11

Transformation-induced mutations in transgenic plants: Analysis and biosafety implications.

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Introduction

Plant transformation has become an essential tool for plant molecular biologists and, almost simultaneously, transgenic plants have become a major focus of many plant breeding programs. The first transgenic cultivar arrived on the market approximately 15 years ago, and some countries have since commercially approved or deregulated (e.g. the United States) various commodity crops with the result that certain transgenic crop plants, such as herbicide resistant canola and soya and pest resistant maize, are currently grown on millions of acres.

Advocates for the use of genetic engineering as a plant breeding tool claim its precision provides a major advantage over other plant breeding techniques. The presumption is that genetic engineering results in (1) only specific and known genotypic changes to the engineered plant (the simple insertion of a defined DNA sequence - the transgene) and (2) only known and specific phenotypic changes [the intended trait(s) encoded by the transgene]. This presumption has strongly influenced biosafety regulation. Regulators typically assume that the plant transformation methods used to introduce a transgene into the plant genome are mostly irrelevant to the risk assessment process and that the major source of risk in transgenic crop plants arises from the transgene itself. The focus of this review is a scientific assessment of the precision of current crop plant transformation techniques.

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Abbreviations: AFLP: amplified fragment length polymorphism; AFRP: amplified fragment random polymorphism; bp: base pairs; *ble*: bleomycin binding protein gene; CaMV: cauliflower mosaic virus; CBI: confidential business information; CP4 EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. Strain CP4 ; dsRNA: double stranded RNA; FISH: fluorescence *in-situ* hybridisation; GFP: green fluorescent protein; *gus*: Beta-glucuronidase gene; kbp: kilobase pairs; LB: left border repeat of T-DNA; *nos*: nopaline synthase gene; *npt*II: neomycin phosphotransferase type II gene; ORF: open reading frame; PCR: polymerase chain reaction; PRVcp: papaya ringspot virus coat protein gene; RAMP: random-amplified microsatellite polymorphism; RB: right border repeat of T-DNA; RFLP: restriction fragment length polymorphism; RAPD: random amplified polymorphic DNA; T_0: primary transformant; T_1: first generation transformant derived from self-fertilisation; T-DNA: transferred DNA; Ti-plasmid: tumor inducing plasmid; USDA: United States Department of Agriculture.

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Agrobacterium-mediated transformation and particle bombardment (biolistic transformation) are the two plant transformation methods most commonly used to produce transgenic plants for both research and commercial purposes. For both *Agrobacterium*-mediated transformation and particle bombardment the mechanism of transgene integration into the plant genome is still poorly understood and has been reviewed elsewhere (Pawlowski and Somers, 1996; Tinland, 1996; Somers and Makarevitch, 2004; Tzfira *et al*., 2004).

If plant transformation were precise, 20 transgenic plants, derived from the same parent plant material and carrying the same transgene, would be identical in phenotype. Furthermore, they would be identical to the non-transgenic parent plant, except for the transgenic trait. This, however, is usually not found in practice. Phenotypic variation is the norm within populations of plants from the same experiment (Hoekema *et al*., 1989; Conner *et al*., 1994; Bregitzer *et al*., 1998; Singh *et al*., 1998; Kaniewski and Thomas, 1999; Shu *et al*., 2002) and breeders and researchers must carefully screen numerous transformed plants to obtain one or a few plants which exhibit only the desired traits (Kumar *et al*., 1998; Dear *et al*., 2003). Despite this, even plants originally selected as having the appropriate phenotype are often found, during later experiments or commercial use, to have unexpected and unintended traits (Kuiper *et al*., 2001; Haslberger, 2003).

One possible cause of unintended phenotypes in transgenic plants is the presence of transformation-induced mutations. The insertion of a transgene into the plant genome inevitably disrupts the sequence of the endogenous plant DNA and may be accompanied by other mutations. A transgene insertion event, as defined here, includes both the desired transgene and any associated insertion-site mutations. In this review we define transformation-induced mutations as: (1) the disruption of plant genomic DNA caused by transgene or superfluous DNA insertion (2) any alterations to plant genomic DNA, including base pair changes, duplications, deletions or rearrangements, caused by the mechanism of transgene insertion or by other aspects of the plant transformation process (such as tissue culture or antibiotic use) and (3) the presence of superfluous DNA (any non-genomic DNA other than a single intact copy of the desired transgene)(reviewed in Smith *et al*., 2001).

In this paper we review what is known about the effect of the plant transformation process on the plant genome. We analyse the extent and frequency of transformation-induced mutations in transgenic plants created by *Agrobacterium*-mediated transformation and particle bombardment. We discuss the potential of such mutations to result in unintended harmful phenotypes and the biosafety and regulatory implications of these findings. We also offer recommendations to researchers and regulators which, if followed, would help prevent or eliminate transformation-induced mutations in transgenic plants. In this review we do not discuss the potential for the transgene itself to result in unintended phenotypic consequences.

To facilitate discussion, we have divided transformation-induced mutations into insertion-site mutations (those created at the site of transgene insertion, which are thus an integral component of the 'insertion event') and genome-wide mutations, those present at other random locations in the plant genome, but which may nevertheless be retained in transgenic cultivars.

Insertion into gene sequences

The ideal transgenic plant for most research and breeding purposes would contain a single intact copy of the desired transgene inserted into a non-functional region of the plant genome, without further alteration of the host plant DNA. However, using current plant transformation techniques, the site of transgene insertion cannot be pre-selected (Puchta 2003). This means that transgenes cannot be targeted to non-functional regions of the genome and that their genomic location must be determined after insertion.

Data accumulated from several large-scale T-DNA tagging experiments in both *Arabidopsis thaliana* (*A. thaliana*) and rice suggest that T-DNA insertion into gene sequences is

frequent using *Agrobacterium*-mediated transformation, occurring at 35-58% of T-DNA insertion events (Jeong *et al*., 2002; Szabados *et al*., 2002; Alonso *et al*., 2003; Chen *et al*., 2003; Sha *et al*., 2004). For example, when researchers mapped more than 1000 T-DNA flanking sequences in rice they found that 58.1% of the T-DNAs had inserted into genic regions (Chen *et al*., 2003). In a T-DNA tagging experiment in *A. thaliana*, 47.8% of 973 T-DNA insertions were into gene coding or known regulatory sequences (Szabados *et al*., 2002). When only single-copy T-DNA insertions were examined in 112 *A. thaliana* lines, researchers found similar numbers: 55.9% of LB sequences and 58.5% of RB were in gene sequences (Forsbach *et al*., 2003). Experiments in other organisms, such as the legume *Medicago truncatula* (Scholte *et al*., 2002), barley (Salvo-Garrido *et al*., 2004), and potato and tobacco (Koncz *et al*., 1989; Lindsey *et al*., 1993) also suggest that frequent T-DNA insertion into gene sequences is the norm.

A more detailed analysis of T-DNA insertion events in *A. thaliana* identified 1010 T-DNAs that had flanking sequences located in or near genes (Qin *et al*., 2003). Based on homologies to known genes, they put these genes into 14 function categories. The majority coded for proteins of unknown function (48.12%), while other categories included metabolism (7.82%), signal transduction (6.93%), transcription (6.04%), disease/defense (4.65%) and intracellular traffic (0.99%). T-DNA insertion thus clearly has the potential to disrupt or alter the function or expression of genes involved in all aspects of plant biology. The disruption of such genes may result in transgenic plants with unexpected, and potentially harmful, phenotypes.

The frequency with which particle bombardment results in transgene insertion into gene sequences is unknown because the necessary experiments have not been done.

It is important here to note that determination of insertion into gene sequences by analysis of short stretches of DNA flanking the transgene insert may underestimate the number of insertion events which disrupt functional plant DNA. This is due to several factors including: (1) the lack of knowledge of the importance of higher order genome structure, gene order and long-range regulatory interactions in plants (all of which are of known importance to gene regulation in other organisms; Carter *et al*., 2002; Hurst *et al*., 2004) and (2) the possible presence of insertion-site mutations such as deletions or rearrangements which disrupt functional DNA and yet are not revealed by analysis of short stretches of flanking DNA. Accurate characterisation of insertion-site mutations requires sequencing the complete transgene insertion event (including the full DNA sequence of the transgene and any superfluous DNA and/or rearranged flanking plant DNA) and comparison with pre-insertion target sequences from the non-transgenic parent plant. Without this comparison, analysis of flanking DNA sequences alone will miss insertion-site mutations such as deletions of chromosomal DNA.

Insertion-site mutations generated by *Agrobacterium*-mediated transformation

It is well documented that, in practice, plant transformation does not result only in the insertion of intact single transgenes (Kohli *et al*., 2003). Southern blot analysis reveals that, as a rule, the majority of T_0 plants produced by *Agrobacterium*-mediated transformation have either multiple copies of the T-DNA inserted at one or more loci, or they have truncated T-DNAs (Hiei *et al*., 1994; Ishida *et al*., 1996; Cheng *et al*., 1997; Dai *et al*., 2001; Dong *et al*., 2001; Forsbach *et al*., 2003; Kim *et al*., 2003; Olhoft *et al*., 2003; Vain *et al*., 2003). This appears to be true for all plant species studied.

In the following discussion, we focus on insertion events identified by Southern blot analysis as carrying a single intact T-DNA. In general, these are the type of insertion event most useful to researchers analysing gene function or breeders creating transgenic crop plants, and for which the most data are available.

FILLER DNA IN T-DNA INSERTION EVENTS

The end of a T-DNA may be joined directly to plant genomic DNA or the T-DNA/genomic DNA junction may also include sequences called filler DNA. Studies of single-copy T-DNA inserts in *A. thaliana* indicate that between 48% and 70% of T-DNA junctions include filler DNA sequences (Windels *et al*., 2001; Meza *et al*., 2002; Forsbach *et al*., 2003). Filler DNA can range in size from 1 bp to several hundreds of bp (Meza *et al*., 2002). In one study, 93 left border (LB) and 94 right border (RB) junctions were analysed for the presence of filler DNA. The authors found that 50 (53.8%) and 59 (62.8%) respectively had insertions of filler DNA, of which 41.9% (LB) and 45.7% (RB) were between 1 and 25 bp and 11.8% (LB) and 13.8% (RB) were between 26 and 100 bp (Forsbach *et al*., 2003). In this experiment, three of the RB filler sequences were greater than 100 bp (Forsbach *et al*., 2003).

In a separate *A. thaliana* study, researchers analysed 67 plant DNA/LB or RB junctions and found each contained between 1 and 51 bp of filler DNA. This study indicated that filler DNA is usually built up from several non-contiguous stretches of DNA, which usually originate from either plant DNA close to the insertion-site and/or sequences from near the T-DNA ends (Windels *et al*., 2001). However, not all filler DNA originates from nearby plant or T-DNA sequences. The insertion of 112 bp of filler DNA from another chromosome (Meza *et al*., 2002) has been documented, as have the presence of filler sequences originating from internal T-DNA sequences (Windels *et al*., 2001; Meza *et al*., 2002; Forsbach *et al*., 2003).

These *A. thaliana* experiments are based on the analysis of fairly large numbers of single-copy T-DNA insertion events (37 events in Meza *et al*. 2002; 112 events in Forsbach *et al*. 2003; and 67 junctions in (Windels *et al*., 2001). There are few such analyses in other plant species and not all are of single-copy T-DNA insertion events. Analysis of 53 RB/plant DNA and 61 LB/plant DNA junctions in rice showed that 32% and 18% respectively had insertions of filler DNA (Kim *et al*., 2003). These were mostly 1-22 bp in size, although at least one 102 bp filler sequence was found (Kim *et al*., 2003). Analysis of 10 T-DNA insertion events in poplar indicated that 6/10 had filler DNA varying in length between 7 and 235 bp located at the LB and/or RB junctions and which originated from nearby plant or T-DNA sequences (Kumar and Fladung, 2002). In *Medicago truncatula*, 4/8 lines analysed had filler sequences of 3, 33, 38 and 392 bp. The 32 bp filler DNA corresponded to internal T-DNA sequence while the others were of unknown origin. Taken together with other analyses of tobacco insertion events, these suggest that filler DNA is likely to be found at T-DNA insertion events in many if not all species (Gheysen *et al*., 1987; Iglesias *et al*., 1997).

VECTOR SEQUENCES AND T-DNA FRAGMENTS IN T-DNA INSERTION EVENTS

When carried by *Agrobacterium*, the T-DNA is located on the Ti (tumor inducing) plasmid. In theory, only DNA within the T-DNA border sequences should be transferred to plant genomes. In practice, however, Southern blot and PCR analyses suggest that plasmid sequences from outside the T-DNA borders (vector backbone) are found in 20-80% of plants. Species examined include populations of transgenic *A. thaliana*, tobacco, rice, soybean, maize and potato (Ramanathan and Veluthambi, 1995; Wenck *et al*., 1997; De Buck *et al*., 2000; Yin and Wang, 2000; Kim *et al*., 2003; Vain *et al*., 2003; Afolabi *et al*., 2004; Rommens *et al*., 2004; Sha *et al*., 2004; Shou *et al*., 2004; Francis and Spiker, 2005). Vector backbone can be found adjacent to either the LB or the RB of T-DNAs integrated into the plant genome. These vector backbone sequences may contain bacterial genes (e.g. *vir* genes, antibiotic resistance genes) and bacterial origins of replication (Tinland, 1996; Tzfira *et al*., 2004).

In *A. thaliana*, 6%-14% of single-copy T-DNA insertion events have been shown, using PCR and sequence analysis, to contain vector backbone sequences (Meza *et al*., 2002; Forsbach *et al*., 2003; De Buck *et al*., 2004). Some of these vector backbone insertions are greater than 5000

bp in size (Meza *et al*., 2002). In one study of T-DNA insertion in rice, Southern blot analysis indicated differences between vectors, such that 11% of single-copy T-DNA insertion events produced using pGreen contained vector backbone, as compared to 67% of single-copy T-DNA insertion events produced using pSoup (Vain *et al*., 2003).

Also in *A. thaliana*, fragments of truncated T-DNA or additional T-DNA border sequences are sometimes integrated adjacent to T-DNAs, even at insertion events which were originally identified by Southern blot analysis as having single-copy T-DNA insertions (Meza *et al*., 2002; Forsbach *et al*., 2003; Guan *et al*., 2003; Pilot *et al*., 2004). In one experiment 1/112 insertion events had an insertion of a 770 bp internal T-DNA fragment (Forsbach *et al*., 2003), while at 3/112 (3%) of the insertion events, partial LB fragments were found adjacent to the RB. In another study of single-copy T-DNA insertion events in *A. thaliana*, 3/37 (8%) insertion events contained additional T-DNA fragments (Meza *et al*., 2002).

Large scale PCR and sequencing analyses have been carried out only in *A. thaliana*. However, it appears that insertions of additional T-DNA border fragments and internal T-DNA fragments also occur at apparent single-copy T-DNA insertion events in other plants (Dong *et al*., 1996; Iglesias *et al*., 1997; Zheng *et al*., 2001; Scholte *et al*., 2002).

DELETIONS AND REARRANGEMENTS OF PLANT GENOMIC DNA AT T-DNA INSERTION EVENTS

PCR and DNA sequence analysis of single-copy T-DNA insertion events in *A. thaliana* reveals that substantial chromosomal deletion and rearrangements are frequent (Meza *et al*., 2002; Forsbach *et al*., 2003; De Buck *et al*., 2004). In one study of 112 single-copy T-DNA insertion events, 64 (87.7%) showed target-site deletions (Forsbach *et al*., 2003). Six deletions were from 1-10 bp, fifty seven were deletions of 11-100 bp and 1 was greater that 100 bp. In addition, two (2%) insertion events had chromosomal translocations (of DNA from different chromosomes) adjacent to the T-DNA and 22 insertion events (20%) were thought to have large deletions, insertions or other rearrangements. The methods employed in this study were not sufficient to determine the full extent of mutation at these 22 insertion events.

In a separate *A. thaliana* study, both LB and RB flanking sequences could be isolated for 22/37 insertion events. When each insertion event was compared to the original genomic target sequence, all events had deletions of the target sequence. These ranged from 1 bp – 1537 bp (Meza *et al*., 2002). Nine were greater than 50 bp and 4 were greater than 100 bp. Furthermore, a complex pattern was found at one insertion event, which had a 35 bp deletion of the target sequence and a second genomic deletion of 825 bp located 60 bp distant from the T-DNA insert. This indicates that insertion-site deletions may not always be located immediately adjacent to the T-DNA. For the remaining 15 (41%) insertion events, the presence of more extensive deletions or rearrangements would explain why it was not possible to isolate both the LB and RB sequences, however these events were not analysed further.

In one final *A. thaliana* experiment, of 21 single-copy T-DNA insertion events analysed, 10 had target-site deletions of 11-317 bp (De Buck *et al*., 2004). Two of 21 events (10%) had T-DNA inserts flanked by DNA from different chromosomes. The presence of target-site deletions in these 2 and in the remaining 9 insertion events was not characterised, presumably again because the large scale of the mutations made analysis difficult.

For *A. thaliana*, it is clear that large-scale mutations are frequently found at single-copy T-DNA insertion events. While many large-scale mutations were not fully characterised in the studies described above, there is evidence that chromosomal deletions and rearrangements at single-copy T-DNA insertions can be substantial (Revenkova *et al*., 1999). For example, the largest insertion-site deletion recorded in the literature is 75.8 kbp. This deletion was predicted to remove 14 genes entirely, as well as part of another gene (Kaya *et al*., 2000). In another case, a 25 kbp deletion at a T-DNA insertion event removed one gene entirely and the 3’ region of a second

gene (Filleur *et al*., 2001). More complex DNA arrangements also occur. Duplications and insertions of greater than 40 kbp of DNA from other chromosomes have been found adjacent to single-copy T-DNA inserts (Tax and Vernon 2001; also see Castle *et al*., 1993; Forsbach *et al*., 2003; De Buck *et al*., 2004; Gutensohn *et al*., 2004). Single-copy T-DNA insertion events can also be associated with reciprocal translocations of DNA (Guan *et al*., 2003; Lafleuriel *et al*., 2004; Pilot *et al*., 2004).

The limited data available from other plant species also indicate that deletions and rearrangements of chromosomal sequences are commonly found at single-copy T-DNA insertion events (Ohba *et al*., 1995). In an experiment in *Medicago truncatula*, researchers were able to isolate the original genomic target sequence from 8/11 insertion events (Scholte *et al*., 2002). The deletions detected at these eight insertion events ranged from 5-404 bp. The remaining three events presumably had larger deletions and/or rearrangements which prevented isolation of the original target sequences using flanking sequence information. An analysis of four insertion events in *Javanica* rice indicated that 2/3 RB junctions analysed had rearranged T-DNA/plant sequences (Dong *et al*., 1996). In tobacco, a few insertion events have been characterised for deletions and rearrangements. In one study, the insertion event was shown to have a 27 bp deletion as well as a 158 bp duplication of plant target sequences (Gheysen *et al*., 1987), while in a separate study, a target-site deletion of 32 bp was identified in one insertion event, while the target-site of a second event could not be determined from flanking sequence information, presumably due to the scrambling of T-DNA and plant sequences (Iglesias *et al*., 1997). In aspen, a study of 10 insertion events found that 7/10 events had deletions of a few bp to 570 bp while 3/10 events had deletions of 0-1 bp (Kumar and Fladung, 2002).

T-DNA INSERTION EVENTS IN COMMERCIAL CROP PLANTS

If a sufficient number of transgenic plants are generated, it should be possible to select insertion events that consist of a single intact T-DNA inserted into DNA with no known function[1] and which are free from deletions, rearrangements and the insertion of superfluous DNA. To our knowledge, there are no complete sequence analyses of deregulated (i.e. commercially approved) T-DNA insertion events. However, we examined the molecular data provided to USDA regulators during the approval process for three different commercial transgenic crop plants (herbicide tolerant LLCotton25[2]; Virus resistant Newleaf® Plus RBMT22-82 Potato[3]; and virus resistant CZW-3 Squash[4]). We found the applicants had provided no sequence data on the genomic DNA flanking the T-DNA and no comparison of flanking sequences with the original genomic target-site DNA (Wilson *et al*., 2004)[5]. The information provided in the applications did reveal that all three insertion events included superfluous DNA. LLCotton25 had superfluous polylinker sequence. Newleaf® Plus RBMT22-82 Potato had three independent T-DNA insertion events (two of which are presumably superfluous and one of which also included superfluous plasmid DNA). CZW-3 squash had a selectable marker gene. However, as the appropriate data were not provided to regulators, it is not possible to know whether any of the insertion events present in these commercial crop plants had additional small insertions of T-DNA or plasmid sequences, or deletions or rearrangements of genomic DNA.

Insertion-site mutations generated by particle bombardment

The majority of transgene insertion events created by particle bombardment are complex, having multiple copies of the transgenic DNA integrated at a single locus (Register *et al*., 1994; Wan and Lemaux, 1994; Pawlowski and Somers, 1996; Kohli *et al*., 1998; Pawlowski and Somers, 1998; Kohli *et al*., 1999; Maqbool and Christou, 1999; Fu *et al*., 2000; Mehlo *et al*., 2000; Svitashev *et al*., 2000; Svitashev and Somers, 2001; Breitler *et al*., 2002; Loc *et al*., 2002; Svitashev and

Somers, 2002; Vain *et al*., 2002; Kohli *et al*., 2003). To illustrate, a single particle bombardment insertion event can include more that 40 copies of the transgene (and the superfluous plasmid DNA used to carry the transgene) (Vain *et al*., 2002). Multiple transgene copies can be arrayed as concatamers or interspersed with small or large segments of plant DNA, and the transgene sequences can be truncated or rearranged (Svitashev and Somers, 2001; Kohli *et al*., 2003).

Most particle bombardment insertion events have been characterised using Southern blot analysis, a technique which, on its own, is unable to identify all of the mutations created at a transgene insertion event (Jakowitsch *et al*., 1999; Mehlo *et al*., 2000; Svitashev and Somers, 2001; Svitashev *et al*., 2002). Only a few studies described in the scientific literature use PCR or DNA sequence analysis to characterise particle bombardment transgene insertion events (Shimizu *et al*., 2001; Windels *et al*., 2001; Svitashev *et al*., 2002; Ulker *et al*., 2002; Makarevitch *et al*., 2003). When DNA sequence analysis is used to characterise particle bombardment events, the results are often surprising. For example, partial sequence analysis of two independent complex insertion events in oat indicated that 'extreme scrambling of non-contiguous transgene and genomic fragments' had occurred at each event, and that many of the scrambled fragments were less than 200 bp (Svitashev *et al*., 2002). Other techniques, for example, fluorescence *in-situ* hybridisation (FISH) analysis, can also uncover surprises. When two transgenic *tritordeum* lines created by particle bombardment were analysed using FISH, three insertion events were identified and all three of the insertion events were associated with translocations (Barro *et al*., 2003).

In addition to extreme scrambling and extensive rearrangement of transgene and plant DNA, particle bombardment insertion events may also include contaminating DNA. Analysis of the DNA sequences between a head to head repeat of the transgene in a complex insertion event in tobacco revealed unidentifiable DNA fragments (which the authors assumed to be tobacco DNA), as well as a 260 bp fragment of chromosomal coding sequence from the *Escherichia coli liv*F gene (Ulker *et al*., 2002). This *E. coli* chromosomal DNA was incorporated into the insertion event despite the fact that, prior to bombardment, the transgene-containing plasmid DNA was purified away from contaminating bacterial DNA using standard methods (Ulker *et al*., 2002). To our knowledge, there are no other reported cases of chromosomal *E. coli* DNA incorporated into particle bombardment insertion events. However, so few particle bombardment events have been analysed using DNA sequencing that it is not possible to determine whether insertion events incorporating contaminating DNA sequences are commonplace or not.

SINGLE-COPY PARTICLE BOMBARDMENT INSERTION EVENTS

We have found only one study in which researchers attempted the complete sequence analysis of transgene insertion events isolated from intact plants transformed by particle bombardment (Makarevitch *et al*., 2003). This study examined three insertion events isolated from two different oat lines. Southern blot analysis indicated that these were relatively simple insertion events.

One particle bombardment insertion event, 3830-1, consisted of a slightly larger than full length copy of the transgenic plasmid in a head to head orientation with a partial copy of the plasmid (Makarevitch *et al*., 2003). There were two regions of extensive scrambling of plant genomic DNA and plasmid DNA fragments, one between the two copies of the plasmid and one near the other end of the truncated copy. The genomic DNA sequences flanking each side of the entire transgenic insertion were also scrambled. A 2182 bp sequence at the 5' end of the inserted transgenes had partial homology to an unknown rice protein and a 250 bp sequence from the 3' flanking DNA had homology to a chloroplast *rsp*7 gene from rice, maize and wheat. Thus the 3830-1 insertion event appears to have resulted in the disruption of at least two plant genes. In this study the researchers were unable to identify a PCR product from wild-type genomic DNA using primers from different sides of the transgene insert, thus they were not able to determine the full extent of insertion-site mutation at event 3830-1. They suggested that large insertions of

scrambled filler DNA and/or a large deletion of genomic target sequence could be responsible for their inability to amplify a product.

A second insertion event, 3830-2, was isolated from the same transgenic line (Makarevitch *et al*., 2003). This event consisted of a 296 bp transgene insert of two noncontiguous fragments of transgene DNA. The 296 bp insert was flanked on both the 5' and 3' ends by scrambled non-contiguous fragments of plant genomic DNA (Makarevitch *et al*., 2003). A PCR product was amplified from wild-type genomic DNA using primers located approximately 950 bp from each end of the transgene insert. When this wild-type target DNA sequence was compared to sequences flanking the 3830-2 insertion event it was found that the insertion event included an 845 bp deletion of genomic DNA and also filler DNA insertions made from fragments of genomic DNA of unknown origin. Beyond this filler DNA, the remaining 360 bp of DNA sequence compared between the insertion event and the wild-type DNA were identical.

The third insertion event, 11929, was a relatively simple insertion event resulting from co-bombardment of 2 different plasmids. The inserted DNA consisted of a truncated copy of each plasmid, interspersed with filler DNA consisting of six small scrambled fragments of transgene and genomic DNA. PCR analysis suggested that the genomic DNA on either side of the inserted DNA was contiguous and unscrambled. However, using primers located on either side of the transgene insertion, the researchers were unable to amplify the original target sequence from wild-type DNA. They suggested this could be explained by a large deletion of genomic DNA at the insertion event.

PARTICLE BOMBARDMENT INSERTION EVENTS IN COMMERCIAL CROP PLANTS

In order to determine whether insertion-site mutations are present in commercial transgenic crop plants created by particle bombardment, we analysed the available molecular data describing five insertion events in the following crops: corn rootworm protected maize MON863[6], virus resistant papayas 55-1 and 63-1[7], insect resistant Maize YieldGard® MON810[8], and herbicide tolerant soybean Roundup Ready® Soybean 40-3-2[9].

Particle bombardment insertion event Mon863 from maize includes two different superfluous transgenic gene sequences, the *npt*II marker gene followed by 153 bp of the *ble* gene, integrated adjacent to the desired *cry*3Bb1 transgene. The *npt*II-*ble* DNA encodes two ORFs: the *npt*II coding sequence and 40% of the *ble* gene. No experimental data analysing whether the *npt*II-*ble* DNA produces RNA or protein products in Mon863 maize were submitted to the USDA. The USDA application states that the 5' and 3' junctions between the Mon863 insert and the genomic flanking sequences were analysed by PCR and DNA sequencing. However, all of the DNA sequence data were designated as confidential business information (CBI) and were thus deleted from the information available to the public. The application did not appear to contain a comparison between the flanking sequences and the original genomic target-site.

Papaya cultivars 55-1 and 63-1 were created by particle bombardment of papaya tissue with whole plasmids containing a transgene. Event 55-1 contains the following superfluous transgenic DNA in addition to the desired PRV *cp* transgene: the *npt*II selectable marker gene; the *gus* reporter gene; and vector backbone sequences including the OriT bacterial origin of replication and part of the tetracycline resistance gene. Northern blot analysis was used to examine mRNA transcripts present in papaya cultivar 55-1 (Fitch *et al*., 1992). This indicated that, in addition to the predicted 1.35 kbp transcript, two larger transcripts (one sized 2.4 kbp and one sized 4.4 kbp) were also present. Neither the presence nor the significance of these transcripts was mentioned in the USDA application. Sequence information on the transgene insert, the genomic flanking sequences and the original genomic target-site were not provided to the USDA.

Papaya cultivar 63-1 contained the following superfluous transgenic DNA sequences in addition to the desired PRV *cp* transgene: the *npt*II selectable marker gene and vector backbone sequences. These included the bacterial gentamycin resistance gene, the OriV and OriT bacterial origins of replication, and at least part of the tetracycline selectable marker gene. Southern blot data presented in both the USDA application and in a separate paper suggest that transgene rearrangements were present in 63-1 (Fitch *et al*., 1992), however no further molecular analysis was done and no further explanation was provided. Sequence information on the transgene insert, the genomic flanking sequences and the original genomic target-site were not provided to the USDA.

Maize YieldGard® event MON810 appears to contain only a single truncated copy of the desired *cry*A(b) transgene, as determined by Southern blot analysis. Sequence information on the transgene insert, the genomic flanking sequences and the original genomic target-site were not provided in the USDA application. However, independent researchers were unable to amplify the original genomic target sequences from wild-type maize using primer sequences derived from the genomic DNA flanking the MON810 *cry*A(b) insert (Hernandez *et al*., 2003). This suggests that the MON810 insertion event includes rearrangement or deletion of genomic sequences.

The Roundup Ready® Soybean 40-3-2 insertion event was described in the original USDA application as having only the desired single intact copy of the CP4 EPSPS gene. Sequence information on the transgene insert, the genomic flanking sequences and the original genomic target-site were not provided in the original USDA application. However, the combined data from documents submitted to the USDA by Monsanto after deregulation and from studies done by independent researchers (Windels *et al*., 2001) indicate that Soybean event 40-3-2 actually consists of the following: (a) the intact CP4 EPSPS gene, followed by a 250 bp CP4 EPSPS fragment which is adjacent to 534 bp of unknown DNA; (b) unidentified deletions and/or rearrangements which prevented target-site amplification from wild-type plants using primers made from DNA flanking the inserted transgenic sequences; and (c) a co-segregating 72 bp CP4 EPSPS fragment which is flanked on both sides by plant genomic DNA.

Genome-wide mutations in transgenic plants produced via *Agrobacterium*-mediated transformation

In addition to insertion-site mutations, transformed plants have heritable unintended genome-wide mutations that are not linked to the transgene. There exist a few studies in which researchers have attempted to quantify the numbers of genome-wide mutations introduced throughout the transgenic plant genome by plant transformation (reviewed in Sala *et al*., 2000). These studies use a combination of restriction fragment length polymorphism (RFLP) and PCR-based techniques to look for random DNA differences (mutations) between transgenic plants and non-transgenic control plants. These genome-wide mutations are visualised as DNA polymorphisms (band differences) between transgenic and non-transgenic plants.

Three papers describe the analysis of genome-wide mutations in transgenic plants created via *Agrobacterium*-mediated transformation. In two, the transformation protocol involved the use of tissue culture (Wang *et al*., 1996; Labra *et al*., 2001), while in one an *in planta* method was used, which avoids the use of tissue culture (Labra *et al*., 2004).

The first, of transgenic poplar, examined polymorphisms between 17 transgenic *P. nigra* plants (derived from 14 independent transformation events), four tissue culture control *P. nigra* plants (regenerated from tissue culture without transformation), and two controls of the original *P. nigra* clone (Wang *et al*., 1996). First, each plant was scored for the presence or absence of 18 different bands using RFLP analysis. No band differences were found between the six untransformed control plants and three of the 14 independent transgenic plants. However, the remaining 11 transgenic plants had between 1 and 8 band differences each, as compared to the control plants. In total 35 polymorphic bands out of 198 were found in the 11 plants combined.

The RFLP data extrapolates to approximately 1000s of polymorphic bands (mutations) per diploid genome. The same plants, and additional poplar clones belonging to different species, were then examined using random amplified polymorphic DNA (RAPD) analysis. Like the RFLP analysis, RAPD analysis indicated numerous genomic differences between control plants and transgenic plants. Using RAPD analysis, there were also polymorphic bands between tissue culture control plants. Finally, the plants were examined for microsatellite sequence differences. Only two of the transgenic lines and the two *P.nigra* control clones showed no microsatellite differences. The authors note that all of the transgenic and non-transgenic plants regenerated from *in vitro* culture showed DNA polymorphisms by one or more of the three techniques used and that, overall, transgenic plants showed greater polymorphism than tissue culture controls. The original *P.nigra* control clones did not show any DNA polymorphisms using any of the techniques.

The second study examined the level of polymorphism in 10 randomly selected transgenic rice (cv. *Taipei* 309) plants as compared to 10 randomly selected seed grown rice (cv. *Taipei* 309) control plants (Labra *et al*., 2001). RAPD analysis of the transgenic plants identified nine polymorphic bands out of 119 bands. Amplified fragment length polymorphism (AFLP) analysis of the same 10 transgenic plants identified 19 polymorphic bands out of 288 bands. No polymorphic bands were found in the control plants, using either RAPD or AFLP analysis. The authors of the study concluded "the genomic similarity value was 100% in the case of the control plants and 96-98% in the case of the transgenic population."

The third study was of *A. thaliana* plants transformed with a T-DNA containing the green fluorescent protein (GFP) transgene (Labra *et al*., 2004). Transformation was done using the floral dip method which avoids the use of tissue culture. This study looked for polymorphisms in the following: (a) 80 transgenic T_1 individuals (eight T_1 from each of 10 independent T_0 plants) selected for kanamycin resistance; (b) 80 transgenic T_1 individuals (eight T_1 from each of 10 independent T_0 plants) selected for GFP fluorescence, (c) 80 transformed T_1 individuals (eight T_1 from each of 10 independent T_0 plants) which did not show fluorescence (i.e. did not contain the transgene); (d) 25 non-transgenic individuals obtained from the seeds of five independent control plants; and (e) 18 plants regenerated from independent callus cultures. AFLP analysis of these plants found 3/80 plants from transgenic population (a) and 5/80 plants from transgenic population (b) had a total of three polymorphic bands and seven polymorphic bands respectively. Non-transgenic populations (c) and (d) had a total of two and three polymorphic bands respectively. The population with the most polymorphic bands was that of the non-transgenic plants regenerated from callus culture, which had 31 polymorphic bands in total. Random amplified microsatellite polymorphism (RAMP) analysis of the five populations showed no polymorphic bands. The authors interpreted these results as indicating that the majority of polymorphisms in transgenic plants derived from *Agrobacterium*-mediated transformation arise from tissue culture procedures, rather than from other aspects of the plant transformation process, such as *Agrobacterium* infection (Labra *et al*., 2004).

Genome-wide mutations in transgenic plants produced via particle bombardment

We have found only one study examining the numbers of genome-wide mutations introduced into transgenic plants by particle bombardment (Arencibia *et al*., 1998). In this study, rice plants were transformed by particle bombardment of immature embryos and transgenic T_0 plants were recovered from hygromycin-resistant embryonic cell clones. Twelve transgenic T_3 plants were analysed from each of three different rice cultivars. Non-transgenic seed-derived control plants and non-transgenic control plants regenerated from calli were also analysed. Using RAPD analysis, no polymorphic bands were found in the 36 transgenic rice genomes. AFLP analysis identified 12 polymorphic bands out of a total of 1711 bands in the 36 transgenic genomes. RAMP and amplified fragment random polymorphism (AFRP) analysis identified 10/566

polymorphic bands and 25/2526 respectively in the 36 transgenic genomes. The callus-derived control plants had polymorphism numbers similar to those of the transgenic plants while the non-transgenic seed-derived control plants showed no polymorphic bands. Extrapolation from the combined AFLP, RAMP and AFRP data suggest that in this experiment, particle bombardment resulted in, on average, many 100's of polymorphisms per diploid rice genome. It is important to note that these are heritable DNA changes as they were identified in the T_3 generation.

Origin and nature of genome-wide mutations

Many of the genome-wide mutations identified by polymorphism analysis probably arise from the use of tissue culture techniques (Arencibia *et al*., 1998; Labra *et al*., 2004). Tissue culture has long been known to be mutagenic and has sometimes been used intentionally as a mutagen to generate novel traits for plant breeding purposes (Larkin and Scowcroft, 1981; Jain, 2001). It has been shown to cause DNA changes ranging from point mutations and methylation differences to transposon induction, gene amplification, chromosomal aberrations and ploidy level changes (Phillips *et al*., 1994; Brown and Thorpe, 1995; Hirochika *et al*., 1996; Kaeppler *et al*., 2000; Jain, 2001; Bregitzer *et al*., 2002). Stresses associated with other aspects of plant transformation, such as the use of antibiotics, may also induce epigenetic and/or genetic changes to the plant genome (Bardini *et al*., 2003; Madlung and Comai, 2004).

Agrobacterium infection is also a potential source of genome-wide mutations. *Agrobacterium*-mediated transformation methods that do not involve tissue culture have been used to create large populations of T-DNA containing plants (Feldman, 1991; McElver *et al*., 2001). When such T-DNA tagging populations are screened for mutations, a large proportion of the identified mutant phenotypes are not linked to a T-DNA insertion event (Forsthoefel *et al*., 1992; McNevin *et al*., 1993; Negruk *et al*., 1996; Budziszewski *et al*., 2001). In one experiment, only 1/3 of the mutant phenotypes identified in the T-DNA tagging population co-segregated with a T-DNA (Budziszewski *et al*., 2001). A few such untagged mutations have been characterised by DNA sequence analysis. For example, two untagged *Cer2* mutant alleles have been isolated from a transformed *A. thaliana* population and sequenced (Negruk *et al*., 1996). One of the two alleles had a 17 bp deletion and the other had a 2 bp substitution and a 2 bp insertion. The authors suggest that such mutations might be the result of unsuccessful T-DNA insertions. It is worth noting that such small mutations would usually be missed by the polymorphism analysis techniques described above, and that therefore such genome sampling methods probably underestimate the numbers of genome-wide mutations in plants transformed by *Agrobacterium*-mediated transformation, and possibly also by particle bombardment. While particle bombardment is a suspected mutagen (Somers and Makarevitch, 2004), the effects of particle bombardment in the absence of tissue culture have not been studied.

Agrobacterium-mediated transformation and particle bombardment can both result in the insertion of small fragments of transgenic DNA at locations unlinked to the primary transgene insertion event. For example, when a single intact T-DNA insertion event was characterised in one plant, an additional T-DNA left border fragment was identified which mapped to another chromosome (Forsbach *et al*., 2003). Likewise, a particle bombardment line originally thought to have a single simple transgene insertion event was subsequently found to have two additional 'minor' insertions at separate genomic locations (Makarevitch *et al*., 2003). Insertions of small fragments of transgenic DNA during *Agrobacterium*-mediated transformation and particle bombardment may be common, and are another potential source of unintended phenotypes, however they are likely to be missed by standard Southern blot analysis of transgenic plants (Makarevitch *et al*., 2003).

The biological and biosafety implications of transformation-induced mutations

Any transformation-induced mutation which affects functional DNA sequences has the potential to result in unexpected phenotypic consequences. This is true for single base pair changes and for large deletions and rearrangements. Thus, in a commercial crop plant, every transformation-induced mutation is a potential hazard.

MUTATIONAL CONSEQUENCES OF PRECISE INSERTION EVENTS

Precise insertion events, those where a T-DNA or transgene inserts into genomic DNA without further genomic disruption, can create loss-of-function mutations which result in unintended phenotypes. These loss-of-function mutations can result from transgene insertion into gene coding or regulatory sequences, such as promoters or enhancers.

Precise insertion events may also result in the mis-expression of endogenous genes by disrupting, for example, a region of a promoter or enhancer that controls tissue-specific expression. The presence of strong transgene promoters, such as the commonly used cauliflower mosaic virus (CaMV) promoter, may also result in mis-expression (especially over-expression) of neighbouring endogenous genes. Such promoters have been shown to alter endogenous gene expression at a distance of up to 12 kbp (Wilson *et al*., 1996; Weigel *et al*., 2000; Jeong *et al*., 2002; Ichikawa *et al*., 2003).

Another possible result of precise transgene insertion is the production of aberrant sense or anti-sense RNAs. This has the potential to result in the silencing of endogenous genes or in the production of truncated or chimaeric proteins. For example, transcriptional read-through and mRNA processing were shown to occur when the *nos* terminator was used in a transgene present in a commercially approved insertion event (Rang *et al*. 2005). In this case, the aberrant transcripts were processed into variants containing open reading frames (ORFs) which could give rise to chimaeric proteins[10].

REARRANGEMENTS, DELETIONS AND SUPERFLUOUS DNA AT TRANSGENE INSERTION EVENTS

Insertion events associated with deletions and rearrangements of genomic DNA and the insertion of superfluous DNA may substantially increase the amount of transformation-induced genomic disruption and thus increase the risk of unintended phenotypes. These types of insertion-site mutations may result in the juxtaposition of transgene and genomic fragments or the juxtaposition of non-contiguous fragments of genomic DNA. Such scrambling can often be extensive, particularly at particle bombardment insertion events. For example, DNA sequence analysis of the simple particle bombardment insertion event 3830-1 from transgenic oat demonstrated the presence of at least 17 non-contiguous fragments of intermixed genomic and transferred DNA (Makarevitch *et al*., 2003).

Loss-of-function mutations

Loss-of-function phenotypes, for example, can arise from deletion of endogenous gene coding or promoter sequences, rather than from T-DNA insertion itself (Filleur *et al*., 2001). Furthermore, insertion-site deletions have been found which remove more than one gene, sometimes resulting in complex pleiotropic phenotypes (Revenkova *et al*., 1999; Kaya *et al*., 2000).

Altered gene expression

Insertion-site mutations may also result in altered patterns of gene expression. Mutations which delete or rearrange regulatory sequences (such as promoter or enhancer sequences) or cause other

genomic alterations which affect gene expression (e.g. to gene order or spacing or to higher order genome structure) could result in increased or decreased gene expression or in the mis-expression of plant genes in inappropriate cell or tissue types or at inappropriate developmental times. Importantly, regulatory mutations may also alter a plant's response to external environmental cues, such as drought or high temperature, for example, by causing inappropriate genes to be activated.

Aberrant RNAs leading to gene silencing

Scrambling of promoter fragments and coding sequence may also result in gene silencing by the creation of either sense or anti-sense transcripts, since both gene over-expression and anti-sense RNAs can trigger silencing mechanisms in plants (Iyer *et al*., 2000). Insertions of duplicated fragments of genomic DNA, including large fragments such as those seen in translocations (Tax and Vernon, 2001) or small fragments such as those present in filler DNA, provide opportunities for creating anti-sense RNA. If the duplications include gene sequences, transcription into or through these sequences may generate anti-sense RNAs, resulting in silencing of the gene from which the duplication originated.

Analysis of a mutation in a non-transgenic rice plant exemplifies how DNA rearrangement may result in RNA silencing of a gene family (Kusaba *et al*., 2003). In this case, a deletion between two highly similar gene family members formed a tail-to-tail repeat and removed a transcription termination signal. The resulting transcript was thought to produce a double-stranded RNA (via a hairpin loop) which activated gene silencing of various family members. Thus, the presence of anti-sense RNA may also result in silencing of entire gene families.

A further possibility is that inadvertently activated gene silencing may have off-target effects, altering the regulation of unrelated genes (Jackson and Linsley, 2004).

Aberrant RNAs leading to truncated or chimaeric proteins

Insertion-site mutations increase the probability of creating aberrant RNAs that encode truncated or chimaeric proteins that have altered regulation or function. Deletions, insertions or duplications could result in proteins that have lost or gained substrate binding sites, active domains, cellular localisation signals or regulatory sites, such as phosphorylation sites. The resulting proteins could be constitutively activated or de-activated, localised to incorrect compartments of the cell or have altered functions. For example, a transformation-induced deletion could remove a part of the coding sequence of a receptor protein, resulting in production of a non-functional protein that that was still able to bind to the other subunits in a multi-protein complex. The presence of the non-functional subunit could inactivate the complex.

Insertion-site mutations and horizontal gene transfer

Insertion-site mutations involving the integration of specific types of superfluous DNA pose an additional risk. Insertions of superfluous bacterial DNA flanking the transgene (e.g. vector backbone, marker DNA, and particularly origins of replication) have the potential to facilitate horizontal gene transfer of transgenes into soil or gut bacteria by providing opportunities for homologous recombination (De Vries and Wackernagel, 2002; Prudhomme *et al*., 2002).

To our knowledge, no one has specifically examined the molecular nature of the genome-wide mutations present in transgenic crop plants. Furthermore, except for the characterisation of a few non-tagged mutant alleles isolated from T-DNA tagging lines, no one has ever identified the molecular basis of a genome-wide mutation in a transgenic plant. As previously discussed, both *Agrobacterium*-mediated transformation and particle bombardment can result in the integration, at sites unlinked to the desired transgene, of small fragments of superfluous DNA, and tissue culture has been shown to result in base pair changes, transposon movement, methylation changes, chromosomal rearrangements and ploidy level changes (Kaeppler *et al*., 2000). Thus, it is likely that the genome-wide mutations found in transgenic plants will result in loss-of-function mutations, altered gene expression and altered protein function by mechanisms similar to those described for insertion-site mutations.

While the molecular mechanisms are unknown, there are, however, examples of genome-wide mutations causing unintended phenotypes. For example, genome-wide mutations have been found in rice that decrease grain size (Wu *et al*., 2002) or alter chlorophyll content, plant height, seedling growth and yield (Shu *et al*., 2002). These mutations were still present in the T_3 and later generations of both the transgenic and non-transgenic progeny of the original T_1 transgenic plants. The heritability of such mutations suggests the presence of genome-wide mutations increases the risk of unintended consequences in transgenic cultivars.

Conclusions

As illustrated in this review, the assumption that transgenic plant breeding methods are precise is undermined by the available scientific data. Transformation-induced mutations are created both at the transgene insertion-site and elsewhere in the genome. Most transgenic plants are likely to have both types of mutations, whether transformed using *Agrobacterium*-mediated methods or particle bombardment.

Insertion-site mutations can include small or large deletions and rearrangements of plant genomic DNA and multiple insertions of superfluous DNA at a single insertion event. Rearrangements may include chromosomal translocations and extensive scrambling of transgenic and genomic DNA, while superfluous DNA insertions may include filler DNA, vector backbone and additional transgene DNA. Particle bombardment insertion events may also include contaminating bacterial chromosomal DNA. In addition to insertion-site mutations, most transgenic plants carry minimally 100s-1000s of genome-wide mutations, unless these have been removed by out-crossing or back-crossing.

While it is clear that current plant transformation methods are mutagenic, more data on the frequency and molecular basis of transformation-induced mutations are still needed. For example, most of the data that describe insertion-site mutations created by *Agrobacterium*-mediated transformation come from a few large-scale analyses in *A. thaliana.* Our knowledge of insertion-site mutations in other species (including important crop plants) is based on studies of, at most, a few transgenic individuals, and most insertion events were analysed incompletely.

The lack of scientific data is even greater for particle bombardment. There are no large-scale studies of insertion-site mutations for any species, as only a handful of particle bombardment insertion events have been (even partially) characterised using DNA sequence analysis. Thus, to date, there are no publicly available data describing the complete characterisation of a functional transgene insertion event produced via particle bombardment.

Similarly, there are few quantitative molecular analyses of genome-wide mutations in transgenic crop plants and there are no analyses of their molecular basis. It is therefore unclear whether plant transformation is more mutagenic in some species than in others.

Genetic damage is not limited to experimental transgenic plants. The insertion events present in transgenic cultivars are not fully characterised prior to commercialisation (Wilson *et al*., 2004) and independent analyses of two commercialised cultivars found uncharacterised and potentially extensive insertion-site mutations (Windels *et al*., 2001; Hernandez *et al*., 2003). This suggests other commercial cultivars are also likely to have undetected insertion-site mutations. Additionally, commercial cultivars will almost certainly have undetected genome-wide mutations, even if most have been removed by genetic segregation.

The seriousness of the risks arising from the presence of transformation-induced mutations in commercial cultivars depends on their phenotypic consequences. There are three general classes of unintended consequences that pose particular risks to the public. The first are alterations to the toxicity or nutritional value of a transgenic cultivar. This class would include mutations that increased the levels of allergens or toxins (known or unknown) or altered the levels of nutrients such as vitamins or antioxidants[11]. The second are changes that have ecological implications, such as mutations that increase out-crossing in transgenic cultivars or mutations which adversely affect beneficial insects (e.g. plant pollinators), soil organisms or other wildlife. The third are changes that have implications for food security. These include mutations that decrease resistance of transgenic crops to stresses, such as disease or pest attack, or which decrease drought or heat tolerance. Under certain environmental conditions, the use of transgenic cultivars carrying such mutations could result in wide-spread crop failures. Such crop failures have occurred in the past. For example, the use of non-transgenic maize that carried a mutation conferring both male sterility and susceptibility to a specific race of corn blight led to wide-spread failure of the 1970 U.S. corn crop. The large-scale production and consumption of crops having unintended mutations that result in one of these three classes of harmful phenotypes could thus result in serious consequences.

In theory, the commercialisation of transgenic cultivars carrying harmful unintended traits could be prevented by extensive pre-market phenotypic testing. This could include extensive mRNA profiling, metabolic profiling and specific analysis of nutrients and plant toxins, as well as extensive greenhouse trials and field trials[12] (Kuiper *et al*., 2001; Freese and Schubert, 2004). However, recent reviews indicate that the current regulatory practices of both the U.S. and Europe are not likely to safeguard the public from unexpected biosafety issues, such as those arising from transformation-induced mutations (Freese and Schubert, 2004; Spok *et al*., 2004; Pelletier, 2005). The significance of these points is underlined by the fact that unexpected traits, including potentially harmful ones, are frequently found in transgenic plants (Kuiper *et al*., 2001; Haslberger, 2003; Cellini *et al*., 2004), including commercialised transgenic cultivars which have already passed through the regulatory process (Gertz *et al*., 1999; Lappe *et al*., 1999; Saxena and Stotzky, 2001; Ridley *et al*., 2002).

The inability of current regulatory practice to identify and prevent the commercialisation of transgenic crop plants with potentially harmful unintended genotypic and phenotypic consequences stems, at least in part, from two factors. The first is that current genotypic and phenotypic analyses of commercial transgenic cultivars are insufficient. At the genotypic level, complete analysis of insertion-site mutations is not required by regulators and no analysis of genome-wide mutations is required. At a phenotypic level, few biochemical analyses are performed and no metabolic or RNA profiling studies are carried out. Furthermore, while some field trials are done, many important unintended consequences would be hard to identify, especially those that are conditional, in that deficiencies would only be apparent under specific conditions, such as drought or pathogen attack.

Secondly, even when unexpected molecular or phenotypic differences are found between commercial transgenic cultivars and control plants, they are ignored by regulators, both pre- and post-commercialisation (CZW-3 squash[11]; Windels *et al*., 2001; Rang *et al*., 2005). This may occur in part because there are currently no standardised species-specific guidelines to help regulators determine which differences are potentially harmful and which are not (Pelletier,

2005). Furthermore, there are no guidelines to indicate which differences merit further testing. The scientific data which would enable regulators to formulate adequate guidelines is currently lacking (Pelletier, 2005).

An emerging risk factor is the production of transgenic plants having multiple transgenes via introduction of several independent transgene insertion events into a single plant. This could be done either by crossing two independently transformed transgenic plants or by re-transforming a transgenic plant with additional transgenes (gene-stacking). In either case, plants with multiple insertion events are likely to have more transformation-induced mutations and thus carry a greater risk of exhibiting unintended consequences.

Transformation-induced mutations are not the only potential cause of the variable and unexpected phenotypes arising in transgenic plants. Unexpected functions of the transgene and off-target effects of transgene silencing may also result in unexpected phenotypes (Schubert, 2002; Jackson and Linsley, 2004; Wilson *et al*., 2004). However, this review makes it clear that the presence of transformation-induced mutations in commercial crop plants poses a potentially large and also unnecessary biosafety risk. The use of more precise plant transformation methods, coupled with improved analysis and selection criteria, and more stringent regulation of commercial transgenic crop plants are urgently needed to decrease the risk of harmful unintended consequences in transgenic crop plants. Specific recommendations are made in the next section.

PREVENTING COMMERCIALISATION OF TRANSGENIC CROP PLANTS CARRYING UNINTENDED TRANSFORMATION-INDUCED MUTATIONS: RECOMMENDATIONS FOR REGULATORS AND TRANSGENIC PLANT BREEDERS

Transformation-induced mutations are an unintended by-product of plant transformation technology and can in theory be either prevented or eliminated from commercial transgenic cultivars.

Preventing insertion-site mutations

Methods should be sought which decrease the number of insertion-site mutations created during plant transformation. One simple improvement would be the use of gene cassettes rather than whole plasmids for particle bombardment. Until recently, researchers and breeders mostly used circularised plasmid DNA[13]. Recent studies show that linear gene cassettes (transgene DNA which has been purified away from plasmid sequences) can be used to generate particle bombardment insertion events that may include less superfluous DNA (Fu *et al*., 2000; Breitler *et al*., 2002; Loc *et al*., 2002; Popelka *et al*., 2003). However, further studies, including DNA sequence analysis and comparison with original target sequences, will be needed to determine whether the use of gene cassettes reduces the number and frequency of genomic deletions and rearrangements at the insertion event. To our knowledge, such experiments have not yet been described in the scientific literature.

Another strategy that could be explored is the development of new T-DNA vectors and/or the modification of T-DNA border sequences, with the goal of introducing fewer mutations during T-DNA insertion.

Changes should be made to transgene regulatory components to minimise their effects on endogenous sequences. For example, effective transcription termination signals must be found, and tested experimentally, to replace ‘leaky’ terminators, such as the *nos* terminator, that allow read-through transcription to occur. In addition, transgene promoter sequences should be analysed to determine their effects on neighbouring genes. Promoters used in commercial lines should be shown experimentally to have no effect on neighbouring genes.

Preventing genome-wide mutations

Methods that avoid the use of known or suspected mutagens, such as tissue culture and antibiotics, could decrease the number of genome-wide mutations in transgenic plants. *In planta Agrobacterium*-mediated transformation methods that do not require tissue culture have been developed for a few species, notably *A. thaliana* (Clough and Bent, 1998), the legume *Medicago trunculata* (Trieu *et al*., 2000), petunia (Tjokrokusumo *et al*., 2000) and radish (Curtis and Nam, 2001). It is also possible that the use of methods which avoid dedifferentiation in tissue culture, such as those which have been developed for peanut (Rohini and Rao, 2001), rice (Park *et al*., 1996) and tobacco (Touraev *et al*., 1997; Aziz and Machray, 2003), or those which use shoot meristematic cultures (Zhang *et al*., 1999) could also decrease the number of genome-wide mutations introduced into transgenic plants.

The use of antibiotic selection during plant transformation can be avoided by the use of PCR-based or protein assays, rather than selectable markers, to identify plants containing transgenes. This would remove another potential source of genome-wide mutations. PCR selection has been shown to be feasible (De Vetten *et al*., 2003) and would have the added benefit that elimination of marker genes would remove a common source of superfluous DNA..

It is unlikely that changes to transformation methods can eliminate all genome-wide mutations, and extensive out-crossing or back-crossing of transgenic plants should be required. Various studies show that genome-wide mutations can remain in later generations, and appropriate methods should be developed to monitor the effectiveness of back-crossing and out-crossing programs (Sala *et al*., 2000; Shu *et al*., 2002; Wu *et al*., 2002).

Analysis of insertion-site mutations

Improved analysis and selection of transgene insertion events is also necessary to prevent plants having unintended consequences from reaching the market. Currently most transgene insertion events found in commercial cultivars have only been analysed using Southern blot techniques. In addition to Southern blot analysis, we recommend the following:

1. Full sequencing of the transgene insertion event including the transgene and a minimum of 50 kbp of flanking DNA on each side.
2. Isolation and sequencing of the genomic target sequences from the untransformed parent cultivar and comparison to the insertion event. Together with Recommendation # 1, this, and only this, will allow the identification of insertion-site mutations such as DNA deletions and rearrangements and superfluous DNA insertion.
3. Production and screening of sufficient numbers of transgenic lines such that only insertion events into non-functional sequences and those lacking insertion-site mutations are chosen for potential commercialisation.

Reducing the risks arising from transformation-induced mutations thus requires (1) improvements to plant transformation methods, (2) the complete characterisation of insertion events and the selection of mutation-free cultivars for commercialisation, and (3) thorough pre-market testing of transgenic crop plants (Kuiper *et al*., 2001; Pelletier, 2005). These changes to current plant transformation and regulatory practice, coupled with the establishment of robust post-market monitoring of the agronomic, health and ecological impacts of transgenic cultivars, are needed to ensure that the biosafety risks arising from the unintended mutagenic consequences of plant transformation are minimised in commercial transgenic cultivars.

Note added in proof

To our knowledge, Roundup Ready maize insertion event NK603 is now the first and only intact transgene insertion event produced via particle bombardment which has been characterised by DNA sequencing of (a minimal amount of) flanking sequence and comparison with the non-transgenic insertion-site [Heck *et al*. (2005), published in *Crop Science* **44:** 329-339]. In addition to a single copy of the transgene insert, the insertion event included a 217 bp inverted duplication of the transgene insert and 301 bp of maize plastid DNA, as well as a 3 bp deletion of the maize insertion-site DNA.

Endnotes

[1] Insertion should be into regions of the genome where there is experimental evidence to support the claim of no known function.
[2] USDA application # 02-042-01p.
[3] USDA application # 99-173-01p.
[4] USDA application # 95-352-01p.
[5] In total we examined the available molecular data describing the insertion events present in 8 deregulated transgenic crop plants: 3 created by *Agrobacterium* -mediated transformation and 5 created by particle bombardment. Most of the data we discuss come from the applications submitted to regulators. When additional data are available in papers published in the scientific literature we note this and provide a reference. The full list of commercial transgenic crop plants currently deregulated or pending deregulation in the United States can be obtained from the following Website: http://www.aphis.usda.gov/bbep/bp/petday.html. The applications submitted to the USDA can be ordered from the Animal and Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) by citing the listed application numbers. Further information on each of the 8 deregulated insertion events can also be found in our original report: in Sections 1.1.7 and 1.2.6; Table 2; and the Appendix. (Wilson *et al*. 2004).
[6] USDA application # 01-137-01p.
[7] USDA application # 96-051-01p.
[8] USDA Application # 96-017-01p.
[9] USDA application # 93-258-01p.
[10] Plants carrying the Roundup Ready® Soybean 40-3-2 insertion event have been shown to transcribe at least 150 bp of the superfluous 250 bp CP4 EPSPS fragment which is inserted adjacent to the functional CP4 EPSPS transgene (Rang *et al*., 2005). A read-through product is made when transcription of the functional CP4 EPSPS transgene fails to terminate at the *nos* promoter. This read-through product is processed to create four different RNA variants. Furthermore, mRNA processing results in the generation of open reading frames which code for putative EPSPS fusion proteins and these fusion proteins also include 24 amino acids derived from the genomic DNA adjacent to the EPSPS fragment. As the *nos* terminator has been used as a regulatory region in transgenes found in other transgenic commercial cultivars, the formation of aberrant read-through transcripts may also occur in other transgenic commercial cultivars. Such read-through transcripts can occur at precise insertion events or, as in the case of the Soybean 40-3-2 insertion event, they can involve insertion-site mutations.
[11] For example, commercial squash cultivar CZW-3 was found to have 67.6 times less Beta Carotene than the control squash (USDA Application # 95-352-01p).
[12] It is important that all such field trials have safeguards to prevent gene flow.
[13] Of the five commercial particle bombardment insertion events analysed in this review, only one, Mon863, was created using purified gene cassette DNA rather than whole plasmid DNA.

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