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**Invited Editorial**

# Unintended consequences of plant transformation: a molecular insight

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**Abstract**. Plant genomes are dynamic structures having both the system to maintain and accurately reproduce the information encoded therein and the ability to accept more or less random changes, which is one of the foundations of evolution. Crop improvement and various uncontrolled stress factors can induce unintended genetic and epigenetic variations. In this review it is attempted to summarize factors causing such changes and the molecular nature of these variations in transgenic plants. Unintended effects in transgenic plants can be divided into three main groups: first, pleiotropic effects of integrated DNA on the host plant genome; second, the influence of the integration site and transgene architecture on transgene expression level and stability; and third, the effect of various stresses related to tissue handling, regeneration and clonal propagation. Many of these factors are recently being redefined due to new researches, which apply modern highly sensitive analytical techniques and sequenced model organisms. The ability to inspect large portions of genomes clearly shows that tissue culture contributes to a vast majority of observed genetic and epigenetic changes. Nevertheless, monitoring of thousands transcripts, proteins and metabolites reveals that unintended variation most often falls in the range of natural differences between landraces or varieties. We expect that an increasing amount of evidence on many important crop species will support these observations in the nearest future.

**Key words**: unintended effects, transgenic plants, position effect, insertion effect, somaclonal variation, DNA methylation, epigenetic variation.

## Introduction

Methods used in modern agriculture to improve plant traits have the potential to generate unintended effects unrelated to the target traits. These methods include such breeding techniques as cross- or self-pollination, generation of haploids, generation of new polymorphisms by chemical or radiation mutagenesis, interspecies crosses, protoplast fusions, somatic embryogenesis, other tissue culture based strategies and, last but not least, plant transformation. Plant transformation techniques have provided the first cases of unintended effects which were of public concern. Development of new genetically modified varieties and the preparation of relevant safety assessment rules have therefore triggered systematic research on unintended effects. Factors contributing to unintended effects in transgenics are the main focus of this review.

Transgenic plants are commonly used in agriculture in several countries, but they still raise the question as to whether a given trait directly linked to a given transgene results in only one difference in the new variety relative to parental non-transgenic plants. According to food safety and environmental protection rules, the agricultural introduction of transgenics requires multidirectional analyses (Kuiper et al. 2001). These analyses include recently developed large scale strategies to monitor changes in a substantial part of genomes, transcriptomes, proteomes

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and metabolomes of transgenics. The primary origins of observed functional fluctuations are traditionally linked to DNA sequence changes. However, it has recently become apparent that epigenetic processes are responsible for a large portion of observed variability.

**The effect of integrated DNA on the host genome – insertion effect**

The specific nature of unintended effects in transgenics may be partly explained using information on the site of transgene integration, copy number, architecture of the inserted fragment and the biochemical and functional nature of the introduced trait. The integration site can influence the transgenic plant in two ways: first, as the influence of the transgene on the functioning of the surrounding sequences (insertion effect) and second, as an effect of surrounding sequences on transgene expression (position effect).

The insertion effect can be of mutagenic nature and can result in null, loss of function, gain of function and other possible phenotypes depending on the specific region of the gene targeted by the insert and elements within the T-DNA (e.g. promoter). T-DNA integrations are found distributed along the entire length of chromosomes (Azpiroz-Leehan and Feldmann 1997). Moreover, it was shown that transformation of the promoterless reporter gene linked to a border sequence yielded transcriptionally active gene fusions at a high frequency indicating that T-DNA preferentially integrates into genomic regions that potentially can be transcribed (Koncz et al. 1992). These data have recently been confirmed after the analysis of numerous sites of T-DNA integration during the evaluation of insertional mutagenesis techniques. For instance, 112 transgenic *Arabidopsis* lines were precisely analyzed, indicating a rather random distribution of integration events (even in heterochromatin regions). The frequencies of insertions into gene sequences (500 bp 5' upstream region, exons, introns, 200 bp 3' downstream regions) and intergenic fragments were proportional to a given sequence length. The only insertion frequency biases were seen within gene regions, where the 5' upstream regions were targeted over two times more frequently than it was statistically expected and within introns where, conversely, the insertion frequency was much lower than expected (Forsbach et al. 2003). A more extensive analysis based on 88,122 T-DNA insertions revealed a slightly non-uniform distribution of integration sites along the *Arabidopsis* chromosomes. The T-DNA density was closely correlated with gene density on each chromosome, being the lowest in regions surrounding centromeres (Alonso et al. 2003). Similar results were obtained for 214 sequenced insertion sites into the rice genome (Sha et al. 2004). The T-DNA location preference for gene-rich regions had been previously postulated in other species. However, these analyses were usually done on a rather low number of transgenic lines with limited sequence information on the surrounding DNA (e.g. Salvo-Garrido et al. 2004; Tagashira et al. 2005). In both large scale experiments on *Arabidopsis* cited above promoters and UTRs were significantly more often targeted by T-DNA (Forsbach et al. 2003; Alonso et al. 2003). The observed bias towards promoters and UTRs could be explained by a possible preferential interaction of Vir proteins with host proteins involved in the initiation or termination of transcription (Tzfira et al. 2001). Consequently, the process of T-DNA integration should be affected by gene activity. However, gene activity assayed on *Arabidopsis* microarrays does not correlate with the observed integration frequency (Alonso et al. 2003).

The mutagenic gene disruption is not the only mechanism, by which transgene insertion may affect the phenotype of a transgenic plant. The surrounding insertion site coding sequences may fall under the influence of transgene promoters leading to sense or antisense transcripts. These molecules can serve as messenger RNAs for recombinant proteins or interfere with the expression of respective host genes via RNA interference (RNAi). Oversized transcripts resulting from inefficient transcription termination by the nos terminator were detected in Roundup Ready soybean (Rang et al. 2005) and in a virus-resistant papaya line (Fitch et al. 1992). The consequences of such events can be largely reduced by a proper construct design. Transgene sequences may also influence more distantly located genes. In activation tagging experiments of *Arabidopsis,* the distant influences of T-DNA located enhancers were observed up to 8.2 kb from the insertion site (Ichikawa et al. 2003).

Apart from mutations caused by insertions, most transgenic plants harbor small or large DNA sequence rearrangements at the RB (right border) or LB (left border) junction sites, including target site deletions, duplications, translocations and insertions of filler sequences representing vector backbone, T-DNA or host sequences (Forsbach et al. 2003). Despite the fact that only single-copy inserts were analyzed, a few inter-chromosomal

rearrangements were also found. Forsbach et al. (2003), in accordance with earlier studies by Herman et al. (1990), showed that in *Arabidopsis* most rearrangements did not span more than 100 bp and occurred near both border sequences with similar frequencies. The frequency of rearrangements is higher in lines carrying multiple copies of T-DNA than in single-copy transgenics, as it was shown for *Arabidopsis* (Wenck et al. 1997). Interestingly, in the same report it was documented that the number of multi-copy transgenics obtained by vacuum infiltration was much higher than after root explant transformation.

With regard to the mutagenic effect of transformation, it should be also noted that, with the exception of selection and regeneration *in vitro,* the plant genome may also be influenced by the infection with *Agrobacterium tumefaciens*. It was shown that fungal pathogens increase the somatic recombination frequency in *Arabidopsis* (Lucht et al. 2002). A similar reaction was observed when reporter plants were treated with salicylic acid analogs, the chemical inducers of the salicylic acid-dependent pathogen response pathway. The authors of this report suggest that an increased somatic recombination represents a general stress response in plants. This could be any abiotic and biotic stress factor influencing plant during transformation.

Most of the research on undesired effects of plant transformation was performed using *Agrobacterium*-treated plants. However, particle bombardment is also a potent and effective transformation procedure, especially when the *Agrobacterium* protocol does not provide satisfactory results. A vast majority of particle bombardment insertion events described in literature are very complex, most often having more than 10 copies and different rearrangements (for a review, see Latham 2006). Particle bombardment generally appears to cause substantial disruptions of plant DNA and rarely gives rise to simple integration patterns (single copy).

These results evoke an important question as to which transformation technique causes the lowest number of disruptions. The answer is needed for any species to be transformed and this consideration is not often incorporated into laboratory practice. There are several examples of attempts to compare different transformation techniques with respect to their impact on the genome, which typically employ the analysis of variability using AFLP (amplified fragment length polymorphism) and RAPD (random amplified polymorphic DNA) techniques. For instance, Labra et al. (2001) compared transgenic rice populations obtained with different transformation techniques with non-transgenic rice. In this study, comparison data were also taken from other experiments reported by Arencibia and coworkers (1998) and Bao and coworkers (1996). Results indicated that transgenic rice produced by *A. tumefaciens* treatment is characterized by fewer genomic changes than observed in plants produced via protoplast treatment, but far more than those produced with particle bombardment or cell electroporation. This problem is well known and widely discussed, mainly in the context of *in vitro* regeneration and somaclonal variations induced therein.

There are a few more possible sources of unintended changes in transgenic plants. The presence of new RNAs and proteins (for marker, reporter or other genes within the introduced DNA fragment) may specifically interact with compatible molecules which are not directly related to the trait being modified. Moreover, the production of transgenic proteins can establish new metabolic sinks for certain amino acids or unspecified substrates for relevant enzymes (Tagashira et al. 2005). Such transgene effects, often influencing plant fitness and productivity, are usually discussed as a balance of costs and benefits of a new trait (Heil and Baldwin 2002; Jackson et al. 2004).

The relatively new method of metabolite profiling has proved to be highly useful by revealing metabolic effects of a genetic modification or mutation (Roessner et al. 2001; Fiehn et al. 2000). This method in combination with large scale transcript profiling and proteomics is recommended for assessment of genetically modified food safety (Kuiper et al. 2001). The power of metabolite profiling using GC/MS (gas chromatography / mass spectrometry) was demonstrated in the analysis of metabolites of *dgd1* and *sdd1-1* mutants of *Arabidopsis thaliana,* (which have mutations in digalactosyldiacylglycerol synthase and the subtilisin-like serine protease involved in stomatal development, respectively), and in their wild type background ecotype counterparts. This extensive approach revealed a number of pleiotropic changes in metabolite levels, not only in the *dgd1* mutant which exhibits a quite severe phenotype, but also in *sdd1-1* which represents a relatively mild phenotype (Fiehn et al. 2000). Fiehn et al. assessed the reproducibility of the method and discovered that since the variability in sample preparation and measurement accuracy can be tolerated, the natural variability of metabolites in plants grown under identical conditions is quite

large. This indicates that plants are capable of great metabolic flexibility. Many metabolite changes were also observed in transgenic potato plants using a similar GC/MS technique (Roessner et al. 2001). The transgenic plants carried bacterial phosphorylase, yeast invertase or bacterial glukokinase transgenes. The analyses revealed a large number of new correlations (often unexpected) between many metabolites. Moreover, Roessner and coworkers showed that some genetic modifications can be pheno-copied in wild type plants under the influence of certain environmental conditions if assayed on the metabolome level.

Monitoring of the variability on transcriptome, proteome and metabolome levels has one more advantage – it reveals the effects of genetic and epigenetic changes. The new large scale methods applied to analyze the "-omic" changes in transgenic plants are very sensitive, therefore the question arises whether the observed changes reflect the pleiotropic effect of the transgene or fluctuations naturally occurring in a living organism. Consequently, special attention should be paid to experiment design, especially concerning uniformity of growth conditions, sampling and the number and type of controls (El Ouakfaoui and Miki 2005; Ruebelt et al. 2006a; Fiehn 2000). Furthermore, it turns out that the range of variation observed in different varieties or landraces of the same species are usually much higher than in transgenic plants (Lehesranta et al. 2005; Ruebelt et al. 2006 b,c).

**Position effect – the influence of the integration site and transgene architecture on transgene expression level and stability**

One more uncontrolled phenomenon, although relatively easy to monitor, has been mentioned, i.e. the copy number of the transgene. The most obvious effect of the number of copies is the level of the transgene expression. It has long been known that independent transgenic lines vary in transgene expression and activity. The differences are sometimes more than 100-fold with respect to reporter or marker protein activity between independent lines and 3–4 fold in clones of the same line (e.g. transgene expression variability in tobacco; Hobbs et al. 1990; Peach and Velten 1991). Moreover, there is often no consistent correlation between expression levels of different reporter or marker genes on the same T-DNA. However, a recent in-depth analysis of 132 independent *Arabidopsis* transgenic lines represented by numerous plants of up to the T7 generation revealed that the site of T-DNA integration does not influence either the silencing of selection marker genes or the expression of *GFP*, *GUS* and *SPT* reporter genes. Twofold differences in transgene expression (transcript level) and activity were highest, when observed for independent single-copy T-DNA lines (Schubert et al. 2004).The observed expression profiles were stable throughout plant development. Similar characteristics were noted for independent lines carrying two copies of the transgene, regardless of the fact whether they were in one locus as a tandem repeat or at two different loci. The specific inverted repeat arrangement of the T-DNA is unlikely to produce a read-through transcript capable of dsRNA formation, which leads to posttranscriptional gene silencing (PTGS; Lechtenberg et al. 2003). Neither the close proximity of *Arabidopsis* repetitive elements nor the presence of a promotorless reporter gene within the T-DNA had an effect on the expression level. The only significant factor leading to the silencing of reporter gene expression driven by the CaMV 35S promoter was a copy number greater than two, which was different for each reporter gene tested (*GFP*, *GUS* and *SPT*) (Schubert et al. 2004). The authors propose that this PTGS is triggered by threshold concentrations of the transgene transcript. This proposal was additionally supported by a failure to observe such silencing when a weak nopaline synthase promoter was used. The copy number-dependent PTGS also indicates the homozygosity-dependence which was observed in other experiments (e.g. Qin et al. 2003).

The possibility exists that, in the above cited results, a subset of T-DNA insertions might have been immediately subjected to TGS (transcriptional gene silencing) and consequently they were not recovered during the selection for expression of the *NPT* gene in primary transformants. TGS is mitotically and meiotically heritable and is characterized by the absence of transgene transcription, predominantly due to cytosine methylation in the promoter region (Vaucheret et al. 1998). Indeed, methylation of transgene sequences has often been found in transgenic lines. It was discovered that TGS can be initiated by double-stranded RNAs possibly directing DNA methylation (Mette et al. 2000). TGS could also be a result of the spread of the nearby heterochromatin (Prols and Mayer 1992) or a result of the strong discrepancy between DNA composition of the transgene and the surrounding genomic sequences (van Blokland 1997). The latter report suggests a correlation of DNA hypermethylation and chromatin condensation. Such a correlation was also observed in the case of repeat induced gene silencing (RIGS) de-

scribed by Ye and Singer (1996). TGS can additionally affect distantly located sequences in a manner similar to paramutation processes, where methylation and stretches of homology are required (reviewed by Vaucheret et al. 1998).

The surrounding sequences can also enhance the expression of transgenes if, for example, they are under minimal promoter influence within the enhancer trap constructs. There are many reports on the beneficial influence of specific S/MARs (scaffold/matrix attachment regions) on transgene expression levels and the percentage of cells expressing the transgene in transgenic plants (Mlynarova et al. 1995; Levin et al. 2005; Halweg et al. 2005; Vain et al. 2002). S/MARs are A/T rich sequences, 300bp to several kb in length, that bind to the nuclear matrix in vitro and are thought to organize chromosomal DNA into loop domains (Allen et al. 2000). Not all results of experiments with MARs provide consistent evidence of their advantages. In *Arabidopsis* there are additional genetic factors needed. A several-fold increase in transgene expression was observed only when MAR-flanked reporter genes were introduced into the posttranscriptional gene silencing mutants *sgs2* and *sgs3* (Butaye et al. 2004). In plant biotechnology, the exogenous chicken lysozyme A element (*chiMAR*; Stief et al. 1989) and tobacco *Rb7* (Hall et al. 1991) are most often used in T-DNA constructs as flanking sequences. It cannot be ruled out that the insertion site can be located close to the endogenous MAR element, where it could possibly influence the transgene expression level. The genome-wide localization of S/MARs sequences was done only in the *Arabidopsis thaliana* genome, showing a density 1 region per 5.5 kb (Rudd et al. 2004). Since there is no effect of MARs on transgene expression in the wild type background, it is difficult to correlate the expression of precisely localized genes with the presence of these regions in this species.

The stability of transgene expression throughout generations of generatively propagated plants is critical for further applications of introduced traits, both in basic research and in biotechnology. One of the obvious effects of having unstable loci is the non-Mendelian inheritance of a given transgene. With the exception of transcriptional and posttranscriptional silencing, there are several other factors which could account for the stability of transgenic plants. These factors include transgene deletion, duplication and rearrangement (reviewed by Yin et al. 2004). These phenomena may be partly explained by rare occurrences of meiotic or mitotic recombination events, which have been observed in tobacco and soybean (Tovar and Lichtenstein 1992; Choffnes et al. 2001). Transgene stability may also depend on the plant species. Large scale studies on 132 homozygous, single copy T-DNA, *Arabidopsis thaliana* transgenic lines did not show any incidence of unstable transgene (*NPTII*, *GUS* and *GFP*) expression throughout the 2–7 generations studied (Schubert et al. 2004). In *Arabidopsis*, the recombination events accounting for the transgene stability are more likely to be related to the copy number than the integration site. A study on 95 independent transgenic rice plant lines obtained by particle bombardment and containing mainly multiple copies of T-DNA also supports the notion that transgene stability is significantly correlated with its structure (Vain et al. 2002).

As it has been shown, transgene expression levels can vary greatly between independently transformed plants. Much of this variation is assigned to copy number, silencing mechanisms and differences in the transgene integration site and locus configuration. Special attention has to be paid for another spurious variation source – uncontrolled differences in experimental protocols. Thus, the development of standard operating procedures for a precise quantification of transgenic expression levels is of key importance (James et al. 2004 a,b). This is especially important when comparing transgene behavior of several generations of transgenic plants.

**The effect of regeneration technique – somaclonal variation**

Most of the transformation protocols apply an *in vitro* selection and regeneration step. These procedures always induce somaclonal variation, which changes numerous plant characteristics, most often as an unintended effect (Karp 1991). At times, plants regenerated *in vitro* may show heritable variability in useful characteristics such as morphological traits, pathogen or stress tolerance, content of specific secondary metabolites and many other characteristics which can be of interest for breeders as a new source of genetic variation (Veilleux and Johnson 1998). It is difficult to ascertain whether recombination and epigenetic imprinting related to the transformation or the regeneration technique contribute most to the unintended variation in transgenic plants. To answer this question Labra et al. (2004) compared the genome-wide variability induced in transgenic *Arabidopsis* plants obtained via the floral dip technique which does not include a tissue culture re-

generation step (Clough and Bent 1998) and in plants regenerated from callus culture. Monitoring of over 650 marker bands obtained after AFLP and RAMP (random amplified multiple polymorphisms) analyses clearly showed that callus culture is the only significant factor inducing variation within DNA relative to controls.

Many factors have been described as potentially responsible for somaclonal variation since the publication of the fundamental article by Larkin and Scowcroft (1981), and generally most can be classified as stress factors. Stresses to which the plant cell is subjected during dedifferentiation and regeneration in tissue culture include wounding, desiccation, osmotic stress, and insufficient nutrient supply/uptake (Carman 1995). Additionally, in order to regenerate the whole plant, transgenic cells are often stimulated by growth regulators and selected on antibiotics which are usually not neutral at the concentration levels employed (LoSchiavo et al. 1989; Schmitt et al. 1997; Bardini et al. 2003). The mutagenic stress-response mechanism can be described as a programmed loss of cellular control, leading to (1) genetic changes such as polyploidy, aneuploidy, chromosome rearrangements, somatic recombination, gene amplifications, point mutations, excisions and insertions of (retro)transposons and (2) epigenetic changes including DNA methylation and histone modifications (Lee and Phillips 1988; Brown et al. 1992; Skirvin et al. 1994; Phillips et al. 1994; Kaeppler et al. 1998; Olhoft and Phillips 1999; Skirvin et al. 2000; Madlung and Comai 2004). Because the molecular basis of somaclonal variation is the subject of many excellent reviews (see list above) we will concentrate here on only a few examples, particularly with regard to the consequences of epigenetic variations.

There is a growing body of evidence that epigenetic changes such as methylation and chromatin modifications induced in tissue culture can lead to direct effects on gene expression (e.g. activation or silencing), or indirectly as a result of point mutations, excisions and insertions of activated transposable elements or leading to chromosome breakage (for reviews, see Kaeppler and Phillips 1993; Kaeppler et al. 2000). Moreover, there is a possibility that methylation and histone modification effects interact. Regulation of gene expression via changes in DNA methylation is a very potent regulatory mechanism often found in nature, e.g. in plant development (Richards 1997; Reyes et al. 2002). Developmental processes are, in turn, the main targets of manipulations in tissue culture techniques. Indeed, tissue culture induced DNA methylation polymorphism is often observed (Anderson et al. 1990; Kaeppler and Phillips 1993; Smulders et al. 1995). These changes occur in both directions, but hypomethylation is much more frequently reported (Kaeppler et al. 1993; Bardini et al. 2003). Methylation changes were quite frequent and stably inherited (Kaeppler and Phillips 1993). The important question arises regarding the randomness of these changes and the possible existence of a specific targeting mechanism. It is easy to accept that there are sequence preferences of methylation machinery. Some examples of tissue culture induced epimutations are more often observed than it would be expected in the case of random site selection (Lund et al. 1995). Nevertheless, Bardini et al. (2003) presented evidence that methylation/demethylation events are not targeted to any specific sequence. However, the MSAP (methylation-sensitive amplification polymorphism) technique used may not have had enough resolving power and the *Arabidopsis* callus lines which were tested may not be homogenous. Theoretically, the targeting mechanism, if any, may depend also on the stress factor. For example, it is known that R1 progeny of regenerated cucumber plants display specific and statistically significant metabolic profiles, which are dependent upon the regeneration system used (Filipecki et al. 2005). We do not know how the genetic and epigenetic changes are processed to result in a particular metabolic profile, but this example shows that the ranges of these changes may not fully overlap. Such a nonrandom genome modification mechanism might be related to the stimulation of somatic recombination by an increased transcription of a locus nearby, which was observed e.g. in yeast (Saxe et al. 2000). Obviously stress is a very potent factor modifying gene transcription. A stress-induced increase of recombination was observed in *Arabidopsis* plants treated with UV irradiation or flagellin, an elicitor of plant defences (Molinier et al. 2006). It was documented that such an increased genome flexibility can be transmitted to the next generation with no need to face the stress factors again.

Studies on mutations induced in tissue culture indicate that changes accumulate sequentially with time in culture (Fukui 1983). This again stresses the importance of adopting optimized transformation techniques to minimize the time in culture and adopting strategies utilizing multigene constructs and simultaneous co-transfor-

mation rather than sequential transformation with different constructs or crosses between independently transformed lines (unless the transformed lines were backcrossed). These observations do not exclude the possibility of pre-existing variations in the explant (Colijn- Hooymans et al. 1994). This is especially important in the case of the epigenetic program. After dedifferentiation and regeneration, this program may not be properly established in regenerated plants. The observation that R0 plants are usually more variable than their progeny supports this hypothesis. Additionally, the degree of differentiation which correlates with the age of an explant and its epigenetic variation should be proportional to the number of observed mutations in regenerated plants. Matzke and Matzke (1996) clearly demonstrated that different epigenetic states of expression ($Hyg^R$) in regenerated plants can be obtained from the same explant. Some of the pre-existing variations observed in this experiment were meiotically heritable. This supports the suggestion to use explants as young as possible in transformation/regeneration experiments. Cell susceptibility for the transformation process could also be a preexisting factor contributing to transgenic/nontransgenic variation. Charlton and coworkers (2004) were able to separate the effects of the transgene from those of the transformation procedure when analyzing metabolite profiles obtained by proton nuclear magnetic resonance spectroscopy of wild type, transgenic and null segregant plants. These results revealed that the primary cause of the observed metabolite differences was the transformation process. The authors hypothesize that the transformation and selection procedures select for a subset of individuals capable of undergoing the entire manipulation.

## Concluding remarks

We have discussed separately unintended effects of transformation and tissue culture. However, there are many stress factors difficult to assign to certain stages of the procedure. Tissue culture induces a majority of changes which are difficult to define. This stresses the importance of generating large quantities of independent transgenics for optimal selection. Consequently, there remains an important need to further improve transformation techniques to be not only effective, but also exhibiting a minimal potential to generate variations. A good example is provided by the floral dip method of transformation which does not include tissue culture and is routinely used for *Arabidopsis* transformation.

When producing a transgenic crop, it is important to consider the architecture of the inserted sequence and the environment of the insertion site, which may cause variable and unstable expression of the transgene (and other sequences) in independent transgenic lines. Many efforts are being made to overcome these obstacles. Some of these efforts include improvement of site directed protocols, improved construct design using tools to suppress gene silencing, and the construction of artificial plant chromosomes (reviewed in Butaye et al. 2005). Additionally, there are alternative transformation protocols such as plastid transformation, which are not devoid of tissue culture, but which circumvent some of problems discussed in this review. Application of protocols which minimize variability among transgenic plants is complicated by widely occurring species or variety specificity with respect to transformation procedures.

The application of new sensitive, non-selective and high performance analytical techniques facilitates a deeper understanding of variability in plants resulting from transformations or other laboratory and breeding practices. This knowledge clearly shows that genetic or epigenetic variations and their consequences are an inherent component of life. It also becomes clear that the use of tissue culture for transformation to obtain a primary transformant, having only the intended modification with no other variations, is not a realistic outcome for most species. More importantly, the induced unintended variation in any character usually falls into the generally acceptable range, naturally occurring in different cultivars or ecotypes of the same or closely related species. Taking into account the large number of primary transformants generated for commercial applications, usually rigorous selection and subsequent breeding programs the significance of unintended changes is rather negligible. In view of the presented studies the concern resulting from unintended effects in genetically modified crops seems largely unjustified.

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