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Genetic Engineering

Principles and Methods





Volume 24

Edited by **Jane K. Setlow**

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Genetic Engineering

Principles and Methods

Volume 24

Edited by

Jane K. Setlow
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FOREIGN DNA: INTEGRATION AND EXPRESSION IN TRANSGENIC PLANTS

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INTRODUCTION

Gene transfer to plants is now a routine procedure, and the number of species amenable to transformation is increasing rapidly. The introduction of foreign DNA into plants allows the creation of engineered plant lines with improved production traits, resistance to pests, pathogens or herbicides, or the ability to synthesize speciality recombinant proteins such as human hormones, enzymes and antibodies. Many of these plants are now reaching commercial status (1). Although the applications of plant genetic engineering are diverse, there are only two basic strategies for stable plant transformation. The first depends on the natural ability of soil bacteria, such as *Agrobacterium tumefaciens*, to transfer a small segment of DNA from a resident plasmid into the plant cell (2). The second is 'direct DNA transfer' and encompasses a range of techniques where DNA is either physically forced into the plant cell, or the cell is persuaded to take up DNA from the surrounding medium. Examples of these techniques include particle bombardment (3), electroporation (4), the transformation of protoplasts mediated by polyethylene glycol (PEG) (5), and transformation using silicon carbide whiskers (6).

Once inside the cell, foreign DNA must integrate into the genome if a stable transgenic plant line is to be produced. Despite the vast number of successful transformation experiments reported in the literature, rather little is known about the actual mechanism of DNA integration, and there is currently no convenient way to control it. Both the position of integration and the structure of the transgenic locus vary considerably among independent transformants, and both these factors have a profound effect on transgene expression. For this reason, producing useful transgenic plants, i.e., plants in which the transgene is expressed

stably and at the level intended by the experimenter, can often depend simply on pot luck. In this review, we discuss the organization of transgenic loci in plants generated by *Agrobacterium*-mediated transformation and direct DNA transfer, and consider models for the mechanism of transgene integration. We also examine the various ways in which transgene structure and organization influence the level and stability of transgene expression, and finally discuss some recent advances that may allow a degree of control over these processes. Methods for studying the structure and organization of transgenic loci are described briefly in **Box 1**.

AGROBACTERIUM-MEDIATED TRANSFORMATION

Principles of Gene Transfer

Agrobacterium tumefaciens is a soil pathogen that colonizes wounded plant cells and induces the formation of a tumor, called a crown gall, at the wound site. The tumor produces special amino acid derivatives called opines, which the bacteria are able to use as a carbon and nitrogen source. The ability of virulent *Agrobacterium* strains to induce tumor growth and opine synthesis, and the capacity to utilize opines, is conferred by a resident tumor-inducing plasmid (Ti-plasmid). During the colonization process, a segment of DNA from this plasmid is transferred to the plant nuclear genome. Genes carried on the transferred DNA (T-DNA) encode enzymes that a) synthesize auxins and cytokinins, resulting in unregulated cell proliferation, and b) synthesize opines from standard amino acids. Importantly, these genes are controlled by plant-like promoters, such that expression is controlled by the plant's transcriptional apparatus. The type of opine synthesized by the plant (e.g., octopine or nopaline) and the type of opine that can be utilized by the bacterium is dependent on the bacterium not the host plant. Therefore, *A. tumefaciens* has carved its own ecological niche by promoting the formation, on the host plant, of a food factory that only the bacteria can utilize (reviewed in 2 and 11).

Vector Design for *Agrobacterium*-Mediated Transformation

The Ti plasmid is a naturally-occurring vector for plant transformation. However, wild-type Ti-plasmids are not suitable vectors for genetic engineering in plants because the oncogenes contained in the T-DNA cause uncontrolled proliferation of transformed plant cells and prevent the regeneration of phenotypically normal and fertile transgenic plants. To be able to regenerate plants efficiently the T-DNA must be *disarmed* by deleting the oncogenes and replacing them with a selectable marker to allow the identification of transformed cells (12). Even disarmed derivatives of wild-type Ti-plasmids are not particularly convenient as experimental vectors because their large size (~200 kb) makes them difficult to manipulate *in vitro*, and there are no unique restriction sites in the T-DNA. Initially, this problem was addressed by subcloning the T-DNA from a Ti-plasmid in a small conventional cloning vector, called an *intermediate vector* (13). Although incapable of replication in *A. tumefaciens*, its introduction by way of a triparental mating or standard transformation process resulted in the presence of both the intermediate vector and a parental Ti plasmid in the same *Agrobacterium* cell. Homologous recombination between the T-DNA sequences of the Ti-plasmid and intermediate vector then resulted in the formation of a large co-integrate plasmid, from which the recombinant T-DNA was transferred to the plant genome.

Box 1: Techniques for the characterization of transgenic loci

1. *Southern blot hybridization*

The technique used most commonly for the analysis of transgenic loci is the Southern blot. Genomic DNA from transgenic plants is digested with one or more restriction enzymes, fractionated by gel electrophoresis and transferred to a nylon membrane. Once denatured and immobilized, the DNA can be hybridized to a suitable labeled probe. Many different types of information can be obtained from Southern blots depending on the restriction enzymes and probes used, for example transgene copy number, the number of independent loci, the presence of concatemers, the presence of genomic DNA between transgene copies at a single locus, the presence of transgene rearrangements, and the extent of vector backbone integration into the genome.

2. *The polymerase chain reaction (PCR)*

The polymerase chain reaction exploits the specificity of pairs of primers annealing at the ends of a particular DNA target to generate a specific amplification product. The information generated by this technique reflects either the presence/absence of a specific product, or the product size. The main advantage of the PCR over Southern blot hybridization is that the former can be used for rapid screening of large numbers of plants. However, in terms of transgene analysis, the PCR can only confirm the presence of a transgene and provide limited information about its structure. It should be noted that a positive PCR result can also be generated by episomal foreign DNA, so other methods must be used to verify stable transgene integration. In conventional PCR, the maximum product size that can be reliably generated is about 5 kb. Long PCR, which employs a mixture of polymerases, can be used to investigate the structure of larger transgenic loci, and may supplement the information provided by Southern blots (e.g., see 7).

3. *DNA sequencing*

DNA sequencing is the highest-resolution transgene analysis method, and permits the precise definition of structural organization and rearrangement. It also allows the nature of transgene-genomic and transgene-transgene junctions to be investigated at the nucleotide level, and the integration site in genomic DNA to be identified. Prior to sequencing, the transgenic locus must be isolated, which can be achieved by standard molecular cloning or through a variety of PCR-based methods (reviewed in 8).

4. *Fluorescence in situ hybridization (FISH)*

Fluorescence *in situ* hybridization involves the use of fluorescently-labeled nucleic acid probes to identify particular target sequences. As with the Southern blot, the use of different probes allows both transgenes and vector backbone sequences to be identified. This technique reveals higher-order transgene organization and the distribution of integration sites. FISH to metaphase chromosomes allows the insertion sites to be mapped cytogenetically and simultaneous analysis in interphase allows the relative position of several integration sites to be determined (9). Fibre-FISH on extended chromatin gives an overview of locus structure, revealing the presence of single-copy inserts, transgene concatemers and interspersed genomic DNA (10). The resolution of FISH lies somewhere between that of genetic segregation and Southern blot hybridization and can provide important correlative data for both techniques.

5. *Genetic segregation*

Segregation analysis involves studying the transmission of particular DNA sequences or the phenotypes thus conferred over several generations of transgenic plants. Closely-linked transgene copies are unlikely to be separated by recombination, while widely-separated loci are likely to segregate at meiosis in some plants. This allows the number of transgenic loci to be determined. Problems with phenotype analysis include complications caused by epigenetic gene silencing, but analysis of DNA sequence segregation by Southern blot hybridization can be highly informative.

T-DNA transfer is controlled by about 30 genes located in the virulence (*vir*) region of the Ti plasmid, which is separate from the T-DNA itself. The *vir* genes function *in trans* and can act on any T-DNA sequence present in the same cell. Therefore, it is possible to use a *binary vector* system in which the T-DNA and virulence functions are present on different plasmids (14, 15). As with the intermediate vector system, the T-DNA can be subcloned on a small conventional cloning plasmid for ease of manipulation, and then introduced into an *Agrobacterium* strain carrying the virulence component of the binary system, typically a Ti plasmid from which the T-DNA has been deleted. Although intermediate vectors are still used today, most contemporary *Agrobacterium* transformation systems are based on the binary vector principle, since this does not rely on homologous recombination to produce a replication-competent plasmid. Typically, the T-DNA is maintained on a shuttle vector, i.e., a plasmid that replicates in both *A. tumefaciens* and the cloning host, *E. coli*. Modern binary vectors are designed with convenience in mind, containing multiple unique cloning sites within the T-DNA, a *lacZ* marker for blue-white selection of recombinants, and a choice of selectable markers to identify transformed plant cells (reviewed in 16).

T-DNA Transfer

The transfer of T-DNA requires three sets of components: a) *cis*-acting elements in the T-DNA itself; b) gene products encoded by the *vir* region of the Ti plasmid (or equivalent vector); and c) certain other gene products encoded by the bacterial chromosome, such as ChvA and ChvB. Transfer is not dependent on any of the genes carried by the T-DNA, hence these can be deleted and replaced with foreign DNA.

In the source plasmid, the T-DNA is flanked by 25-bp imperfect direct repeats known as *border sequences*. These sequences are not transferred to the plant genome intact, but they are required for the transfer process. Executive control of T-DNA transfer is mediated by the *virA* and *virG* genes. These are constitutively expressed at a low level and regulate the other *vir* genes. VirA is a receptor kinase that spans the inner bacterial membrane, and binds phenolic molecules such as acetosyringone that are released by wounded plant cells (17). Activated VirA transphosphorylates the VirG protein, which upregulates the other *vir* genes. The induction of *vir* gene expression results in the synthesis of proteins that form a conjugative pilus through which the T-DNA is transferred to the plant cell. The components of the pilus are encoded by genes in the *virB* operon (reviewed in 18). DNA transfer itself is initiated by an endonuclease formed by the products of the *virD1* and *virD2* genes. This introduces either single-strand nicks or a double-strand break at the 25-bp borders of the T-DNA. It is thought that the intermediate formed (a double-stranded T-DNA or a single T-strand) may depend on the virulence functions particular to the *Agrobacterium* strain (19). Either the left or right border sequence can initiate T-DNA transfer, although it is more usual for initiation to occur at the right border due to the presence of an adjacent *overdrive sequence*, which is recognized by the VirC1 and VirC2 proteins and acts as a transfer enhancer (20, 21). For this reason, deletion of the right T-DNA border severely reduces the efficiency of transfer, whereas deletion of the left border has little effect (22). The VirD2 protein remains covalently attached to the 5' end of the processed T-DNA strand and has been proposed to protect the T-DNA against nucleases, to target the DNA to the plant cell nucleus, and to help integrate it into the plant genome (23, 24). T-DNA transfer has been discussed in more detail in several comprehensive reviews (23-27).

T-DNA Integration

Distribution of Transgenic Loci

Early studies in petunia and tomato indicated that T-DNA integration is essentially random (28, 29). However, later investigations suggested there may be preferential integration into transcription units (30-32). Lindsey et al. (33) showed that over 90% of all integration events occurred within transcription units, but there seems to be no preference for integration into particular genes. Random distribution of T-DNA inserts has also been suggested by several studies of fluorescence *in situ* hybridization (FISH) to metaphase chromosomes (34-37). However, in petunia, FISH analysis showed that T-DNA inserts were found preferentially at distal chromosome sites (38, 39). This may reflect the distribution of gene-rich regions in this species rather than any sequence-dependent insertional bias, and a recent comparative study supports this. Barakat et al. (40) compared the distribution of T-DNA inserts in *Arabidopsis* (where genes are distributed over 85% of the genome and there is little repetitive DNA) and rice (where genes are restricted to 10-25% of the genome and there is much repetitive DNA). They found essentially random distribution in *Arabidopsis* but in rice the integration events correlated well with the 'gene space'. It has also been suggested that T-DNA integration occurs preferentially in regions showing microhomology to the T-DNA borders (41), which may also be enriched in the transcribed part of the genome. The nonselectivity of T-DNA integration in genes is valuable for gene tagging experiments (31, 42), particularly in functional genomics strategies where the aim is to assemble an unbiased genome-wide collection of mutants (43-47).

Organization of transgenic loci

The notion that *Agrobacterium*-mediated transformation gives rise to clean, intact, single-copy or low-copy-number integration events has been widely accepted and has become part of molecular biology 'folklore'. However, several detailed studies have shown that this is not necessarily the case, and that the structure and complexity of the transgenic locus may depend on the *Agrobacterium* strain used, variations in the transformation method and the species and explant type of the host plant. For example, Sarmento et al. (48) used *Agrobacterium* to transform pickling cucumber and showed that three of the 21 transformants they obtained contained more than 10 T-DNA copies. It is generally the case, however, that *Agrobacterium* transformation gives rise to lower transgene copy numbers than direct transformation methods. An informative experiment was performed by Cheng et al. (49) to demonstrate this. These investigators transformed wheat using both *Agrobacterium* and particle bombardment. Of 26 *Agrobacterium*-mediated transformants, more than one-third contained a single T-DNA insert, half contained 2-3 copies and the remainder (about 15%) contained 4-5 copies. There were no transformants containing more than five T-DNAs. In contrast, from the population of 77 bombarded transformants, only 13 (17%) contained a single copy of the transgene. The maximum number of transgene copies in this population was not reported.

Even in nature, the organization of integrated T-DNA sequences differs among *Agrobacterium* strains. For example, the structure and organization of nopaline plasmid T-DNA sequences is usually simple, i.e., there is a single segment. Conversely, octopine T-DNA comprises two segments, T_L (which carries the genes required for tumor formation and

octopine synthesis) and T_R (which carries genes for the synthesis of other opines). The two segments are transferred to the plant genome independently and may be present as single or multiple copies. The significance of this additional complexity is not clear but strain-dependent locus complexity is also a feature of recombinant T-DNAs. In early studies, Jorgensen, Jones and colleagues (50, 51) investigated the structure of transgenic loci generated by *A. tumefaciens* strain C58 derivatives, which contain a nopaline-type Ti plasmid. In both cases, integrated T-DNA preferentially formed dimers in an inverted repeat organization, linked either at the left or right borders. Similar findings were reported by De Block and Debrouwer (52). These investigators transformed *Brassica napus* with two T-DNAs carried by different C58-derived *Agrobacterium* strains. Each T-DNA carried a separate selectable marker, allowing them to identify cotransformation events. In the majority of such cases, the different T-DNAs were present as 'heterodimer' inverted repeats, preferentially around the right border. In contrast, Spielmann and Simpson (53) carried out transformation using an octopine strain of *Agrobacterium*, LBA4404. They found that only two integration events of the 22 characterized resulted in dimer formation, while most of the rest were single-copy integrations. When cotransformation experiments were carried out with this strain (54), three double transformants were obtained and in all cases the two T-DNAs were genetically unlinked. These results suggest that the virulence functions carried by a particular *Agrobacterium* strain may strongly influence the structure of the transgene locus.

Grevelding et al. (55) have investigated transgene locus structure following different methods of *Agrobacterium*-mediated transformation. They transformed *Arabidopsis thaliana* using a standard leaf disk method as well as a root transformation method. Surprisingly, nearly 90% of leaf disk transformants showed multiple T-DNA inserts, whereas this proportion dropped to about one-third for the root transformants. In a previous report, van der Graaff and Hooykaas (56) found that 68% of leaf disk transformants contained a single insert. Therefore, transgene locus structure may be extremely sensitive to variations of a particular transformation method.

Transgene Rearrangement

Occasionally, it has been reported that T-DNA undergoes spontaneous rearrangement prior to or during integration (57-59). Indced the presence of multiple, rearranged T-DNAs has been directly shown in the case of transgenic potato with the technique of fibre-FISH (60). As discussed below for particle bombardment, there is evidence that rearrangements can be induced by particular sequences, such as the CaMV 35S promoter. T-DNA constructs containing an enhanced CaMV 35S promoter were found to undergo frequent rearrangements in potato (61). The CaMV 35S promoter contains an imperfect palindrome with the ability to form a cruciform secondary structure, which may stimulate recombination events (62).

Integration Mechanism

A number of groups have investigated the structure of genomic/T-DNA junctions in plants and have concluded that integration occurs by illegitimate recombination (63-66). This process is ubiquitous, and involves either the end-to-end joining of unrelated DNA strands (blunt-end ligation), or the joining of strands at regions of 'microhomology' – three to ten nucleotides in common which would presumably allow single-strand annealing if both the transgene and broken genomic DNA have 'ragged ends' (reviewed in 67). T-DNA has sometimes been shown to be flanked by short direct repeats, suggesting it has inserted precisely at the point of a staggered break in the genomic DNA, with subsequent repair over

the single-stranded gaps. In other cases, some genomic DNA has been deleted at the site of integration (23).

While a great deal of research has been carried out to elucidate the mechanism of T-DNA transfer, comparatively little information is available concerning the next stage of the transformation process, integration itself. Tinland and Hohn (68) proposed a 'strand invasion' model for T-DNA integration, whereby the 3' end of the T-strand initiates the integration process by hybridizing to a short region of homology in the plant genome, the second strand being completed by 'primer extension' of the plant DNA. Other models suggest conversion of the T-strand into a double-stranded intermediate which integrates at the site of naturally-occurring chromosome breaks *via* double-strand DNA break repair. Agents that cause DNA breaks such as UV irradiation increase the frequency of integration, while *Arabidopsis* DNA repair mutants show impaired transformation efficiency (see below). However, integration still occurs in such mutants and the structure of the loci generated is the same as normal. It is therefore possible that both strand invasion and DNA break repair mechanisms occur.

DNA repair models argue that proteins encoded by the host plant have a much more important role in T-DNA integration than *Agrobacterium* proteins, such as VirD2, which are imported into the plant with the T-DNA. However, since VirD2 protein remains covalently attached to the 5' end of the T-strand during transfer (69) it is also likely to influence integration. In addition to its endonuclease domain, which is essential for nicking the T-DNA prior to export, the VirD2 protein has a nuclear localization signal and an omega sequence, which is proposed to be involved in the integration process. Mysore et al. (70) showed that deletion or substitution of the omega sequence resulted in a lower frequency of transgene integration in *Arabidopsis*, although the structures of the integration events that did occur in the absence of this sequence were no different from those generated by wild-type *A. tumefaciens*. In an *in vitro* assay, VirD2 can ligate together a cleaved T-DNA border sequence (71) but cannot ligate T-DNA to other genomic targets unless plant cell extracts are also present (72).

Plant proteins are certainly required for integration, as a number of *Arabidopsis* mutants have been identified that are deficient for T-DNA insertion. The role of DNA strand-break repair in T-DNA integration was supported by the discovery of *Arabidopsis* mutants *uvh1* and *rad5*, which are hypersensitive to UV and gamma radiation, respectively, and show a low frequency of stable transformation by *Agrobacterium*. Since these mutants showed normal levels of transient expression, it was suggested that they caused deficiencies in the repair of radiation-induced breaks and that break repair is essential for T-DNA integration (73). However, more recent work (74) showed that *uvh1* is no less transformation proficient than wild-type plants and that *rad5* is deficient in both transient and stable transformation, indicating that the dysfunction affects a process occurring much earlier than T-DNA integration. Other mutants resistant to *Agrobacterium* transformation (*rat* mutants) have been identified, and five are thought to be blocked at the point of T-DNA integration (75). One of these, *rat5*, encodes a histone protein, suggesting that efficient T-DNA integration is dependent on chromatin structure at the integration site.

It has been proposed that the formation of dimers and higher-copy-number transgenic loci may involve either duplication of a single T-DNA insert during the integration process or ligation of separate T-DNAs prior to integration. De Block and Debrouwer (52) and De Neve et al. (76) clearly showed that two separate T-DNAs, derived from different *Agrobacterium* strains, could co-integrate to form a 'heterodimer', which is strong evidence in favour of the ligation model. These multimers are likely to form inside the plant cell, rather than during the transfer from *Agrobacterium* to the plant, because no multimers have been isolated from *Agrobacterium* cells (77). However, the investigation of T-DNA/T-DNA junctions in

multicopy transgenic loci provides some evidence against a direct ligation mechanism. While inverted repeats around the right border are often precise, as would be expected in a ligation reaction occurring prior to integration, inverted repeats around the left border and direct T-DNA repeats are often characterized by the insertion of variable sized regions of filler DNA, which may be derived from the T-DNA sequence or from plant genomic DNA (78-80). This suggests either the simultaneous integration of multiple T-DNAs at a single locus, or a two-phase mechanism in which a primary T-DNA integration event stimulates further secondary integrations in the same area. As discussed later, remarkably similar models have been suggested for the integration of transgenes following particle bombardment (81, 82).

Cotransfer of Vector Backbone Sequences by *Agrobacterium*

Agrobacterium was once believed to result in 'clean transformation', i.e., the introduction of a more-or-less precisely-defined segment of DNA, the T-DNA, into the plant genome. This is considered desirable because superfluous DNA sequences may have unpredictable effects on the plant's phenotype. Generally, it has been observed that cleavage occurs at a precise position within the right border repeat and somewhere around the left border that varies by about 100 bp. However, it has become clear over the last 15 years that the T-DNA transfer mechanism is 'leaky', resulting in the frequent cotransfer of vector sequences lying outside the T-DNA borders. The first report of this nature (83) showed that up to 30% of transformants contained vector sequences linked to the T-DNA insert, indicating that the cleavage reaction during T-DNA transfer was rather inefficient. In these cases, the selectable marker was within the T-DNA, so only transformants containing T-DNA in addition to vector backbone sequences were identified. The leakiness in such transfer events is probably caused by limiting amounts of the endonuclease encoded by *virD1* and *virD2*. Gene transfer by *virD2* mutants, for example, leads to the integration of concatemers of the entire binary vector (84).

Ramanathan and Veluthambi (85) constructed binary vectors in which the selectable marker was located outside the left T-DNA border. In accordance with the T-DNA transfer mechanisms discussed above, it was considered likely that this strategy would catch those transfer events in which transfer, initiated at the right border, overran the left border and terminated somewhere along the plasmid backbone. Surprisingly, these investigators found that none of the transformants contained any T-DNA sequences, indicating that transfer had initiated at the left border and had proceeded around the plasmid, presumably breaking off before reaching the right border sequence.

Further investigations have shown that vector sequence transfer is probably a very common event, in some cases involving the entire plasmid backbone with or without the T-DNA (86-88). Concatemers of the entire binary vector have also been seen, indicating that in some cases transfer does not terminate at the T-DNA border, even after one or more complete circuits of the vector (89). The exact structure of the insert and the presence or absence of T-DNA in recovered transgenic plants depends of course on the position of the selectable marker. In the strategy of Ramanathan and Veluthambi (85), the external position of the marker allowed non-T-DNA transformants to be recovered. The experiments carried out by Kononov and colleagues (88) are particularly informative because they constructed binary vectors in which a selectable marker was present within the T-DNA and a screenable marker gene was present outside either the left or the right borders of the T-DNA. Over 200 transformants were obtained under selection and 75% were shown to carry the external screenable marker gene, *gusA*. Interestingly, both vectors appeared to transfer *gusA* to the plant genome with equal efficiency, suggesting that T-DNA transfer could be initiated

nonselectively at either the left or right borders. It is also notable that Kononov and colleagues used three alternative *Agrobacterium* strains: LBA4404, GV3101 and EHA105, representing octopine, nopaline and agropine-type virulence functions, respectively. There were no significant differences among the strains in terms of the frequency of vector sequence transfer. Finally, these investigators reported that they could also detect independent integration events involving plasmid backbone sequences alone. Since the selectable marker in these experiments was located within the T-DNA, such vector-only integrations must have occurred in addition to the T-DNA-linked integration events. This indicates that in the natural course of transformation, many vector-only integration events may occur, but will not be recovered under selection. It is also likely that vector-only integration events occur, undetected, in many plant transformation experiments.

Factors affecting the frequency of vector cotransfer have been studied by Wenck and co-workers (89). These investigators found that particular parameters in the transformation method strongly influence the fidelity of the transfer mechanism. Specifically, a traditional root-transformation procedure in *Arabidopsis thaliana* resulted in vector transfer in 33% of all transformants, whereas the more popular and rapid technique of vacuum infiltration approximately doubled this figure (62%). *Agrobacterium*-mediated protoplast transformation in *Nicotiana plumbaginifolia* also produced more than 50% vector transfer events, although the sample in this case was just five, so it is difficult to compare this result with other methods. It is clear, however, that the frequency of vector backbone integration is influenced by species, explant type and variations in the chosen transformation method.

PARTICLE BOMBARDMENT

Principles of Gene Transfer

Particle bombardment involves the acceleration of small DNA-coated metal particles (either gold or tungsten) into plant tissue with sufficient force to break through the cell wall and membrane (3, 90, 91). Some of the particles reach the nucleus, where the DNA is released, probably by a simple diffusion mechanism. Notably, the foreign DNA entering a bombarded cell is naked, double-stranded and competent for both transient episomal expression or integration into the genome. Transient expression also occurs in the process of *Agrobacterium*-mediated transformation, but the T-strand must first be converted into a double-stranded intermediate (92). As discussed above, it is not clear whether the T-strand or the double-stranded intermediate is the normal substrate for integration.

Vector Design for Particle Bombardment

While *Agrobacterium*-mediated transformation involves a number of virulence gene products that must be supplied either on the same plasmid as the T-DNA or on a separate binary vector, particle bombardment has no such requirements because the introduction of DNA is governed entirely by external physical factors (reviewed in 93 and 94). For convenience, therefore, vectors used for direct transfer are generally based on bacterial cloning plasmids, and incorporate a selectable marker and origin of replication functional in bacteria. In *Agrobacterium*-mediated transformation, the T-DNA is naturally excised from the vector during the transformation process, and vector backbone transfer results from inefficient processing. In contrast, there is no such processing in particle bombardment. Therefore, all parts of the vector – transgene and backbone – have the same chance of integrating into the genome.

Although plasmid backbone sequences are considered undesirable, it is practical and convenient in most cases to use supercoiled plasmid DNA to coat the metal particles. However, it has been shown that linear DNA is at least as efficient as circular plasmid DNA for transformation in bombardment experiments (95). Therefore, an acceptable strategy to eliminate vector backbone integration altogether is to purify the transgene as a short linear cassette prior to transformation (96). As discussed below, this strategy also has a significant impact on the structure and complexity of the transgenic locus.

Transgene Integration

Distribution of Transgenic Loci

There have been few studies in which integration sites generated by particle bombardment have been carefully mapped, so it is not clear whether there is any insertional bias for foreign DNA in this or any other direct transfer method. As discussed above, it has been suggested that T-DNA preferentially integrates at genomic sites showing microhomology to the border sequences (41). Such a bias would be unlikely in direct DNA transfer because the end-sequence and structure of transgenes varies from experiment to experiment. In support of this, Chen et al. (97) noted that in rice plants cotransformed with up to 13 plasmids, there was no preference for the integration of particular transgenes, indicating that the insertion mechanisms operated independently of gene sequence. There is some evidence that, as with T-DNA, foreign DNA introduced by particle bombardment preferentially integrates into transcription units. Somers and colleagues (98) showed by FISH analysis of transgenic oat that integration occurred randomly with respect to the A/D and C genomes, and there was no preference for particular chromosomes in any of the genomes. However, the majority of integration events in oat occurred at telomeric and subtelomeric regions, which are typically gene rich. It is also possible that this preferential integration may reflect some aspect of the nuclear architecture in oat rather than the distribution of genes, since FISH analysis of a limited number of transgenic wheat plants generated by particle bombardment showed no preferential integration in terms of the chromosome region (9, 10).

Organization of Transgenic Loci

Unlike the situation with *Agrobacterium*-mediated transformation, a vast literature has accumulated on the structure and complexity of transgenic loci generated by direct DNA transfer, particularly particle bombardment. As discussed above, T-DNA integration usually occurs with a low-copy number, rarely exceeding five copies, and the T-DNA is generally intact. In contrast, direct DNA transfer often generates much larger transgenic loci. Typically, these contain from 1-20 transgene copies (e.g., 99-101) , although higher-copy numbers are possible (102). The structure of such loci is highly variable, comprising single copies, tandem or inverted repeats, concatemers, intact transgenes, truncated and rearranged sequences, and interspersed genomic DNA. The complexities of transgene integration can be probed quite informatively through the use of Southern blot hybridization (**Box 2**) and fluorescence *in situ* hybridization (FISH). Recent analysis of transgenic plants by FISH to extended DNA fibres, metaphase chromosomes and interphase chromatin, has revealed a higher-order level of organization where discrete integration events are interspersed by large fragments of genomic DNA, up to several hundred kilobases in size. This organization, which generates immense (megabase) transgenic loci, appears unique to particle

bombardment, and could thus reflect the nature of the transformation process itself (see below).

Box 2: What Southern blots tell us about transgene integration

As discussed in Box 1, Southern blot hybridization can be one of the most informative techniques for transgene analysis, and this reflects the careful use of different restriction enzymes and different hybridizing probes. This box briefly summarizes how different enzyme/probe combinations can reveal information about transgene structure.

1. Dual cutter with transgene probe

In this approach, genomic DNA from a transgenic plant is digested with an enzyme that cuts twice in the transforming plasmid, releasing a diagnostic cassette of a defined size. If a probe that hybridizes to this cassette is used, information is provided concerning both transgene copy number and transgene rearrangements. If all copies of the transgene are intact, there should be only one hybridizing band corresponding to the size of the diagnostic cassette. The signal intensity is representative of the transgene copy number, since one cassette should be released from each integrated copy. Copy-number determination is best achieved by 'spiking' genomic DNA from an untransformed plant with a known amount of transforming plasmid DNA, and then digesting this and the genomic DNA from genuine transgenic plants. It may be useful to set up a series of control DNA samples, containing e.g., one, five and ten copies of the transforming plasmid per genome equivalent of DNA. In this way, a 'calibration curve' of signal intensities relative to copy number can be generated, onto which any transgenic plant can be mapped. Band sizes greater or smaller than the diagnostic fragment indicate truncations or rearrangements.

2. Single cutter with transgene or vector probe

In this approach, genomic DNA from a transgenic plant is digested with an enzyme that cuts once in the transforming plasmid. In the case of single-copy integrations, a unique band will be generated whose size depends on the distance between the restriction site in the plasmid and the adjacent matching site in the genomic DNA. For multicopy integrations, several outcomes are possible. If the transforming plasmid has integrated as a concatemer, the copy adjacent to the genomic DNA will generate a unique band as above. However, all remaining copies will generate a fragment that is equivalent in size to the transforming plasmid. This, of course, is assuming that the concatemer comprises a series of identical linear copies of the plasmid in the same orientation. Since this is unlikely to be the case, single-cutter digestions tend to generate a complex mixture of bands of different sizes, providing a unique 'fingerprint' of a given transgenic line which can be used to identify clonal relatives and follow particular transgene copies in segregation analysis. Since in most cases, each transgene would generate a unique band, the number of bands is approximately equivalent to the number of copies. The sequential use of transgene and vector probes on the same sample of plants can reveal 'vector only' integration events.

3. Non-cutter with transgene probe

In this technique, genomic DNA is digested with an enzyme (or panel of enzymes) that do not cut in the transforming plasmid. If all copies of the transgene have integrated at a single locus as a concatemer, digestion with such enzymes should liberate the locus as a single, high-molecular-weight fragment. Thus, the presence of two or more bands suggests either: a) the presence of two or more independent transgenic loci (this can be confirmed by segregation analysis); or b) the presence of interspersed genomic DNA between transgene copies at a single locus (this can be confirmed, if required, by fibre-FISH).

A useful overview of transgene organization in wheat has been reported by Jackson et al. (10) using the technique of fibre-FISH. This study showed that transgene loci in bombarded wheat plants can be organized in three ways. The simplest arrangement, described as a type

III locus, is characterized by a single discrete fibre-FISH signal corresponding approximately to the length of the transforming plasmid. This represents an intact, single-copy integration. Type III loci may be present uniquely in a plant, or there may be two or more unlinked inserts representing multiple genetic loci. These two possibilities can be distinguished by FISH to metaphase chromosomes and genetic segregation analysis.

Other loci, described as type I loci, are longer than the single-plasmid copy yet still generate a continuous signal along the extended chromatin fibre. For example, Jackson et al. reported type I transgenic loci with a continuous signal of 77 kb, representing 11 contiguous plasmid copies (10). These loci represent concatemers of the transforming plasmid and are characterized by the absence of intervening genomic DNA. The presence of concatemers can also be confirmed by Southern blot analysis and sequencing across plasmid/plasmid junctions. Loci thus characterized have been described by Kohli and colleagues as 'transgene arrays' (62, 81). Until the late 1990s, both head-to-head and head-to-tail concatemers had been sporadically reported in the literature, but it was unusual for the structure of a transgenic locus to be examined in such detail. Concatemerization is probably quite a common phenomenon. Extensive concatermerization, for example, has been reported by Hadi et al. (103) in transgenic soybean simultaneously transformed with 12 different plasmid vectors.

The remaining class, termed type II loci (10), are the most complex. They are characterized by fibre-FISH signals that extend for a significant distance (> 100 kb) over the chromosome, but which are punctuated regularly by intervening segments of genomic DNA. Such loci have also been identified in transgenic oat, rice and maize (7, 81, 82) by Southern blot hybridization with non-cutter enzymes (**Box 2**) and by DNA sequencing across plasmid junctions. Kohli and colleagues have named such loci 'transgene clusters' (81). It should be noted, however, that the detection of type II loci by Southern blot hybridization depends on the fortuitous choice of an enzyme that recognizes a site in the intervening genomic DNA. Svitashev and Somers (104) showed, in one transgenic oat line, that a locus identified by Southern analysis as concatemeric (type I) was in fact interspersed with genomic DNA (type II) when examined by fibre-FISH.

Type II loci contain genomic interspersions ranging from a few tens of base pairs to approximately 10 kb. Although dispersed over a distance of up to 100 kbp, such loci would still be expected to generate a single discrete signal if FISH analysis was applied to metaphase chromosomes, due to the low resolution of this technique. However, the analysis of metaphase wheat chromosomes by FISH has revealed an unexpected third level of organization, involving the dispersion of transgene arrays and/or clusters over a larger area comprising megabase pairs of DNA (9). Instead of discrete spots for each transgenic locus, two or more separable FISH signals were often observed, restricted to a particular chromosome region. To be separable at the cytogenetic level, each signal must be interspersed by hundreds of kilobase pairs of genomic DNA. Similarly large genomic interspersions have been seen in transgenic oat (104).

Interestingly, FISH analysis of interphase chromatin and metaphase chromosomes in the same transgenic wheat plants showed that the dispersed metaphase FISH signals could come together at interphase (9). Occasionally, the signals clustered at the same region of the nucleus but remained discrete. However, in other cases they converged to form a single signal. The ramifications of these data are far reaching, and are discussed below.

Mechanisms of Transgene Integration

The analysis of plasmid/plasmid and plasmid/genomic junctions in transgenic plants generated by particle bombardment reveals several features characteristic of illegitimate recombination. These include the following: a) regions of microhomology at the

recombination junctions, i.e., 4-8 nucleotides in common between the recombining partners; b) the presence of filler DNA at the junctions, i.e., several nucleotides that are not recognizable as belonging to either recombining partner; c) deletions of a few nucleotides in one or other of the partners, probably reflecting nuclease 'nibbling' of the foreign DNA prior to joining; and d) purine-rich elements surrounding the junction site, with similarity to topoisomerase I binding/cleavage sites. In the analysis of multiple plasmid/plasmid junctions in 12 transgenic rice lines, Kohli et al. (62) observed 10 plants with microhomology at the junctions and two where junctions appeared to be generated by blunt ligation, with no overlap. A similar ratio of end-joining to microhomology-mediated recombination was observed by Gorbunova and Levy (105) and Salomon and Puchta (66). Topoisomerase I sites were also observed adjacent to 10 out of 12 junctions characterized in *Arabidopsis* transgenics generated by particle bombardment (106). Illegitimate recombination therefore appears to be responsible both for the integration of foreign DNA into the plant genome, and the linking of multiple plasmid copies, which is similar to the mechanism proposed for T-DNA integration discussed above.

Any model for transgene integration following particle bombardment must take into account the three-tier organization revealed in transgenic cereals: contiguous arrays, interspersed clusters and widely-dispersed FISH signals. Two-phase transgene integration mechanisms have been proposed to explain the first two levels of organization, and in such models concatemerization is proposed to occur prior to integration, while interspersion occurs during the integration process (81, 82). In each model, penetration of the cell is proposed to elicit a wound response, which would include the induction of DNA repair enzymes such as nucleases and ligases. The presence of these enzymes and an excess of foreign DNA would result in the linking together of several copies to form concatemers, which would be the substrates for integration. This might be stimulated by homology between individual copies of transforming plasmids, and 'backbone' homology might also result in the concatemerization of plasmids carrying different transgenes in cotransformation experiments. However, cotransformation and co-integration were also shown to occur when two nonhomologous minimal cassettes were used for transformation, so homology might not be as important as the presence of free DNA ends (96). Kohli and colleagues (81) then suggest that transgene clusters arise in a second phase where a primary integration event occurring by illegitimate recombination at a chromosome break generates a 'hot spot' for further integration events in the same area. This might be due, for example, to the presence of local repair complexes that slide along the DNA and introduce nicks which can be exploited by more foreign DNA (107). Pawlowski and Somers (82) suggest an alternative second phase where a number of discrete transgene concatemers integrate simultaneously at a site containing multiple replication forks. Although there is no direct evidence for either mechanism, it is interesting to note that DNA integration is stimulated in rapidly-dividing cells, and is blocked in *Arabidopsis* mutants lacking essential components of the DNA recombination machinery (see above).

The higher-order organization of transgenic loci observed by metaphase FISH is apparently unique to particle bombardment and demands a model which takes into account the three-dimensional structure of the nucleus. In one scenario, it is possible that the transformation event affects a local region of the interphase nucleus. For example, it is possible that the metal particle causes damage to a particular area of chromatin, which is arranged in loops attached to the nuclear matrix. If the particle 'skims' several loops, there will be regions of DNA damage close together *in trans*, but widely separated in the *cis* configuration were the DNA to be stretched out. Each of these sites could act as a nucleation point where foreign DNA diffusing from the metal particle is used to patch up double-strand breaks, generating widely-separated arrays and/or clusters (9, 104). In support of this

induced break and repair model, Svitashev et al. (98) have shown that in six of 25 transgenic oat plants generated by particle bombardment, transgene integration sites were associated with rearranged chromosomes. This suggests that DNA breaks caused by incoming particles are repaired with foreign DNA and may also result in deletions, inversions and translocations involving genomic DNA. Chromosomal rearrangements have also been seen associated with T-DNA integration (108, 109).

The model above suggests that dispersed metaphase signals come together at interphase due to the physical position of the transgenic loci at the moment of transformation. In another scenario, the bringing together of transgene sites at interphase could represent recruitment, e.g., to a common transcription factory in the nucleus. Recent studies on the spatiotemporal organization of replication, transcription, RNA processing and RNA transport in the nucleus indicate that established models of replication and transcription, in which polymerases slide along DNA templates, may not be appropriate. Increasing evidence supports an alternative model in which DNA and RNA polymerases are immobilized in large complexes, or factories, in specific regions of the nucleus, and that the DNA is reeled through (110-112) Promoter similarity may be one factor that determines the particular factory at which a given transcript is synthesized, and in the experiments carried out so far, all the transgenes have been driven by the same promoter, so it is not inconceivable that such co-recruitment could occur. It would be interesting to carry out further experiments in which different promoters were used, as this could result either in the same coincidence of signals, or perhaps in recruitment to separate sites.

A further scenario involves transgenes that are brought together by virtue of their homology, perhaps as a consequence of their initial placing in the same region of the nucleus. This is an exciting prospect because the coincidence of FISH signals observed in wheat nuclei could represent a physical basis of the postulated DNA-DNA interactions that precede transcriptional transgene silencing in plants (see below). A range of exciting experimental opportunities is generated by these results. Do the discrete interphase signals occur in expressing plants and the coincident signals in silenced plants? What happens to transgenes and homologous endogenous genes on the same chromosome – do they interact in the same way? These and other similar questions emphasize the point that transgene organization in the context of higher-order genome structure and nuclear architecture must be taken into account to provide a full understanding of the effects of transgene organization on expression.

Transgene Rearrangements

Transgene rearrangements following particle bombardment have been widely reported in the literature and in countless anecdotal accounts of transformation. The number of rearrangements that can be detected depends entirely on the resolution of the method being used. Thus, careful analysis of locus structure by Southern blot hybridization, PCR and DNA sequencing has recently shown that rearrangements may be more widespread than first envisaged. The analysis of transgenic oat loci by Somers and colleagues has shown that transgene rearrangements can be extensive and extremely complex, with multiple small insertions, inversions and deletions within any transgene, plus the presence of 'filler DNA' of uncertain origin (David Somers, personal communication). In an analysis of transgenic maize plants, Mehlo and colleagues noted that every single plant showed some form of rearrangement, and they speculated that undetected 'minor' rearrangements may be responsible for many instances of transgene silencing, which are currently attributed to epigenetic effects (7). In particular, certain transgene rearrangements were not detectable by Southern blot hybridization because they were too subtle, but they could be picked up by PCR and sequencing. Since in most cases, Southern blot hybridization is used to determine

whether a given locus is intact or rearranged, this suggests caution should be used in relying on such results, since only 'major' rearrangements can be detected.

Why should particle bombardment generate so much more transgene rearrangement than other transformation methods, particularly *Agrobacterium*-mediated transformation? The answer probably reflects several factors in combination, but most importantly the relatively large amount of DNA introduced into the cell (compared to a small number of T-strands) and the immense physical forces involved in the transformation process. Although high-quality supercoiled plasmid DNA is used to coat the metal particles, it is possible that many of the plasmids are sheared during particle preparation and bombardment itself, and these damaged molecules are then exposed to nucleases in the plant cell, resulting in further degradation. Cell penetration is known to trigger a wound response (113), and the presence of large amounts of fragmented episomal DNA is likely to induce the activity of nucleases and ligases as the cell fails to discriminate this from its own DNA and attempts to repair damage to its own nucleus. Small wonder then that multiple plasmid fragments become ligated together in complex arrays prior to integration.

Few researchers have characterized transgene rearrangements in detail, but work by Kohli and colleagues (62) has shown that rearrangements may involve recombinogenic sequences present in the transforming plasmid. These investigators characterized 12 transgenic rice lines, transformed by particle bombardment, which had been shown to contain rearranged transgenes. Interestingly, they found that an imperfect palindrome in the cauliflower mosaic virus 35S promoter was involved in one-third of all rearrangements, i.e., the sequence of this palindrome was adjacent to the rearrangement junction. As discussed above, this sequence has the ability to adopt a cruciform secondary structure, which may stimulate recombination events.

'Clean DNA' Transformation

The use of whole plasmid molecules for particle bombardment inevitably results in the integration of vector backbone sequences along with the transgene(s) of interest. Fu and colleagues (96) have addressed this problem by carrying out particle bombardment using minimal expression cassettes, i.e., the transgene bracketed by its promoter and polyadenylation site, which is isolated from the plasmid and used to coat the metal particles. Interestingly, they found that the use of such minimal cassettes not only prevented vector backbone integration, but also increased the proportion of low-copy-number, structurally-intact transgenic loci, resulting in improved stability of expression. The reasons for this are not clear, but probably reflect a combination of the following: a) the foreign DNA is shorter and in a relaxed configuration, and is therefore less likely to succumb to shear forces during transformation; b) removal of the plasmid backbone removes some potential recombination hot spots, such as the plasmid origin of replication; c) prokaryotic sequences can act as a trigger for *de novo* methylation (see below) so their absence increases the chance of stable transgene expression.

OTHER DIRECT DNA TRANSFER METHODS

There has been a limited number of transgene organization studies in plants generated by direct transfer methods other than particle bombardment. Kartzke et al. (114) carried out an interesting series of experiments to demonstrate that transgene organization in tobacco protoplasts varied according to the stage of the cell cycle at the time of transformation. Non-synchronized protoplast cultures were transformed first, and showed a preponderance for single-copy insertions of intact transgenes. In contrast, the transformation of protoplasts

synchronized in S-phase resulted in high-copy-number transgenic loci in which there were a large number of truncations and rearrangements. Transformation during M-phase and G_2 resulted in a different organization once more – this time with multiple copies of the transgene dispersed at different loci. These results go some way to explaining the diverse range of structures reported by different groups. Many have reported extensive concatemerization of transgenes, presumably prior to integration, with both PEG-mediated transformation and electroporation (e.g., 115, 116). Such multicopy loci typically contain both intact and rearranged transgenes (117, 118). Conversely, Ling and Binding (119) have reported that PEG-mediated transformation of *Linum usitatissimum* results predominantly in single-copy transgenic loci, typically 1-2 loci per transformant. It is likely that host species, transformation method and the characteristics of the protoplast culture all play a role in determining the structure of the locus. Nonspecific carrier DNA is often used in protoplast transformation techniques, and this can also form part of the transgenic locus. In animals, large concatemers, up to 2 Mb in length, have been observed in cells transformed by calcium phosphate along with carrier DNA (120). It is not clear if such complexes form in plants, but the integration of calf thymus carrier DNA has been confirmed in tobacco protoplasts (121) and transgene locus structure in rice transformed with calcium phosphate has been analyzed in detail (122).

The transgene copy number reported for protoplast transformation varies from one to 25 or more, with a moderate amount of transgene rearrangement in higher-copy-number plants. Similar observations have been made in transgenic plants generated by whisker-mediated transformation (123) although other reports indicate that the copy number rarely exceeds five (124, 125). A more reliable picture of the structure of transgenic loci generated by whisker-mediated transformation will probably arise as larger numbers of transgenic plants are generated by this method.

CONTROLLING TRANSGENE EXPRESSION

Factors Influencing Transgene Expression

Transgenic plants offer many benefits, but they are only useful if the transgene(s) they contain are expressed strongly and stably over many generations. Transgene expression in plants is obviously influenced by the design of the expression construct, reflecting such factors as promoter choice, the presence of an intron, the presence of a polyadenylation site, the presence of a translational enhancer in the non-coding region of the transgene, codon optimization in line with the host plant, and targeting the recombinant protein to a suitable intracellular compartment. These factors have been widely reviewed elsewhere (126-136) and since they are ultimately under the precise control of the experimenter, they are not discussed further.

Transgene expression is also influenced by a variety of other factors, which are host-dependent and very difficult to control. The most relevant of these, in the context of transgene integration, are the position of integration and the locus structure. As discussed earlier, integration is essentially random and the structure of the locus varies widely, often containing multiple transgene copies some of which are truncated or rearranged. The unpredictable nature of transgene integration has an equally unpredictable effect on expression.

Position of Integration

Position Effect

'Position effect' is an umbrella term used to describe the influence of surrounding DNA at the site of transgene integration on the level and pattern of expression (reviewed in 137). Position effects can be specific or nonspecific, and they may be constitutive or triggered by the transgene itself.

Specific position effects result from the influence of local regulatory elements on the transgene. For example, an integrated transgene may come under the influence of a nearby enhancer, such that its expression profile is modified. The effect is transgene-specific because the enhancer interacts with regulatory elements in the transformation construct to control transcription, hence the final expression pattern reflects the combined influence of both regulatory elements. Such effects are clearly revealed by entrapment constructs, which contain minimal control sequences linked to a visible marker gene and therefore 'report' the activity of local regulatory elements (e.g., 138, 139).

As well as specific position effects governed by local regulatory elements, nonspecific position effects can also be generated by the surrounding chromatin architecture. In this context, nonspecific means that any transgene will be affected in the same way. Where the local environment is favourable for transgene expression, i.e., a positive position effect, it is generally taken for granted. However, nonspecific and repressive position effects reflect the integration of the transgene into a chromosomal region containing repressed chromatin (heterochromatin). The molecular features of heterochromatin, including its characteristic nucleosome structure, deacetylated histones and hypermethylated DNA, spread into the transgene causing it to be inactivated (140, 141). In some cases, variegated transgene expression has been reported due to cell-autonomous variations in the extent of this spreading process (reviewed in 142). Analysis of the genomic context of silenced transgenes suggests that integration in the vicinity of certain repetitive DNA sequences, such as microsatellites and retrotransposon remnants, may predispose the transgene to silencing (122, 143). The chromosomal location is important, since in many plants, the genes are restricted to a small portion of the genome known as gene space, and the majority of the DNA is taken up by repetitive sequences. Thus, stable transgene expression has been associated with telomeric and subtelomeric integration sites whereas mosaic expression and silencing occurs at centromeric loci (37).

A third type of position effect reflects the tolerance of the surrounding DNA for 'invasion' by foreign DNA. In this case, the effect is not automatic (as above) but is set off by the presence of the transgene. It appears that certain sequences can trigger *de novo* methylation, perhaps because the GC-content or sequence architecture is recognized as abnormal (reviewed in 144). Prokaryotic DNA may be recognized in this manner, since prokaryotic sequences act as a strong trigger for *de novo* methylation in animals (145). In plants, silencing is often associated with the presence of prokaryotic vector backbone DNA, particularly binary vector sequences joining T-DNA to genomic DNA (37). The absence of vector DNA may explain the improved stability of transgene expression seen in transgenic plants generated by bombardment with minimal transgene cassettes (96).

Overcoming position effects: buffering the transgene

As discussed above, analysis of the genomic context of transgene integration sites has shown that silenced transgenes are often surrounded by repetitive elements, which are sequestered into repressed chromatin. The same studies have also shown, however, that stably-expressed transgenes are often associated with matrix attachment regions (MARs) (37). These are AT-rich elements which attach chromatin to the nuclear matrix and organize it into topologically-isolated loops (reviewed in 146 and 147). A number of highly-expressed endogenous plant genes have also been shown to be flanked by matrix attachment regions (e.g., 148, 149). One strategy that has been proposed to overcome position effects is therefore to protect or buffer the transgene by flanking it with MARs prior to transformation. In this way, it is hoped that the transgene will form a discrete chromatin loop which will be isolated from surrounding chromatin.

Several experiments have been carried out in which a reporter gene such as *gusA* has been flanked by MARs. Such constructs have been introduced into transgenic plants and compared to populations containing the same reporter gene without MARs (e.g., 148, 150-152). The general impression from such experiments is that MARs do have a positive effect on transgene expression and can significantly reduce position effects. However, the results are variable and in some cases contradictory. In most cases, a 2-3-fold reduction in expression variability has been observed, although with a soybean MAR in tobacco, a 15-fold decrease in variability was recorded (153). Similarly, the level of expression is generally increased up to about 5-fold, but in a few reports much higher increases have been recorded, e.g., 25-fold with a yeast MAR and 140-fold with a tobacco MAR in tobacco callus (154, 155). There is still some variability in expression even with the use of MARs, but the same amount of variation is seen in genetically-identical transgenic plants, i.e., with the transgenic locus in the same position (152). Thus, it is likely that the remaining variability is due to other factors such as developmental and environmental noise, which are very difficult to control.

Overcoming position effects: homologous recombination

Transgene integration in higher plants occurs almost universally by illegitimate recombination, which may involve short regions of homology but is not actually homology dependent. Since there is only minimal sequence relationship between the transgene and the genomic region into which it integrates, the experimenter has little control over the integration site. In other systems, notably yeast, transformation can result in an alternative process called homologous recombination, where a particular genomic sequence is replaced by a homologous segment of foreign DNA. This depends on the design of the transformation vector: if it carries a homology region that matches the yeast genome, then homologous recombination is most likely to occur; if the construct has no relationship to the yeast genome, it can only integrate illegitimately (156). Homologous recombination is a very useful phenomenon because it allows the introduction of precise sequence changes into genes in a predetermined manner (gene targeting). In the context of controlling transgene integration, it also allows transgenes to be inserted at specific loci, a strategy that should allow favorable sites for transgene integration to be chosen, theoretically abolishing position effects.

Although widely used in microbial systems, homologous recombination occurs with a very low efficiency in higher eukaryotes. In most animal and plant cells, illegitimate recombination occurs 10^4-10^5 times more frequently than homologous recombination, making genuine targeting events difficult to isolate. There are some exceptions. In mouse

ES cells, for example, homologous recombination occurs at a frequency of approximately 10^{-3} compared to illegitimate recombination, allowing targeted cells to be identified by suitable selection strategies (e.g., 157) or high-throughput screening by PCR (158, 159). In the case of plants, several reports have been published in which integrated transgenes have been modified by gene targeting with either *Agrobacterium*-mediated transformation or direct DNA delivery (160-163). There have also been a few reports in which endogenous gene loci have been successfully targeted (164, 165). In each case, the frequency of targeting was extremely low, and it is presently not clear whether this strategy can be routinely used either for gene targeting or transgene integration. Strategies such as positive-negative selection, and optimizing the size of the donor homologous sequence, which have been successfully applied in mouse ES cells, appear to have little effect on gene targeting in plants (166, 167). We do not discuss the use of RNA-DNA chimeric oligonucleotides for gene targeting in plants because this technique cannot be used to control the integration of transgenes.

It might be possible to exploit homologous recombination for transgene integration if a way could be found to improve the targeting frequency in plant cells. It is unknown why certain cells, such as mouse ES cells, are more amenable to homologous recombination than others, but it is likely to reflect the presence of active recombination machinery in the cell, perhaps because of the early stage of development from which the cells arise. Similarly in plants it has been suggested that the frequency of homologous recombination could be increased by importing recombination genes from other species or identifying plant cells at a stage of development where the homologous recombination machinery is more active than usual (reviewed in 168). The moss *Phycomitrella patens* is almost as efficient as yeast in terms of homologous recombination (169), and this suggests that higher plant cells at a similar stage of the life cycle (i.e., haploid and gametophytic) could be useful transformation targets. It has been suggested that the successful targeting of the *Arabidopsis AGL5* gene (165) might reflect the introduction of DNA into rapidly-dividing meristematic tissues or cells undergoing meiosis (170).

Overcoming position effects: chloroplast transformation

The plant cell contains not only a nuclear genome, but also organellar genomes in the chloroplasts and mitochondria. The chloroplast is a useful target for gene transfer because tens of thousands of chloroplasts may be present in a single plant cell, and each chloroplast may contain multiple copies of its chromosome (171). If foreign DNA can be integrated into the chloroplast instead of the nuclear genome, extremely high levels of transgene expression are therefore possible (172-174). This reflects not only the fact that the transgene number is highly amplified, but also that transgenes integrated into chloroplast DNA do not appear to suffer position effects or any other form of transgene silencing. Chloroplast transformation also provides a natural containment method for transgenic plants, since for most species the transgene cannot be transmitted through pollen (reviewed in 175).

Chloroplast transformation was first achieved serendipitously, and the integration events were found to be unstable. In an early experiment, tobacco protoplasts were co-cultivated with *Agrobacterium* and one transgenic plant line was recovered in which the transgene was transmitted maternally. Southern blot analysis of chloroplast DNA showed directly that the foreign DNA had become integrated into the chloroplast genome (176). *Agrobacterium* is not particularly useful for chloroplast transformation, since the T-DNA complex is targeted to the nucleus (see above). Therefore, research into stable chloroplast transformation has focused on direct DNA transfer. This was first achieved with particle bombardment to transform the alga *Chlamydomonas reinhardtii*, which has a single large chloroplast occupying most of the volume of the cell (177). The principles established with

this simple organism were extended to tobacco, allowing the recovery of stable *transplastomic* tobacco plants (178). These principles included the use of vectors containing long chloroplast homology regions, allowing targeted integration into the chloroplast genome, and use of a selectable marker gene such as *aad* (aminoglycoside adenyltransferase), which confers resistance to streptomycin and spectinomycin (179). Efficient chloroplast transformation has been achieved both through particle bombardment (e.g., 174, 180, 181) and PEG-mediated transformation (182-184). More recently, a novel technique has been explored in which DNA is introduced directly into chloroplasts with a fine needle (185).

Overcoming position effects: site-specific recombination

A final strategy for addressing the problem of position effects is site-specific transgene integration, which takes advantage of the phenomenon of site-specific recombination. As with the homologous recombination strategy discussed above, the idea is to introduce foreign DNA at a specific, favourable locus. However, there are several important differences between homologous and site-specific recombination. First, homologous recombination is a ubiquitous process that relies on endogenous recombinase enzymes present in every cell, whereas site-specific recombination systems are very specialized, and different systems are found in different organisms. Homologous recombination occurs between DNA sequences with long regions of homology but no particular sequence specificity, whereas site-specific recombination occurs at short, specific recognition sites. This means that target sites for site-specific recombination can be introduced relatively easily into transgenic plants, but recombination will only occur in a heterologous cell if a source of recombinase is also supplied.

A number of different site-specific recombination systems have been identified and several have been studied in detail (reviewed in 186 and 187). The most extensively used are Cre recombinase from bacteriophage P1 (188) and FLP recombinase from the yeast *Saccharomyces cerevisiae* (189). These have been shown to function in many heterologous eukaryotic systems including transgenic plants (190-192). Both recombinases recognize 34 bp sites (*loxP* and *FRP*) comprising a pair of 13 bp inverted repeats surrounding an 8 bp central sequence. *FRP* possesses an additional copy of the 13 bp repeat sequence, although this has been shown to be nonessential for recombination.

Site-specific integration of transgenes can occur if the genome contains a recombinase recognition site. Thus, the target site for the recombinase, e.g., *loxP*, must be introduced into the genome by conventional transformation. With an unmodified Cre-*loxP* system, transgene integration occurs at a low efficiency because the equilibrium of the reaction favours excision due to the degradation of episomal DNA fragments. Initial attempts to overcome this problem involved transient *cre* expression, but these were generally unsuccessful (193, 194). However, high-efficiency Cre-mediated integration has been achieved in plants with mutated *loxP* sites (195). Although this strategy is effective in introducing foreign DNA into a pre-determined locus, independent transgenic plants still show variability of transgene expression. Thus, as with MARs, the abolition of position effects removes only some of the factors that control the level and stability of transgene expression.

Structure of the Transgenic Locus

Homology-dependent gene silencing

It is natural to assume that increasing the number of copies of a particular transgene will lead to an increase in the level of its product. However, even from the earliest plant

transformation experiments, it was appreciated that multiple transgene copies could lead to loss of expression (transgene silencing) and that the phenomenon was associated with DNA methylation at the transgenic locus (196-199). A strikingly visual demonstration of this effect was provided by introducing the maize *A1* gene into mutant petunia plants with white flowers (200, 201). Expression of the transgene resulted in pelargonidin production, generating a red pigment. However, it was shown that red flowers generally appeared on plants with single-copy transgenes, while plants with multiple transgene copies had white or variegated flowers. Where transgene silencing had occurred, increased methylation of the foreign DNA was observed (202).

In a similar vein, it was thought that the amount of pigment in wild-type petunia flowers could be increased by introducing extra copies of the chalcone synthase (*chs*) gene (203). Chalcone synthase converts coumaryol-CoA and 3-malonyl-CoA into chalcone, a precursor of anthocyanin pigments. The presence of multiple transgene copies was expected to increase the level of enzyme and hence cause stronger flower pigmentation. However, in about 50% of the plants recovered from the experiment, exactly the opposite effect was observed. The flowers were either pure white, or variegated with purple and white sectors. It appeared that integration of multiple copies of the transgene led not only to the suppression of transgene expression, but also *co-suppression* of the homologous endogenous gene.

Transgene silencing and co-suppression are two manifestations of a complex phenomenon known as homology-dependent gene silencing (HDGS), which occurs not only in plants, but also in fungi and animals. A full discourse on this fascinating subject is beyond the scope of this article, and the interested reader is referred to several excellent reviews which have been published in the last few years (204-210). Herein, we briefly summarize what is known about HDGS and how this relates to transgene organization and expression.

Two forms of HDGS have been recognized. The first is termed transcriptional gene silencing (TGS), and is characterized by the reduction or abolition of transcription from one or more copies of the transgene. This is often correlated with increased methylation in the promoter regions of affected loci, and both the methylation and silencing tend to be heritable through meiosis. The second is termed post-transcriptional gene silencing (PTGS). In this case transcription is actually required for silencing to take place, and induces the degradation of mRNA so that very little accumulates in the cytoplasm. Homology is required in the transcribed regions, which may become methylated, and the silencing effect is usually reset at meiosis. Most cases of co-suppression are post-transcriptional, although this is not always the case. Thus, there may be some cross-talk between the transcriptional and post-transcriptional silencing pathways.

The mechanism by which repeated transgenes cause silencing is not precisely understood. In the case of transcriptional silencing, it is envisaged that the transgene repeats somehow act as a trigger for *de novo* DNA methylation. It has been shown that inverted repeats can form secondary structures that attract methylation, and thus it is likely that *cis* DNA-DNA pairing may be involved in such processes. However, transgene silencing can also occur *in trans*, i.e., silencing interactions may occur between unlinked loci. This has been shown, for example, in sequential transformation events with homologous transgenes, or where two plant lines carrying homologous transgenes have been crossed (211, 212). In this situation, it is suggested that physical interactions between transgenes may occur to mediate silencing, and that DNA methylation may somehow be transferred from one site to another (205-208). As discussed above, FISH studies in transgenic wheat provide tantalyzing evidence for such interactions in the interphase nucleus (9).

A recurring theme in post-transcriptional silencing is the presence of double-stranded RNA. Recent experiments in the nematode worm *Caenorhabditis elegans* have shown that dsRNA is a potent and specific inhibitor of gene expression at the post-transcriptional level, a

phenomenon termed RNA interference (213). Double-stranded RNA introduced into the plant cell can trigger the catalytic degradation of homologous RNA molecules and the methylation of homologous DNA sequences in the genome (214-216). It is envisaged that complex multicopy transgenic loci could also generate dsRNA, e.g., if two transgenes are present as inverted repeats. Transcription through one transgene into the other would produce an RNA molecule that could fold into a hairpin structure. The presence of truncated and otherwise rearranged transgene fragments would provide more opportunity for such aberrant RNAs to be generated (208)

Post-transcriptional silencing has also been observed in plants containing single-copy transgenes, which at first glance would appear to be protected from homology-dependent silencing effects (217). However, even single-copy transgenes might be able to produce dsRNA if counter-transcription was initiated from an adjacent selectable marker gene or perhaps even an endogenous plant promoter in the opposite orientation. It is also possible that partial transcripts could be produced by single-copy transgenes, which could be converted into small dsRNA fragments.

Overcoming locus structure effects: site-specific recombination

Transformation currently involves the introduction of an unknown amount of foreign DNA into the plant cell, which is at the mercy of plant-produced enzymes that digest and ligate individual DNA fragments. Locus structure is therefore the most difficult factor to control when producing transgenic plants. However, site-specific recombination as discussed above may provide a way to generate simple loci that are less likely to induce silencing. In animals, the effect of repeat-induced silencing has been addressed in the following manner (218): a transgene construct containing the human β-globin cDNA was modified to contain a single *loxP* site, which is recognized by Cre recombinase. Transgenic mice were generated carrying multiple copies of the transgene, and in these animals the locus was highly methylated and β-globin expression was low. However, when Cre recombinase was expressed, recombination between the *loxP* sites resulted in the excision of all copies of the transgene except one. The reduction in the transgene copy number resulted in increased expression accompanied by reduced methylation at the transgenic locus. A similar strategy has now been used in plants. Recombination between the outermost sites in a multicopy transgenic wheat line deleted all the repeats and resolved the locus into a single copy, containing a single recombinase site (219). This can potentially provide a shortcut to the normal method of obtaining such lines, which is the screening of large numbers of transformants, however practical limitations in terms of efficiencies still limit the wider adoption of this method.

CONCLUSIONS

It is now clear that transgene integration following *Agrobacterium*-mediated transformation and direct DNA transfer occur by essentially the same mechanisms. The actual integration event is mediated by illegitimate recombination, either following strand invasion by the foreign DNA, or the repair of double-strand breaks in the host genome. There is often microhomology between the recombining partners, although direct blunt-end ligation also occurs. Both transformation methods induce a wound response, resulting in the activation of nucleases, ligases and recombinases in the host cell. The foreign DNA is simultaneously degraded and concatemerized resulting in transgene arrays containing intact and/or truncated and rearranged copies. Several integration events may occur simultaneously at a cluster of replication forks, or a primary integration event may stimulate further

integrations in the local area. Regardless of the mechanism, the result is a transgene cluster interspersed with genomic DNA. In the case of particle bombardment, clusters and arrays may be widely dispersed, generating very large transgenic loci. The position of transgene integration is essentially random within the 'gene space' of the plant species. The transgene is thus subject to position effects which may influence its expression and cause silencing. Similarly, the structure of the locus may cause silencing, particularly if there are multiple transgene copies, intact or rearranged, organized as inverted repeats. Position effects may potentially be reduced by buffering the transgene with matrix attachment regions or controlling the site of integration, however no conclusive results to validate this have been reported in the literature. Methods to control transgene integration include homologous and site-specific recombination, both of which aim to target the transgene to a specific locus. Site-specific recombination can also be used to simplify locus structure, by reducing the number of repeats.

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