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Minireview

Transgene integration, organization and interaction in plants

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Abstract

It has been appreciated for many years that the structure of a transgene locus can have a major influence on the level and stability of transgene expression. Until recently, however, it has been common practice to discard plant lines with poor or unstable expression levels in favor of those with practical uses. In the last few years, an increasing number of experiments have been carried out with the primary aim of characterizing transgene loci and studying the fundamental links between locus structure and expression. Cereals have been at the forefront of this research because molecular, genetic and cytogenetic analysis can be carried out in parallel to examine transgene loci in detail. This review discusses what is known about the structure and organization of transgene loci in cereals, both at the molecular and cytogenetic levels. In the latter case, important links are beginning to be revealed between higher order locus organization, nuclear architecture, chromatin structure and transgene expression.

Introduction

The production of transgenic plants is achieved using two alternative strategies. One exploits the natural ability of *Agrobacterium tumefaciens* to transfer DNA from a resident plasmid into the plant genome (Kado, 1998). The other is a group of unrelated techniques collectively referred to as 'direct DNA transfer', which includes methods such as microinjection (Crossway *et al.*, 1986), transformation of protoplasts mediated by polyethylene glycol or calcium phosphate (Datta *et al.*, 1990; Jongsma *et al.*, 1987), particle bombardment (Christou, 1992), electroporation (Fromm *et al.*, 1994) and transformation using silicon carbide whiskers (Frame *et al.*, 1994). These and other transformation techniques have been reviewed by Twyman *et al.* (2002). The only similarity among the direct transfer techniques is that external physical or chemical factors mediate DNA delivery into the cell.

The integration mechanism of the foreign DNA is poorly understood, both in the case of *Agrobacterium*-mediated transformation and direct DNA transfer. The position of integration and the structure of the transgene locus can vary considerably among independent transformants, and each of these factors may have a profound effect on the level and stability of transgene expression. Of particular interest is the role of transgene organization in silencing phenomena, and

the relationship between locus structure and *de novo* methylation. Until recently, transgene organization in plants had not been widely investigated. Studies that had been carried out on plants such as *Arabidopsis thaliana*, tobacco, petunia, rice and maize relied predominantly on Southern blot hybridization. This technique is more useful for the characterization of plants generated by *Agrobacterium*-mediated transformation than direct transfer methods because in a large number of transformants the right and left border sequences define the insert. Over the last few years, however, there have been a number of detailed studies involving combined molecular and cytogenetic analysis, which have provided more insight into the role of locus organization on transgene expression. In particular, the use of Southern blot hybridization in combination with fluorescence *in situ* hybridization (FISH), methods that discriminate between methylated and non-methylated DNA, and methods that identify open and repressed chromatin, have been useful in examining higher order transgene organization in the context of nuclear architecture. Cereals have been at the forefront of this research. Some cereals, such as wheat, are ideal for cytogenetic analysis because of the large nucleus. Results from experiments with rice, barley and oat have also contributed to our understanding. In this review, we discuss the organization of transgene loci generated by particle bombardment and *Agrobacterium*-mediated transformation, and consider how recent studies have helped to develop models of transgene integration and an understanding of factors controlling the level and stability of transgene expression.

Particle bombardment-mediated transformation

Particle bombardment is the predominant direct transfer method for cereal transformation and involves the acceleration of DNA-coated metal particles through the cell wall and membrane (Christou, 1995). All major cereals and many other plant species have been transformed using particle bombardment (Dubey *et al.*, 1997). The metal particles are usually coated with supercoiled plasmid DNA, hence all parts of the vector – transgene and plasmid backbone – have the same chance of integrating into the genome. However, because DNA transfer is governed entirely by external physical factors (reviewed by Sanford *et al.*, 1993), the plasmid backbone is dispensable. Linear DNA is at least as efficient as supercoiled plasmid DNA for transformation in bombardment experiments (Nandadeva *et al.*, 1999). Therefore, an acceptable strategy to eliminate vector backbone integration all together is to purify the transgene as a short linear cassette prior to transformation (Fu *et al.*, 2000).

Distribution and organization of transgene loci

The position of transgene integration can influence expression (Kumar and Fladung, 2001) so it is pertinent to ask whether there is any insertional bias for foreign DNA introduced into plants by particle bombardment. The integration mechanism does not appear to be sequence-dependent. No preferential integration was observed in transgenic rice when 13 different non-selected plasmids were introduced into rice simultaneously (Chen *et al.*, 1998). It is possible, however, that foreign DNA may integrate preferentially into transcribed regions of the genome. In transgenic oat, FISH analysis revealed that the A/D and C genomes and their different chromosomes are equally amenable to foreign DNA integration (Svitashev *et al.*, 2000). However, the majority of integration events occurred at telomeric and subtelomeric regions, which are typically gene rich. Earlier, transgenic wheat, barley and triticale plants were analyzed by FISH (Pedersen *et al.*, 1997) and the majority of integration events were found to have occurred near the ends of chromosomes. In transgenic oat, 18 out of 26 transgenic inserts were localized in telomeric and sub-telomeric regions (Svitashev *et al.*, 2000). It is possible that the preferential distal integration reflects aspects of the nuclear architecture, because more recent FISH analysis of a limited number of transgenic wheat plants showed no preferential integration in terms of the chromosome region (Abranches *et al.*, 2000; Jackson *et al.*, 2001).

Particle bombardment often generates very large, high-copy-number transgenic loci. There are usually 1-20 transgene copies (Klein *et al.*, 1987; Register *et al.*, 1994) per locus. Locus structure varies from a single intact copy to complex configurations comprised of intact copies, tandem or inverted repeats, concatemers, truncated and rearranged sequences, and interspersed genomic DNA. Jackson *et al.* (2001) used the technique of fiber-FISH to place such loci into three categories. The simplest were termed type III loci and are characterized by a single discrete fiber-FISH signal corresponding to the length of the transforming plasmid, indicating an intact, single copy integration event. Type I loci are continuous but longer than the transforming plasmid and correspond

to concatemeric arrays. Plasmid concatemerization is a common phenomenon during transformation, e.g. Hadi *et al.* (1996) described transgenic soybean lines simultaneously transformed with 12 different plasmids, in which long arrays were present. Arrays were also described by Kohli *et al.* (1998; 1999), who used Southern blot analysis and sequencing over plasmid/plasmid junctions to confirm the absence of intervening genomic DNA.

Type II loci are the most complex, and generate discontinuous fiber-FISH signals that can extend for several hundred kilobases. The discontinuities represent intervening segments of genomic DNA. Such loci have also been identified in transgenic oat, rice and maize (Pawlowski and Somers, 1998; Kohli *et al.*, 1998; Mehlo *et al.*, 2000). Type II loci, also known as 'transgene clusters' can be identified by Southern blot hybridization using a panel of restriction enzymes that do not cut in the transforming plasmid. However, caution should be exercised in interpreting such results because the size of the intervening segment can vary and an appropriate restriction site may not be available. For example, Svitashev and Somers (2001) showed, in one transgenic oat line, that a locus identified by Southern blot analysis as concatemeric (type I) was in fact interspersed with genomic DNA (type II) when characterized by fiber-FISH.

Despite genomic interspersions, type II loci still generate a single discrete signal when FISH analysis is applied to metaphase chromosomes. However, the analysis of metaphase wheat chromosomes by FISH revealed two or more separate signals on the same chromosome (Pedersen *et al.*, 1997; Abranches *et al.*, 2000). Similarly, separate FISH signals were found in barley (Pedersen *et al.*, 1997; Salvo-Garrido *et al.*, 2001) and triticale (Pedersen *et al.*, 1997). To be separable at the cytogenetic level in metaphase spreads, megabase pairs of DNA would be present between the transgene arrays and/or clusters. Interestingly, comparison of FISH analysis of interphase chromatin and metaphase chromosomes in the same transgenic wheat plants showed that the dispersed metaphase FISH signals came closer together at interphase (Abranches *et al.*, 2000). In most cases, clustered but discrete interphase signals were observed at a specific region of the nucleus. However, in other cases they were juxtaposed, forming one signal. The importance of these observations is discussed below.

Structure of transgene and integration junctions

The analysis of plasmid/plasmid and plasmid/genomic junctions in transgenic plants generated by particle bombardment suggests that integration occurs by illegitimate recombination. Characteristics of illegitimate recombination include regions of microhomology at the recombination junctions (i.e. 4–8 nucleotides in common between the recombining partners), the presence of filler DNA (short sequences that are not recognizable as belonging to either recombining partner), small deletions probably reflecting nuclease 'nibbling' of the foreign DNA prior to joining, and motifs with similarity to topoisomerase I binding/cleavage sites (Ehrlich *et al.*, 1993). Kohli *et al.* (1999) characterized 12 transgenic rice lines and observed microhomology at the junctions in 10 lines. Two junctions appeared to be generated by blunt end ligation. Topoisomerase I sites have also been observed at the integration junctions formed by such illegitimate recombination events in transgenic *Arabidopsis* plants generated by particle bombardment (Sawasaki *et al.*, 1998).

Transgene rearrangements following particle bombardment have been widely reported although the rearrangements that can be detected depend on the resolution of the detection method. The analysis of transgene loci in oat (Svitashev *et al.*, 2000; Svitashev and Somers, 2001) has revealed 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. Mehlo *et al.* (2000) studied seven transgenic maize lines with multicopy transgene loci and found that every line showed some form of transgene rearrangement in at least one copy. Importantly, some of these rearrangements could be detected by sequencing and/or PCR, but were too subtle to be picked up by Southern blot analysis, the predominant technique used to characterize transgene loci. The authors speculated that undetected 'minor' rearrangements might be extremely common and may be responsible for many instances in which expression is lost from seemingly intact transgenes. Aberrant RNA originating from subtly rearranged transgenes may contribute to silencing of intact, expressing transgenes. However, sequencing and PCR analyses by themselves would provide an incomplete picture of transgene organization because, depending on the location of the sequencing and PCR primers, some major rearrangements might not be detected. Therefore,

PCR, sequencing and hybridization provide complementary information regarding locus structure.

Rearrangements may involve recombinogenic sequences present in the transforming plasmid. Kohli *et al.* (1999) examined 12 transgenic rice lines and found that an imperfect palindrome in the cauliflower mosaic virus 35S promoter was involved in one third of all rearrangements. This sequence forms a hairpin secondary structure, which may stimulate illegitimate recombination events. Muller *et al.* (1999) also noted the importance of palindromic DNA sequences in the promotion of illegitimate recombination events. Of the 12 illegitimate recombination products they analyzed in tobacco, all involved sites of palindromic structures or AT rich DNA elements. Notably, the 35S palindrome is GA-rich, but it does include the TATA box of the promoter.

Clean DNA transformation

As noted above, 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. The use of minimal cassettes (containing the promoter, coding region and polyadenylation signal of the transgene) eliminates this problem and results in 'clean DNA transformation' (Fu *et al.*, 2000; Breitler *et al.*, 2002). Interestingly, Fu *et al.* (2000) found that the use of such minimal cassettes also increased the proportion of low-copy-number, structurally intact transgene loci compared to whole plasmid transformations, and this resulted in improved stability of expression. How the use of short cassettes rather than whole plasmids has such a profound affect on locus organization and expression is unclear but probably reflects the fact that the foreign DNA is in a relaxed configuration (and is therefore less likely to succumb to shear forces) and that prokaryotic sequences in the vector backbone are absent. Such sequences have been shown to provide recombination hotspots that stimulate transgene rearrangements (Kohli *et al.*, 1999).

It will be interesting to find out how general these improvements are as the clean DNA transformation technique is applied to a wider range of plant species. For example, transformation of barley using minimal cassettes did not generate a high proportion of low-copy-number transgenic plants (Fredy Altpeter, personal communication) but further experiments using rice confirmed the trend towards low-copy-number transgenics (Pawan Agrawal and Ajay Kohli, unpublished results). Additionally, these experiments revealed that after co-transformation with five different transgenes, transgenic plants had either low, moderate or high copy numbers of all transgenes present. There were no instances in which one transgene was present at a low copy number but others at a high copy number. This suggests that the state of the cell may regulate transgene copy numbers more than any feature of the transgene itself.

Transgene integration models

Any model for transgene integration following particle bombardment must take into account the three-tier transgene organization revealed in cereals: contiguous arrays, interspersed clusters and widely dispersed metaphase FISH signals (Figure 1). Two-phase integration mechanisms have been proposed to explain the first two levels of organization (Kohli *et al.*, 1998; Pawlowski *et al.*, 1998). Following penetration of the cell by the metal particles, the plant's wound response leads to the presence of DNA repair enzymes (nucleases, topoisomerases, and ligases) which together with large amounts of exogenous DNA would sponsor both degradation of the incoming DNA and joining of free ends. This generates complex and variable arrays containing both intact and rearranged transgenes. Such arrays would then act as the substrates for integration. Integration is proposed to occur at naturally-occurring chromosome breaks. This is supported by the prevalence of chromosome rearrangements, e.g. translocations and deletions, at transgene integration sites (e.g. Svitashev *et al.* 2000).

Kohli *et al.* (1998) suggest that interspersed transgene clusters arise in a second phase where a primary integration 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 that are patched using additional foreign DNA (Gelvin, 1998). Pawlowski and Somers (1998) suggest an alternative second phase where a number of discrete transgene concatemers integrate simultaneously at a site containing multiple replication forks. Evidence supporting the replication model includes the fact that transgene integration is stimulated in rapidly dividing cells, but the existence of transformation-resistant *Arabidopsis* mutants lacking essential components of the DNA recombination machinery could support both models (see below).

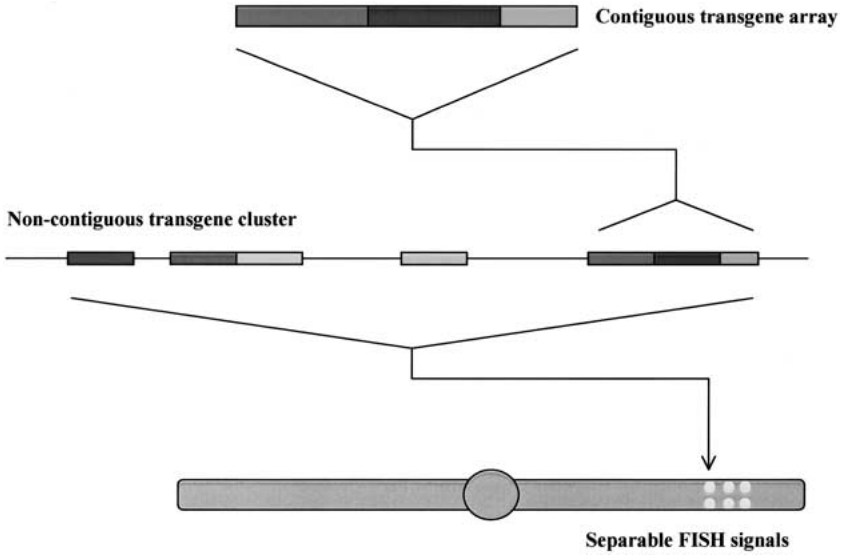

Figure 1. Hierarchical organization of transgene loci: a single FISH signal on metaphase spreads indicates a single transgene locus made up of a contiguous array or non-contiguous cluster. Multiple FISH signals close together may also behave as a single genetic locus or they may segregate depending on the distance of separation on the chromosome. Each of the separable FISH signals may comprise either an array or cluster.

FISH analysis and models for higher-order transgene locus structure

Abranches *et al.* (2000) reported widely separated transgene FISH signals on individual chromosomes in wheat metaphase spreads. Interestingly, they also showed that such dispersed signals could come together at interphase, in some cases remaining discrete but in others resolving to one signal. The higher order organization of transgene loci revealed by comparative metaphase and interphase FISH analysis demands a model which takes into account the state of chromatin condensation within the three-dimensional structure of the nucleus (Figure 2). In one scenario, it is possible that the metal particle causes damage to a particular area where chromatin is arranged in loops attached to the nuclear matrix. If the ends of several peripheral loops were damaged, this would result in strand breaks that were close together in *trans* but widely separated in the *cis* configuration. Each of these sites could act as a focus for two-phase DNA integration, generating widely-separated arrays and/or clusters (Abranches *et al.*, 2000; Svitashev and Somers, 2001). This model confirms the distinct tertiary organization of metaphase and interphase chromatin, and suggests that the dispersed metaphase signals come together at interphase to restore the physical positions of the transgene loci at the moment of transformation.

Another possibility is that transgene loci come together at interphase because they are recruited to a common transcription factory in the nucleus. Recent studies of the spatiotemporal organization of replication, transcription, RNA processing and RNA transport in the nucleus indicate that DNA and RNA polymerases are immobilized in large complexes, or factories, in specific regions of the nucleus, and that the DNA is reeled through (Cook, 1999; Szentirmay and Sawadogo, 2000; Pombo *et al.*, 2000). For example, rRNA genes dispersed throughout the genome are recruited to the nucleolus. Promoter similarity may be one factor that determines the particular factory at which a given transcript is synthesized. It would be interesting to carry out experiments in which different promoters were used, as this could result either in the same coincidence of signals (co-recruitment), or perhaps in recruitment to separate sites.

Yet another possibility is that the transgenes are brought together at interphase due to homology, perhaps as a consequence of their position influenced by local chromatin (de)condensation. This is an exciting prospect because the coincidence of FISH signals observed in wheat nuclei could represent a physical basis for the postulated DNA-DNA interactions that precede transcriptional transgene silencing in plants. It is therefore important to determine whether discrete interphase signals occur in plants with stable

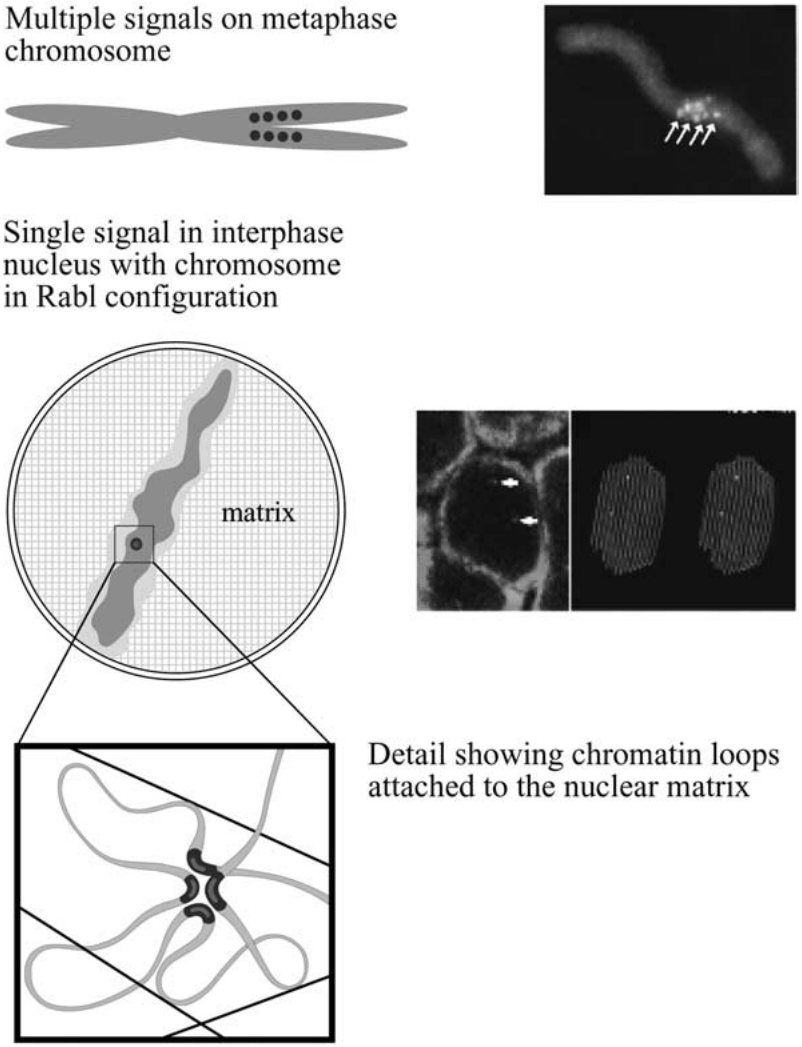

Figure 2. Multiple FISH signals visible in metaphase spreads as indicated by white arrows on the top right panel may juxtapose to give reduced number of signals in the interphase preparations of the same transgenic plant as indicated by white arrows in the lower right panel. The schematic figure on the left indicates the possible mechanism, which may lead to reduction of FISH signals in the interphase preparations.

transgene expression and coincident signals occur in silenced plants, and whether homologous endogenous genes could also be involved, because this would suggest a physical basis for the induction of cosuppression. Transformants containing a single transgene copy are also known to undergo silencing (Elmayan and Vaucheret, 1996) and homologous endogenous sequences may be important in explaining such silencing. However, endoreduplication has also been invoked as a mechanism capable of supplying homologous sequences in transformants containing a single copy of the transgene. These and other similar questions emphasize the point that transgene organization must be studied in the context of higher order genome structure and nuclear architecture to achieve a full understanding of how organization affects transgene expression.

Agrobacterium-mediated transformation

The soil pathogen *Agrobacterium tumefaciens* can transfer a small segment of DNA (known as T-DNA) from a resident Ti plasmid into the plant genome. T-DNA transfer by *Agrobacterium* has been discussed in detail in several reviews (Tinland, 1996; Gelvin, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). Cereals are not natural hosts for *Agrobacterium* species and

only recently has it been possible to achieve cereal transformation by this method (reviewed by Komari *et al.*, 1998). Five cereal species have so far been transformed by *Agrobacterium*. The first was rice (Chan *et al.*, 1992). Later, transformation was reported for maize (Ishida *et al.*, 1996), barley (Tingay *et al.*, 1997), wheat (Cheng *et al.*, 1997) and sorghum (Zhao *et al.*, 2000).

Distribution and organization of transgene loci

Early studies in dicot species suggested that T-DNA integration is a random process (Wallroth *et al.*, 1986; Chyi *et al.*, 1986) and this has been generally supported by FISH analysis (Fransz *et al.*, 1996; Papp *et al.*, 1996; Park *et al.*, 1996; Iglesias *et al.*, 1997). However, preferential integration into transcription units has been reported, and in some cases it has been shown that nearly all integration events occur in genes (Feldmann, 1991; Lindsey *et al.*, 1993). These results can be reconciled by comparing the distribution of T-DNA inserts with the distribution of genes in different species (Barakat *et al.*, 2000). In *Arabidopsis*, for example, gene density is high whereas in rice, genes are restricted to particular regions representing only 10-25% of the genome. The distribution of T-DNA inserts in *Arabidopsis* is essentially random whereas in rice T-DNA integration events are restricted and correspond predominantly to the regions known as 'gene space'. A recent FISH analysis of rice metaphase chromosomes showed that T-DNA inserts were located at distal sites (Dong *et al.*, 2001). Similar results were found in *Allium cepa* (Khrustaleva and Kik, 2001). In petunia, T-DNA was also localized to the distal regions of the chromosomes (Wang *et al.*, 1995; ten Hoopen *et al.*, 1996).

Generally *Agrobacterium*-mediated transformation gives rise to lower transgene copy numbers compared to direct transformation methods. Cheng *et al.* (1997) 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 contained 4–5 copies. In contrast, from the population of 77 bombarded transformants, only 13 (17%) contained a single copy of the transgene. Several detailed studies in dicots have shown that the structure and complexity of the transgene locus may depend on the *Agrobacterium* strain used (Spielman and Simpson 1986; Jorgensen *et al.*, 1987; Jones *et al.*, 1987; McKnight *et al.*, 1987; Deblock and Debrouwer, 1991), the transformation method (Grevelding *et al.*, 1993; Vandergraaff and Hooykaas,1996), the species or ecotype (Sarmento *et al.*, 1992; Nam *et al.*, 1997) and explant type of the host plant (Grevelding et al., 1993). The locus may comprise a single T-DNA copy, but dimers are preferentially obtained with certain strains of the bacterium and complex multicopy loci containing intact and rearranged T-DNA sequences have also been documented. Although studies in cereals have yet to be carried out in such detail, it is clear that locus structure can vary widely in monocots as well as dicots.

Co-transfer of vector backbone sequences by Agrobacterium

Gene transfer using *Agrobacterium* was initially considered a clean transformation system in which only sequences between the T-DNA borders were introduced into the plant genome. However, it was shown that many transformants contained vector sequences linked to the T-DNA insert (Ramanathan and Veluthambi, 1995). The leakiness in such transfer events is probably caused by limiting amounts of *virD* gene products (Tinland *et al.*, 1995). This results in inefficient cleavage of border sequences, and in the most extreme cases leads to integration of concatemers of the entire binary vector (Wenck *et al.*, 1997). Further investigations have shown that vector sequence transfer is probably a common event (Cluster *et al.*, 1996). Wenck *et al.* (1997) also found that traditional root transformation in *Arabidopsis thaliana* resulted in vector transfer in 33% of all transformants, whereas vacuum infiltration resulted in 62% vector transfer. Kononov *et al.* (1997) constructed binary vectors containing the screenable marker gene *gusA*, present outside either the left or the right borders of the T-DNA. Of the 200 tobacco transformants obtained, 75% were shown to carry the external screenable marker gene. Such co-transfer of the vector was also reported in rice after *Agrobacterium*-mediated transformation (Yin and Wang, 2000). These authors showed that out of 226 independent transgenic plants, 33% of the transformants contained non-T-DNA sequences. There was no major difference in the frequency of non-T-DNA transfer among three *Agrobacterium tumefaciens* strains used.

Structure of T-DNA integration junctions: T-DNA integration models

As with particle bombardment, transgene rearrangement occurs during T-DNA integration, but its prevalence is lower perhaps because the T-DNA is protected from degradation by its association with the *Agrobacterium* virulence proteins. There have been several reports of T-DNA rearrangements in dicots (Deroles and Gardner, 1988; Offringa *et al.*, 1990; Puchta *et al.*, 1992). The presence of multiple rearranged T-DNAs has been shown directly in the case of transgenic potato using fiber-FISH (Wolters *et al.*, 1998). Turning to monocots, Azhakanandam *et al.* (2000) recently analyzed the left and right T-DNA border/plant DNA junction fragments in 20 lines from three rice cultivars. Only two of these contained single non-rearranged inserts. Truncated T-DNA inserts were also observed in 14% of the rice transformants analyzed by Yin and Wang (2000). Dong *et al.* (2001) used a plasmid which contained three different genes (*hpt*, *bar* and *gusA*) on the same T-DNA. Their analysis of 18 transgenic rice lines revealed a discrepancy in the copy number of one or more of the three genes in 38% of cases suggesting rearrangements and truncations. As with particle bombardment, the CaMV 35S promoter has been shown to be involved in T-DNA rearrangements (Porsch *et al.*, 1998).

A number of groups have investigated the structure of genomic/T-DNA junctions in plants and have concluded that integration occurs by illegitimate recombination (Gheysen *et al.*, 1991; Meyerhofer *et al.*, 1991) as it does for particle bombardment. Illegitimate recombination junctions at integration sites were studied in detail in transgenic tobacco plants (Gorbunova and Levy, 1997; Salomon and Puchta, 1998).

Both DNA replication and repair have been implicated in the integration process (Tinland, 1996, Gelvin, 2000). It has been suggested that there may be preferential integration at sites with homology to the T-DNA border sequences (Matsumoto *et al.*, 1991). 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* (Sonti *et al.*, 1995). More recent work has argued that these particular mutants are blocked at an earlier stage of transformation than T-DNA integration (Nam *et al.*, 1998) but further mutants resistant to *Agrobacterium* transformation (*rat* mutants) have been identified, and five are thought to be blocked at the T-DNA integration stage (Nam *et al.*, 1999). One of these, *rat5*, encodes a histone protein, suggesting that chromatin structure at the integration site is important for the integration process (Mysore *et al.*, 2000)

It has been proposed that the formation of dimers and higher-copy-number T-DNA 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 (1991) and De Neve *et al.* (1997) clearly showed that T-DNAs derived from different *Agrobacterium* strains could co-integrate, which is strong evidence in favor 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 (Zambryski, 1988). However, the investigation of T-DNA/T-DNA junctions in multicopy transgene loci provides some evidence against a direct ligation mechanism. While inverted repeats around the right border are often precise, direct T-DNA repeats and inverted repeats around the left border are often characterized by the insertion of variable sized segments of filler DNA (Debuck *et al.*, 1999; Krizkova and Hrouda, 1998; Kumar and Fladung, 2000). 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. These models are remarkably similar to those suggested for transgene integration following bombardment (Kohli *et al.*, 1998; Pawlowski and Somers, 1998).

FISH analysis of transgenic monocots generated by Agrobacterium*-mediated transformation*

FISH studies have highlighted the importance of chromatin structure at the site of integration in another monocot, *Allium cepa*. Khrustaleva and Kik (2001) analyzed a single copy T-DNA insert in transgenic *A. cepa* using tyramide-FISH (Tyr-FISH). The single copy insert was located at the distal end of the chromosome, and colocalized with a satellite DNA probe on metaphase chromosomes. This result presented a difficulty, i.e. if there were a single copy of the insert located in an non-transcribed heterochromatic region, how was the transgenic line recovered without the expression of the selectable marker? However Tyr-FISH results with the same probes on prophase and interphase chromosomes clearly revealed that T-DNA

signals were located some distance from the satellite DNA, in less condensed euchromatin. A difference between T-DNA localization on metaphase and interphase chromosomes was also noticed in petunia (ten Hoopen *et al.*, 1999).

Transgenic rice lines generated by *Agrobacterium*-mediated transformation have recently been analyzed by metaphase FISH (Dong *et al.*, 2001). The FISH data were consistent with molecular data supporting single or multiple locations of the inserts. One transgenic rice line containing T-DNA at separate locations did not express the *gusA* reporter gene. Molecular analysis and transgene expression studies on progeny plants of this line revealed that one of the two segregating loci contained a single copy of the T-DNA that expressed *gusA*, while the other contained multiple copies of the T-DNA and lacked *gusA* expression. This suggested that epistatic interaction between the two loci led to silencing of the active locus. It was not reported if all copies of the insert at the silencing locus were intact or rearranged.

FISH analysis of plants generated by both *Agrobacterium*-mediated transformation (ten Hoopen *et al.*, 1999; Khrustaleva and Kik, 2001) and particle bombardment (Abranches *et al.*, 2000) reveal different results at different stages of the cell cycle. In petunia, three transgenes located near telomeres on three different metaphase chromosomes appeared to be distributed internally in interphase root meristem (ten Hoopen *et al.*, 1999). Khrustaleva and Kik (2001) studied both metaphase and interphase spreads, but the transgenic *Allium* line contained a single insert. Unfortunately, apart from the study of Abranches *et al.* (2000) there have been no experiments to determine whether widely separated metaphase FISH signals come to lie close together during interphase. Additional FISH results obtained in future experiments with monocots or dicots transformed by *Agrobacterium* should indicate the uniqueness, if any, of the hierarchical pattern of transgene organization revealed by FISH in cereals.

Conclusions

Plant transformation depends both on external and internal (host-dependent) factors. External factors are responsible for introducing DNA into the plant cell. In the case of *Agrobacterium*-mediated transformation, these factors are biological in nature and are controlled by the *vir* gene products. In direct DNA transfer, these are physical or chemical parameters. Once inside, the integration of foreign DNA is largely dependent on host proteins involved in DNA replication, repair and recombination. The exact way in which these proteins interact with exogenous DNA and the plant's own genome is unpredictable, leading to wide variation in locus structure. In both transformation methods, however, an initial two-phase integration mechanism can be proposed in which exogenous DNA is first linked into concatemeric arrays and then integrated into the genome with the concurrent interspersion of genomic DNA. For *Agrobacterium*-mediated transformation, transgene rearrangement and genomic DNA interspersion is less frequent. This is perhaps because less foreign DNA is present in the cell and that DNA is coated with *Agrobacterium* proteins that protect it and direct it to the nucleus. In particle bombardment, a third level of organization has been observed in which transgene arrays and clusters are dispersed widely over the chromosome generating discrete FISH signals. There appears to be a link between this hierarchical organization and the higher order architecture of chromatin in the nucleus. Thus 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.

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