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Technical Report
October 2004

Genome Scrambling - Myth or Reality?

Transformation-Induced Mutations in Transgenic Crop Plants

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Summary of Report

Internationally, safety regulations of transgenic (genetically modified or GM) crop plants focus primarily on the potential hazards of specific transgenes and their products (e.g. allergenicity of the *B. thuringiensis cry*3A protein). This emphasis on the transgene and its product is a feature of the case-by-case approach to risk assessment. The case-by-case approach effectively assumes that plant transformation methods (the techniques used to introduce recombinant DNA into a plant) carry no inherent risk. Nevertheless, current crop plant transformation methods typically require tissue culture (i.e. regeneration of an intact plant from a single cell that has been treated with hormones and antibiotics and forced to undergo abnormal developmental changes) and either infection with a pathogenic organism (*A. tumefaciens*) or bombardment with tungsten particles. It would therefore not be surprising if plant transformation resulted in significant genetic consequences which were unrelated to the nature of the specific transgene. Indeed, both tissue culture and transgene insertion have been used as mutagenic agents (Jain 2001, Krysan *et al.* 1999).

In this report we examine the mutations introduced into transgenic crop plants by plant transformation. We have searched and analysed the relevant scientific literature for *Agrobacterium*-mediated transformation and particle bombardment, the two most frequently used plant transformation methods. We have also analysed the molecular data submitted to the USDA in applications requesting commercial approval for transgenic cultivars. Lastly, we have examined whether mutations arising from plant transformation have the potential to be hazardous and whether current safety tests are robust enough to detect hazardous mutations before they reach the market.

Transformation-induced mutations: In theory, plant transformation could result in exact insertion of a single transgene without further genomic disruption. In practice, this rarely, if ever, occurs. As we demonstrate in this report, in addition to the transgene, each transformed plant genome contains a unique spectrum of mutations resulting from (a) tissue culture procedures, (b) gene transfer methods such as *Agrobacterium*-mediated or particle bombardment transfer, (c) transgene insertion and (d) superfluous DNA insertion[1]. These transformation-induced mutations can be separated into two types: those introduced at the site of transgene insertion, which we refer to as *insertion-site mutations* and those introduced at other random locations, which we refer to as *genome-wide mutations*.

Insertion-site mutations: Our search of the primary literature revealed that remarkably little is known about the mutations created in crop plants at the site of transgene insertion. This is true both for transgene insertion via *Agrobacterium*-mediated transformation (**Section 1.1**) and for particle bombardment (**Section 1.2**). This lack of understanding is caused in part by a lack of large-scale systematic studies of insertion-site mutations (**Sections 1.1.5** and **1.2.4**). Additionally, much of the available data comes from research on a non-crop plant, *Arabidopsis thaliana*, and it is not clear whether such results apply to crop plants.

Agrobacterium-mediated transformation: *Agrobacterium*-mediated transformation has been used to create commercial cultivars for over 10 years and is known to create insertion-site mutations (**Table 2**, **Section 1.1**). However, there has been only one large-scale study of the mutations created at insertion events[2] containing single T-DNA[3] inserts (the type of event preferred for commercial purposes; Forsbach *et al.* 2003). In this study of 112 single-copy T-DNA insertion events in *A. thaliana*, the researchers found that exact T-DNA integration almost never occurred (Forsbach *et al.* 2003). Most of the T-DNA insertions resulted in small (1-100 base pair) deletions of plant genomic sequences at the insertion-site. However, for a significant number (24/112) there was evidence for large-scale rearrangement of plant genomic DNA at the insertion-site. Two of these insertion events contained chromosomal translocations. The rest had rearrangements which were not fully characterised. It is known, however, that rearrangements of genomic DNA at T-DNA insertion sites can be very substantial. A 78Kbp deletion (removing 13 genes) is the largest recorded for T-DNA insertion (Kaya *et al.* 2000) and other researchers have reported duplication and translocation of a segment of DNA at least 40 Kbp in size (Tax and Vernon 2001). Superfluous DNA insertion is also a common feature of T-DNA insertion-sites (**Sections**

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1 Superfluous DNA is defined as any transferred DNA other than a single copy of the desired transgene and includes: marker gene sequences, bacterial plasmid sequences, fragments of bacterial genomic DNA, and additional whole or partial copies of the transgene.
2 A transgene insertion event consists of the transgene and its flanking sequences.
3 The T-DNA is the segment of DNA bounded by the T-DNA borders which is transferred to a plant via *Agrobacterium*-mediated transformation. The T-DNA contains the desired transgene and often contains marker DNA. It is carried on the Ti plasmid and sometimes plasmid DNA outside the T-DNA borders is also transferred.

1.1.1-**1.1.3**). For example, Forsbach *et al.* (2003) found that 8 of their 112 single copy T-DNA insertion events also had large insertions of superfluous plasmid or T-DNA sequences. The majority of the remaining lines had insertions of 1-100 bp of DNA of undefined origin.

The results of these and other studies suggest that the vast majority of T-DNA insertion events include small or large genomic DNA disruptions and insertions of superfluous DNA.

Particle bombardment transformation: Particle bombardment has also been used to create numerous commercial cultivars (**Table 2**). Although it can result in large scale genomic disruption, there are few studies detailing the insertion-site mutations resulting from particle bombardment (**Section 1.2**). Furthermore, there have been no large-scale systematic studies of such mutations.

Most of the particle bombardment insertion events that are described in the scientific literature are extremely complex (Pawlowski and Somers 1996). Multiple copies of delivered DNA are often interspersed with small or large fragments of plant genomic DNA (Kohli *et al.* 2003). One paper even reported the insertion of bacterial chromosomal DNA at a particle bombardment insertion-site (Ulker *et al.* 2002).

Without the use of PCR and DNA sequencing, analyses of insertion-site mutations are likely to be incomplete. We have found only two particle bombardment studies where PCR and DNA sequence analyses were used to characterise mutations created at single-copy insertion events which had been isolated from intact plants. In one paper (Makarevitch *et al.* 2003), 3 insertion events were analysed, in the other (Windels *et al.* 2001), the commercialized Roundup Ready® soybean insertion event 40-3-2 was analysed. The mutations present at each of these four 'simple' insertion events appeared to include large-scale genomic deletions and/or rearrangements, in addition to stretches of scrambled genomic and transferred DNA (Makarevitch *et al.* 2003, Windels *et al.* 2001). For example, in addition to the intended EPSPS[4] transgene described in the original application, soybean event 40-3-2 included a 254 bp EPSPS gene fragment, a 540 bp segment of unidentified DNA, a segment of plant DNA and another 72 bp fragment of EPSPS, as well as additional unspecified genomic alterations (Windels *et al.* 2001, USDA petition 93-258-01p). These insertion event mutations were only reported after commercialisation of Roundup Ready® soybean insertion event 40-3-2. It is interesting that independent analysis of another commercialized cultivar suggested that Maize YieldGard® insertion event Mon810 also includes additional unspecified and previously unreported insertion-site mutations (Hernandez *et al.* 2003).

For particle bombardment insertion events, we could find no study in which the sequence of a transgene insertion-site was successfully compared to the original undisrupted site (**Section 1.2.4**). Thus the full extent of mutation at a transgene-containing particle bombardment insertion-site has never been reported, either in the scientific literature or in applications submitted to regulators[5]. The existing sequence data describing particle bombardment insertion events are thus extremely limited. However, these data suggest that transgene integration at particle bombardment insertion events is always accompanied by substantial genomic disruption and superfluous DNA insertion.

Southern blot analysis is insufficient to identify all insertion-site mutations: Another limitation to the understanding of insertion-site mutations is that Southern blot hybridisation is the technique most commonly used to analyse transgene insertion events for both research and regulatory purposes (Kohli *et al.* 2003). Analysis of transgene insertion-sites by other techniques such as FISH, PCR or DNA sequencing indicates that Southern blot analysis is not sufficient to reliably determine either the presence of superfluous DNA or the extent of genomic disruption at the transgene insertion-site (**Sections 1.1.4** and **1.2.3**). For example, Mehlo *et al.* (2000) used both PCR and Southern Blot analysis to analyse particle bombardment insertion events and concluded that Southern blotting was useful only in detecting large-scale features of the transgene insertion-site. In another study, fiber-FISH techniques were used to analyse a particle bombardment insertion event which was predicted by Southern blotting to contain tandem repeats of a transgene (Svitashev and Somers 2001). Their analysis revealed that there were actually 3-10 Kbp of chromosomal DNA between most of the repeats. This suggests that, in some cases, Southern blot analysis is inadequate for identifying even large-scale rearrangements.

These and other reports lead us to draw various conclusions. Firstly, that analysis of transgenic lines based solely or primarily on Southern blot data can miss many of the mutations present at insertion-sites. Thus, the plant genome is probably more disrupted by transgene insertion than commonly supposed. Secondly, that, as almost all commercial approvals of transgenic events or cultivars are based primarily on Southern blot analysis of transgene insertion (**Table 2**, **Appendix**), it is likely that most transgenic events currently approved for commercial use harbour unreported large and small-scale transgene insertion-site mutations.

Genome-wide mutations: In this report we also examine what is known about mutations which are introduced as a result of tissue culture and gene transfer procedures but which are not associated with insertion of the transgene (**Section 2**). There are 5 studies in which researchers have attempted to quantify the number of mutations introduced during plant transformation (reviewed in Sala *et al.* 2000). These researchers used DNA polymorphism analysis (based on RFLP, AFLP and other PCR techniques) to compare the genomes of transformed plants to the genomes of non-transformed control plants. Their results suggest that many hundreds or thousands of such genome-wide mutations are likely to be present in plants transformed using typical plant transformation methods, especially those involving the use of plant tissue culture techniques (**Section 2.3**). In one study, Labra *et al.* (2001) 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. Even though the numbers of mutations found in these studies were high, the nature of the analytical techniques used in these experiments suggests that these

4 The EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene from *Agrobacterium* sp. Strain CP4 confers tolerance to the herbicide glyphosphate.

5 Makarevitch *et al.* (2003) were able to compare the insertion-site of a 296 bp transgene fragment to its target site. They found the insertion event included rearrangement of the genomic DNA flanking the fragment and an 845 bp deletion of genomic DNA.

figures may underestimate the extent of mutation to the plant genome (**Section 2.5**). Also, such studies do not address the nature of these mutations, such as whether they are small scale or large-scale genomic changes and whether they occur in functional regions of the genome.

Depending on the extent of outcrossing or backcrossing undergone by the primary transformant, many and sometimes all of the mutations created in the primary transformant are likely to be retained in commercialised cultivars (**Section 4.3**). Even where backcrossing has been extensive, genome-wide mutations genetically linked to the transgene insertion-site probably remain in the commercial cultivar.

Genome-wide mutations have been found in all transformed plants examined and such mutations have been shown to be heritable (Sala *et al.* 2000). However, current safety regulations do not require any testing or analysis of genome-wide mutations in commercial cultivars.

Significance of transformation-induced mutations: Insertion-site and genome-wide mutations can be hazardous if they occur in a functional region of plant DNA (**Section 3**). Mutations in functional plant DNA, including gene coding sequences or regulatory sequences, may have implications for agronomic performance or environmental interactions or for animal or human health. For example, a transformation-induced mutation might disrupt a gene whose product is involved in nutrient biosynthesis, resulting in altered nutrient levels, or it might disrupt or alter a gene involved in the regulation or synthesis of compounds toxic to humans. Disruption of a gene encoding a regulatory protein, such as a transcription factor, could result in the miss-expression of numerous other genes. Such biochemical changes would be unpredictable and difficult to identify even with extensive biochemical testing (Kuiper *et al.* 2001). Typically, only a few biochemical tests are required by regulators. Therefore, using current safety assessments, many of the harmful phenotypes which could arise from transformation-induced mutations would be unlikely to be identified prior to commercialisation.

Frequency of disruption of functional DNA by transformation-induced mutations: The limited amount of data available suggests that transgenes frequently insert into or near gene sequences[6] (**Section 1.1.6**). In the few plant species studied, DNA sequence analysis of T-DNA insertion-sites suggests that approximately 35-58% of transgene insertions disrupt plant gene sequences (Forsbach *et al.* 2003, Jeong *et al.* 2002, Szabados *et al.* 2002). Similar studies of transgenes delivered via particle bombardment have never been conducted (**Section 1.2.5**).

Despite its importance for safety assessment, it is usually not clear whether transgenes in commercial lines have inserted into or near gene sequences. Most applications submitted to the USDA requesting permission to commercialise a transgenic line provide neither the sequence of the genomic DNA flanking the inserted transgene nor a comparison with the original target-site sequence (**Table 2***,* **Appendix**). An added difficulty in determining the significance of an insertion event is that it is currently not possible to know with certainty that a region of the genome is non-functional[7].

The frequency with which genome-wide mutations disrupt functional DNA has never been specifically investigated. However, the successful use of tissue culture to induce mutations for research and breeding purposes (**Section 2.1**) and the isolation, from populations of transformed plants, of mutant phenotypes which are not linked to a transgene insert (**Section 2.2**) both suggest that genome-wide mutations do frequently occur in functional DNA sequences.

Even if no functional sequences are disrupted, transgene and superfluous DNA insertions are not necessarily harmless or inert. Promoter sequences may alter the expression of neighbouring genes (Weigel *et al.* 2000), while bacterial chromosomal or plasmid sequences (bacterial origins of replication in particular) inserted adjacent to the transgene may enhance the probability of horizontal gene transfer (**Section 3.2**). Of the 8 commercial cultivars and events that we analysed for this report, 6 had insertions of superfluous bacterial and/or viral DNA at the insertion event (**Table 2, Appendix***,* **Sections 1.1.7** *and* **1.2.6**).

Appropriate safety assessment of transgenic crop plants: In support of the case-by-case approach to regulation and risk assessment, it is often suggested that genetic engineering is as safe as other modern plant breeding technologies. We analyse the assumptions behind this assertion with respect to the plant transformation techniques used to genetically engineer a transgenic plant (**Section 4**). First we note that the hazards arising from other types of plant breeding technology are not well characterised (**Section 4.1**). Second we note that 'safety' has never been measured either absolutely or relatively for any method of plant breeding, making comparisons between breeding methods difficult, if not impossible (**Section 4.4**). Therefore, we suggest that to try and determine the risks arising from plant transformation by comparing it to other plant breeding methods is neither logical nor even possible. We argue instead that proper safety assessment of transgenic crop plants requires scientific analysis of the specific hazards and risks arising from genetic engineering (**Section 4.5**). As well as the specific risks arising from the transgene, these risks would include risks which arise from plant transformation methods.

Conclusions: This report identifies the insertion-site and genome-wide mutations created by plant transformation procedures as potentially major, but poorly understood, sources of hazard associated with the production and use of commercial transgenic cultivars.

We suggest that an understanding of the implications of transformation-induced mutations urgently needs to be incorporated into regulatory frameworks (**Section 5**). To facilitate this, we make various recommendations (**Section 6**), including a requirement for complete analysis of insertion-site and genome-wide mutations in transgenic cultivars prior to commercialisation. We suggest that

6 It should be noted that because transgene-containing cells or plants are usually identified by selecting for the expression of a marker gene, current plant transformation methods are actively selecting for insertion events occurring in functional transcribed (and thus gene-rich) regions of the genome.

7 Other factors increase the difficulty in determining whether insertion into a particular region of the genome or the presence of a particular insertion-site mutation is without consequence. In other higher eukaryotes, long-range regulatory interactions are common (Carter *et al.* 2002). In other words, regulatory sequences can be hundreds of Kbp away from the gene coding sequences or even act *in trans.* There is also evidence in many cases that genes are clustered in the genome and that gene order can be important for gene regulation (Hurst *et al.* 2004).

changes to both transgenic plant breeding practices and to the regulation of transgenic crop plants are required so that hazardous mutations are either prevented, or identified and removed, prior to commercialisation.

As discussed in this report, food crops are not inherently safe. All plants produce harmful substances and many food crops are derived from inedible ancestors and may contain toxic tissues or organs. They therefore have within them the genetic potential to cause harm. Consequently, the genetic stability of cultivars in the plant breeding pool is crucial if plant breeders are to produce reasonably safe cultivars. The presence of transformation-induced mutations poses a threat to this stability that is potentially very serious and that is also entirely unnecessary. In addition, the pool of cultivars available to farmers is declining and certain cultivars are grown on a large scale worldwide. Consequently, ensuring the safety of commercial transgenic cultivars presents a major challenge for governments and institutions involved in biosafety regulation.

Abbreviations: AFLP: amplified fragment length polymorphism, **bp:** base pairs, **FISH:** fluorescent *in situ* hybridisation, **Kbp:** Kilobase pairs, **PCR:** polymerase chain reaction (DNA amplification method), **RFLP:** restriction fragment length polymorphism, **T-DNA:** transferred- DNA, the DNA sequences contained between left and right border repeats of the Ti plasmid of *Agrobacterium* that is transferred to plant genome during *Agrobacterium*-mediated transformation, **Ti-Plasmid:** tumour inducing plasmid, **USDA:** United States Department of Agriculture.

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