this is no more of an issue in *Agrobacterium*-mediated transformation than that found in plants transformed using direct DNA transfer methods that by the very nature of their transformation procedures are more likely to transfer superfluous vector sequences to the plant. In all instances, regardless of transformation procedure, it is good practice to establish whether superfluous vector sequences are present in transgenic plants. Where there are such vector sequences present in the plant genome, it must be evaluated as to whether this is acceptable or not according to the plant's intended use.

B. Microprojectile Bombardment and Other Direct Gene Transfer Methods

Published evidence for the integration of superfluous vector sequences into plant genomes using direct gene transfer methods is limited to reports of integrated plasmid concatamers. Although not reported, patterns of superfluous integrations can be speculated as being similar to those reported for selectable marker genes and genes of interest, remembering that selection pressure will influence the pattern of integrated selectable marker gene/s and therefore possibly the pattern of other gene sequences integrated into the genome. Again, the vast resource of transgenic seed created using direct transfer methods is ideal for reanalysis to establish the extent and patterns of superfluous vector DNA in transgenic plants. Silicon carbide-mediated transformation has the potential for using small amounts of DNA during the transformation process. It is also worthy of attention because of its accessibility as a transformation method.

Cell cycle and therefore explant type and developmental stage seem to play a role in the extent and pattern of transgene integration that will surely extend to superfluous integration events. However, the greatest development in the understanding and prevention of superfluous sequence integration is that of Agrolistic transformation (Hansen and Chilton, 1996; Hansen et al., 1997). A combination of *Agrobacterium*-mediated transformation and microprojectile bombardment, it contains a number of limitations. However, it is the first step on the ladder to thoughtful, well-designed experiments. Reduction in the integration of superfluous sequences during plant transformation is likely to be improved to the greatest extent through the refinement of sequences introduced into the plant cell, selective nuclear targeting, reduced amounts of foreign DNA used, homologous recombination, and the integration of direct transfer and *Agrobacterium*-mediated transformation methods.

Where superfluous DNA transfer to the plant genome is unavoidable or is present in a transgenic line exhibiting good expression of the required transgene, it may then be possible to eliminate the superfluous DNA at a later stage if necessary. Better practice in the transformation and analysis of plants will ultimately lead to a reduction in the amount of superfluous foreign DNA present in transgenic plants.

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