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Review Article

The Mutational Consequences of Plant Transformation

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Plant transformation is a genetic engineering tool for introducing transgenes into plant genomes. It is now being used for the breeding of commercial crops. A central feature of transformation is insertion of the transgene into plant chromosomal DNA. Transgene insertion is infrequently, if ever, a precise event. Mutations found at transgene insertion sites include deletions and rearrangements of host chromosomal DNA and introduction of superfluous DNA. Insertion sites introduced using *Agrobacterium tumefaciens* tend to have simpler structures but can be associated with extensive chromosomal rearrangements, while those of particle bombardment appear invariably to be associated with deletion and extensive scrambling of inserted and chromosomal DNA. Ancillary procedures associated with plant transformation, including tissue culture and infection with *A tumefaciens*, can also introduce mutations. These genome-wide mutations can number from hundreds to many thousands per diploid genome. Despite the fact that confidence in the safety and dependability of crop species rests significantly on their genetic integrity, the frequency of transformation-induced mutations and their importance as potential biosafety hazards are poorly understood.

PLANT TRANSFORMATION

Plant transformation is the set of procedures used to introduce a transgene into a plant genome. Commercially, this is carried out by either *Agrobacterium*-mediated transformation: infecting plant cells with a disarmed pathogenic organism (*Agrobacterium tumefaciens*) containing the transgene; or particle bombardment: bombardment of cells with metal particles carrying the transgene. Both of these methods are also used in research applications. The molecular mechanisms by which transgenes insert into host DNA are poorly understood [1–3].

With the exception of the model plant *Arabidopsis thaliana*, transgene insertion usually involves a tissue culture step. Tissue culture (as used in plant transformation) induces dedifferentiation of plant tissues in the presence of hormones and antibiotics or other selective agents and allows selection and regeneration of an intact plant from a single genetically modified cell containing the transgene. Once regenerated, transgenic crops can be incorporated into standard plant breeding programmes.

PLANT TRANSFORMATION AS A MUTAGEN

Individual components of plant transformation are often mutagens. T-DNA insertion (using *Agrobacterium*) and tissue culture have both been used as such, either in plant breeding or to identify (by disruption) functional gene sequences in model organisms [4–6]. Less well known is that pathogen infection and antibiotics may also cause mutations in plant genomes [7–9].

Perhaps the clearest evidence implicating *A tumefaciens* infection as a mutagen comes from large-scale T-DNA tagging experiments which use plant transformation without tissue culture [10, 11]. In these experiments, often only one third of the mutations identified by their phenotype are found later to have been tagged with a T-DNA. Mutagenicity of *Agrobacterium* infection provides the most likely explanation for the remaining two thirds. It is not known whether particle bombardment results in chromosomal mutations.

Primary transformants (T_0 plants) emerging from plant transformation procedures have therefore been exposed to various known or suspected mutagens. For the following discussion, transformation-induced mutations have been separated into two types: those introduced at the site of transgene insertion, which we call *insertion-site mutations*, and those introduced at other random locations, which are *genome-wide mutations*.

INSERTION-SITE MUTATIONS RESULTING FROM *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Agrobacterium-mediated transformation has been used in research for over 15 years and has frequently been applied to

create commercial transgenic cultivars. A few studies have examined unselected T-DNA insertions for chromosomal rearrangements and deletion of host DNA [12–14]. Only one large-scale study has investigated the chromosomal mutations created by single-copy transgene insertions, the type of event usually selected for commercial purposes [15]. This study is examined in detail since it is the largest and appears to be broadly typical.

Forsbach and his colleagues studied 112 independent insertions into the *A thaliana* genome that they had selected by southern blotting as containing only single copies of the transgene [15]. They found that most insertions resulted in small deletions of plant DNA at the insertion site. However, 21% (24/112) appeared to be associated with large-scale rearrangement or deletion of plant chromosomal DNA. Of these, two were confirmed as chromosomal translocations. The rearrangements in the remaining 22 were not fully characterised. Similar results were obtained for T-DNA insertions into aspen, in which 7 of 10 events contained deletions at the insertion site, the largest being 580 bp [14]. A survey of nine unselected T-DNA insertions in rice reported that they were associated with deletion of up to 76 bp [13].

The results of Forsbach and his colleagues are reinforced by T-DNA mutagenesis screens which regularly find rearrangements and deletions of genomic DNA at T-DNA insertion-sites in *A thaliana*. A 78 Kbp deletion is the largest recorded for *Agrobacterium*-mediated transformation [16]. Also reported are a 1287 bp deletion [17]; a deletion of 1,980 bp [18]; and a 25 Kbp deletion [19]. More complex rearrangements have included a duplication of at least 40 Kbp [20] and a reciprocal translocation and 1.4 Kbp deletion associated with the same T-DNA insertion [21]. Lastly, among 36 *A thaliana* embryonic mutants, five involved probable chromosomal translocations [11]. This is by no means an exhaustive list of such rearrangements.

Insertion of superfluous DNA is also a consistent feature of *Agrobacterium*-generated insertion sites. This superfluous DNA may consist of extra whole or partial copies of the transgene, vector backbone DNA, or filler DNA. Filler DNA is DNA newly created at DNA-DNA junctions. It usually has some homology to the T-DNA or the transgene, alternatively it may resemble nearby chromosomal DNA or it may be of unknown origin [14]. For example, Forsbach and his colleagues found that 8 of their 112 single copy transgene insertions contained large segments of superfluous vector backbone DNA. The majority of the remaining transgenic plant lines had insertions of small (< 100 bp) segments of vector backbone or transgene DNA or DNA of undefined origin. Studies in rice have also shown that a high proportion of T-DNA insertion events feature superfluous DNA. In one study, 147 of 361 unselected loci contained superfluous DNA [22]; in another, 53%–66% of loci contained superfluous DNA [23]; and in another, 30% of all T-DNA insertions were associated with superfluous DNA [24].

INSERTION-SITE MUTATIONS GENERATED BY PARTICLE BOMBARDMENT

Particle bombardment has also been used to create numerous cultivars for research and commercial use. Most of the particle bombardment insertion events described in the scientific literature are extremely complex and insertion of multiple copies (often more than 40) of delivered DNA, sometimes interspersed with fragments of plant DNA, appears to be the norm (eg, [25–28]).

Only a handful of studies have provided detailed data on the chromosomal mutations resulting from particle bombardment insertion [29–32]. None of these have been large scale or systematic and all chose to examine relatively "simple" insertions identified by southern blotting as containing only a single copy of the transgene. Although relatively simple insertion events from particle bombardment are rare, they are important because they are more likely to be relevant to events presented for regulatory approval.

Analysis of insertion-site mutations requires DNA sequence analysis of large stretches of flanking DNA and a careful comparison with the original target site. Without this, deletions or rearrangements will probably not be detected. We have found only two studies where detailed analyses (ie, PCR, cloning and DNA sequencing) were used to characterise single-copy particle bombardment insertion sites from regenerated plants [29, 31]. Since so few studies are available, it is worth detailing their findings. One analysed the commercialised Roundup Ready soybean insertion event 40-3-2 [29]. In addition to the intended EPSPS (enoylpyruvate shikimate synthase) transgene described in the original application for commercial approval, the authors found a 254 bp EPSPS gene fragment, a 540 bp segment of unidentified DNA, a segment of plant DNA, another 72 bp fragment of EPSPS, and evidence for additional alterations to flanking plant DNA [29], (USDA Application # 93-258-01p). These insertion-site mutations were reported only after commercialisation of Roundup Ready soybean insertion event 40-3-2. Interestingly, independent analysis of another commercialised event, Maize YieldGard (event Mon810), also found evidence for previously unreported insertion-site mutations [32]. Again, these were not characterised further.

In the most complete study of particle bombardment loci performed to date, three insertion events (all from oat) were sequenced [31]. One event was nonfunctional and contained 296 bp comprising two noncontiguous fragments of delivered DNA flanked by probable rearranged genomic sequences of approximately 300 bp, 500 bp, and 800 bp. A deletion of 845 bp of chromosomal DNA was also detected at this site. At the second, a functional locus, 18 DNA/DNA junctions were detected among multiple juxtaposed sequences of genomic and transgene DNA [31]. The authors also found evidence for still further rearrangements which were not analysed further. These authors also sequenced a third locus, which they had again selected as "simple." It contained one truncated copy of each of two codelivered plasmids "interspersed with six small scrambled fragments of transgene

and genomic DNA" as well as probable additional rearrangements that were not investigated further [31].

The sequence of a functional transgene insertion site resulting from particle bombardment has therefore never been definitively compared to its undisrupted site of insertion, either in the scientific literature or in applications submitted to US regulators. Consequently, the minimal extent of mutation possible at a functional particle bombardment insertion site is unknown. Due to the small number of events analysed (even partially), any conclusions regarding particle bombardment insertion events can only be provisional. However, it appears that transgene integration resulting from particle bombardment is usually or always accompanied by substantial disruption of plant DNA and insertion of superfluous DNA.

Given the relative lack of research describing insertion sites resulting from particle bombardment, it is interesting that there is a single report of the insertion of contaminating bacterial chromosomal DNA adjacent to a transgene [33]. It is as yet too early to say whether insertion of contaminating DNA is a common outcome of particle bombardment.

THE MOLECULAR CONSEQUENCES OF TRANSGENE INSERTION

It is apparent that small and large-scale deletions, rearrangements of plant DNA, and insertion of superfluous DNA are each common occurrences at *Agrobacterium*-mediated transgene insertion sites. Particle bombardment insertion sites, however, appear always to be associated with genome disruption, rearrangements, and superfluous DNA.

Mutations at insertion sites have the potential to result in inadvertent loss, acquisition, or misexpression of important traits, in part because transgenes insert into or near functional gene sequences. In the plant species most studied (*A thaliana* and rice), approximately 27%–63% of T-DNA insertions disrupt known gene sequences [15, 24, 34–37]. Large-scale studies of insertion patterns of transgenes delivered by particle bombardment have never been conducted in any species. Deletions or rearrangements associated with transgene insertion further increase the likelihood of alterations to the plant phenotype. Among many examples, the 78 Kbp deletion recorded in *A thaliana* resulted in loss of 13 genes and disruption of two others [16]. The 1,980 bp deletion also noted earlier resulted simultaneously in upregulation and altered transcript sizes of an adjacent gene [18].

Gene disruption and deletion are not the only mechanisms by which transgene insertion may affect the phenotype of a transgenic plant. When transgene insertion is associated with rearrangements or insertion of superfluous DNA then juxtaposition of promoter sequences and coding fragments may lead to sense or antisense transcripts which, similarly to siRNAs and miRNAs, can interfere with the expression of genes containing homologous or similar sequences [38, 39]. A naturally occurring instance of this phenomenon has been reported in the nontransgenic rice *low glutelin content* mutation. Here, a deletion resulted in transcription into a neighbouring member of the glutelin gene family and was thought to have caused gene silencing of the entire glutelin gene family [40]. Obviously, the more the present scrambling is, the higher the probability that an aberrant phenotype will result is. Alternatively, complex insertion sites may generate aberrant transcripts coding for fusion proteins that can also lead to mutant phenotypes.

Studies of transcription patterns at insertion sites are rare. To our knowledge, only two papers describe transcription patterns at transgene integration sites and both studied events approved for commercial release [41, 42]. Although neither focussed on flanking DNA, both showed evidence for aberrant transcription. At the Soybean Roundup Ready 40-3-2 locus, transcription originating in the transgene continues into scrambled DNA at the 3′ end of the locus. This is a consequence of inefficient transcription termination by the nopaline synthase transcription terminator—termination sequence widely used in transgenic plants [42]. Rang et al predicted that the oversized transcripts detected at the Roundup Ready locus might express fusion proteins containing the EPSPS gene and the unidentified sequence flanking the scrambled transgene [42]. However, they made no attempt to detect any protein product. In the second report, several abundant and oversized mRNA transcripts originating in the transgene were detected by Northern blotting of a commercial virus-resistant papaya line. The origin and significance of these aberrant viral transcripts was never investigated [41].

There are other mechanisms by which insertion-site mutations may affect plant phenotypes. Sequences carried on T-DNAs can alter the expression of neighbouring genes at least 12 Kbp from the transgene [18, 35, 43]. Such distant effects are thought to be mediated by multiple promoters and therefore it is plausible that scrambled or complex transgene insertion sites that accumulate multiple promoter sequences may also influence the expression of linked genes.

Lastly, bacterial chromosomal DNA, plasmid sequences (bacterial origins of replication in particular), or antibiotic resistance genes accidentally inserting adjacent to the transgene may significantly enhance the probability of horizontal gene transfer. Availability of sequence homology is considered one of the major obstacles to horizontal gene transfer from plants to bacteria. By providing adjacent regions of bacterial sequence homology, researchers have shown that horizontal gene transfer from bacterial replicons to bacterial chromosomes can be elevated 10^5 fold [44, 45]. Most (US approved) commercial transgenic cultivars analysed in a previous publication had insertions of superfluous bacterial DNA at the insertion site [46].

GENOME-WIDE MUTATIONS

The second class of mutations associated with plant transformation are genome-wide mutations. These are not necessarily genetically linked to the transgene insertion site but arise as a consequence of tissue culture, probably *Agrobacterium* infection and possibly particle bombardment and antibiotic use [3, 10, 47, 48]. There are 5 studies in which researchers have attempted to quantify mutations introduced

throughout the plant genome during plant transformation. These researchers used DNA polymorphism analysis (based on RFLP, AFLP, and other PCR techniques) to sample and compare the genomic DNA of transformed plants with that of nontransformed control plants [49–53].

Their results are broadly consistent. They suggest that plant transformation procedures typically introduce many hundreds to thousands of genome-wide mutations into the DNA of transgenic plants. For example, Labra et al [52] estimated that the "genomic similarity value" of control plants was 100%, but only 96%–98% for the transgenic plants. In other words, very extensive genetic mutation had resulted from the plant transformation procedures. These studies suggest that most genome-wide mutations are caused by passage through tissue culture, which in plant transformation is used in a particularly mutagenic form [54].

Genome-wide mutations were found in all analysed plants transformed using tissue culture and the mutations have been shown to be heritable [55]. Even though the mutations found in these studies were numerous, the analytical techniques used may underestimate the true numbers because they are likely to miss most point mutations and small deletions. The degree of underestimate will depend on the precise mutation spectrum of plant transformation, which is unknown.

UNINTENDED PHENOTYPES IN TRANSGENIC PLANTS

Both insertion-site and genome-wide mutations may result in transgenic plants with unexpected traits. Despite the supposed precision of genetic engineering, it is common knowledge that large numbers of individual transgenic plants must be produced in order to obtain one or a few plants that express the desired trait in an otherwise normal plant. Even after selection, there are many reports of apparently normal transgenic plants exhibiting aberrant behavioural or biochemical characteristics upon further analysis. These unexpected traits range from altered nutrient or toxin levels to lower yields under certain environmental conditions, see references in [46, 56, 57]. Among others are altered interactions with soil microorganisms [58], susceptibility to pathogens [59], altered insect resistance [60], and plant reproductive characteristics [61]. Despite the paucity of publicly accessible data and lack of monitoring of commercial transgenic crop varieties, commercial (ie, approved) transgenic plants have also been observed with unintended traits. Verified examples include stem splitting and decreased yields in transgenic soybean plants [62] and a 67-fold reduction in beta-carotene content in a transgenic squash variety engineered for virus resistance (USDA Application # 95-352-01).

These examples show that unexpected transformation-induced phenotypes can affect any aspect of plant phenotype, including those of value or concern to humans. Furthermore, the incidence of unintended phenotypes in transgenic plants seems to be high, indicating that plant transformation is currently not predictable. Few unexpected phenotypes have been followed up but we propose that an important source of unpredictability is likely to be the mutational consequences of plant transformation.

Interestingly, phenotypes sometimes arise from the transformation process that cannot have been caused by the transgene because it transpired that the transgene was not present in that particular line. Examples include the most virus-resistant line arising from a transformation experiment, and a plant giving a 7–10 fold increase in insectivore mortality [51, 63]. These cases in particular suggest that transformation-induced mutations were the cause of the unexpected phenotype.

THE IMPLICATIONS OF TRANSFORMATION-INDUCED MUTATIONS

Transformation-induced mutations are significant for two reasons. Firstly, they can obscure the results of scientific experiments. For example, they may generate spurious results in metabolic engineering experiments [64] or obscure the cause of a phenotype in T-DNA tagging experiments [18].

The second and more important significance of transformation-induced mutations is that they may affect the safety or performance of transgenic crops intended for commercial release.

Effects such as these are collectively termed biosafety effects. Biosafety recognises that crops are part of an extensive and fragile web of ecological and human interactions. It therefore covers a wide range of potential consequences that crops may have, either while growing, as food or feed, or as waste or residues. These include nontrivial effects on the wholesomeness of food or feed, on soil processes, pollination or other biotic interactions as well as crop failures that may have food security, agronomic, or economic consequences. Transformation-induced mutations have the potential to affect any biosafety phenotype that is under genetic control. The frequency with which phenotypes will occur is the chief unknown, but in crops approved for commercial use this will depend greatly on the extent to which applicants present, and regulators accept, transgenic lines carrying these mutations.

CURRENT TRANSGENIC CROP BIOSAFETY ASSESSMENTS

Biosafety risk assessments are intended to prevent crops with hazardous traits reaching the market. Many authors however have argued that present risk assessments are flawed. Some have criticised the concept of risk assessment based on substantial equivalence [65], others have identified "serious deficiencies in both regulatory oversight and corporate testing procedures" [66], and others have pointed out an overreliance on assumption-based reasoning and a tendency to rationalise away potential sources of harm [67]. These criticisms are primarily directed at the principal purpose of current transgenic risk assessments: the detection of known and predicted hazards resulting from the specific transgene. However, transformation-induced mutations are likely to have unpredictable rather than predictable effects on the phenotype. These are even less likely to be detected than predicted effects because regulators do not systematically examine all potential hazards arising from each transgenic plant.

The above criticisms primarily concern safety assessment of the phenotype of transgenic plants. But, despite their biosafety implications, risk assessments typically fail to effectively examine the genetic aspects of transformation. Risk assessments do not analyse genome-wide mutations and we have found that assessments of flanking DNA are inadequate for the likely extent of genetic damage at insertion sites [46]. As noted previously, two independent evaluations of commercial transgene insertion sites have been published [29, 32]. Both found that the respective insertion sites were much more complex than was apparent from the data supplied in the application for commercial release. Consequently, they support the contention that complex integration patterns are common, that the insertion site analysis accepted by the regulator was inadequate and consequently transgenic varieties containing complex insertion sites reach the market.

IMPROVING RISK ASSESSMENT AND ERADICATING TRANSFORMATION-INDUCED MUTATIONS

The phenotypic consequences arising from transformation-induced mutations may be avoided in two ways. The first approach required is to ensure that transgenic plants are as identical to their parent as possible. This can be achieved by changes to transformation procedures such as (1) eliminating tissue culture, (2) effective backcrossing, (3) targeted insertion and possibly, and (4) development of alternative transformation methods able to create insertion events without superfluous DNA or chromosomal damage. Some of these, such as transformation without tissue culture, have already been achieved in a few species (see references in [46]). Other improvements will require research and development, but all will require analysis to ensure that they are not themselves mutagenic.

The second requirement is to improve genetic analysis and selection of transgenic plants. From the perspective of preventing unintended mutations, a principal defect in current analyses is the inadequate examination of transgene insertion sites [46]. We recommended that after extended backcrossing, the transgene and extensive flanking regions must be sequenced (we suggest 50 Kbp on each side) and compared to parental DNA to ensure there are no alterations. Insertion sites disrupting gene sequences and those with superfluous DNA insertions, deletions or rearrangements should be discarded. Insertion sites that result in aberrant transcripts or altered regulation of neighbouring genes should also be discarded. Current knowledge suggests that, if rigorously applied together with the strategy outlined in (1–4) above, these precautions could ensure that transformation-induced mutations will not impact on biosafety. It is important to note however that current knowledge is always provisional and also that other potential sources of risk (eg, from the transgene itself) require their own specific risk assessment, mitigation, and monitoring measures.

To retain public and institutional confidence, biosafety decisions need to be clearly grounded in evidence. This review is an attempt to determine the degree to which the genetic consequences of transgene insertion contribute to uncertainty and risk in transgenic plants. We conclude that much remains to be discovered about genome-wide and insertion-site mutations. In particular, lack of information, especially for crop plants and particle bombardment, means that plant transformation may be even more damaging than is apparent from this review. Even with the limited information currently available it is clear that plant transformation is rarely, if ever, precise and that this lack of precision may cause many of the frequent unexpected phenotypes that characterise plant transformation and that pose a significant biosafety risk. It is also clear that implementation of the steps outlined above can greatly decrease that risk.

Ultimately, it should not be forgotten that though transformation-induced mutations magnify the risks of genetic engineering, they bring no benefits and are unnecessary for the production of transgenic crops.

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