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Genome Scrambling - Myth or Reality?

Transformation-Induced Mutations in Transgenic Crop Plants

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Summary

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

[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.

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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 78 Kbp 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 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.

[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.

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 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 mis-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

[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).

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 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, **AFRP:** amplified fragment random polymorphism, **bp:** base pairs, **CaMV:** Cauliflower Mosiac Virus, **CBI:** confidential business information, **CP4 EPSPS:** 5-enolpyruvylshikimate-3-phosphate synthase, **FDA:** Food and Drug Administration, **FISH:** fluorescent *in situ* hybridisation, **GUS:** Beta-glucuronidase (enzyme encoded by bacterial *uid*A gene which can be assayed for using a chromogenic substrate), **IPCR:** inverse polymerase chain reaction, **Kbp:** Kilobase pairs, **LB:** left border (of T-DNA), **PCR:** polymerase chain reaction (DNA amplification method), **RAMP:** random-amplified microsatellite polymorphism, **RB:** right border (of T-DNA), **RFLP:** restriction fragment length polymorphism, **RAPD:** random amplified polymorphic DNA, **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

Introduction

Regulatory background

Current safety regulation of transgenic (often known as 'genetically modified' or GM) crop plants focuses on the risks incurred by the presence of a particular transgene and its product (Kessler *et al.* 1992). The safety assessments provided to regulators include only a limited amount of data which would enable the identification of unexpected mutations introduced during plant transformation. These include at most a very limited molecular analysis of the integrated transgene and its insertion-site and limited biochemical and agronomic analyses of the transgenic cultivar (Spok *et al.* 2003). For example, transgene insertions are usually characterised at the molecular level using Southern Blot techniques to test for the presence of the transgene and for the presence of superfluous marker and plasmid DNA. Data detailing the sequences of the plant DNA flanking the transgene insertion are usually not submitted to regulators, nor is a sequence comparison between the transgene insertion-site and the original undisrupted site.

In theory, comparative biochemical tests between transgenic and non-transgenic parent cultivars might be able to identify differences resulting from unintended mutations in the plant genome. In practice few nutritional or compositional tests are performed. Many of these are tests of complex mixtures (such as total amino acids or ash content), which are of limited use in detecting whether changes in the levels of specific nutrients or other biochemicals have occurred. Furthermore, it is clear that regulators accept transgenic plants which have nutrient levels that differ greatly from parental control plants, without questioning what caused the difference. In the case of transgenic squash line CZW-3, a nutritional analysis showed that CZW-3 squash had 67.6 times less Beta Carotene[8] than the control, however CZW-3 was accepted as being 'substantially equivalent' to its traditional counterparts and granted commercial approval.

Thus, the molecular and biochemical data currently submitted to regulators have a very limited ability to reveal the presence of mutations introduced during the process of creating a transgenic cultivar. The acceptability of such limited safety assessments is based on a number of crucial assumptions, one of which is that plant transformation methods are not a significant source of risk. In this report we review and analyse the scientific literature in order to determine whether this assumption can be justified on scientific grounds. In this report we do not address risks arising from the nature of specific transgene sequences or their products.

Plant transformation techniques

Plant transformation techniques are used to transfer specific DNA sequences (often from another species) into the DNA of a host plant. While there are a number of different plant transformation protocols, they all have the same basic objective: to produce a viable plant carrying a stably heritable DNA insertion that can be passed to future generations by normal plant breeding methods. For genetically heritable DNA insertion to occur, the transferred[9] DNA must pass through the host plant cell wall and the plant cell membrane, enter the cell nucleus, and chemically integrate into the plant's genomic DNA[10]. Upon insertion into plant genomic DNA, the transferred DNA is called transgenic or transgene DNA.

Once DNA transfer has occurred, tissue culture techniques are then used to regenerate an individual transgenic plant cell into a genetically uniform plant[11]. The aim is that all cells of the regenerated plant, including the reproductive cells, will carry a copy of the transgene DNA[12]. Plants containing one or more insertions of transgenic DNA are called transgenic plants. After regeneration the transgenic plant (called the primary transformant) can be used in plant breeding programmes.

The two procedures most commonly used to transform plants (for both research and commercial purposes) are *Agrobacterium*-mediated transformation and particle bombardment. Both have been used to transform a wide variety of commercially important crop plants including oilseed rape (canola), potato, squash, rice, wheat, soybean, cotton, maize (corn), and barley (Dai *et al.* 2001, Repellin *et al.* 2001, Wilkins *et al.* 2000, Jauhar and Chibbar 1999). Electroporation of intact tissue and direct DNA uptake by protoplasts are less widely used plant transformation alternatives (Hansen and Wright 1999). Brief descriptions of plant transformation via *Agrobacterium*-mediated transformation and particle bombardment are found below.

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation utilizes the natural ability of the soil bacterium and phytopathogen *Agrobacterium tumefaciens* to transfer a segment of its DNA, the T-DNA, into the host plant genome (see **Figure 1**). The T-DNA of naturally occurring *A. tumefaciens* contains DNA sequences coding for proteins involved in the synthesis of tumour-inducing plant growth factors and bacterial nutrients. When *A. tumefaciens* is used for making transgenic plants, these genes are replaced in the laboratory with the desired transgene and selectable marker sequences (for reviews see Gelvin 2003, Tinland 1996).

The T-DNA is located on the Ti plasmid (tumour inducing plasmid or pTi) of *A. tumefaciens*. The T-DNA is bounded by left and right border sequences, which are transferred along with the DNA sequences contained between them (the left border is a 25 bp imperfect direct repeat of the right border). In theory, the rest of the pTi is not transferred (but see **Section 1.1.2**), however, it does contain genes necessary for T-DNA transfer (Tinland 1996). For details see **Figure 1** and footnote[13] .

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[8] Beta carotene was measured by HPLC analysis. The actual amounts were 0.3mg/100g for CZW-3 and 20.3mg/100g for the control. The CZW-3 measurement was based on a composite sample of 20 fruits and the control measurement was based on a composite sample of 11 fruits (p19, USDA Application 95-352-01p)

[9] When particle bombardment is used to transform plants, the term 'delivered' is often substituted for 'transferred' and the transferred DNA is often referred to as 'delivered' DNA.

[10] Transferred DNA sequences can also be inherited if they insert into plastid (e.g. chloroplast) DNA (Maliga 2003). Plastid transformation technology is still not practical for most plant species and is not discussed in this report.

[11] If plant tissue culture is used during transformation, it is possible for regenerated plants to be composed of genetically non-identical cells (chimaeric). Genetically uniform transgenic plants can be selected for in future generations.

Little is known about the mechanism of T-DNA integration into the plant genome. It has been proposed that single-stranded T-DNA integrates into the plant genome via illegitimate (i.e. non-homologous) recombination (Kohli *et al*. 2003, Tinland 1996).

From a commercial and biosafety perspective, the ideal transformed plant would have a single insertion of a single intact T-DNA. However, in practice, independently-derived primary transformants resulting from *Agrobacterium*-mediated transformation vary in the number, nature and genomic location of their T-DNA insertion events. Identification and selection of primary transformants with simple insertion patterns is - or should be - an important component of the commercialisation process.

Particle bombardment transformation

Particle bombardment (biolistic or gene gun) transformation relies on the delivery of tungsten or gold particles into the plant cell nucleus. These particles are coated with the particular DNA to be inserted into the plant genome. The delivered DNA is usually plasmid DNA carrying the gene of interest or, alternatively, excised gene-cassette DNA[14]. The DNA-coated particles are accelerated or 'fired' into the plant cell. It is thought that DNA dissociates from those particles that have reached the nucleus, and then is free to integrate into the plant genome. As is the case with *Agrobacterium*-mediated transformation, the mechanism of transgene integration using particle bombardment is not well understood (Kohli *et al*. 2003, Pawlowski and Somers 1996).

Kohli *et al.* (1998) speculate that integration of DNA during particle bombardment occurs in a similar way to T-DNA integration: both *Agrobacterium*-mediated transformation and particle bombardment inititate a wound response in the plant cell. This induces plant enzymes involved in DNA repair and in foreign DNA degradation (e.g. nucleases, ligases, topoisomerases) and these enzymes may help integrate the DNA into the plant genome. As with *Agrobacterium*-mediated transformation (Iglesias *et al.* 1997, Dong *et al.* 1996, Gheysen *et al.* 1987), integration of DNA during particle bombardment is thought to occur via illegitimate recombination (Kohli *et al.* 2003).

As with *Agrobacterium*-mediated transformation, independently-derived primary transformants resulting from particle bombardment vary in the number, nature and genomic location of their transgene insertion events.

[12]Alternatively, reproductive cells of the plant can be transformed directly using *in planta* transformation methods and avoiding the use of tissue culture. Currently, *A.thaliana* is the only species with a well-established *in planta* transformation system.

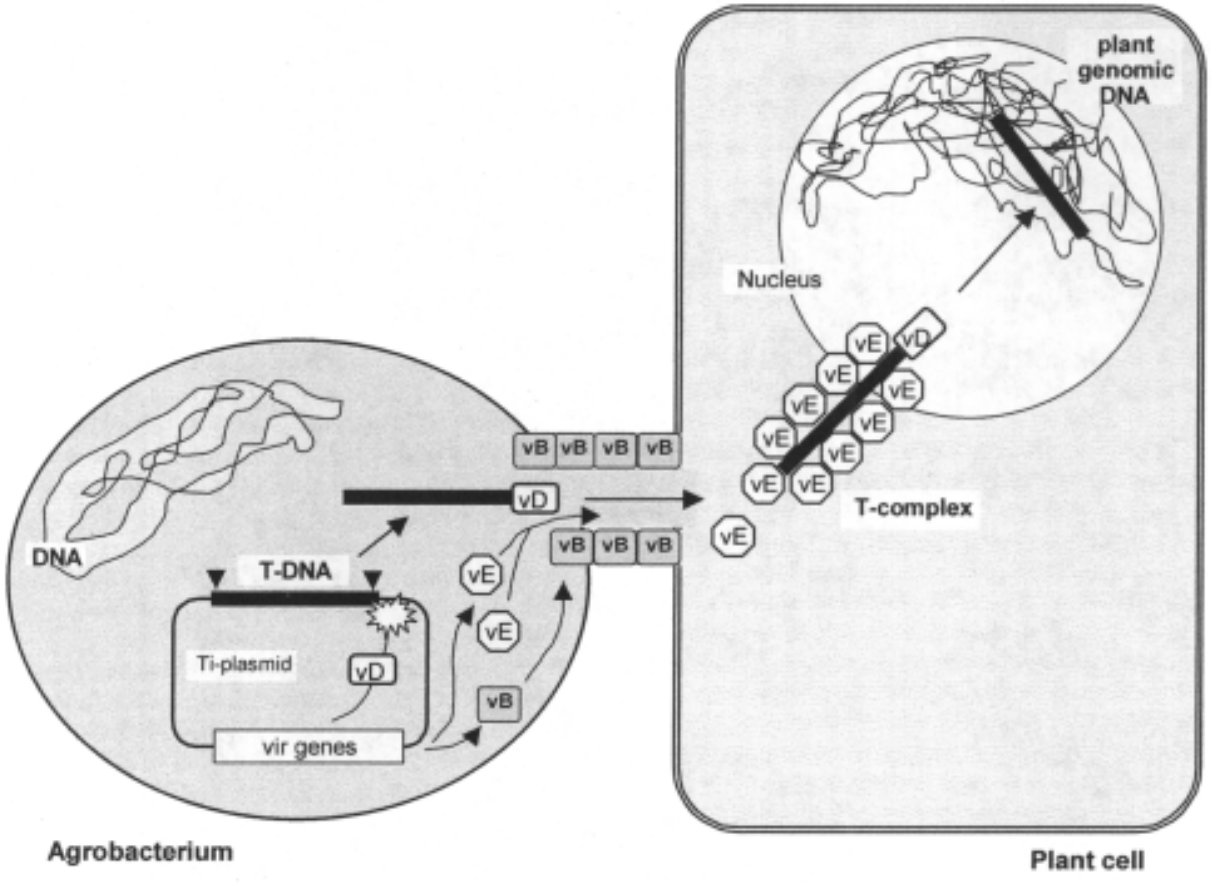

Figure 1. Schematic representation of *Agrobacterium tumefaciens*-mediated transfer of a T-DNA into the genomic DNA of a plant cell.

The use of plant tissue culture and selectable marker genes

Although plant transformation protocols that do not involve the use of a tissue culture step are available for a few species (**Table 1**), in practice all established crop plant transformation protocols involve the use of plant tissue culture (Hansen and Wright 1999, Walden and Wingender 1995).

Plant tissue culture is used to maintain or proliferate undifferentiated plant cells (or intact plant organs) on artificial media containing salts, sugars and plant hormones. Two kinds of plant tissue culture can be distinguished. The first involves plant production from pre-formed meristems[15]. This type of tissue culture is used for propagation purposes (e.g. of potatoes or cassava; Bhojwani 1990). The goal is to produce large numbers of genetically identical plants that exhibit minimal phenotypic variation, and techniques are adjusted accordingly (Skirvin *et al.* 1994, Cailloux 1984). Some authors prefer the term 'vegetative propagation' rather than 'tissue culture' to describe such procedures.

The second form of plant tissue culture involves the dedifferentiation of previously differentiated plant cells and the regeneration of these dedifferentiated cells into intact plants. This type of tissue culture is associated with high levels of somaclonal variation[16], and therefore mutations (Skirvin *et al.* 1994, Cailloux 1984, Larkin and Scowcroft 1981). It has occasionally been used as a mutagen during plant breeding programs (**Section 4.2**, Jain 2001). This latter sort of tissue culture is used for crop plant transformation.

Tissue culture is useful during plant transformation for several reasons. First, the use of plant tissue culture permits various plant tissues (e.g. callus[17] or protoplasts[18]) or organs (e.g. meristems, embryos, leaves, anthers) to be used as the starting material for plant transformation (Walden and Wingender 1995).

Tissue culture also permits the preferential regeneration of plants containing the transgene. Not all of the plant cells that have been subjected to the transformation process will contain integrated transgene sequences. If an antibiotic is included in the tissue culture medium and an antibiotic resistance gene is linked to the transgene, then transgenic cells will regenerate preferentially. Use of such selectable marker genes greatly increases the chance that regenerated plants will contain the desired transgene. Once a marker gene has been used to identify a transformed plant, it is superfluous. Marker genes are unnecessary and possibly hazardous if maintained in transgenic crop plants used for research or commercial purposes (Jelenic 2003, Hohn *et al.* 2001, Sawahel 1994).

[13]Various *A. tumefaciens* proteins (the products of the virulence *vir* genes) are involved in transferring the T-DNA to the plant cell and in integrating it into the plant genome. Once the bacterium has attached itself to the plant cell, the T-DNA is excised from the Ti plasmid as single-stranded T-DNA, which becomes attached to the VirD2 protein. It is then exported from the bacterial cell into the plant cell through a channel composed of VirB proteins. Once inside the plant cell, the single stranded T-DNA is coated with VirE2 proteins. The T-DNA-VirD2-VirE2 complex (T-complex) enters the nucleus through a nuclear pore complex.

Plant species	Transformation Method	Reference
Peanut (*Arachis hypogaea* L.)	Inoculation of embryo meristems with *Agrobacterium* using a 28 gauge needle	Rohini VK and Rao KS (2001) Plant Science 160: 889-898
Rice (*Oryza sativa* L. Tropical Japonica, cv. Maybelle)	Inoculation of isolated shoot apices with *Agrobacterium*	Sung HP *et al.* (1996) P Mol Biol 32: 1135-1148
Radish (*Raphanus sativus* L.)	Dipping of floral tissues in *Agrobacterium* culture	Curtis IS and Nam HG (2001) Transgenic Research 10 : 363-371
Arabidopsis thaliana	Dipping of floral tissues in *Agrobacterium* culture	Clough SJ and Bent AF (1998) Plant J 16(6): 735-743
Model legume *Medicago truncatula*	Infiltration of seedlings or flowering plants with *Agrobacterium*	Trieu AT *et al.* (2000) Plant J 22 (6):531-541
Petunia hybrida	Vacuum infiltration of pollen with *Agrobacterium* or application of *Agrobacterium* to stigma prior to pollination	Tjokrokusumo D *et al.* (2000) P Cell Reports 19:792-797
Tobacco (*Nicotiana tabacum* L.)	Particle bombardment of microspores	Aziz N and Machray GC (2003) P Mol Biol 51: 203-211

Table 1. Methods for direct germ line plant transformation that do not require dedifferentiation in tissue culture.

Risks arising from the plant transformation process

The ideal plant transformation system for both research and commercial purposes would reliably produce transgenic plants with (a) an unaltered genotype[19] except for the insertion of a single intact copy of the desired transgene and (b) an unaltered phenotype[20] except for the trait encoded by the transgene. However, by definition, transgene insertion disrupts the continuity of genomic sequences. Additionally, plants transformed using current methods exhibit unintended genetic changes such as the insertion of superfluous DNA [21] and various changes to the plant genomic DNA including base pair changes, small or large deletions, duplications, insertions, translocations and other DNA rearrangements.

We have divided these transformation-induced mutations into two categories. The first category we call **insertion-site mutations**. These consist of the insertions, deletions, duplications and rearrangements of plant genomic DNA and superfluous DNA created during the process of insertion of the desired DNA sequences into the plant genomic DNA. These mutations are genetically linked to the desired transgene sequences and are part of the transgene insertion-site. This combination of the inserted transgene and any associated superfluous DNA and/or genomic rearrangement is also called the 'transgene insertion event' or 'transformation event' and is genetically heritable. Insertion-site mutations are discussed in **Section 1** of this report.

The second category of transformation-induced mutations we call **genome-wide mutations**. Genome-wide mutations include all mutations not specifically associated with the insertion of the desired DNA. Some of these mutations result from the mutagenicity of the tissue culture procedures that are used in conjunction with current plant transformation protocols. Others may result from the imprecision and/or mutagenicity of the DNA transfer procedures themselves (e.g. *A. tumefaciens* infection or particle bombardment of cells). For example, some of these genome-wide mutations may be caused by failed insertion events. As their name implies, genome-wide mutations can occur anywhere in the plant genome, at sites genetically linked or unlinked to transgene insertion-sites. Genome-wide mutations are discussed in **Section 2** of this report.

The data we review in **Sections 1** and **2** suggest that the presence of both insertion-site mutations and genome-wide mutations is to be expected in plants transformed using current methods. Indeed, we show that such mutations are present in transgenic cultivars that have been granted commercial approval[22] by the USDA (**Sections 1.1.7** and **1.2.6**).

Outline

This report is divided into six sections:

1. **Insertion-site mutations in transgenic plants**
2. **Genome-wide mutations in transgenic plants**
3. **Significance of insertion-site and genome-wide mutations**
4. **Can transgenic plant breeding methods be compared to modern non-transgenic plant breeding methods?**
5. **Conclusions**
6. **Recommendations**

[14]Gene cassette DNA refers to the desired transgene sequences purified away from superfluous plasmid sequences prior to particle bombardment.

[15] A plant meristem is a plant tissue consisting of actively dividing cells that give rise to cells that differentiate into the new tissues of the plant.

[16]Somaclonal variation is the genotypic and phenotypic variation seen in plants which have been through tissue culture. Much of somaclonal variation results from genetic mutation.

[17]Callus refers to disorganized undifferentiated cell proliferation. Callus is often induced at the edges of wounded tissues.

[18]A protoplast is a plant cell which has had the cell wall removed, leaving the nucleus and the cytoplasm bounded by the cell membrane.

[19]The genotype of a plant is its genetic composition, i.e. the combination of alleles it posses.

[20]The phenotype of a plant consists of all aspects of the plants character (including its biochemistry, physiology, appearance and behaviour).

[21]In this report we define superfluous DNA as any DNA introduced into the plant genome via plant transformation *other than a single functional copy of the desired transgene(s)*. Thus, superfluous DNA includes any additional intact, rearranged or fragmented transgene sequences, as well as any inserted plasmid DNA, marker gene, or bacterial genomic (or other contaminating DNA) sequences.

[22]In the USA the federal government does not use the phrase 'commercial approval'. Rather, it grants transgenic crop plants 'non-regulated status' based on an assessment of data submitted by the applicants. Once a transgenic cultivar or a transgenic event has been granted non-regulated status, the cultivar or plants carrying the non-regulated event can be sold and grown commercially without further restriction. In this report we will use the phrase 'commercial approval' rather than 'non-regulated status'.

Section 1. Insertion-site mutations in transgenic plants

1.1 Transgene insertion events created using *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation can result in the integration of three types of superfluous DNA at the T-DNA insertion event: extra intact or partial copies of T-DNA sequences, *A. tumefaciens* binary vector (plasmid) sequences and selectable marker sequences (Smith *et al.* 2001). *Agrobacterium*-mediated transformation also usually results in small or large-scale deletion, duplication or rearrangement of nearby plant DNA sequences and in the integration of filler DNA[23] or non-contiguous plant DNA at the site of T-DNA insertion (Scholte *et al.* 2002, Iglesias *et al*. 1997, Dong *et al*. 1996, Tinland 1996, Gheysen *et al.* 1991, Gheysen *et al.* 1987). Recent data indicate that substantial rearrangement of plant sequences upon T-DNA insertion may be common (Forsbach *et al.* 2003, Tax and Vernon 2001, Kaya *et al.* 2000, Ohba *et al.* 1995).

1.1.1 *Superfluous T-DNA insertion*

Agrobacterium-mediated transformation usually results in transgenic plants having insertions of incomplete T-DNAs or insertions of more than one T-DNA (or T-DNA fragments) at one or more location(s) in the plant genome (Forsbach *et al.* 2003, Kim *et al.* 2003, Kohli *et al.* 2003, Dai *et al.* 2001, Dong *et al.* 2001, Zheng *et al.* 2001, Wolters *et al.* 1998, Cheng *et al.* 1997, Bhattacharyya *et al.* 1994). For example, Dai *et al*. (2001) transformed rice with a single T-DNA containing three different transgenes. In the 13 independent transformed lines examined by Southern blot[24] analysis, copy numbers for the three transgenes ranged from 1-5 per gene per transformed plant. In at least three lines there were differences in copy numbers between the three genes, indicating that truncation or rearrangement of the T-DNA had occurred. Only 4/13 lines appeared to have a single intact copy of the T-DNA (and thus a single copy of each of the three transgenes, the desired transformation product in this case). Forsbach *et al*. (2003) found that 80% of their *Arabidopsis thaliana* (thale cress) primary transformants had a single T-DNA insertion event in their genome, as determined by segregation analysis. Of these insertion events, just 22% had only a single intact copy of the T-DNA (as determined by Southern blot analysis). Thus, most of the T-DNA insertion events were composed either of an incomplete T-DNA or of multiple T-DNAs or T-DNA fragments.

These rice and *A. thaliana* examples are representative of the data in the scientific literature, in that insertion of incomplete or multiple T-DNAs (and/or T-DNA fragments) is more common than insertion of a single intact T-DNA.

1.1.2 *Superfluous plasmid DNA insertion*

Originally, it was assumed that only the T-DNA region of the Ti plasmid was transferred to the plant genome. However, Martineau *et al*. (1994) showed that Ti plasmid sequences outside the T-DNA borders (often referred to as plasmid 'backbone' sequences) could also frequently be transferred. These results have been confirmed by other researchers. Wenck *et al*. (1997) found the frequency of plasmid transfer to the *A*. *thaliana* genome ranged from 33% (6/18), using *A*. *thaliana* root transformation procedures, to 62% (39/63), using vacuum infiltration of intact plants. De Buck *et al*. (2000) found that the frequency of plasmid DNA incorporation varied from 20%-50% for *Agrobacterium*-mediated transformation of *A*. *thaliana* and tobacco. Kim *et al*. (2003) found that 77/171 (45%) of rice transformants contained integrated plasmid DNA. *Agrobacterium*-mediated transformation can sometimes result in complex T-DNA insertion events, in which numerous stretches of plasmid DNA are interspersed with T-DNA and plant DNA (Wolters *et al*. 1998).

1.1.3 *Superfluous marker DNA*

Crop plants currently produced via *Agrobacterium*-mediated transformation usually contain integrated marker DNA, in addition to the desired transgene. This is because T-DNAs usually contain a selectable marker gene located between their T-DNA borders[25].

1.1.4 *Southern blot analysis is not sufficient to detect all mutations at T-DNA insertion events*

Southern blot analysis is the technique most commonly used to determine both transgene copy number and the presence or absence of plasmid DNA. It is thus employed to identify transgenic lines with single-copy T-DNA insertion events suitable for plant breeding purposes. However, when used on its own, Southern blot analysis of transgene insertion events often gives misleading results (Makarevitch *et al.* 2003, Svitashev and Somers 2001, Zheng *et al.* 2001, Mehlo *et al.* 2000, Wolters *et al.* 1998). For example, Zheng *et al.* (2001) used adapter ligation PCR (AL-PCR) to analyse three transgenic shallot lines that had been shown by Southern blot analysis to have single copy T-DNA inserts. They would thus all be expected to show one right border (RB) AL-PCR fragment and one left border (LB) AL-PCR fragment. This was the case only for shallot Line 3. Line 1 had two RB fragments and one LB

[23] Filler DNA refers to DNA inserted between transgene DNA (either delivered DNA or T-DNA) and the flanking plant genomic DNA. It consists of short sequences that are not recognisable as belonging to either recombining partner (Kohli *et al*. 2003).

[24] Southern blot analysis relies on cutting test DNA with a restriction enzyme, separating the different size DNA fragments on an agarose gel by electrophoresis, transferring the separated test DNA to a membrane, and hybridising the membrane-bound DNA to a labeled fragment of known DNA (the probe). The labeled probe will only attach (hybridise) to fragments of the test DNA that have sequences in common with the probe sequences. Unbound probe is removed and the bound probe is visible as a distinct band. This technique can be used to examine test DNA (such as the genomic DNA from a transformed plant) for the presence and copy number of specific DNA sequences (such as T-DNA sequences or plasmid sequences).

[25] Transgenic plants produced using *Agrobacterium*-mediated transformation usually contain a marker gene within the same T-DNA as the desired transgene (Frary and Hamilton 2001, Ye *et al.* 2000, Cheng *et al.* 1997, Ishida *et al.* 1996). Therefore, unless the T-DNA has been truncated or rearranged, the transformed plant will contain the selectable marker gene in addition to the transgene.

fragment and Line 2 had three RB and three LB fragments. To explain these results, the authors suggest that Line 1 had a small fragment of T-DNA next to a full length T-DNA and that Line 2 had either a single T-DNA integrated next to several very small T-DNA fragments or else a complex integration event with various T-DNA rearrangements. Zheng *et al*. (2001) suggested that the cloning and sequencing of each insertion event, and fluorescence *in situ* hybridisation[26] (FISH) analysis were needed to clarify the organisation of these insertion events.

Wolters *et al*. (1998) also found discrepancies between methods when comparing the results of Southern blot analysis of complex T-DNA insertion events in potato to FISH analysis on extended DNA fibres (fiber-FISH). Fiber-FISH analysis revealed additional T-DNA and plasmid sequences at the insertion events which were not indicated by Southern blot analysis, and fiber-FISH analysis enabled determination of the order of T-DNA, plasmid and plant genomic DNA within the insertion event.

Therefore, Southern blot analysis alone is unlikely to result in an accurate description of either simple or complex T-DNA insertion events.

1.1.5 *Systematic analysis of single-copy T-DNA insertion events reveals frequent insertion-site mutations*

It is theoretically possible for exact integration of the T-DNA into the plant genome to occur (i.e. where the genomic target-site[27] remains intact except for insertion of the T-DNA). In practice this almost never happens (Forsbach *et al.* 2003, Tinland 1996). DNA sequence comparisons of the target-site before and after T-DNA integration indicate that target-site deletion and/or the insertion of filler, superfluous or plant genomic DNA nearly always occur during T-DNA insertion (Forsbach *et al.* 2003, Scholte *et al.* 2002, Iglesias *et al.* 1997, Dong *et al.* 1996, Tinland 1996, Gheysen *et al.* 1987). However, until very recently, a large-scale systematic analysis of T-DNA target-site mutations had not been carried out in any species (Forsbach *et al.* 2003).

In order to address this issue in *A. thaliana*, Forsbach *et al.* (2003) analysed target-site disruption at randomly generated single-copy T-DNA insertion-sites. First, they used genetic segregation and Southern blot analysis to identify transformed *A. thaliana* plants containing only a single-copy T-DNA insert. Then, using a combination of inverse polymerase chain reaction (IPCR) and DNA sequencing, they identified the DNA sequences flanking the left border (LB) and/or the right border (RB) of these T-DNA inserts. Aligning the flanking sequences to the known *A. thaliana* genome sequence[28] allowed them to position 112 independent T-DNA inserts in the *A. thaliana* genome. Then they made PCR primers homologous to the deduced flanking sequences and to the LB and RB T-DNA sequences in order to amplify and sequence the actual flanking DNA from the transformed plants.

Using this method, they found that 29% (32/112) of the transgenic plants contained insertion events associated with large scale mutations. Two lines had a chromosomal translocation[29] adjacent to the T-DNA insert, seven had large insertions of Ti plasmid sequences adjacent to one border, and one had a 770 bp internal T-DNA fragment adjacent to the RB. The remaining 22 lines (20% of the total) were thought to have had large deletions, insertions, duplications and/or rearrangements at the insertion event. However, the full extent of genome disruption was not determined for these lines.

The remaining 71% (80/112) of the transgenic lines had small-scale mutations at the T-DNA insertion event. These included insertions of filler DNA (ranging from 1-100 bp) and/or small deletions of target-site sequence (usually ranging from 1-100 bp).

Forsbach *et al*. (2003) found that filler DNA sequences could originate from plant DNA, including gene sequences, or from transferred DNA. They also found that insertion-site sequences could be duplicated. Finally, they found that 3/112 lines had additional LB fragments adjacent to the RB of the T-DNA insert and 1 line had an additional fragment of LB sequence which mapped to a different chromosome from the T-DNA insert.

In conclusion, 80 lines had both left and right T-DNA border sequences which could be aligned with a single region of the known *A. thaliana* genome sequence. In those, the deletions, insertions and rearrangements which occurred at the insertion-site were mostly limited to relatively small-scale (1-100 bp) changes. Ten other lines had either genomic translocations or large inserts of plasmid vector sequence adjacent to a T-DNA border. In the remaining 22 lines, where the full extent of genome disruption was not determined, the deletions, insertions and rearrangements are likely to be on a much larger scale.

Based on their results, Forsbach *et al*. (2003) conclude that, “even single-copy T-DNA insertions are frequently associated with small or large rearrangements” of the target plant DNA.

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[26] FISH (fluorescent *in situ* hybridisation) techniques enable specific DNA sequences (e.g. transgene sequences) to be localized to individual plant chromosomes. The labelled probe (e.g. transgene DNA) is allowed to bind to (hybridise with) homologous sequences on chromosome spreads. This can give a visual picture of where transgene loci are located in the plant genome, and an idea of the number of transgene loci present. Fiber-FISH, using extended DNA fibres, can be used to give an idea of how many copies of a transgene containing plasmid are located at a single locus, and whether the transgene copies are interspersed with plant DNA or integrated as concatamers. Fiber-FISH can also be used to determine the order of plasmid sequences and transgene sequences within a locus.

[27] In the context of plant transformation, the ‘target-site’ refers to the undisrupted plant DNA sequences present at the T-DNA or delivered DNA insertion-site *before* the insertion of T-DNA or delivered DNA. While it is theoretically possible for ‘exact integration’ of the T-DNA or delivered DNA to occur, in practice this almost never happens. Only DNA sequence comparison of the target DNA sequences (present in the untransformed parent plant) with the sequences flanking the T-DNA or delivered DNA after insertion will accurately reveal the extent of plant genomic DNA disruption which has occurred during insertion.

[28] The entire *Arabidopsis thaliana* genome sequence has been determined and is available to the public (*Arabidopsis* Genome Initiative 2000). Thus, once the position of a T-DNA insert has been determined in the *A. thaliana* genome by aligning the T-DNA flanking sequence for either the RB or the LB, the entire sequence of the target-site prior to T-DNA insertion is known.

[29] DNA translocation is the transfer of genomic DNA from one location in the genome to another.

Some reports from other laboratories also indicate that large-scale rearrangements associated with single-copy T-DNA inserts may be common. Tax and Vernon (2001) characterised two *A. thaliana* T-DNA mutants at the molecular level and found in both cases that a T-DNA had inserted into a target-site along with sequences originating from a different chromosome. In one line the translocated LB genomic flanking region was at least 40 Kbp, in the other it was at least 1020 bp. In both cases the translocated plant DNA seemed to have been duplicated, as copies of the translocated DNA also appeared to be present at their original location in the T-DNA tagged lines. Kaya *et al.* (2000) analysed the T-DNA tagged *A. thaliana* mutant called *hosoba toge toge* and found a 75.8 Kbp deletion (predicted to have deleted 13 genes) at the T-DNA insertion site. In tobacco, Ohba *et al.* (1995) analysed the target-site of a single T-DNA insertion into repetitive genomic sequences. They found a short deletion of target DNA, duplication of a fragment of the T-DNA and duplications of three different stretches of the target DNA.

1.1.6 *T-DNAs frequently insert into functional genomic sequences*

Various experiments indicate that a large proportion of T-DNAs insert in or near endogenous plant genes (Jeong *et al.* 2002, Szabados *et al.* 2002, Dong *et al.* 1996; Koncz *et al.* 1989). In fact, this is the basis of T-DNA insertional mutagenesis (Forsthoefel *et al*. 1992) and activation tagging experiments (Weigel *et al.* 2000), which rely on the insertion of T-DNAs into or near to functional gene sequences to create novel phenotypes for research purposes.

Systematic DNA sequence analysis of 1000 T-DNA insertions in *A. thaliana* indicated that 35.4% of the T-DNAs inserted into gene coding sequences (47.8% if integrations into regulatory sequences were added, Szabados *et al.* 2002). Similar results were obtained by Forsbach *et al.* (2003), who estimated that 55.9% of LB and 58.5% of RB sequences in their *A. thaliana* transformants were inserted into gene sequences. In other experiments T-DNAs inserted 39.4% of the time (28/71 insertions) into plant gene coding or regulatory sequences in rice (Jeong *et al.* 2002) and 42% of the time (5/12 insertions) in the legume *Medicago truncatula* (Scholte *et al.* 2002). Further experiments in *A. thaliana*, tobacco and potato also indicate a high frequency of T-DNA insertion into or near plant genes (Campisi *et al.* 1999, Lindsey *et al.* 1993, Koncz *et al.*1989).

1.1.7 *Insertion-site mutations are present in commercial transgenic cultivars created via Agrobacterium-mediated transformation*

We wanted to determine the extent to which transformation-induced mutations are present in commercial transgenic cultivars produced using *Agrobacterium*-mediated transformation. We have analysed the data submitted-to the USDA in the applications requesting commercial approval for a representative group of three such transgenic cultivars. A summary of the data available for these cultivars and the supporting references can be found in **Table 2** and in the **Appendix**. All of the cultivars listed in **Table 2** and the **Appendix** have been granted commercial approval by the USDA.

Superfluous DNA in commercially approved cultivars

All of the T-DNA-containing commercial transgenic cultivars presented in **Table 2** contain superfluous transgenic DNA sequences.

As determined by Southern blot analysis: herbicide tolerant cultivar LLCotton25 contains a 79 bp polylinker sequence; the virus-resistant squash cultivar CZW-3 carries a 35S CaMV promoter-regulated *npt*II selectable marker gene; and Newleaf® Plus potato RBMT22-82 has three T-DNA insertion events, all three of which contain intact copies of both *Cry3A* and *PLRVrep.* Two of the Newleaf® Plus potato RBMT22-82 insertion events have intact copies of the CP4-EPSPS selectable marker/herbicide resistance gene and one insertion event has a rearranged copy of CP4-EPSPS. One insertion event also includes integrated plasmid sequences containing the bacterial *aad* gene and the *ori*322 bacterial origin of replication (**Table 2** and **Appendix**).

Target-site disruption in commercially approved cultivars

For the three T-DNA-containing commercial transgenic cultivars described in **Table 2** and in the **Appendix** (LLCotton25, Squash CZW-3 and potato Newleaf® plus RBMT22-82), no data derived from either PCR or DNA sequencing were provided to the USDA for the purpose of analysing the effects of T-DNA insertion on the plant genome. No comparison was made between the T-DNA insertion-sites and the original target-sites.

1.2 Transgene insertion events created using particle bombardment

Particle bombardment insertion events are poorly understood. One reason is because such insertion events tend to be difficult to analyse, as they are often large and complex, with multiple copies of transgene sequences. A second reason is that most of the particle bombardment insertion events described in the scientific literature have been analysed by Southern blot analysis rather than DNA sequencing. Southern blot analysis can offer only a limited understanding of particle bombardment insertion events (**Sections 1.1.4** and **1.2.3**).

Particle bombardment appears to create three distinct types of transgene insertion event (called Type I, Type II, and Type III, Kohli *et al.* 2003). The vast majority of the insertion events created via particle bombardment are extremely complex, with multiple copies of transgenic DNA inserted at a single insertion-site (Kohli *et al.* 2003, Breitler *et al.* 2002, Loc *et al.* 2002, Svitashev and Somers 2002, Vain *et al.* 2002, Svitashev and Somers 2001, Fu *et al.* 2000, Mehlo *et al.* 2000, Svitashev *et al.* 2000, Kohli *et al.* 1999, Maqbool and Christou 1999, Kohli *et al.* 1998, Pawlowski and Somers 1998 and 1996, Register *et al.* 1994, Wan and

Lemaux 1994). The transgenic DNA is arranged either as multiple copies of intact or fragmented transgene DNA (Type I) or as multiple copies of transgene DNA interspersed with small or large fragments of plant genomic DNA (Type II). Both Type I and Type II insertion events contain large amounts of superfluous DNA and appear to result in large-scale genomic DNA disruption.

Relatively simple insertion events (Type III), having only one intact copy of the transgene, are difficult to obtain using particle bombardment. Additionally, when fully characterised, such insertion events turn out to contain fragments of superfluous DNA and/or they appear to be associated with large deletions and/or rearrangements of the target plant DNA (**Sections 1.2.4** and **1.2.6**). While single-copy transgene insertion events are the type most likely to be selected for transgenic plant breeding purposes, there are few studies describing single-copy particle bombardment insertion events (**Section 1.2.4**).

1.2.1 *Superfluous transgene DNA insertion*

In a direct comparison with *Agrobacterium*-mediated transformation (**Section 1.1.1**), Dai *et al.* (2001) used particle bombardment to transform rice with a plasmid carrying a T-DNA containing three transgenes, the *htp* gene, the *uid*A gene, and the *npt*II gene. Southern blot analysis of 14 lines indicated that copy numbers for each of the three individual transgenes ranged between 0-8 per gene per plant (the average *htp* transgene copy number

Transformed line or transformation event	Transformation method*	USDA application number and other relevant references	Date of approval for deregulation	Superfluous DNA at or near the transgene locus	Sequence analysis of DNA flanking the inserted transgene
LLCotton25 Herbicide tolerant	*Agrobacterium*-mediated transformation	02-042-01p	March 2003	Polylinker sequence	n.d.
Newleaf® Plus RBMT22-82 Potato Virus resistant and Colorado Potato Beetle resistant	*Agrobacterium*-mediated transformation	99-173-01p	July 2000	Three independent insertion events; one event also included plasmid sequences	n.d.
CZW-3 Squash Virus resistant	*Agrobacterium*-mediated transformation	95-352-01p	June 1996	Selectable marker gene	n.d.
Maize MON863 Corn rootworm protected	Particle bombardment with gene cassette	01-137-01p	October 2002	Selectable marker gene and a fragment of a superfluous gene	Yes, but sequence is not publicly available
63-1 Papaya **55-1 Papaya** Virus resistant	Particle bombardment with whole plasmid	96-051-01p Fitch *et al.* 1992	September 1996	Both lines included selectable marker genes and plasmid DNA sequences	n.d.
Roundup Ready® Soybean 40-3-2 herbicide tolerant soybean line	Particle bombardment with whole plasmid	93-258-01p Windels *et al.* 2001	May 1994	Superfluous transgene sequences and unknown DNA sequences	n.d. (probable deletion and/or rearrangement at insertion site)
Maize YieldGard® MON810 Lepidopteran insect resistant	Particle bombardment	96-017-01p Hernandez *et al.* 2003	March 1996	none	n.d. (probable deletion and/or rearrangement at insertion site)

Table 2. Commercial transgenic crop cultivars or events discussed in this report.

was 2.5 copies per plant). None of the 14 lines had only a single intact copy of all three genes, the desired transformation product. These results are representative of particle bombardment transformation experiments in that primary transformants harbouring only one intact single-copy transgene insertion event are rare (Kohli *et al.* 2003, Pawlowski and Somers 1996).

1.2.2 *Superfluous plasmid, marker gene and bacterial genomic DNA insertion*

All DNA sequences present on the bombarded particles are equally likely to be integrated into the plant genome during transformation (Chen *et al.* 1998). Although it is possible to use gene cassettes (transgene and marker sequences that have been purified away from superfluous plasmid DNA sequences) for particle bombardment (Breitler *et al.* 2002, Loc *et al.* 2002, Fu *et al.* 2000), it has been standard practice to use entire plasmids. It is also standard practice to use a selectable marker gene to identify transformed plants. Thus most particle bombardment insertion events, whether created for research or commercial purposes, include both superfluous integrated plasmid and selectable marker DNA.

Bacterial genomic DNA, or any other contaminating DNA, that is inadvertently included in particle bombardment transformations may also integrate into the plant genome. Ulker *et al.* (2002) used DNA sequencing to analyse the DNA located between a head to head repeat of a GUS transgene at a complex insertion event in tobacco. They found 260 bp of *E. coli* chromosomal DNA integrated between the two GUS genes. Ulker *et al.* (2002) noted that, prior to bombardment, the plasmid DNA they used for transformation had been purified using standard methods, thus no bacterial chromosomal DNA should have been present.

To our knowledge, this is the only reported example of unintentional integration of bacterial genomic DNA during plant transformation. However, it is not possible to say whether or not integration of contaminating DNA is a common event, because so few particle bombardment insertion events have been analysed using techniques capable of identifying such DNA.

1.2.3 *Southern blot analysis is not sufficient to identify all insertion-site mutations created via particle bombardment*

For particle bombardment insertion events, as for insertion events created via *Agrobacterium*-mediated transformation, the data presented in the scientific literature (and in applications for commercial approval) are usually derived solely from Southern blot analysis. However, in order to accurately describe the transgene insertion and its associated genomic mutations, it is necessary to use several different techniques. This becomes clear when researchers use more than one technique to analyse a transgene insertion event.

When Svitashev and Somers (2001) used fiber-FISH analysis to examine transgene insertion events in six transgenic oat lines created via particle bombardment, they found that the number of plant genomic DNA interspersions within the insertion event was greater than predicted by Southern blot analysis. At one insertion event, Southern blot analysis predicted that the transgene copies were arranged as tandem repeats. Fiber-fish analysis indicated that there were, in fact, 3-10 Kb of genomic DNA between most or all of the transgene copies.

Mehlo *et al.* (2000) used PCR and Southern blot analyses to characterise seven particle bombardment insertion events in maize. They found that Southern blot analysis was useful only in detecting large-scale genomic changes (such as large deletions) while PCR analysis was necessary to detect more subtle rearrangements.

Svitashev *et al.* (2002) analysed two complex transgene insertion events identified in two different transgenic oat lines, line 803 and line 3801, using DNA sequencing techniques. They sequenced non-overlapping plasmid rescue clones (line 803) or clones obtained by screening a genomic library with plasmid sequences (line 3801). They did not sequence either insertion event in its entirety. After sequencing 160 Kb of DNA obtained from the two insertion events, they found “60 unique plasmid-plasmid and 35 plasmid-genomic junctions involving 155 breakpoints in the delivered DNA”. These data indicate a huge amount of scrambling of transgene and plant genomic sequences. Their data also revealed that many of the inserted transgene sequences were less than 200 bp in length. This is important because such small fragments would be hard to detect using only Southern blot analysis of transformants. When PCR and DNA sequencing have been used in addition to Southern blot analysis, small transgene fragments have several times been found in and/or near presumed single-copy insertion events in experimental and commercial transgenic lines (**Table 2**, **Appendix**, Makarevitch *et al*. 2003, Windels *et al*. 2001).

1.2.4 *DNA sequencing of particle bombardment insertion events reveals unexpected complexity*

DNA sequencing of the entire insertion event, including the delivered DNA and flanking genomic DNA, provides the definitive description of an insertion event. When the nucleotide sequence of the insertion event is then compared to the nucleotide sequence of the undisrupted target-site, it is possible to also determine the extent of genome disruption that occurred during transgene insertion.

Analyses of particle bombardment insertion events by DNA sequence analysis are extremely rare in the scientific literature. The authors of a 1996 review could find no published DNA sequence analyses of particle bombardment insertion events (Pawlowski and Somers 1996). Below (and in **Section 1.2.6**, Windels *et al.* 2001) we summarise the findings of the three papers available in the scientific literature which use DNA sequence analysis to describe ‘simple’ particle bombardment insertion events.

Shimizu *et al.* (2001) used DNA sequence analysis to examine a single insertion event from a transgenic tobacco cell line (the insertion event was not isolated from a transgenic plant). They found that an intact copy of the delivered plasmid had integrated adjacent to a partial copy. Associated disruption of the plant genome included inter-

spersion of a 1 Kb fragment of plant DNA with plasmid sequence and the creation of inverted repeats (of 1.3 Kb of plant genomic DNA and a small fragment of plasmid DNA) on either side of the inserted plasmid sequences. The sequence of the original target-site DNA was not determined.

Makarevitch *et al.* (2003) analysed three particle bombardment insertion events from two oat primary transformants (called transgenic line 3830 and transgenic line 11929) using DNA sequencing. When first analysed using fiber-FISH and Southern blot techniques, line 3830 was thought to contain only a single transgene at a single site in the genome. This insertion event was called 3830-1 (Svitashev and Somers 2001, Svitashev *et al*. 2000). DNA sequencing showed that insertion event 3830-1 contained the full sequence of the delivered plasmid, a small stretch of interspersed genomic and plasmid sequences, a partial copy of the delivered plasmid, another stretch of scrambled genomic and plasmid sequences and a stretch of scrambled plasmid sequence (Makarevitch *et al.* 2003). Further analysis indicated that line 3830 actually contained two other 'minor' insertion events, in addition to 3830-1 (Makarevitch *et al.* 2003). Sequence analysis of minor insertion event 3830-2 indicated that it consisted of 296 bp of rearranged plasmid DNA. The second minor insertion event was not analysed.

Insertion event 11929 was isolated from transgenic oat line 11929, which had been created by co-bombardment with two different plasmids (Makarevitch *et al.* 2003). Sequence analysis revealed that insertion event 11929 contained a truncated copy of each plasmid. These were separated by scrambled genomic and plasmid DNA sequences (Makarevitch *et al.* 2003).

Makarevitch *et al.* (2003) compared the disrupted transgene insertion-sites to their pre-insertion target-site sequences. When they compared the plant genomic sequences flanking insertion event 3830-2 to the target-site in the non-transformed parent oat line, they found an 845 bp deletion of plant genomic DNA had occurred, as well as rearrangement of plant genomic DNA sequences on both sides of the 296 bp plasmid DNA insert. For both insertion event 3830-1 and insertion event 11929, they were unable to PCR amplify the target-site from DNA obtained from the non-transformed parental oat plants, using primers homologous to the plant DNA flanking the transgene insertions. They suggested that additional rearrangement and/or deletion of plant genomic DNA had occurred at the site of transgene insertion. They did not further analyse these rearrangements.

1.2.5 *Frequency of insertion into gene sequences is not known for particle bombardment insertion events*

For particle bombardment, there are not enough sequence data available to determine the frequency of delivered DNA insertion into gene sequences. However, it is interesting to note that in one of the three oat insertion events (insertion event 3830-1) analysed by Makarevitch *et al.* (2003), the delivered DNA is flanked on either side by known plant gene sequences, each from a different gene.

Although *Agrobacterium*-mediated transformation has frequently been used to identify functional sequences in gene tagging experiments, this has not been the case for particle bombardment. A recent paper by Salgueiro *et al.* (2002) is the first to describe the use of particle bombardment in a gene tagging experiment. *Tritordeum* (a hexaploid cereal species) was transformed with a promoterless *uid*A gene. Eight transformants were identified that contained the *uid*A gene. Five of these eight expressed *uid*A, indicating that the transgene may have inserted into plant gene regulatory sequences. This very limited experiment suggests that particle bombardment, like *Agrobacterium*-mediated transformation, may also result in a high frequency of transgene insertion into plant gene coding and/or regulatory sequences. However, the plant DNA flanking the *uid*A insertions was not sequenced, so this hypothesis remains unconfirmed (Salgueiro *et al.* 2002).

1.2.6 *Insertion-site mutations are present in commercial cultivars created via particle bombardment*

To determine the nature and extent of insertion-site mutations present in commercial transgenic cultivars created via particle bombardment we examined the data submitted to the USDA for five representative transgenic crop cultivars or transgene insertion events. Data and references referred to in the text are summarized in **Table 2** and more information can be found in the **Appendix**.

Superfluous DNA in commercially approved cultivars

Transgenic maize event Mon863 consists of a single copy of the desired transgene *Cry3*Bb1 and the following superfluous DNA: a single copy of the *npt*II selectable marker gene, and a 153 bp fragment of the *ble* gene (which forms part of a dicistronic mRNA transcript).

Transgenic papaya cultivar, Papaya 55-1, has an intact copy of the desired CMV-PRV transgene and the following superfluous DNA sequences: the GUS visible marker gene, the *npt*II selectable marker gene and OriT/Tet bacterial plasmid sequences.

Transgenic papaya cultivar, Papaya 63-1, contains an intact copy of the desired CMV-PRV transgene and the following superfluous DNA sequences: the *npt*II gene and the GENT, OriV/Tet and OriT/Tet bacterial plasmid sequences. In Papaya 63-1, the GUS gene has been lost and other unspecified rearrangements have occurred, as indicated by unexpected bands on Southern blots.

Roundup Ready® soybean event 40-3-2 has two superfluous fragments of the CP4 EPSPS herbicide tolerance gene as detailed below.

Target-site disruption in commercially approved cultivars

Applications for approval rarely contain an analysis of the DNA sequences flanking transgene insertion sites and never a comparison with the undisrupted target-site. Of the applications for the 8 transgenic cultivars

that we examined (**Table 2, Appendix**), only one (for event Mon863) actually provided any sequence data.

Maize event Mon863: The submitted application claims to have provided a DNA sequence analysis of the DNA flanking the transgene insert. However, these data were classed as confidential business information (CBI) and were thus not available for public examination.

Roundup Ready® Soybean event 40-3-2: No DNA sequence analysis of soybean event 40-3-2 was present in the original application granted approval for deregulation in 1994. However in 2000, Monsanto provided further information to the USDA on soybean event 40-3-2. They described the presence of an additional 254 bp CP4 EPSPS fragment adjacent to the desired CP4 EPSPS transgene and the presence of a 72 bp fragment of CP4 EPSPS sequence that was genetically linked to the intact CP4 EPSPS gene, but separated by plant genomic DNA.

Windels *et al.* (2001) used DNA sequencing to analyse the genomic DNA flanking the EPSPS transgene in transgenic plants containing insertion event 40-3-2. They found the adjacent 254 bp fragment of the CP4 EPSPS gene reported by Monsanto and an additional 540 bp sequence of unidentified origin (**Table 2** and **Appendix**).

Using PCR, Windels *et al.* (2001) were unable to amplify the genomic target-site for the CP4 EPSPS insertion from the non-transformed parental soybean line. This indicated that soybean event 40-3-2 probably resulted in the rearrangement and/or deletion of plant genomic sequences at the insertion-site. The actual nature of these insertion-site rearrangements was not determined.

Maize YieldGard® event Mon810: No DNA sequence analysis of maize event Mon810 was present in the original application granted approval for deregulation. However, Hernandez *et al.* (2003) and Holck *et al.* (2002) cloned the plant genomic DNA flanking the transgene insertion event Mon810 in maize YieldGard®. Hernandez *et al.* (2003) were unable to PCR amplify the equivalent target-site sequences from untransformed parent plants and they suggested that plant genomic DNA had been rearranged and/or deleted at the site of insertion of the truncated *Cry*IA(b) transgene. They did not determine the exact nature of the event Mon810 insertion-site rearrangements.

Papaya cultivars 63-1 and 55-1: Despite the availability of Southern blot data indicating that transgene rearrangements had occurred, no DNA sequence analyses of the transgene insertion, its flanking DNA and/or target-site DNA were carried out for transgenic Papaya cultivars 63-1 or 55-1.

1.3 Summary

a) The only systematic analysis of transgene insertion-site mutations is an analysis of 112 single copy T-DNA insertions in *A. thaliana.*
b) No systematic analyses of transgene insertion-site mutations have been carried out for particle bombardment insertion events or for transgenic crop plants transformed using any method.
c) Superfluous DNA insertion and both small and large genomic deletions and rearrangements are common at T-DNA insertion sites.
d) Approximately 35%-58% of T-DNAs insert into gene sequences[30].
e) DNA sequence analyses of delivered DNA insertions and/or their flanking DNA are available for only a handful of particle bombardment insertion events.
f) The very limited data available suggest that particle bombardment always creates substantial deletions and rearrangements of genomic DNA at the transgene insertion-site.
g) It is not known with what frequency transgenes insert into gene sequences using particle bombardment.
h) Superfluous DNA insertions and genomic DNA deletions and rearrangements are present at the insertion events found in commercially approved transgenic cultivars created by both *Agrobacterium*-mediated and particle bombardment transformation.
i) Applications submitted to the USDA do not contain the PCR and DNA sequence data necessary to determine the full extent of the insertion-site mutations present at the transgene insertion events in commercial lines.
j) The data submitted to the USDA are not sufficient to determine whether plant gene sequences have been disrupted at the transgene insertion-site.

Section 2. Genome-wide mutations in Transgenic Plants

In addition to the mutations created at the transgene insertion-site (**Section 1**), heritable mutations are introduced throughout the genome by the standard methods of plant transformation. This is because both the tissue culture procedures used during plant transformation and the specific DNA transfer methods (e.g. *A. tumefaciens* infection, particle bombardment and electroporation) introduce mutations into the plant genome (Jain 2001, Forsthoefel *et al.* 1992). In **Section 2**, we discuss these genome-wide mutations created during plant transformation.

2.1 Tissue culture induces genome-wide mutations

It has long been known that plant tissue culture is mutagenic (Larkin and Scowcroft 1981)[31]. Plant tissue culture induces base pair changes (point mutations), DNA deletion and rearrangement, gene amplification and de-amplification, movement of transposons, methylation changes and ploidy level changes (Bregitzer *et al.* 2002, Jain 2001, Kaeppler *et al.* 2000, Hirochika *et al.* 1996, Brown and Thorpe 1995, Phillips *et al.* 1994).

When such mutations affect functional gene sequences, they can result in heritable mutant phenotypes (Dennis *et al.* 1987, Brettell *et al.* 1986). Observed tissue culture induced phenotypes include variations in height, morphology, seed yield, essential oil content, disease resistance, and other important traits (Jain 2001, Godwin *et al*. 1997, Brown and Thorpe 1995, Phillips *et al.* 1994). For this reason tissue culture has sometimes been used intentionally as a mutagen to create phenotypic variation for use in plant breeding programs (Jain 2001).

The mutagenicity of the tissue culture procedure depends on a host of poorly characterised plant-related and cell culture-related factors (**Section 4.2**). Species, cultivar type, explant[32] source and explant ploidy level can all influence the number of mutations found in regenerated plants (Skirvin *et al.* 1994). Likewise, cell culture conditions, such as longer amounts of time spent in tissue culture, faster cell proliferation, the presence of antibiotics and higher hormone levels have been shown to result in increased tissue culture mutagenicity (Skirvin *et al.* 1994).

2.2 Gene transfer methods can cause genome-wide mutations

Populations of primary transformants frequently contain numerous plants with visibly aberrant phenotypes.

Primary transformants exhibit a spectrum of mutant phenotypes similar to those seen in tissue culture derived plants (Shu *et al*. 2002, Bregitzer *et al.* 1998, Kaniewski and Thomas 1999, Hoekema *et al.* 1989, Singh *et al.* 1998). More subtle heritable mutant phenotypes (e.g. altered lipid levels or differences in disease resistance, nodulation phenotype, grain cooking quality or cuticular wax formation), can also be identified in populations of primary transformants when such phenotypes are tested for specifically (Wu *et al.* 2002, Schroder-Pontoppidan *et al.* 2000, Schauser *et al.* 1998, Presting *et al.* 1995, McNevin *et al.* 1993).

Many of these are heritable and not genetically linked to the transgene insertion site. These genome-wide mutations could be due either to plant tissue culture or to the DNA transfer method. Some plant populations transformed by either *Agrobacterium*-mediated or particle bombardment transformation methods appear to have more unintended phenotypic variation than non-transformed tissue culture derived control populations (Bregitzer *et al.* 1998, Dale and McPartlan 1992). These particular experiments, however, are far from conclusive[33].

The mutagenicity of *A. tumefaciens* infection during plant transformation is also suggested by a small body of evidence based on T-DNA tagging experiments. When *A. thaliana* plants are transformed via *A. tumefaciens* infection without the use of tissue culture, the resulting transformants frequently exhibit mutant phenotypes which are unlinked to a transgene insertion (Coury and Feldman 1998, Negruk *et al.* 1996, McNevin *et al.* 1993, Forsthoefel *et al.* 1992). Some or all of these phenotypes may be due to mutations (such as small deletions, substitutions and insertions) resulting from unsuccessful T-DNA insertion events (Coury and Feldman 1998, Negruk *et al.* 1996, Marton *et al.* 1994, McNevin *et al*. 1993).

Negruk *et al.* (1996) sequenced two mutant alleles of the *CER2* gene that had been generated during *Agrobacterium*-mediated seed transformation[34], but which were not linked to a T-DNA. They found a 17 bp deletion in one allele and a 2-bp substitution and a 2-bp insertion in the other.

No studies, based on the analysis of populations of plants transformed in the absence of tissue culture, have specifically been carried out to determine the mutagenicity of other DNA transfer methods.

It should also be noted that small stretches of superfluous DNA can be inserted at random genomic locations during both *Agrobacterium*-mediated and particle bombardment transformation. Such small insertions could be responsible for some of the genome-wide mutations present in transgenic plants. Forsbach *et al.* (2003) found the insertion of a T-DNA left border fragment which mapped to a different chromosome than the full length T-DNA insert in one of 112 lines. Makarevitch *et al.* (2003) found that a line which had been previously classified as having only a single transgene insertion event, in fact, had 2 additional 'minor' insertion events. They analysed one of the minor insertion events and found it contained a 296 bp fragment of non-contiguous transgene DNA. These small insertions of superfluous DNA are not readily detected by standard Southern blot analysis.

[30] Given the current state of knowledge, it is not possible, using sequence analysis alone, to determine whether a segment of DNA is truly non-functional. Thus an estimate of frequency of insertion into gene sequences may be an understimate of the frequency of insertion into functional sequences.

[31] Tissue culture induced mutations are also known as somaclonal variation or somaclonal mutations. A 'somaclone' is a plant derived from any form of tissue culture and 'somaclonal variation' thus refers to the variation (mutations) seen among plants regenerated from tissue culture (Larkin and Scowcroft 1981)

[32] The explant refers to the part of the plant (e.g. leaf, stem section, apical meristem) excised and transferred to the tissue culture media.

2.3 Quantitative molecular analysis suggests numerous genome-wide mutations are present in transformed plants

Recently, researchers have begun using molecular techniques to quantify genome-wide mutations in transgenic plants. Using techniques such as RFLP[35], RAPD[36], AFLP[37] and RAMP[38], mutations in the genomes of individual transformants can be identified and displayed as polymorphic bands[39] on a gel or Southern blot (**Table 3**, Sala *et al*. 2000).

Use of these techniques to analyse transgenic plants is quite new, but they have an established history of use in documenting the mutagenic nature of tissue culture (Gesteira *et al.* 2002, Polanco and Ruiz 2002, Afza *et al.* 2001, Matthes *et al.* 2001, Godwin *et al.* 1997, Kaeppler and Phillips 1993, Brown *et al.* 1991). To date, relatively few quantitative molecular analyses of transformation-induced mutations have been published. We discuss all of the available papers below, including those analysing less widely used transformation methods such as cell electroporation and protoplast transformation. In all of these studies, the plant transformation methods incorporated a tissue culture step.

The results of these experiments suggest that current plant transformation techniques create new mutations in a significant proportion of the total genome of a transformed plant.

[33]Bregitzer *et al.* (1998) compare their transgenic population to a tissue culture-derived population from a previous experiment, and it is not clear from Dale and McPartlan (1992) whether their tissue culture derived controls were created at the same time and within the same experiment as the transgenic plants they analysed.

[34]Seed transformation is an '*in planta*' procedure which does not involve a tissue culture step.

[35]RFLP analysis: Genomic DNA is digested with a restriction enzyme, the DNA is separated on a gel by electrophoresis and analysed using Southern blot techniques. Mutations (RFLPs) appear as differences in the presence or absence of bands of different sizes on the Southern blot.

[36]RAPD analysis: Random oligonucleotide primer pairs are used to amplify genomic DNA and the resulting products are run on agarose gels. Genomic differences appear as bands of different sizes. The RAPD technique allows detection of genomic differences due (a) point mutations at primer binding sites or (b) rearranged genomic regions (due to recombination, transposition, insertion/deletion, inversion or amplification) which occur within the region amplified by the primers.

Plant species	Transformation method	Technique used to analyse variation	Reference
Rice	*Agrobacterium*-mediated transformation of scutellar- derived calli	RAPD AFLP	Labra *et al.* 2001
Sugarcane	Electroporation of embryogenic calli	RAPD AFLP RAMP	Arencibia *et al.* 1999
Rice	Particle bombardment of immature embryos Electroporation of embryogenic calli	RAPD AFLP RAMP AFRP	Arencibia *et al.* 1998
Rice	Protoplast transformation	RAPD	Bao *et al.* 1996
Poplar	*Agrobacterium*-mediated transformation of leaves	Leaf shape and growth RFLP RAPD Microsatellites	Wang *et al.* 1996
Rice	Protoplast electroporation	Phenotypic analysis	Schuh *et al.* 1993
Potato	*Agrobacterium*-mediated transformation of tuber discs	Phenotypic analysis	Dale and McPartlan 1992
Barley	Particle bombardment of immature embryos	Phenotypic analysis	Bregitzer *et al.* 1998

Table 3. Studies of genome-wide mutations in transgenic plants

2.3.1 *Agrobacterium-mediated transformation*

Labra *et al.* (2001) examined DNA polymorphism in 10 independently derived transgenic rice plants produced via *Agrobacterium*-mediated transformation of callus. The pre-transformation callus was derived from a single parental line. With RAPD analysis, using seventeen primers, they found 9 bands to be polymorphic (i.e. different from those in the parent line) out of total of 119 bands amplified from the ten transgenic genomes.

Using AFLP analysis and using four pairs of primers, they found 19 polymorphic bands out of 288 amplified from the same ten transgenic genomes. There were no polymorphic bands amplified from 10 control plants[40]. Their data led Labra *et al.* (2001) to conclude that, for both RAPD and AFLP analysis, "the genomic similarity value was 100% in the case of control plants and 96-98% in the case of the transgenic population".

Wang *et al.* (1996) observed both phenotypic (e.g. leaf shape differences) and genotypic changes in *Populus nigra* (poplar) plants that had undergone *Agrobacterium*-mediated transformation and tissue culture regeneration. Using RFLP, RAPD, and microsatellite analysis, they analysed 17 transformed *P. nigra* plants (from 14 independent transformation events), 4 control *P. nigra* plants regenerated without transformation, and 2 *P. nigra* controls for the presence of DNA polymorphisms.

For RFLP analysis, each individual transformant was scored for the presence or absence of 18 different bands. No band differences were found between the 6 untransformed control plants. Three of the 14 independent transformants also showed no band differences. Each of the remaining 11 transformants had between 1-8 bands which differed from the pattern shown by the control plants. The total number of polymorphic bands in these 11 transformants was 35, out of a total of 198 bands. This extrapolates to approximately 1000s of polymorphic bands per diploid genome.

The same plants were analysed using RAPD analysis. In this analysis, it was found that most of the transgenic *P. nigra* plants exhibited greater polymorphism than the tissue culture control plants (Wang *et al.* 1996).

Microsatellite analysis of the same plants showed that all four of the tissue culture derived controls and all but two of the 14 independent *P. nigra* transformants showed microsattelite size differences as compared to the two *P. nigra* control plants. All of the *P. nigra* transformants showed DNA polymorphisms by one or more of the three methods used (Wang *et al.* 1996).

2.3.2 *Particle Bombardment*

Arencibia *et al*. (1998) examined mutations arising in rice plants transformed via particle bombardment of immature embryos. They analysed 12 transgenic T_3[41] plants from each of three different rice cultivars. In these 36 transgenic genomes, they found no polymorphic bands using RAPD analysis. When they used AFLP analysis, they found 12 polymorphic bands out of a total of 1711 bands amplified from the 36 genomes. Polymorphisms were also found using RAMP and AFRP analysis. Extrapolation from the combined data derived from AFLP, RAMP and AFRP analysis suggests that, in this experiment, transformation via particle bombardment generated, on average, many hundreds of changes per transformed rice plant genome. The number of polymorphisms found in the tissue culture control plants was approximately the same as the number found in the transformed plants. This suggests that, in this experiment, most of the genome-wide mutations in the transformed lines are attributable to tissue culture.

2.3.3 *Cell electroporation and protoplast transformation*

Arencibia *et al.* (1998) also examined rice transformed via electroporation of embryogenic callus. They analysed 15 independent transgenic T_0 plants that had been transformed via cell electroporation. Using RAPD analysis, no polymorphisms were found. Using AFLP analysis, six polymorphic bands out of 639 were seen in the 15 transgenic genomes. Polymorphisms were also found using RAMP and AFRP analysis. The combined data suggest that, in this experiment, transformation via cell electroporation also generated, on average, many hundreds of changes per transformed diploid genome. The number of polymorphisms found in the tissue culture control plants was approximately the same as the number found in the transformed plants. This suggests that, again, most of the genome-wide mutations are attributable to tissue culture (Arencibia *et al.* 1998).

In a separate study, Arencibia *et al.* (1999) examined the genomes of five transgenic sugarcane plants that had been transformed via cell electroporation of embryogenic callus. Using both AFLP and RAMP analysis, they found a total of 51 polymorphic DNA bands, out of 1237 amplified. They estimated that they had examined approximately 814 Kbp of genomic DNA. Extrapolated to the entire genome, this represents, depending mainly on the estimated genome size, many 100s to 1000s of mutations per regenerated plant.

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[37] AFLP analysis: Genomic DNA is cut with a restriction enzyme (e.g. *PstI* or *MseI*), biotinylated adapters (e.g. *PstI* or *MseI* adapters) are ligated to restricted genomic fragments, biotinylated fragments are isolated by binding to streptavidin particles, and used as template DNA in PCR reactions with primers appropriate to the restriction site used. Resulting PCR products are run on polyacrylamide gels. Polymorphisms appear as band differences.

[38] RAMP analysis: PCR analysis where genomic DNA is amplified using primers that specifically recognise microsatellite DNA. Microsatellite polymorphisms appear as band differences on polyacrylamide gels.

[39] A polymorphic band is a band which is present in the DNA sample analysed from one plant (e.g. parental line) but absent in another (e.g. transformant). It represents a genomic difference (such as a nucleotide difference or a small or large DNA deletion, insertion or rearrangement) between the 2 plants being compared.

[40] These control plants had not undergone plant tissue culture or plant transformation.

[41] The original transformed plant, the primary transformant is called the T_0, the progeny of self-fertilised T_0 plants are called T_1 plants, and the progeny of self-fertilised T_1 plants are called T_2 plants and so on.

Bao *et al.* (1996), using RAPD analysis, examined three independent transgenic rice plants produced via protoplast transformation. They found 2-4% of the bands analysed were polymorphic between the transformed plants and untransformed control plants.

2.4 Genome-wide mutations are likely to be found in transgenic crop plants granted commercial approval

The data discussed in **Section 2.3** indicate that plant transformation methods can induce mutations at many 100s to 1000s of different sites in the plant genome. These numbers are likely to be underestimates of the true number. This is due to the fact that all the analytical methods used (e.g. RFLP and RAMP) are able to detect only a subset of the possible mutations. For example, some methods fail to detect most base pair substitutions, short deletions and insertions. As a consequence, the total number of mutations is likely to be greater than detected in the experiments described above. The exact degree of the underestimate will depend on the mutational spectrum of plant transformation and this is not known.

Sala *et al.* (2000) state that, in their experience, such mutations can be found in every transformed plant analysed, if the right molecular tools are used. Large numbers of these mutations are heritable, either clonally and/or sexually (Sala *et al.* 2000, Arencibia *et al.* 1998, Bao *et al.* 1996). Thus, using similar methods of analysis, mutations resulting in polymorphic bands should be identifiable in commercial transgenic crop plants, unless such mutations have been eliminated in later generations by backcrossing or outcrossing. At present, there is no published study using such molecular techniques to look for genome-wide mutations in commercially approved transgenic lines or cultivars.

2.5 Summary

a) Few studies have examined the nature and extent of the genome-wide transformation-induced mutations found in transgenic crop plants.
b) DNA polymorphism analysis indicates that very large numbers of heritable mutations are created in primary transformants (and can be retained in their progeny) using the standard methods of plant transformation. The best estimate is that these number many 100s to 1000s per plant genome.
c) These mutations are caused by tissue culture techniques and possibly by *Agrobacterium*-mediated transformation and particle bombardment.
d) The numbers of heritable mutations suggested by the available data (many 100s to 1000s per genome) are likely to be underestimates of the true number.
e) Genome-wide mutations are likely to be found in commercial transgenic cultivars unless they have been removed using extensive backcrossing or out crossing.
f) There is no published study examining genome-wide mutations in commercial transgenic cultivars.

Section 3. Significance of insertion-site and genome-wide mutations

In this section we demonstrate how the presence of transformation-induced insertion-site or genome-wide mutations in transgenic crop plants (**Sections 1** and **2**) might result in potentially harmful consequences. In order to facilitate the following discussion, we illustrate it with the simple example of altered nutrient, allergen or toxin accumulation. However, all of the genetic consequences described in **Points I-V** below could equally well result in altered crop plant ecology, thus posing environmental risks, rather than risks to consumers.

3.1 Insertion-site mutations can result in transgenic crop plants having hazardous phenotypes

Insertion of the transgene and superfluous DNA and/or the deletion or rearrangement of plant genomic DNA at the insertion-site may have the following consequences:

I. Loss of gene function: Insertion into, deletion or rearrangement of gene coding sequence is likely to cause a loss of plant gene function. If the disrupted gene codes for an enzyme involved in nutrient biosynthesis (or for a regulatory protein controlling the activity of such an enzyme) this may result in reduced nutrient levels. Similarly, loss of function of a regulatory protein that controls the levels of a substance that is harmful to humans could result in the accumulation of toxins, allergens or anti-nutrients. Some insertion-site deletions or rearrangements are large enough to result in the loss of function of many genes (e.g. Kaya *et al.* 2000).

II. Altered protein function: Insertion of a transgene (or superfluous DNA) and/or the associated deletion or rearrangement of genomic DNA may result in the production of a truncated or chimaeric protein. Such proteins may be mis-localised or mis-regulated or they may exhibit otherwise inappropriate activity. An example would be the creation of a non-functional truncated version of a protein that normally forms a multi-protein complex with other proteins. If the protein complex normally acts to regulate genes in a nutrient biosynthesis pathway, and the truncated protein displaces functional subunits, this could inactivate the complex, resulting in reduced nutrient levels.

Alternatively, truncated or chimaeric proteins may acquire or lose sequences necessary for cellular localisation (e.g. nuclear localisation signals) or for protein activation or inactivation (e.g. phosphorylation sites). If the altered proteins are involved in the synthesis of toxins or nutritional compounds (or in the regulation of their accumulation) the level of these toxins or nutritional compounds may be altered.

III. Loss of gene expression or altered gene expression: Altered patterns of gene expression (e.g. loss of gene expression, increased expression, or mis-expression of genes in new tissue or cell types may occur due to: (a) deletion or disruption of promoter or enhancer sequences, (b) altered spacing between genes and the

removal of 'boundary' sequences that prevent the regulatory sequences of one gene from influencing another gene, (c) altered higher order genome structure resulting from DNA rearrangement, or (d) sense or anti-sense suppression of an endogenous gene, leading to reduction or loss of expression. For example, a duplicated gene, created during transgene insertion, could insert next to an endogenous promoter (or one originating from the transgene, see **Point IV** below) and create an antisense transcript. This antisense transcript could silence the homologous endogenous gene[42].

Mutations in some functional sequences can have effects on numerous genes. For example, a mutation in a gene which encodes a transcription factor could result in the mis-regulation of more than one gene.

Alterations in gene expression may have negative consequences if they result in the over-expression or mis-expression (e.g. in the edible parts of plants) of genes producing substances toxic to humans, or, alternatively, if they result in the loss of expression of genes involved in nutrient biosynthesis.

Mutations of the types mentioned in **Points I-III** above are well known to molecular geneticists and they are not confined to transgenic organisms. The most common mutations are loss-of-function mutations. Precedents for the genetic consequences resulting from the more complicated types of genetic rearrangements and disruptions (such as those described in **Points II-III**) exist in non-transgenic cultivars (Kusaba *et al.* 2003[43]). Additionally, transgenic plants with antisense, over-expressing or dominant-negative mutant genes or with constitutively activated mutant proteins have all been produced for experimental purposes (Branen *et al.* 2003, Li *et al.* 2002, Markel *et al.* 2002, Tao *et al.* 2002). This reinforces the fact that such mutations are all plausible consequences of transgene insertion, especially those insertions associated with large-scale rearrangement of host DNA and/or superfluous DNA.

Even for simple mutations (such as base pair changes or small deletions), predicting the consequences of genetic change is not easy. For example, knowledge of the sequence of the target-site is not currently sufficient to determine whether insertion into a certain target-site sequence is without consequence. It is known that regulatory regions may regulate more than one gene and also that, in many higher eukaryotes, regulatory sequences can be distant from gene coding sequences (Carter *et al.* 2002). It is also clear that in higher eukaryotes genes are often clustered and that genomic position may have consequences for gene regulation (Hurst *et al.* 2004). Therefore, while it is clear that transgene insertion into known gene sequences is problematic, it is not clear how to determine when a transgene insertion is without consequence.

3.2 Potential sequence-specific consequences of superfluous DNA insertion include the creation of hazardous phenotypes and increased risk of horizontal gene transfer

The discussion above has largely assumed that the nature or origin of inserted superfluous DNA does not add to the risk of hazardous consequences. However, this may not always be true.

IV. Transgene promoter-induced mis-expression of plant genes: Insertion of promoter DNA, such as the 35S CaMV promoter (which is routinely used in making transgenic crop plants) can cause over-expression or mis-expression of neighbouring genes, with the consequences described in **Point III** above. Experiments carried out in transgenic *A. thaliana* and rice plants indicate that strong transgene enhancers or promoters can influence endogenous gene expression, even at a distance of several Kilobase pairs (Jeong *et al.* 2002, Weigel *et al.* 2000, Wilson *et al.* 1996).

V. Horizontal gene transfer: Insertion of superfluous DNA (e.g. bacterial plasmid DNA, bacterial marker DNA, or transgene DNA[44]) into transgenic plants could facilitate horizontal gene transfer of the transgene DNA (e.g. to soil bacteria or human or animal gut bacteria) by providing opportunities for homologous recombination (Nielsen *et al.* 2001, Gebhard and Smalla 1998, Nielsen *et al.* 1998, 1997a, 1997b, Lorenz and Wackernagel 1994). For example, in the human gut bacterium, *E. coli*, a minimal length of 20 bp of homology is required for recombination to occur (Nielsen *et al.* 1998). Plasmid origins of replication, in particular, may facilitate horizontal gene transfer.

3.3 Genome-wide mutations also pose safety risks

Much of the spectrum of genome-wide mutations (deletions, rearrangements etc.) is probably similar to that of insertion-site mutations. Where these mutations (**Section 2**) occur in functional plant sequences they can result in aberrant phenotypes by the mechanisms described in **Points I-V** above (Negruk *et al.* 1996, Dennis *et al.* 1987). Therefore, genome-wide mutations introduced during plant transformation have the same potential for hazardous toxicological, nutritional, or environmental consequences as insertion-site mutations.

3.4 Summary

a) Plant transformation (i.e. tissue culture techniques, DNA transfer techniques and DNA insertion) can introduce a wide spectrum of potentially deleterious mutations into the genome of a transformed plant.
b) When mutations occur in functional DNA sequences they can result in loss of gene activity, altered gene function and altered gene expression, and may impact on proteins involved in complex gene regulation systems and biochemical pathways.
c) Both insertion-site and genome-wide mutations have the potential to alter the behaviour and/or the biochemical characteristics of the plants that carry them.
d) Even though their effects may not be immediately apparent or obvious, these mutations may have profound consequences for the environment or for those who produce or consume such plants.
e) Mutations involving insertion of superfluous DNA sequences may also have consequences for the potential for horizontal gene transfer of transgene sequences.

[42] There are precedents for the type of mutation that could give rise to such a transcript. Duplication and reinsertion of an endogenous gene next to a T-DNA was reported by Tax and Vernon (2001) and Kusaba *et al*. (2003) report a mutation in a non-transgenic plant that likely confers a mutant phenotype via gene silencing.

Section 4. Can transgenic plant breeding methods be compared to modern non-transgenic plant breeding methods?

It is common for advocates of transgenic plant breeding to argue that transgenic plant breeding methods pose no greater risks than those arising from other 'modern' plant breeding methods[45] developed during the 20th century (Prakash 2001, Stewart *et al.* 2000, Conner and Jacobs 1999, Kessler *et al.* 1992). This assumption is the basis for FDA regulation of transgenic crops in the United States (Kessler *et al.* 1992). Setting aside the question of whether a comparison with existing worst practice (i.e. the presumed most hazardous breeding technology) is an appropriate baseline, they claim that: (1) modern non-transgenic breeding techniques create mutations in plant genomes that are comparable to those created by transgenic plant breeding, (2) modern plant breeding techniques are used on a wide scale and that (3) modern methods have a history of safe use, brought about in part because commercial breeders can and do detect and discard hazardous varieties (Prakash 2001, Stewart *et al.* 2000, NRC 2000; Conner and Jacobs 1999, Kessler *et al.* 1992). These and other authors also compare transgenic breeding with the use of wide crosses and they argue that the products of wide crosses can exhibit hazardous traits (Prakash 2001, Stewart *et al.* 2000, NRC 2000, Conner and Jacobs 1999, Kessler *et al.* 1992). With one of these claims we *partially* agree: that modern plant breeding techniques do produce unpredictable changes in plant genomes (claim 1). Whether these mutations are of a similar magnitude and type to those introduced by transgenic plant breeding methods is still an open question.

It is known that non-transgenic plant breeding programmes can produce cultivars with hazardous properties. A small number of clearly hazardous cultivars have been developed accidentally during the course of 20th century plant breeding programmes. For example, potato and celery cultivars with high levels of substances toxic to humans (i.e. glycoalkaloids and linear furanocoumarins respectively) have been inadvertently created using non-transgenic plant breeding methods (NRC 2000). Similarly, cultivars may show altered environmental interactions such as increased susceptibility to diseases (NRC/IOM 2004). Thus, unexpected phenotypes arising from modern plant breeding techniques do present a real risk of adverse consequences. However, we argue that the existence of these hazardous cultivars should be used to justify vigilance, rather than complacency, when regulators are confronted by a novel plant breeding technique such as plant transformation, the full extent of whose mutagenicity is still unknown.

In the rest of this section, we argue that the advocates of transgenic plant breeding fail to adequately substantiate any of their claims. We argue that the suggestion that genomic changes created during non-transgenic plant breeding are equivalent to those created using transgenic methods (claim 1) lacks sufficient supporting evidence. This is due to a lack of research into the nature and extent of the mutations induced in plants bred using either transgenic or non-transgenic methods. There is also a lack of evidence supporting claims of the wide-scale use of modern non-transgenic plant breeding methods (claim 2). Indeed, for some major crops for which transgenic methods are being used, widespread use of other modern breeding methods can be specifically ruled out. Finally, there is also lack of evidence for the scientifically established safe use of modern methods (claim 3).

In the following sections, we examine the mutagenicity of non-transgenic plant breeding methods and their history of use in plant breeding programs.

4.1 Mutations introduced by modern non-transgenic plant breeding methods

Induced mutation, tissue culture, somatic hybridisation and wide crossing are all modern techniques which can be used to create or introduce novel variation for use in plant breeding programs. Tissue culture can also be used for the clonal propagation of certain plants and to manipulate plant cells or tissues during plant breeding programs. In this section, we discuss what is known about the unpredictable genetic consequences of these methods.

Induced mutagenesis: Mutagenesis of plant genomes induced by application of chemicals (EMS, ENU) or irradiation can be used to increase the amount of variation available to plant breeders. Over-application of mutagenic procedures renders the plant genome non-functional or loads it with so many mutations that the useful variation cannot be identified or separated from deleterious mutations. Breeders aim therefore to strike a balance between creating sufficient variation and causing excessive damage. Little information exists on the genetic consequences of various mutagenic treatments for particular crops and the actual application rates used by breeders. However, it seems reasonable to conclude that the mutagenic effect on a crop plant genome could be considerable.

Tissue culture: The mutagenic potential of tissue culture depends on various factors (Cailloux 1984). Tissue culture methods relying on dedifferentiation are widely considered to cause greater genetic damage than those which do not (e.g. vegetative propagation or embryo rescue[46]; Skirvin *et al.* 1994, Karp 1989, Cailloux 1984). Consequently, dedifferentiated tissue culture has been used occasionally as a form of induced mutagenesis

[43] Kusaba *et al.* (2003) show that rice plants carrying the *Lgc*1 mutation have a 3.5 Kbp deletion between two highly similar *glutelin* genes, *Glu*B4 and *Glu*B5. The deletion results in a tail-to-tail repeat and removal of the transcription termination signal from *Glu*B5. A read-through product is produced which contains sequences from both genes and which they speculate may produce a double-stranded RNA molecule. Such a molecule may induce gene silencing and the suppression of the *Glutelin* multigene family seen in plants carrying the *Lgc*1 mutation.

[44] The cauliflower mosaic virus 35S promoter is routinely used to express transgenes. The cauliflower mosaic virus 35S promoter has been shown to be active not only in plants but also in the gut bacterium *Escherichia coli* (Jacob *et al.* 2002, Lewin *et al.* 1998, Assaad and Signer 1990), the gut pathogen *Yersinia enterocolitica*, the soil bacterium *Agrobacterium rhizogenes* (Jacob *et al.* 2002, Lewin *et al.* 1998), and in fungi (Pobjecky *et al.* 1990) and cell extracts of human HeLa cells (Burke *et al.* 1990, Cooke and Penon 1990, Guilley *et al.* 1982).

[45] 'Modern' plant breeding methods cited include plant tissue culture, radiation and chemical mutagenesis and the use of wide crosses such as between sexually incompatible species.

(Jain 2001). Again, as with chemical and irradiation mutagenesis techniques, the details of the tissue procedure are crucial. Plant cells which spend extensive periods in dedifferentiating and regenerating conditions are likely to exhibit more DNA mutations than those that have not (Birch 1997, Skirvin *et al.* 1994, Cailloux 1984). Similarly, species differences, hormone levels and use of antibiotics all have apparent effects on the number of mutations which accumulate in the plant DNA (Skirvin *et al.* 1994, Cailloux 1984).

It is important to emphasise the contrast between those tissue culture procedures which necessitate dedifferentiation and therefore new meristem or organ formation, and those tissue culture procedures associated with plant propagation (e.g. of potato). The former are usually used to make transgenic plants and are associated with somatic variation, while the latter are far more widely used for propagation purposes but are generally less mutagenic (Skirvin *et al* 1994, Cailloux 1984).

Somatic hybridisation and wide crossing: Somatic hybridisation by protoplast fusion is a tissue culture-based method for fusing genomes which cannot be brought together by conventional sexual crossing. Somatic hybridisation can also be used to put genomic DNA into new cytoplasmic backgrounds (Orczyk *et al.* 2003, Akagi *et al.* 1989). While the majority of successful somatic hybridisations have been between related species within the same genus (Solomon-Blackburn and Barker 2001, Brown and Thorpe 1995), researchers have produced fertile somatic hybrids between plants as distantly related as dicots and monocots (Kisaka *et al.* 1997, Kisaka *et al.* 1994). The genetic outcomes of somatic hybridisation are unpredictable and the use of plant tissue culture and the introduction of very distantly related genetic material into one genome both have the potential to create large-scale genetic differences between hybrids and their parents. Concern over the safety of this technique has been expressed because importing genetic material using somatic hybridisation techniques has been shown to result in unpredictable phenotypic consequences (NRC 2000).

Clearly, some non-transgenic modern plant breeding techniques are potentially highly mutagenic. As is the case for plant transformation, the magnitude and nature of the mutations induced by different breeding methods is not easily measured and is likely to vary widely depending on the specific cultivars and treatments used. It is therefore difficult to compare the mutagenicity of modern transgenic and non-transgenic breeding methods in the straightforward manner implied in claim 1.

4.2 Non-transgenic plant breeding methods in the 20th Century: History and extent of use

Until the 20th century, farmers everywhere grew crops derived from 'traditional' plant breeding methods. The differences between cultivars depended mainly on the selection and propagation of natural variants (arising from spontaneous mutation, polyploidisation, and sexual crossing) and occasionally on the selection and propagation of the progeny from intentional crosses (Koornneef and Stam 2001). The rediscovery of Mendel's work accelerated a change in plant breeding methods used in the West, the major one being the routine use of intentional sexual crosses by professional plant breeders (Koornneef and Stam 2001, Hayward *et al*. 1993).

The 20th century also saw the development of additional techniques able to create or introduce genomic variation for breeding purposes (Koornneef and Stam 2001). It became possible to create novel genetic variation using mutagenic radiation, chemical or tissue culture treatments and to access new sources of genetic variation by producing 'wide crosses' via techniques such as embryo rescue or somatic hybridisation (Sharma 1995, Hayward *et al.* 1993).

Traditional plant breeding methods (selection and intentional crosses) are still responsible for the vast majority of crop plant cultivars available for use in agriculture today (Koornneef and Stam 2001, Maluszynski *et al*. 2000, Brown and Thorpe 1995, Hayward *et al.* 1993). Modern non-transgenic techniques (e.g. intentional mutagenesis and somatic hybridisation) are used much less often to produce commercialised cultivars. Nevertheless, various commercial cultivars incorporate genes derived from radiation mutagenesis programmes (although many of these are not edible crop plants) and some cultivars carrying induced mutations (e.g. dwarf cereal and rice varieties) have been grown on a large scale (Maluszynski *et al.* 2000). There are even fewer cultivars with tissue culture-derived mutant genes (Jain 2001, Li *et al.* 2001, Skirvin *et al.* 1994). Similarly, because the vast majority of the products derived from somatic hybridisation are genetically unstable or infertile, this method has not been widely used to produce commercial cultivars (Orczyk *et al.* 2003, Collonnier *et al.* 2001, Koornneef and Stam 2001, Solomon-Blackburn and Barker 2001, Wolters *et al.* 1994). Consequently, in only a few crops (e.g. tomatoes, wheat and potatoes) do non-transgenic commercial cultivars commonly incorporate any genes derived from other plant species (Hayward *et al.* 1993, Jauhar and Chibbar 1999).

Thus, the claim that modern non-transgenic plant breeding methods are used on a large scale is not true for most crops. Some modern non-transgenic methods (e.g. wide crosses and somatic hybridisation) have only recently become incorporated into commercial breeding programmes. Others have at best made only a modest or even minor contribution (e.g. chemical and irradiation mutagenesis), and this for only a few crops (e.g. wheat), to the total number of cultivars introduced worldwide (Maluszynski *et al.* 2000). Indeed, some major crops for which transgenic varieties are now commercially available (e.g. maize) have a negligible history of modern non-transgenic plant breeding (Smartt and Symonds 1995, Darrah and Zuber 1986). Nevertheless, there will always be some uncertainty on the exact extent of use of modern non-transgenic methods (claim 2) because plant breeding for many species is now a commercial and somewhat secretive activity and the genetic heritage of cultivars can be obscure.

[46]Embryo rescue is used to culture hybrid embyos which have been excised from seeds. This permits sexual hybridisation between plants that show post-fertilisation incompatibility (e.g. due to endosperm defects).

4.3 Role of backcrossing and outcrossing in plant breeding programmes

The number of unintended mutations retained in commercial cultivars depends on two factors: (1) the mutagenic nature of the initial breeding programme and (2) the steps taken by breeders to remove unwanted mutations. Non-transgenic plant breeding programmes typically use backcrossing and/or outcrossing to remove unwanted variation and to introduce new variation into elite cultivars. However, since this is an area of primarily commercial interest, little concrete information is publicly available detailing backcrossing programmes for specific commercial cultivars.

Extensive backcrossing significantly reduces the risk of commercial cultivars retaining mutations introduced during modern plant breeding procedures. Genomic exchange occurs with each backcross, so that the mutations accumulated (e.g. during tissue culture, mutagenesis or wide crosses) are progressively lost during backcrossing programmes. Lack of necessity to back-cross extensively has been cited as a reason why breeding programmes featuring plant transformation are more rapid than non-transgenic breeding programmes (GM Science Review Panel 2003). Furthermore, plant transformation is often applied to or proposed for crops in which backcrossing or outcrossing are difficult or slow, such as potato, banana and papaya (**Table 2**).

If transformed plants are not backcrossed or outcrossed then all of the mutations introduced during plant transformation will be present in future generations of the transgenic plant. For example, the genome of commercially approved Newleaf® Plus Potato RBMT22-82 has superfluous marker, plasmid and transgene DNA sequences inserted at several different insertion-sites (**Section 1.1.7**). It therefore appears that this cultivar has not been backcrossed. If so it will retain all of the mutations introduced during transformation.

Because the transgene is always being selected for in backcrosses, it is difficult to remove DNA sequences (and mutations contained within them) that are linked to the transgene. These linked sequences may constitute a considerable portion of the total genome (Stam and Zeven 1981). The exact proportion depends, among other factors, on the crop species, the frequency of recombination and the location of the insertion site. This difficulty in recombining away linked sequences applies equally to transgenic and non-transgenic traits introduced by mutagenic modern plant breeding methods and to traits introduced by wide crosses (Gepts 2002).

4.4 Is there evidence of safe use?

In order to argue that modern non-transgenic plant breeding methods have a history of safe use (claim 3), it is also necessary to demonstrate that crops bred by modern non-transgenic means are in fact 'safe', and if 'safe' is not an absolute term, to define what is meant by it in this specific context. It is beyond the scope of this report to do this. However, it is worth noting that transgenic plant breeding advocates make no attempt to do this (Prakash 2001, Stewart *et al.* 2000, Conner and Jacobs 1999, Kessler *et al.* 1992). This may be a reflection on the extreme difficulty of the task. Many foods (e.g. peanuts, cow's milk, soybeans, wheat, meat), for many individuals, are not safe to consume at all. Even for foods with no specific evidence for harm, it is unclear whether the human diet would be superior without them, since our ability to identify sources of delayed or cumulative negative health effects in the human diet is very limited. Where and how widely a crop is grown, as well as how the products are processed and consumed also impact on the ultimate 'safety' of a crop plant and its products. All of these factors are hard to predict and control. Thus any assertion that modern non-transgenic breeding methods deliver safe food has very limited scientific validity.

4.5 Regulation of cultivars derived from modern plant breeding technologies

We have argued that there are not enough scientific data to support claims that (1) modern non-transgenic breeding techniques create mutations in plant genomes that are comparable to those created by transgenic plant breeding, (2) modern non-transgenic plant breeding techniques are used on a wide scale and that (3) modern methods have a history of safe use. Therefore we do not consider modern plant breeding methods an appropriate benchmark from which to determine the safety of transgenic plant breeding techniques. Additionally, we believe that it is neither rational nor scientific to suggest that transgenic plant breeding should be deemed safe if it can be shown to be no worse than the current presumed most hazardous practice. To condone arguments in which a new technology is compared only with its least safe equivalent is not a positive step forward for consumer, producer or environmental protection.

We reject a comparative approach in favour of a rational and scientific enquiry into the specific risks arising from each particular plant breeding method (e.g. plant transformation, somatic hybridisation). For example, a scientifically based risk assessment of a transgenic crop cultivar would take into account risks arising from:

A. Insertion-site and genome-wide mutations created during the plant transformation process

B. Effects of the transgene DNA sequences

C. The potential for horizontal gene transfer

D. The transgene products (RNA or protein) and their effects on plant biochemical pathways

E. The expected and unexpected traits conferred by the transgene product

Such a safety assessment would be more accurate than the current method of relying on the presumed safety of other modern non-transgenic plant breeding methods and the presumed equivalence of transgenic plant breeding to these methods. In the past, commercial cultivars derived from modern non-transgenic plant breeding techniques have entered the food supply without specific regulation. However, in the interests of consumer and

environmental safety, it may be necessary to devise safety guidelines or regulations for cultivars derived from other modern plant breeding methods. We argue that, if needed, such regulations should be based on the specific risks arising from the specific technology.

4.6 Summary

a) Plant breeding has the potential to result in harmful cultivars.
b) Some modern non-transgenic plant breeding techniques have the potential to frequently introduce harmful mutations into commercial cultivars.
c) Crop varieties bred using these modern non-transgenic methods represent a small minority of the total number of commercial crop cultivars.
d) Backcrossing and/or outcrossing further reduce the likelihood that harmful mutations will be retained within released non-transgenic cultivars.
e) No commercial crop cultivars, especially those produced by modern transgenic or non-transgenic methods, can be claimed to have an unequivocal and scientifically supported history of safe use.
f) There is no scientific justification for claiming that transgenic plant breeding methods are as safe as modern non-transgenic plant breeding methods.
g) A rational approach to risk assessment for any modern plant breeding technology would take into account the specific risks arising from that specific technology.
h) In the interests of environmental and consumer safety, cultivars derived from other modern non-transgenic plant breeding technologies may also require regulation.

Section 5. Conclusions

This report has drawn together what is known about the mutagenic effects of the plant transformation process and its potential consequences for transgenic cultivars. In this report we have shown that current plant transformation methods are neither precise nor predictable and that the genomes of transformed plants typically contain many unintended mutations.

Transformation-induced mutations

The two most frequently used crop plant transformation methods, *Agrobacterium*-mediated transformation and particle bombardment, create both insertion-site and genome-wide mutations in transformed plants. Transgene insertion, by its very nature, disrupts the sequences into which it inserts. It is usually accompanied by additional rearrangement, duplication or deletion of plant genomic DNA and by insertion of superfluous DNA, such as rearranged or truncated transgene, plasmid, selectable marker or bacterial genomic DNA (**Section 1.1**). Such insertion-site mutations can be substantial, including:

- translocation of plant genomic DNA from other regions of the genome (translocations of up to 40 Kbp have been reported, Tax and Vernon 2001)
- deletion of plant genomic DNA (a 75.8 Kb deletion predicted to have deleted 13 genes was reported by Kaya *et al.* 2000)
- duplications of plant gene sequences (e.g. Forsbach *et al*. 2003)
- insertions of large amounts of superfluous transgene or plasmid DNA
- scrambling of transgene and plant genomic DNA (even at 'simple' particle bombardment insertion events, transgenes can be flanked by numerous plant and/or transgene DNA fragments, Makarevitch *et al.* 2003)

These characteristics of transgene insertion are significant for several reasons. First, mutations created at the transgene insertion-site will be genetically linked to the transgene. Therefore, a specific transgene 'insertion event' or 'transformation event' includes not only the transgene but any associated insertion-site mutations. A commercial transgenic cultivar carrying a particular insertion event will therefore also harbour a characteristic spectrum of insertion-site mutations.

Second, transgenes and superfluous DNA sequences frequently insert into functional plant genomic DNA (**Section 1.1.6** and **1.2.5**), such as regulatory sequences (e.g. gene promoters) or coding sequences, potentially destroying or altering gene function. It is for this reason that *Agrobacterium*-mediated transformation has often been used experimentally as a 'mutagen' (Jeong *et al.* 2002, Weigel *et al*. 2000, Krysan *et al.* 1999, Schauser *et al.* 1998, McNevin *et al.* 1993, Forsthoefel *et al.* 1992).

Third, superfluous DNA insertions that include bacterial chromosomal and plasmid sequences may increase the possibility of horizontal gene transfer to microbes or viruses (**Section 3.2**).

Notwithstanding the above, the frequency and full extent of insertion-site mutations in transgenic crop plants is only partially understood. This is partly due to a surprising lack of systematic studies (**Sections 1.3** and **2.5**). Another reason is the equally surprising lack of studies using methods such as PCR and DNA sequencing to characterise insertion events and target-site sequences, and thus allow a full comparison between them. These studies are alone in having the capability to detect the full extent of mutation at the transgene insertion-site. For these reasons, virtually nothing is known about the general characteristics of single-copy transgene insertion events resulting from particle bombardment, including the extent of the resulting genome disruption or insertion-site preferences (**Sections 1.2.4** and **1.2.5**).

The situation is only marginally better for T-DNA insertion events created via *Agrobacterium*-mediated transformation. Much of the most useful data on T-DNA insertion events comes from studies conducted on the non-crop plant *A. thaliana*. It is not certain whether the results of such studies can be extrapolated to crop plants. A further complication is that crop transformation in different laboratories is carried out using different strains of bacteria, different transformation conditions and different plasmids. Even the best studies cannot therefore be certain of replicating the conditions found in the laboratories of commercial developers of transgenic crops. Without detailed studies of the insertion-site mutations found in commercial cultivars, it is not possible to be sure whether the results of studies reported in the scientific literature are also relevant to and representative of commercial cultivars.

The tissue culture and gene transfer procedures used to transform plants also introduce mutations at numerous locations throughout the genome (**Section 2**). These mutations can also occur in functional plant DNA (**Section 2.2**). Little is known about the specific molecular nature of these genome-wide mutations. For every individual transformed plant, the magnitude and nature of these mutations will depend on the crop plant, the plant transformation protocol and on chance events. The number of genome-wide mutations present in a commercial transgenic cultivar will additionally depend on the extent of backcrossing or outcrossing prior to commercial release. Due to their abundance, these mutations have the potential to be highly significant.

Health, Farming and Environmental Implications of transformation-induced mutations

It is not usually disputed that both transgenic and non-transgenic plant breeding methods can result in crop plants having harmful characteristics as a result of unexpected genomic changes or that such characteristics could cause serious harm to consumers, growers or the environment (NRC/IOM 2004, NRC 2000).

It is therefore of great concern that the plant transformation techniques currently used to produce commercial transgenic cultivars introduce both insertion-site and genome-wide mutations which have such potential to cause harmful genetic consequences if they occur in functional DNA. This potential for harm arises principally because crop plants produce a multiplicity of compounds that are individually associated with positive or negative nutritional qualities when consumed by other organisms (Ames *et al.* 1990). Alterations in the abundance of these compounds, such as those caused by mutations in the genes which synthesise or regulate them, could result in major changes in the nutritional quality or health implications of that crop, especially if it is a staple for humans or livestock.

As well as providing resources for humans and livestock, crop plants have multifaceted roles within their semi-natural farmland environment. They are food for a diverse array of herbivores and seed-eating animals (including mammals, birds, and insects), pathogens, detritivores and saprophytes. In addition, they form mutualistic relations with pollinators, microbes and fungi. They also affect the growth of neighbouring plants, fungi and microorganisms. Phenotypes arising from unintended genetic changes could have a significant impact on one or more of the ecological roles of a particular cultivar. A cultivar could, for instance, become vulnerable to a pathogen it was previously resistant to, it might cease to form mycorrhizal associations or it might have adverse effects on specific soil organisms.

There is an additional reason for concern over the safety of modern commercial cultivars that is rarely mentioned. Due to a range of factors, there is decline in the range of crop plant cultivars available to producers. Consequently, certain modern commercial cultivars are grown on a vast scale world-wide. Thus commercialisation of a hazardous cultivar could result in harm to the environment on an unprecedented scale and many consumers are likely to find a single cultivar forming a large proportion of their diet.

Insertion-site mutations in commercial crop cultivars

Regulators appear to accept that analysis of the transgene insertion-site is important. We have shown, however, that the data contained in applications for commercial approval are invariably incomplete. Commercial applicants often provide only data from Southern blot analyses, they do not normally sequence the transgene insertion site, nor do they sequence or reconstruct the undisrupted target-site (**Table 2, Appendix**). Complete analysis requires sequencing the entire transgene insert and its flanking DNA, and should include a comparison with the original target-site sequence. This is necessary to show the full extent of genomic damage at the insertion-site and to determine the extent of superfluous DNA insertion.

Even the limited data available are sufficient to show that commercial transgenic plants currently approved for commercialisation in the US exhibit transgene insertion-site rearrangements and insertion of superfluous DNA (**Table 2, Appendix**). We predict that a complete analysis

of the transgene insertion-site in previously approved commercial transgenic cultivars would uncover further mutations, as occurred when commercially approved maize event MON810 and soybean event 40-3-2 were subjected to independent analysis after commercialisation (Hernandez *et al.* 2003, Windels *et al.* 2001).

Our report underlines both the importance of a full analysis of transgene insertion events and the inadequacy of current efforts.

Genome-wide mutations in commercial cultivars

Genome-wide mutations are likely to be present even in commercial transgenic cultivars where extensive outcrossing and/or back-crossing programmes have been carried out (**Section 4.3**). The potential for these mutations to cause harm is considerable, not least because the numbers reported here are highly likely to be a substantial underestimate of their actual abundance. However, analysis of genome-wide mutations is not required or recommended by any regulatory regime (**Section 2.4** and **3.3**). This is presumably because the significance of these mutations has yet to be recognised. We very much hope that this situation will be quickly remedied. Two immediately useful advances would be to require extensive backcrossing of transgenic cultivars and to eliminate the use of plant tissue culture during plant transformation. Other approaches are suggested in the recommendations (**Section 6**).

Safety assessment of commercial transgenic cultivars: molecular analysis

Current safety assessment of transgenic crop plants is focused (however imperfectly) on the potential hazards encoded by the transgene. Implicit in regulatory processes worldwide is the assumption that the plant transformation process itself does not have any impact on safety (NRC/IOM 2004, Kessler *et al.* 1992). The data reviewed in this report suggest strongly that this assumption is wrong.

Given the potential magnitude of transformation-induced mutations in commercial lines, and their potential hazardous consequences, we recommend immediate changes to the procedures used to evaluate and regulate commercial transgenic crop plants (**Section 6**). Such changes include:

- Requiring a complete molecular analysis of transgene insertion events, including sequencing the transgene insert and flanking DNA and a comparison with the original genomic target-site
- Rejection of transgenic events associated with superfluous transgene, marker or plasmid DNA
- Rejection of transgenic events associated with deletion and rearrangement of genomic DNA
- Rejection of transgenic events inserting into or near gene sequences
- Extensive backcrossing or outcrossing of the transgenic cultivar prior to commercialisation
- Analysis of genome-wide mutations remaining in the transgenic cultivar

As long as plant transformation continues to be mutagenic, and while the genomic location of transgene insertion is not able to be controlled, we feel that it is unacceptable and inaccurate for transgenic plant breeders to claim that either plant transformation or its products are precise, predictable or innately safe.

Safety assessment of commercial transgenic cultivars: biochemical and phenotypic analysis

In addition to a molecular analysis of the transgene insertion, safety assessments of transgenic cultivars usually include a limited number of biochemical and agronomic analyses. However, the limited genotypic and phenotypic analyses (including 'substantial equivalence' studies) which are submitted to regulators are an inadequate substitute for controlled and well-understood technologies. This is because it is hardly possible to anticipate and test for all possible harmful phenotypes, and, to quote Kuiper *et al.* (2001):

"It is plausible, but not proven, that expected changes in the metabolism as a possible result of genetic modification will be identified by analysis of a great number of components, but unexpected changes are merely identified by chance. The targeted approach has severe limitations with respect to unknown anti-nutrients and natural toxins, especially in less well known crops."

It is not just biochemical analyses which have severe limitations. Agronomic tests and animal feeding studies of transgenic cultivars or their products suffer from the same limitations. In agronomic tests based on field trials, for example, varying environmental conditions can have a large impact on outcome and it is not possible to carry out trials under all relevant growing conditions. As another example, when animal feeding trials are conducted, it is not clear what end points should be measured to ensure cultivar safety. Also, unless long-term studies are carried out, such studies have a limited ability to detect harmful cultivars.

Finding a clear biochemical, agronomic or quality difference between a transgenic cultivar and its parent (or between their products) should warrant either rejection or further analysis. A recent report from the NRC/Institute of Medicine of the USA claims that this is the proper role of substantial equivalence - that it is merely a starting point in the safety analysis of a transgenic cultivar (NRC/IOM 2004). However, at least in the past, regulators in the US have used it as an endpoint. For example, transgenic squash cultivar CZW-3 was found to have 68 times less beta-carotene than the non-transgenic control plants, as well as four times more sodium. However, it was approved for commercialisation and regulators did not require an explanation of these differences (USDA Application 95-352-01p).

Given the potential of both the transgene and transformation-induced mutations to result in unexpected hazardous phenotypes, we support calls for more extensive biochemical and physiological testing of transgenic plants prior to commercialisation. We also strongly support the formulation of specific guidelines for how to interpret the results of such analyses, so that unexpected differences are no longer ignored or rationalised away.

We believe that, while not the only source of risk arising from the use of transgenic crop plants, transformation-induced mutations are a very significant, but currently ignored, source of risk. Unless current regulatory procedures are changed to ensure that transformation-induced mutations are either prevented or removed from transgenic cultivars, commercial transgenic crop plants will continue to harbour such mutations and will continue to pose unnecessary risks to growers, consumers and the environment.

In this context it should not be overlooked that transgenic crop plants are not a necessity. Solutions to the problems for which transgenic traits are proposed as the answer can be achieved by other methods. These include the use of traditional plant breeding, making changes to agricultural practices and using alternative methods of food production. If a modern plant breeding technique is really to join or replace traditional plant breeding methods, it should surely be precise and controlled and we should understand the real risks arising from its use. At present we are still far from a proper process of identifying and analysing all of the real risks arising from the use of genetic engineering and transgenic crop plants.

Section 6. Recommendations

6.1 Overview

Current safety regulations do not adequately protect against the health and environmental risks arising from the plant transformation process. Regulations are needed that either prevent the occurrence of harmful mutations or permit identification and rejection of final commercial cultivars that have, or are likely to have, unpredictable or harmful characteristics. Such cultivars need to be identified before they reach the market. In this section we recommend improvements whose goals are to prevent or eliminate the mutations that occur during current transgenic plant breeding programmes. Some of these improvements necessitate more rigorous regulatory standards while others require research advances.

6.2 Recommendations for regulatory improvements

6.2.1 *Recombining away transformation-induced mutations*

As mentioned in **Section 4.3,** genetic backcrossing (or outcrossing) can remove many of the mutations introduced during the plant breeding process.

➢ We recommend that all transformed plants intended for field-scale trials or commercial release be subjected to extensive backcrossing programmes, followed by testing for effective removal of transformation-induced mutations.

Effectiveness of backcrosssing depends on the number of backcrosses carried out. However, they are not efficient at removing mutations at or near the site of transgene insertion.

➢ We recommend that the problem of inefficient backcrossing be remedied by deliberate selection for recombination events close to transgene insertion-sites using molecular markers (Note: this would require crossing a transgenic event into a non-parental line, in order to identify appropriate markers and to identify recombinants).

6.2.2 *Testing for mutations at transgene insertion events*

At present, the data provided by commercial applicants are not sufficient to determine whether transgene insertion has disrupted important host genomic sequences. This is because usually only Southern blot analyses of transgene insertions are provided in applications for commercial approval.

➢ We recommend that transgene insertion events into sequences which are, or may be, functional DNA sequences should be rejected from plant breeding programmes.

➢ We recommend that transgenic lines containing genomic alterations at the site of transgene insertion be rejected.

➢ We recommend that the insertion of superfluous DNA be considered unacceptable.

➢ In order to establish that such insertion-site mutations are not present, we recommend that the full extent of DNA disruption at the site of transgene insertion be determined before commercial approval is granted.

➢ To accomplish this we recommend that both the transgene insertion event (including all transferred DNA and a large stretch of flanking DNA) and the original target-site be sequenced and compared as the only known way to definitively determine whether gene sequences have been disrupted.

➢ We recommend that as well as DNA sequencing, techniques such as FISH, fiber-FISH, Southern blot analysis and PCR be used to detect transformed plants containing rearranged transgenes and superfluous DNA at or near the transgene insertion event. This combination of techniques should be sufficiently sensitive to detect lines with insertions of small fragments of transgene or superfluous DNA at some distance from the transgene insertion-site.

Even with these simple criteria, interpretation of the significance of a transgene insertion event is likely to be controversial because our current understanding of crop plant molecular genetics is insufficient to determine whether insertion into a particular genomic region is phenotypically inconsequential.

6.2.3 *Testing for phenotypic changes*

Biochemical, environmental impact and toxicological tests are one way of identifying transgenic cultivars which carry harmful mutations and thus need to be eliminated from breeding programmes (Kuiper *et al.* 2001). They should nevertheless be considered a second line of defence against transformation-induced mutations. These tests are likely to be expensive, inadequate and difficult to interpret. However, given the mutagenicity of current plant transformation processes such tests are necessary.

➢ We recommend extensive biochemical tests be required for all transgenic crop plants prior to commercial release. Such tests should include testing for all known toxins and anti-nutrients, major metabolites and all vitamins and minerals.

➢ We recommend that mRNA microarray techniques be used to look for alterations in gene expression by comparing the mRNA expression patterns in transgenic cultivars to those in non-transgenic parent lines (Kuiper *et al.* 2001).

➢ We recommend that significant differences in biochemical or microarray results between transgenic lines and parental lines should be evaluated not just in terms of the specific molecule or mRNA which deviates from its normal value but also in the sense that it may be a biomarker for other changes in the transgenic line.

Transgenic lines differing biochemically from non-transformed parent lines should either be eliminated from plant breeding programmes or analysed further (**Section 5**). More research is needed to determine the significance of both biochemical differences and microarray differences, in order to establish their usefulness as indicators of health or environmental hazards during risk assessment.

6.2.4 *Improvements to transformation technology*

Some mutations can be prevented by requiring transgenic plant breeders to adopt the following procedures:

➢ We recommend that tissue culture induced mutations be eliminated by the adoption of *in planta* transformation methods (**Table 1**).

➢ We recommend the insertion of selectable markers into the plant genome be eliminated by the use of PCR to identify primary transformants (de Vetten *et al.* 2003).

➢ We recommend that the insertion of plasmid DNA sequences be eliminated by using purified gene cassettes instead of entire plasmids during particle bombardment (Breitler *et al.* 2002, Loc *et al.* 2002, Fu *et al.* 2000).

PCR identification of transformants and the use of purified gene cassettes can immediately replace current transformation methods. Several plant species have already been experimentally transformed using *in planta* transformation methods, indicating that the development of such methods may be feasible for all species. Nevertheless, any methods would have to be evaluated in detail (e.g. to determine their mutagenicity) before their use could be considered.

6.3 Recommendations for further research

There are still many scientific uncertainties pertaining to plant transformation. If transgenic crop plants are to be grown commercially, further research is needed both to derive more accurate estimates of the risks discussed in this report and to seek and ensure ways of eliminating them.

- **Transgene insertion events:** For transgenic crop plants produced using any of the currently available plant transformation methods, we do not know what the typical arrangement is of a transgene insertion event. However, this information is necessary for anticipating the risks of transgene insertion which arise from endogenous gene disruption and transgene rearrangement. Large-scale

studies need to be carried out using various transformation procedures and in different crops. These studies need to be carried out on large numbers of loci classified as single transgene inserts by Southern blot analysis, as single transgene inserts are the kind most often used for commercial purposes (see Forsbach *et al.* 2003 for the first example of such a study in any plant). DNA sequence analysis needs to be carried out on numerous transgene loci as well as large tracts of their flanking genomic DNA to determine the full extent of target-site disruption. Such large-scale studies of random single-insert loci will help to clarify the nature and extent of genomic and transgene DNA rearrangement during transgene insertion. They will also help clarify the extent and nature of superfluous DNA insertion (including bacterial genomic DNA insertion, **Section 1.2.2**) in the vicinity of the desired transgene(s).

- **Effects of transgene promoters on endogenous plant genes:** Research is needed to determine whether transgene promoters influence expression of genes on either side of the inserted transgene. This is especially relevant when strong viral promoters are used to regulate transgenes, as such promoters are known to have the ability to affect nearby genes (**Section 3.2, Point IV**).

- **Higher order genome structure and function:** It is important to understand the effects of transgene insertion on genes in the vicinity of the transgene insertion event. Research is also needed to determine the effects of transgenes on higher order genome function.

- **Occurrence of genome-wide mutations:** Unless non-mutagenic methods of plant transformation are developed, further research is needed (using RFLPs, AFLPs etc., see **Section 2.3**) to quantify the magnitude of genomic change produced in different species and by various transformation methods.

- **Mutagenicity of gene transfer methods in the absence of tissue culture:** Research is needed to quantify the genome-wide mutagenicity of plant transformation methods that do not use tissue culture and to determine the nature of such mutations. It is also important to develop methodology that permits more accurate comparisons between transformation methods and between different experiments.

The quantitative techniques which have been used to measure genome-wide mutations suggest that genome damage is extensive, but individual techniques are typically only competent to detect gross abnormalities and may fail to detect mutations that may be more common and more important, such as small deletions or insertions. For example, techniques based only on analysis of band size differences could fail to detect nucleotide substitutions or inversions. Additional methods must be developed to improve the accuracy of assessment of genome-wide genetic damage in transformed plants.

The following developments are needed if transgenic plant breeding is to become a precise and less mutagenic technique:

- **Improved precision of T-DNA transfer:** Plant transformation techniques are needed that eliminate or reduce the probability of insertion of plasmid and other superfluous DNA during *Agrobacterium*-mediated transformation and that do not result in rearrangement of the T-DNA and the target-site DNA. This may be possible using different *Agrobacterium* strains or by redesigning the plasmids used for transformation.

- **Improved precision of particle bombardment:** Transgene insertion events created during particle bombardment are typically complex. If particle bombardment is to be used, particle bombardment procedures should be developed that reduce or eliminate transgene and target-site rearrangements and the insertion of superfluous DNA.

- **Targeted insertion of transgenes:** Homologous recombination or some other method of targeted transgene insertion should be developed to reduce the chance of disrupting functional sequences by random transgene insertion (Britt and May 2003).

- **Prevention of transformation-induced mutations:** Plant transformation methods should be developed which do not introduce genome-wide mutations.

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Appendix

Transgenic crop plants granted 'Non-regulated status' by the USDA which were analysed for this report.

The appendix provides further information about the commercial transgenic crop lines or 'events' discussed in this report and listed in **Table 2**. All of the commercial transgenic lines or events listed in **Table 2** and in the **Appendix** have been granted approval for 'non-regulated status' by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). The full list of commercial transgenic crop plants currently approved or pending approval in the US can be obtained at the following Website: (http://www.aphis.usda.gov/bbep/bp/petday.html). The applications submitted to the USDA can be ordered from APHIS on request by citing the listed application numbers. The list of transgenic crop plants currently approved or pending approval by the European Union can be obtained at the following Website: (http://www.defra.gov.uk/environment/gm/regulation/euconsent.htm).

USDA approval is based on a review of the data presented by each company or organisation in a formal application for deregulation. The applicants choose which data to present, although the regulatory bodies can ask for additional data. We have examined the applications submitted for a representative sample of commercial transgenic events/cultivars in order to determine the extent and nature of transformation-induced mutations remaining in transgenic lines once they reach this stage in the commercialisation process. Data and references pertaining to the commercial transgenic lines referred to in the text are summarized in both **Table 2** and in the **Appendix**.

For the transgenic events/cultivars we discuss in the body of the report, the **Appendix** summarises all of the data which are publicly available on the genotypic changes introduced in or near the transgene insertion event during the plant transformation. Some of the data submitted by the companies are considered 'confidential business information' and have been deleted from the applications available to the public. When relevant data have been deleted, this is noted in **Table 2** or the **Appendix** as having been 'CBI-deleted'. In some cases, additional relevant data are available in the scientific literature. In these cases, we have included the relevant references.

Abbreviations: ***bar* gene:** bialaphos resistance gene encoding a PAT enzyme conferring resistance to the herbicide glufosinate ammonium, **CaMV**: cauliflower mosaic virus, **CBI:** Confidential Business Information, **CP4 EPSPS:** 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. Strain CP4 (conferring tolerance to glyphosate), ***cry*3A** gene: encodes the *cry*3A protein (an endotoxin from the soil bacterium *Bacillus thuringiensis*), **FMV:** figwort mosaic virus, ***npt*II:** neomycin phosphotransferase type II selectable marker gene, **ORF:** open reading frame, **PAT:** phosphinothricin-N-acetyltransferase

Commercial transgenic events and cultivars produced via *Agrobacterium*-mediated transformation and granted 'non-regulated status' by the USDA

I. Glufosinate-ammonium herbicide-tolerant transgenic cotton event LLCotton 25, Application # 02-042-01p. Southern blot analysis indicated the presence of a single intact copy of a T-DNA containing the 35S CaMV promoter-regulated *bar* gene and the absence of plasmid backbone sequences. According to the application, the only superfluous DNA included in the T-DNA appears to be 79 bp of polylinker sequence.

No DNA sequence analysis of the T-DNA insert, its flanking DNA or the target-site DNA was carried out. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

II. Newleaf® Plus Colorado potato beetle and virus resistant potato line RBMT22-82, Application # 99-173-01p (expedited review of one specific transgenic line from Application # 97-204-01p). As determined by Southern blot analysis, potato line RBMT22-82 contains three T-DNAs incorporated at three different insertion events. Each of the three T-DNAs has an intact copy of the *cry*3A transgene and an intact copy of the PLRV *rep* gene. Two of the T-DNAs have an intact-copy and one has a truncated copy of the CP4 EPSPS transgene. One insertion event also contains plasmid backbone sequences from outside the right border of the T-DNA including the *aad* gene (a bacterial antibiotic resistance selectable marker gene) and ori322 (origin of replication) sequences.

No DNA sequence analyses of the T-DNA inserts, their flanking DNA or their target-site DNA were carried out. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

III. Virus resistant squash line CZW-3, Application # 95-352-01p. The presence of a single intact T-DNA insert and the absence of plasmid backbone sequences were determined by Southern blot analysis alone. The T-DNA contained a copy of the *npt*II selectable marker gene, in addition to three transgenes conferring resistance to different viruses.

No DNA sequence analyses of the T-DNA inserts, their flanking DNA or their target-site DNA were carried out. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

Commercial transgenic cultivars and events produced via particle bombardment-mediated transformation and granted 'non-regulated status' by the USDA

I. Corn rootworm protected maize event MON863, Application # 01-137-01p. The transgene DNA originated from a purified gene cassette that had been separated from the remainder of the plasmid sequence. The gene cassette was transferred to the plant via particle bombardment. Southern blot analysis indicated that Event MON863 consisted of the insertion of an *npt*II gene, followed by 153 bp of the 378 bp *ble* gene integrated adjacent to the *cry*3Bb1 transgene. The mRNA transcribed from the superfluous transgenic *npt*II-*ble* DNA encodes two ORFs: the *npt*II coding sequence and 40% of the ble gene. The inclusion of the superfluous 153 bp *ble* gene fragment appears to have been an artifact of restriction site choice during the cloning of the transgene.

The application states that PCR and DNA sequencing were performed on genomic DNA to confirm the unique junction sequences at the 5' and 3' ends of the MON863 insert. The information pertaining to the flanking DNA sequences was CBI-deleted. There was no indication that the plant target-site sequences were determined and compared to the DNA flanking the transgene insert. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

II. Transgenic virus resistant papaya cultivars 55-1 and 63-1, Application # 96-051-01p. Both cultivars were created by particle bombardment of papaya tissue with a whole plasmid containing a T-DNA. The genomic insertion events in both cultivars were analysed by Southern blot techniques.

Papaya cultivar 55-1: Southern blot analysis indicated that in addition to the desired *PRVcp* (papaya ringspot virus coat protein) transgene, Papaya cultivar 55-1 contained the following superfluous DNA: an *npt*II selectable marker gene, the gus reporter gene and plasmid backbone DNA, including the OriT sequence and part of the tetracycline resistance gene. Using Northern blot analysis to examine mRNA transcripts, Fitch *et al.* (1992) found two larger transcripts (a 2.4 Kbp and a 4.4 Kbp transcript) which hybridised to a PRV cp probe, in addition to the predicted 1.35 Kbp PRV cp transcript. The presence or significance of the unexpected transcripts was not discussed in the application for deregulation.

No DNA sequence analyses of the transgene DNA, genomic flanking DNA or the target-site DNA were carried out. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

Papaya cultivar 63-1: Southern blot analysis indicated that Papaya cultivar 63-1 appeared to contain the following superfluous DNA: the *npt*II gene and the following bacterial plasmid backbone sequences: the bacterial gentamycin resistance gene, the OriV and Ori T bacterial origins of replication, and some (or all) of the tetracycline selectable marker gene. The application states that in line 63-1 "some rearrangements of genes may have occurred during integration since multiple bands are observed on this line following *Hind*III/*Bam*HI digests, and the hybridisation bands are located at a much higher molecular weight than expected". Fitch *et al.* (1996) report the presence of multiple bands in genomic DNA from papaya line 63-1 probed with *npt*II DNA sequences. No further description or analysis of these unexpected bands was provided in the application.

No DNA sequence analyses of the transgene DNA, genomic flanking DNA or the target-site DNA were carried out. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

III. Roundup Ready® glyphosate tolerant soybean event 40-3-2, Application # 93-258-01p. Particle bombardment was performed with a whole plasmid (PV-GMGt04). Transgenic soybean event 40-3-2 consisted of the genomic integration of one intact CP4 EPSPS gene as determined by Southern blot analysis. According to the application, no other sequences contained in the bombarded plasmid were present in the 40-3-2 soybean line. In 2000, Monsanto updated the file on RR Soya event 40-3-2 and described event 40-3-2 as the genomic integration of: the intact CP4 EPSPS transgene followed by a 250 bp CP4 EPSPS fragment and a co-segregating 72 bp CP4 EPSPS fragment (the 72 bp CP4 EPSPS fragment is flanked on both sides by plant genomic DNA). The application does not comment on the sequence of the flanking plant genomic DNA.

Windels *et al.* (2001) independently analysed the genomic DNA flanking the 40-3-2 insertion event. They also found that adjacent to the intact CP4 EPSPS transgene was a 254 bp fragment of CP4 EPSPS. Adjacent to this was 534 bp of unknown DNA, followed by plant genomic DNA. Windels *et al.* (2001) were unable to PCR amplify the equivalent genomic insertion-site from non-transformed plants using primers to the genomic DNA sequences flanking the transgene. Therefore, they suggested that further deletion and/or rearrangement of plant genomic DNA must have occurred during transgene insertion.

The combined data indicate that transgenic soybean event 40-3-2 consists of the following: (a) insertion of an intact copy of CP4 EPSPS followed by a 250 bp fragment of CP4 EPSPS followed by 534 bp of unknown DNA and, (b) deletions and/or rearrangement of the plant genomic DNA at the insertion-site, and (c) insertion of a 72 bp fragment of CP4 EPSPS which is flanked on both sides by plant genomic DNA but is genetically linked to the insertion-site of the intact CP4 EPSPS gene.

IV. Maize YieldGard® event MON810, Application #96-017-01p. Southern blot analysis indicated that only a single copy of the *cry*A(b) transgene was present in event MON810 and no superfluous sequences were inserted.

No DNA sequence analysis of the transgene DNA, the genomic flanking DNA or the target-site DNA were present in the application given to the USDA. No other molecular genetic data relevant to the presence of transformation-induced mutations were presented in the Application.

However, sequence data and PCR analysis reported by Hernandez *et al*. (2003) for event MON810, suggest that insertion of the truncated copy of the *cry*IA(b) transgene resulted in substantial deletion and/or rearrangement of plant genomic DNA at the insertion-site. Using primers homologous to the plant genomic sequences flanking the *cry*IA(b) transgene, Hernandez *et al*. (2003) were unable to amplify the corresponding target-site sequences from genomic DNA from the non-transgenic parent plant.

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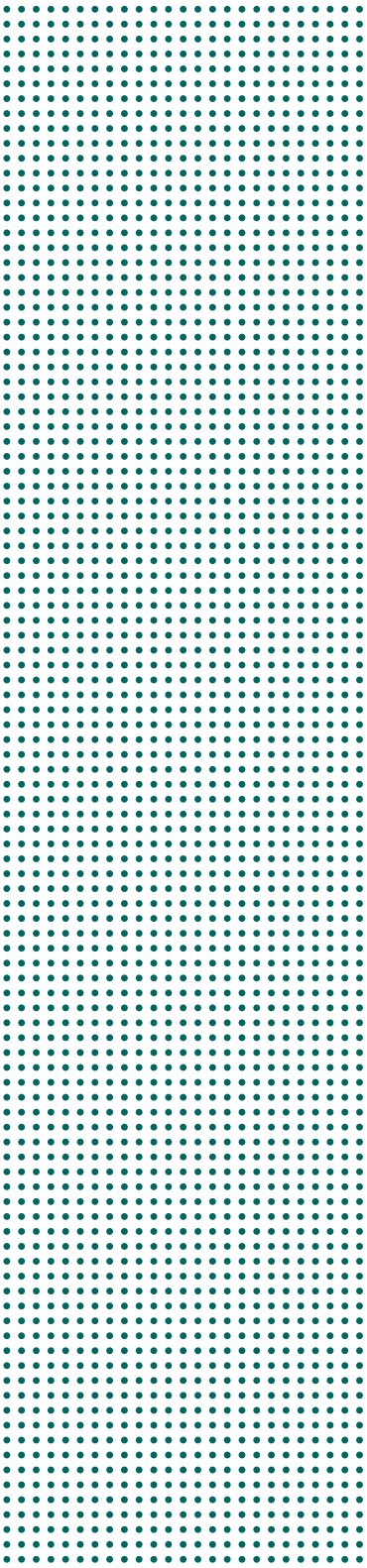

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