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REVIEW

Genomic stability and stability of expression in genetically modified plants

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CONTENTS

SUMMARY

This review provides a survey of those factors that might influence genetic stability of genetically modified organisms and somatic hybrids in breeding programmes. In this respect several aspects may be distinguished: (i) host genomic factors that might influence genetic stability, (ii) events related to the introduction of new DNA into the genome and their effect on genetic stability, (iii) stability of gene expression of newly introduced DNA, and (iv) stability of the modified genome. In our view a gene is defined as being stable if it inherits according to Mendelian laws. Obviously, this can be valid only for nuclear genes. Non-Mendelian inheritance may be caused by intrinsic genomic factors or be the result of skewed segregation during meiosis. Newly introduced DNA may be stably integrated into the genome, yet data on its site of integration is limited. The level of expression and, thus, the strength of the related trait, may vary. Variation in expression may depend on the construct, such as the promoter or additional sequences such as MAR elements or the coding sequence itself, the site of integration and the species used. Another, and undesired, phenomenon is the silencing of expression of introduced genes. The kinds of silencing described depend on the relative position in the genome of the genes involved, *cis* vs. *trans* and whether only one or all genes are silenced. Instability of expression generally becomes visible within a few generations, but once expression is stable it is supposed to remain so provided the environment does not change dramatically. Although the production

of somatic hybrids seems to be a promising technique to obtain new genetic material and even though numerous hybrids have been made, only a few follow-up studies have been published. Therefore the use of somatic hybrids in breeding programmes is limited.

Key-words: genomic stability, risk assessment, position effect, silencing, transgene, transposable element.

INTRODUCTION

The possibility of introducing new genetic material, and thus new traits, via DNA transfer techniques may enable the introduction of traits from phylogenetically distinct taxons and promises to reduce the time necessary to create cultivars with new traits.

An increasing number of such genetically modified (transgenic) plants have been made, and some of them have already been introduced into the market. The genetic modification of plants may fit several purposes, such as the improvement of agronomic behaviour (for example higher yield, resistance), the production of specific molecules (for example antibodies), or the use of transgenic plants in cross-breeding programmes. The aim of crossing a transgenic plant with other plants will be the improvement of a cultivar with a trait of interest (cultivar+) or the creation of a line where the introduced trait is of such importance that other traits remain of minor interest (a new cultivar). Hybrids may be of particular interest as they are superior to the parents with respect to vigour and other characteristics, such as high yield, resistance and performance in different environments (for example see Sneep *et al.* 1979). To obtain specific hybrids genetically modified plants may be used as a tool, as in the sterile and fertility restorer lines of tobacco (for example see Mariani *et al.* 1990, 1992).

Several aspects of transgenic plant biosafety may be considered. First, the trait(s) introduced should not vary in expression over generations and between cultivars. Secondly, the potential effects of introduced DNA, such as selection markers and reporter genes, to facilitate the selection of the transgene (Metz & Nap 1997, this issue, p. 25). Thirdly, the interaction of transgenic plants with their environment, such as information of the species used (for example see Metz *et al.* 1997, this issue, p. 51), the dispersal of transgenic plants in wild populations (van Raamsdonk & Schouten 1997, this issue, p. 69) and the influence of the expression of antibacterial and antifungal genes in transgenic plants on the saprophytic soil microflora (Glandorf *et al.* 1997, this issue, p. 85). Currently, various aspects of genomic stability and stability of expression of genetically modified plants in breeding programmes will be discussed in more detail.

To obtain a stably useful transgenic plant three criteria should be met. First, the introduced gene should be stably integrated into the genome; secondly, the expression of the gene should be detectable or recognizable; and thirdly, the level of expression should show no variation (the trait of interest should be stable). Additionally, the transgene should be stably inherited over a large number of generations and its expression should be stable under different environmental conditions. These factors are, of course, also a prerequisite for successful marketing of a new variety. It may be assumed that transgenic plants, genetically, behave differently from their parents due to changes in the genome caused by the introduction of the transgene. The aim of the present study is to investigate whether such changes in behaviour may occur. The premise in all the evaluations must be that any gene is stable if it is inherited in a

Mendelian way. In fact Mendelian inheritance is the only valid genetic criterion with respect to the stability of a gene in its genomic environment.

In the investigation described here we made a survey of various factors that might influence the stability of transgenes in the genome (see also Maessen & Derksen 1995a,b). These factors include characteristics of the host genome, the transgene itself, the mechanism of introduction and the site of integration. An inventory of these factors should allow determination of whether the genome of a transgenic plant is likely to be stable. If the gene of interest is introduced into the extrachromosomal part of the genome, a non-Mendelian type of inheritance will be observed. However, this does not imply that the trait introduced is not stably incorporated. As mitochondria and chloroplasts are maternally inherited, it may be desirable to insert DNA into the organelle DNA (for example see Mcbride *et al.* 1995). The advantage of the transgenic plants thus obtained is that gene flow by pollen distribution will not occur and gene regulation is different. Such constructs may be of interest for use in breeding programmes, but so far they have hardly been used.

GENETIC STABILITY

Recombination, the aim of sexual reproduction, occurs in otherwise completely stable genomes and is a highly regulated process. Genes should not be changed or lost during recombination. The prerequisite for Mendelian inheritance is location of the gene in the chromosomal DNA and not in the extrachromosomal DNA, i.e. in the mitochondrial and chloroplast genomes. If deviations in Mendelian inheritance occur they may be caused by genomic factors, such as incorrect crossing-over during meiosis, spontaneous or induced mutations or other factors such as B chromosomes, polyploidy, aneuploidy, sex chromosomes and transposable elements.

Introduction of new stable traits not only depends on intrinsic genomic factors but also on interactions with the environment during cultivation. For example, plant cell cultures are known to produce new varieties due to intrinsic genomic instability during cultivation (for a review see Phillips *et al.* 1994; Ronchi 1995). Changes in the genome, however, do not necessarily also result in different phenotypes; these plants are regarded as being phenotypically stable. When Mendelian inheritance is used as a criterion, it is necessary to make an inventory of those factors that can disturb the inheritance of any trait in successive generations.

B chromosomes

Supernumerary chromosomes, mainly called B chromosomes (Bs), are dispensable extra chromosomes that give rise to numerical chromosomal polymorphism. The B chromosomes share a number of common features. For example, during meiosis the B chromosomes do not pair with members of A (normal) chromosomes. Often they carry a large amount of heterochromatin, but this is not always the case. B chromosomes are not homologous with members of the basic diploid or polydiploid complement (As). Normally they hardly contain genes and nucleolar organizers. Bs are considered selfish and are believed not to result from the introduction of foreign DNA. In mitosis pairing and non-disjunction of Bs occurs regularly. Pairing of Bs and their subsequent distribution at meiosis is often less efficient than that of A chromosomes. Inheritance of B chromosomes is independent of As and their frequencies can rise in populations. The inheritance is irregular and non-Mendelian.

Bs may be restricted to a particular tissue of the plant and are often confined to a few individuals in a population; subspecies or races may also strongly differ in their B composition. Reproductive tissues for breeding and somatic tissues for somatic transformation will therefore be the targets of B chromosome investigation (for reviews see Jones & Rees 1982; Jones 1991; Mcvean 1995; Hackstein *et al.* 1996). A comprehensive survey of most species with B chromosomes has been described by Jones & Rees (1982). Any trait dependent on a gene on a B chromosome is not suitable for propagation, because the trait will be inherited in a non-Mendelian manner.

Polyploidy

Polyploids, organisms with more than two basic sets of chromosomes per somatic cell, are highly common in cultivated species. Spontaneous polyploidization can occur and is thought to be due to the formation of unreduced microspores (non-disjunction during male meiosis) (Harlan & Dewet 1975). Abnormal chromosome pairing during meiosis of the newly formed polyploid plant can lead to irregularities such as trivalents and thus abnormal segregation. Additional problems are not expected if an inserted DNA is present in a newly induced polyploid except that abnormal segregation of the chromosomes can lead to aberrant segregation. If triploids are the result of such an abnormal segregation, they may be expected to be infertile.

Aneuploidy

The occurrence of aneuploidy may also cause non-Mendelian inheritance. Aneuploids are individuals (cells, organisms, species) with a chromosome number other than that of the basic number of chromosomes (n) or multiples thereof, i.e. not an exact multiple of a monoploid (n). Aneuploidy may result from abnormal segregation of the chromosomes at meiosis. The point at issue is that in aneuploids inheritance may occur in a non-Mendelian manner, especially if the gene of interest is located on the aneuploid chromosome (for reviews see Khush 1973; Sybenga 1992). As in polyploids, despite their difference in segregation, the genes may inherit in a predictable manner.

Sex chromosomes

In plants, sex chromosomes have also been identified, for example, as in *Rumex* and in cultivated species of *Spinacea* (spinach) and *Asparagus* (asparagus) (see Sybenga 1992). The most intensively studied sex chromosomes are those found in *Melandrium album* (for example see Vyskot *et al.* 1993). The inheritance of sex chromosomes differs from that of the autosomes (non-sex chromosomes), but sex-linked inheritance is highly predictable and follows the rules for hemizygotic inheritance like the genes located on the X chromosomes of the fruit fly *Drosophila.*

Transposable elements

Transposons or transposable elements (TEs) are defined as genetic entities that are able to insert into the genome at different positions and to affect the function of genes with which they become associated (for reviews see Grandbastien 1992; Gierl & Saedler 1992; Flavell *et al.* 1994). Because they are highly mobile they frequently alter their chromosomal position. They often induce mutations and consequently somatic instability. TEs consist of autonomous elements (encode their own transposase) and non-autonomous elements (depletive elements, which can not transpose without a transposase encoded by an autonomous element). In only a few species it has been

Table 1. Comparison of mutation, recombination and transposition frequencies

Phenomenon	Species	Target gene	Frequency per locus per generation
Mutation rate	*Zea mays*	Shrunken seeds, purple seeds	10^{-5}–10^{-6} mutations
Intra-chromosomal recombination*	*Arabidopsis thaliana* *Nicotiana tabacum*	Introduced defective gene like GUS	10^{-4}–10^{-7} events
Transposition	*Zea mays*	Waxy, Opaque-2	10^{-4}–10^{-7} insertions

For mutation rates see Ayala & Kiger (1980); intra-chromosomal recombination frequency see Swoboda *et al.* (1993, 1994); Lebel *et al.* (1993); Peterhans *et al.* (1990); for review see also Puchta *et al.* (1994); Lichtenstein *et al.* (1994); transposition frequencies see Walbot (1992).
*No data are available from *Zea mays*.

shown that TE really transpose, these include *Zea mays* (Fedoroff 1989; Michel *et al.* 1995), *Anthirrhinum* (Coen *et al.* 1989, *Petunia* (Gerats *et al.* 1990; Huits *et al.* 1995), *Arabidopsis* (Tsay *et al.* 1993) and *Nicotiana* (Grandbastien *et al.* 1989; Casacuberta *et al.* 1995). Many TEs have been found in plants and animals and because of present techniques, the possibility of identifying a TE has strongly increased.

TEs occur in plants both with small genomes, such as *Arabidopsis thaliana* (e.g. Athila (Pelissier *et al.* 1995), Tal (Voytas *et al.* 1988), and Tagl (Tsay *et al.* 1993); 1C nuclear DNA content of 0·2 pg, Bennett & Leitch 1995) and plants with large genomes such as *Triticum aestivum* (e.g. Wis2 (Harberd *et al.* 1987; Moore *et al.* 1991); 1C nuclear DNA content of 17·8 pg, Bennett & Leitch 1995). The large number of TEs found in some species, as in *Zea mays*, mainly reflects scientific interest in those species and is not a specific characteristic of the plant compared with other species.

TEs are supposed to insert preferentially in low-copy-number DNA regions (the transcriptionally active region), as has been described for Mu (Capel *et al.* 1993; Cresse *et al.* 1995), Ac, Tal, Tntl and Cin4 transposable elements (Capel *et al.* 1993). However, some observations indicate that TEs are also present in the heterochromatic part of the genome, as has been shown for *Drosophila* (Charlesworth *et al.* 1994). It is, therefore, expected that TE sequences are present in dispersed repeats of the genome (Flavell 1985; Smyth 1991). Several TEs such as the members of the Tourist family (Bureau & Wessler 1992, 1994a), Stonaway (Bureau & Wessler 1994b) and Wis2-1A (Monte *et al.* 1995), have been shown to be ubiquitous in plants. TEs may regularly change their position in the genome and thus the inheritance of the trait altered is unpredictable. The frequency of transposition can be measured as the probability that a specific transposon shall insert into a certain gene. TEs and mutations are important factors which determine the basic level of genetic changes in a plant. In Table 1 the intra-chromosomal recombination frequency, transposition frequency and the mutation rates are shown. With respect to intra-chromosomal recombinal frequency the results were obtained with genetically modified plants (Lichtenstein *et al.* 1994; Puchta *et al.* 1994), while the other results were obtained with non-genetically modified species. The results presented in the table suggest that genetically modified plants do not behave any differently from non-genetically modified plants.

TEs are supposed to be present in all species and, therefore, contribute to the stability of the line used. If an autonomous TE is present it may cause a change in the trait studied and, therefore, a non-Mendelian and unpredictable inheritance of that gene.

Other factors

A number of other factors causing non-Mendelian inheritance due to skewed segregation during meiosis are known to occur, but their effects are difficult to predict and their incidence is difficult to measure. The mechanisms and modes of these processes are largely enigmatic.

Skewed segregation during meiosis may be caused by various mechanisms such as: meiotic drive, linkage to a recessive lethal gene, paramutation and genomic imprinting. All these factors occur in both wild-type and cultivated plants. In addition, these features transgress Mendelian inheritance and are largely unpredictable.

GENOME SIZE

The genome size of plants can differ by three orders of magnitude (see, for example, Cavalier-Smith 1985; Bennett & Leitch 1995). Small genomes have proportionally small amounts of heterochromatin, while large genomes contain a high percentage of mainly non-coding regions (heterochromatin) in addition to the coding regions (Sybenga 1992). It means that in large genomes there are more potential sites of integration allowing the introduced DNA to insert percentage-wise more often in the non-coding regions, thereby resulting in inactive transgenes. Experimental evidence, however, is lacking.

In small genomes the insertion of foreign DNA in the genome can easily result in a destabilization of the chromosome. From T-DNA and transposon-tagging experiments in *Arabidopsis* (a species with a relatively low DNA content; 1C nuclear DNA content of 0·2 pg, Bennett & Leitch 1995), it is known that failure to produce progeny may be caused by the insertion of DNA in an obligatory gene (see Walbot 1992). This failure to produce progeny may be caused by the inactivation of the host gene at the site of insertion or by rearrangements caused by the insertion. However, any insertion may result in a destabilized genome, lower viability, etc.

SITE OF INTEGRATION

Since the first examples of plant cell transformation (Marton *et al.* 1979), a number of different methods have been used to introduce DNA into the plant genome. Most experience has been gathered by two methods, namely, DNA transfer by *Agrobacterium*, a natural genetically modifying soil bacterium, and by particle gun bombardment, a mechanical introduction of DNA into a host. Other methods such as electroporation, polyethylene glycol-mediated gene transfer, microinjection and silicon carbide fibres mediated DNA transfer have been described as being promising techniques (for review see Potrykus 1990, 1993) but have not yet been widely used. The *Agrobacterium*-mediated DNA transfer technique is mainly limited to dicotyledonous plants (for review see Potrykus 1993). In both the *Agrobacterium* and the particle gun transformation techniques the DNA is thought to insert into the genome by illegitimate recombination. In the *in vivo Agrobacterium*-mediated transformation single-stranded DNA is

incorporated into the nucleus, while in the particle gun normally double-stranded DNA will be shot into a cell. Therefore, different recombination mechanisms may be involved, but positive evidence for such conclusion is absent.

The process of transformation and regeneration of a genetically modified plant may cause instabilities or abnormalities in the plant genome. For example, DNA integration into an essential gene may result in lethality, while integration into constitutive heterochromatic regions may result in a destabilized chromosome and subsequent lethality. Thus, the introduction of a foreign gene may result in non-viable or unstable lines. The sensitivity of the plant to these phenomena depends on the species or lines used.

One of the approaches used for characterizing the DNA integration site in the genome has been to introduce a promoterless gene and measure the activity of the introduced reporter gene (Koncz *et al.* 1989; Herman *et al.* 1990; Kertbundit *et al.* 1991). The results of such studies indicate that incorporation mainly occurs randomly in the transcriptionally active part of the genome. This type of integration suggests that the gene of interest is inserted in a region of the genome that presumably has a chromatin conformation favoring transcription (Allen 1995). However, the possibility that transgenes are inserted into heterochromatic or heterochromatic-adjacent regions or in the neighbourhood of silencing (see below) cannot be excluded.

Randomness of integration suggests that there is no preference for certain chromosomes. Conclusions drawn from the data for the *Agrobacterium*-mediated DNA transfer technique for *Crepis capillaris* (Ambros *et al.* 1986), *Petunia* (Wallroth *et al.* 1986), tomato (Chyi *et al.* 1986; Thomas *et al.* 1994), potato (Jacobs *et al.* 1995; Van Eck 1995) and *Arabidopsis* (Liu *et al.* 1995; Franzmann *et al.* 1995) (Table 2) suggest randomness for the site of integration. Close examination of reports investigating the location of the inserted genes shows that only a few species have been investigated and that a limited number of independent transformants have been used. In general, the numbers are too low for a statistical evaluation. Therefore, no definite conclusion can be drawn about the randomness of the site of integration.

Despite an increase in the number of publications describing transgenic plants obtained by the particle gun method, results are not available which describe the DNA integration site into the genome after transformation. The insertion sites indicated by the particle gun method may differ from the results obtained with the *Agrobacterium*-mediated DNA transfer technique. In the *Agrobacterium*-mediated DNA transfer technique, the DNA of interest is cloned between two borders of the disarmed T-DNA and transferred in the bacterium *Agrobacterium tumefaciens*. Transfer of the DNA into the plant cell is performed by co-cultivating the bacteria with host plant cells. Then the transformed cells are selected and plants regenerated.

The T-DNA, a copy of a segment of a Ti (tumour inducing) plasmid, is flanked by 25 base pair repeats. In *Agrobacterium* T-strand production starts at the 25 base pair right border and normally stops at the left border of the T-DNA. Therefore, a complete T-DNA sequence might be expected in the genome after integration (for review see Zupan & Zambryski 1995). However, in 20–30% of the transgenic plants investigated, sequences that extend beyond 25 base pair borders of the plasmid have been found (Denis *et al.* 1993; Martineau *et al.* 1994; Denis 1994). The right border of the T-DNA is generally retained, while the left border quite often is not faithfully utilized. In some cases even integration of the almost complete Ti plasmid has been found (Denis *et al.* 1993; Denis 1994). These observations show that in *Agrobacterium* read-through of

Table 2. T-DNA localization sites

Species	Diploid chromosome number	Chromosome number	Number of T-DNA integrations on chromosome (*)	Number of T-DNA integrations on chromosome (*)
			(A)	
Crepis capillaris	$2n=6$	1	xxx	
		2	x	
		3	xx	
			(B)	(C)
Arabidopsis thaliana	$2n=10$	1	xxxxxxxxx	xxxxx
		2		xxxx
		3	xxxxxxx	xxxxx
		4	xxxxxxx	xxxxxx
		5	xxx	xxxxxx
			(D)	
Petunia hybrida	$2n=14$	1	xxxxx	
		3	xxxx	
		4	x	
		5	x	
			(E)	(F)
Lycopersicon (tomato)	$2n=24$	1	x	xxxx
		2	xxx	xxxxxxxxx
		3		xxxx
		4		xx
		5	x	x
		6		
		7		xxxxxx
		8	x	xxxx
		9		x
		10		x
		11		xxx
		12	x	xxx
			(G)	(G)
Solanum (potato)	$2n=2x=24$	1	xx	xx
		2	xxxx	xxxxxx
		3	xxxxxxx	xxx
		4	xxxxxxx	xxxxxxxx
		5	x	xxx
		6	xx	xxxx
		7	xx	xxxx
		8	xxxxxx	xxxx
		9	xx	xxxxx
		10	xxxxx	xxxx
		11	x	xx
		12	xxxx	xxxxxx

(*)x indicates one insertion found on a chromosome. (A) from Ambros *et al.* (1986). (B) from Liu *et al.* (1995b). (C) from Franzmann *et al.* (1995). (D) from Wallroth *et al.* (1986). (E) from Chyi *et al.* (1986). (F) from Thomas *et al.* (1994). (G) from Jacobs *et al.* (1995); Van Eck (1995).

T-DNA molecules beyond the border is possible. Insertions shorter than those expected (for example see Denis *et al.* 1993) or missing the left or right border sequences (for example see Deroles & Gardner 1988a) have also been found. This may result in transgenic plants that do not contain the trait of interest because the gene of interest may be absent or incomplete and thus not functional.

The particle gun or biolistic DNA transfer method is a mechanical method, in which DNA-coated particles (mostly gold or tungsten) are shot into the cell (for a review see Klein *et al.* 1992) and presumes the complete incorporation of the transferred DNA. However, there is evidence that incomplete genes are also inserted into the genome (Register *et al.* 1994). Various observations indicate that rearrangements and or deletions of the transgene may occur (Tomes *et al.* 1990; Jähne *et al.* 1994; Register *et al.* 1994; Schulze *et al.* 1995). It has been suggested that rearrangements of the transgene expression cassettes are more likely to occur if non-selectable markers are used than when selectable markers under conditions of selection are used (Register *et al.* 1994). It must be borne in mind that DNA transfer with the particle gun method occurs in a non-preferential manner. This means that contamination of the DNA construct by other DNA may result in transgenic plants containing both the gene(s) of interest and non-specified DNA sequences.

Potential effects of the introduced DNA are unlikely to influence segregation. Some reports describe the inheritance of the introduced trait as Mendelian. The inheritance of introduced traits has mainly been studied for antibiotic resistance markers, such as in *Petunia* (Deroles & Gardner 1988a,b; Ulian *et al.* 1994), *Arabidopsis* (Feldmann & Marks 1987; Scheid *et al.* 1991; Kilby *et al.* 1995), tobacco (Matzke & Matzke 1991; Matzke *et al.* 1993), pea (Puonti-Kaerles *et al.* 1992), maize (Walters *et al.* 1992) and rice (Schuh *et al.* 1993). Some traits in transgenic plants inherit in the proper Mendelian way and some do not. It is unclear whether this inheritance depends on the DNA construct used. It will also depend on the site of integration, on the genomic stability of the plant after transformation and regeneration.

STABILITY OF EXPRESSION

Successful introduction of the transgene into the genome is a prerequisite for obtaining transgenic plants. Only plants displaying the desired level of expression of the new gene during several generations will pass the selection procedure. It is important to bear in mind that two co-introduced genes may differ in the individual stability of their expression as well as in their sensitivity to the selection procedure (for example see Register *et al.* 1994; Van Der Hoeven *et al.* 1994; Schulze *et al.* 1995). Variations in the levels of transgene expression of independent transformants have been observed (Peach & Velten 1991; Nap *et al.* 1993).

Variability of expression does not occur at the genomic level, yet affects the phenotype of the plant. Thus variability of expression may be considered a pseudo-instability of the transgene. Variability in expression has been described as a result of differences between integration sites, the T-DNA copy number of R-DNA organization (Hobbs *et al.* 1993; Breyne *et al.* 1992a) or the environmental circumstances. Stability of expression depends on several factors, such as type of construct used, the gene inserted, the occurrence of silencing and the level of expression. Even a mutation in the T-DNA may cause loss of expression (Stephens *et al.* 1996).

Table 3. MAR (matrix-associated regions) elements used in plants

Element	Origin	Increase in expression	Reduction in variation	Reporter gene	
Lysozyme-A	Chicken	No	Yes	GUS	(A)
Heat shock	Soybean	Yes	No	GUS	(B)
Beta phaseolin	Bean	Yes	Yes	GUS	(C)
Seed-specific lectin	Soybean	No	Yes	GUS	(D)
Beta globin	Human	No	No	GUS	(D)
ARS-1	Yeast	Yes	No	GUS	(E)
Lysozyme-A	Chicken	No	No	NPT II	(F)

(A) Mlynarova *et al.* (1994, 1995); (B) Schöffl *et al.* (1993); (C) Van der Geest *et al.* (1994); (D) Breyne *et al.* (1992); (E) Allen *et al.* (1993); (F) Mlynarova *et al.* (1995).

Position effects

From plants little direct information on the effect of the site of integration is available, but from *Drosophila* (Henikoff 1990; Dorer & Henikoff 1994) and yeast (Gottschling *et al.* 1990; Allshire *et al.* 1994; Aparicio & Gottschling 1994) it is known that integration into transcriptionally inactive heterochromatin or heterochromatin-adjacent regions may result in silencing or in a reduction in intact transgenes expression levels. This effect is called position effect variegation (for a recent review see Henikoff 1994). For tomato it has been suggested that position effect variegation may occur (Wisman *et al.* 1993). It is expected that this mechanism is generally present in plants.

Moreover, variation in expression is also thought to result from the influence of host DNA sequences or chromosomal organization at or near the site of integration and is called position effect variation (Peach & Velten 1991). Introduction of inserts containing additional DNA fragments such as matrix-associated regions (MAR), also known as scaffold-associated regions (SAR), appears to be a very promising approach to stabilize the levels of expression (for a review about plant MAR elements see Breyne *et al.* 1994). MAR elements are thought to demarcate a regulatory domain of gene expression by forming DNA loop boundaries that bind to the matrix and thus insulate the gene encoded within these loops (Laemmli *et al.* 1992). MAR elements are A/T rich and thought to bind topoisomerase II (an enzyme involved in the removal of knots or the unwinding of excessive chromosomal DNA twists). In this way MAR elements might protect gene activity from the influence of surrounding chromatin such as nearby positive regulatory elements (Allen *et al.* 1993). Another explanation for this event may be that insertion of the transgene into the heterochromatic part of the genome is prevented (Dorer & Henikoff 1994). Several plant MAR elements have been identified, for example, the soybean heat shock gene (Schöffl *et al.* 1993), the seed lectin gene (Breyne *et al.* 1992b) and the bean β-phaseolin gene (Van der Geest *et al.* 1994). For only one plant gene, the β-phaseolin gene, MAR elements have been described, both at the 5′ and the 3′ side (Van der Geest *et al.* 1994).

Investigation of the role of MAR elements in transgene expression is usually performed by measuring the activity of a reporter gene (normally GUS), either in the presence or absence of MAR elements. To be effective, MAR elements need to be incorporated stably into the genome. The introduction of a reporter gene flanked

by MAR elements by either a stable or a transient transformation technique showed that extrachromosomal copies are hardly effective (Allen *et al.* 1993; Frisch *et al.* 1995).

The use of MAR elements may enhance the expression level and or transformation efficiency and/or reduce the expression variability. The expression level was enhanced using the yeast ARS-1 MAR element (Allen *et al.* 1993), the soybean heat shock gene MAR element (Schöfl *et al.* 1993) and the bean β-phaseolin gene MAR element (Van der Geest *et al.* 1994). Reduction in the variability of transgene expression has been found using the GUS reporter gene placed between the MAR elements from the soybean seed lectin gene (Breyne *et al.* 1992b), the chicken lysozyme-A gene (Mlynarova *et al.* 1994, 1995) and the bean β-phaseolin gene (Van der Geest *et al.* 1994). In contrast, the expression of the NPT II gene, placed together with the GUS reporter gene between chicken lysozyme-A MAR elements, did not show a significant reduction in expression variability (Mlynarova *et al.* 1995).

Apart from the fact that MAR elements are thought to establish independent chromatin domains, reports exist which suggest that using these elements results in higher transformation efficiencies, as for the chicken lysozyme-A MAR element in tobacco (Mlynarova *et al.* 1995) and the *Petunia* transformation booster sequence (Buising & Benbow 1994), which is supposed to contain a MAR element (Galliano *et al.* 1995). It is unknown whether MAR elements introduced inherit in a Mendelian manner over several generations or contribute to unpredictable segregation.

Variability of expression

Factors other than position effects may also cause a variable expression, such as the promoter used. Although the generally used 35S CaMV promoter was originally considered to be constitutive, it has been shown to possess elements that can facilitate developmental and tissue-specific regulation of the promoter (Benfey 1989) and thus variation in expression in different parts of the plant may occur. The use of heterologous organ-specific promoters, which should avoid variation in expression in organs, does not necessarily result in organ-specific expression. For example, the introduction of a GUS construct driven by the root-specific *par* (*Parasponia*) haemoglobin promoter in tobacco showed that expression of the gene was also found in the leaves as well as in the roots (Van der Hoeven *et al.* 1994). Even the use of promoters from highly conserved proteins does not allow constitutive expression in the same organ. For example, the ubiquitin promoter Ub-1 responded independently to environmental changes, such as heat or physical stress, in individual cells of the same plant; its expression appeared to be cell-cycle dependent (Takimoto *et al.* 1994).

Expression of a trait is not only determined by the construct or the species used, but may also be influenced by environmental factors during plant growth. Environmental factors have been described to be responsible for variation in transgenic plant expression. One example is the field test of the maize A1 gene (dihydroflavone reductase) in transgenic *Petunia* (Meyer *et al.* 1992). This test showed that in June the flowers were predominantly salmon red (caused by the introduction of the maize A1 gene), while in August the entire field was weakly coloured. Other examples of variation in expression caused by the environment include the use of a GUS construct in transgenic *Nicotiana* (Peach & Velten 1991) and the csr-1 gene (Sulphonylurea herbicide resistance gene) in

Nicotiana (Palmer 1995). Thus, variation in expression may contribute to a surprisingly unpredictable segregation.

Silencing

Gene silencing was only recognized when plants not exhibiting the desired level of expression were used as a subject of study. It is often visible only in part of the independent transformants and may vary among individual transgenes (for reviews see Kooter & Mol 1993; Matzke & Matzke 1993, 1995a,b; Finnegan & McElroy 1994; Flavell 1994). Originally the term silencing was intended to describe a repression mechanism that acts across several kilobases of DNA and affects heterologous promoters (Brand *et al.* 1985). This definition is limited to *cis* elements only. Currently, the term silencing is used in a much broader sense, namely, a repression of expression of homologous and/or heterologous genes. Several distinct types of silencing may be recognized: first, mutual suppression of transgenes (also called *cis* inactivation); secondly, co-suppression or sense suppression (also called reciprocal ectopic *trans* inactivation); and thirdly, unidirectional silencing (also called unilateral ectopic or epistatic *trans* inactivation).

The first type of silencing, mutual suppression of transgenes, is a phenomenon in which the transgenes are inactivated by co-insertion at a single locus, and the sequences show no homology with endogenous genes. Methylation of the transgene is often found, but its role in the inactivation process is unclear. For example, introduction of the *uidA* gene under the 35S CaMV promoter showed that multiple transgene insertions gave low expression levels, while the expression levels of single-copy insertions were high (Hobbs *et al.* 1990). For other examples of mutual suppression see Linn *et al.* (1990); Scheid *et al.* (1991); Kilby *et al.* (1992); and Assaad *et al.* (1993).

The second type, co-suppression or sense suppression, is a phenomenon in which the silencing of a gene is caused by mutual inactivation of the transgene and a partial homologue, being either a transgene or an endogenous gene. An example of co-suppression of an homologous gene is the introduction of extra copies of the dihydroflavenol reductase gene in *Petunia*, which resulted in a reduction in expression of the gene (Van der Krol *et al.* 1990). Even part of a gene may be sufficient to silence expression, as has been shown for the polygalacturonase gene in tomato (Smith *et al.* 1990). For other examples of this type of silencing see Napoli *et al.* (1990); Hart *et al.* (1992); Fray & Frierson, 1993b; and Seymour *et al.* (1993).

The third type of silencing is unidirectional silencing, a phenomenon in which a gene is silenced following the introduction of a transgene. This can be either the transgene itself or a partially endogenous homologue (Matzke & Matzke, 1995b). The silenced gene can be present at either an allelic or non-allelic (ectopic) locus. For example, the activity of a single copy of the *uidA* gene is inhibited when it is crossed with a plant carrying poorly expressed duplicate copies of this *iudA* gene under the 35S CaMV promoter (Hobbs *et al.* 1993). Elimination of the duplicated copies by segregation always showed reactivation of the single-copy *uidA* gene (Hobbs *et al.* 1993). For other examples of unidirectional silencing see Matzke & Matzke (1991); Matzke *et al.* (1993b); Meyer *et al.* (1993); Vaucheret (1993) and Matzke *et al.* (1994b). Allelic *trans* inactivation of the transgene closely resembles paramutation which has been observed in transgenic *Petunia* (for example see Meyer *et al.* 1993). Paramutation-like effects (see below) have also been observed for homologous sequences on non-homologous chromosomes (Matzke *et al.* 1994a; Neuhuber *et al.* 1994; Vaucheret 1994). Although

silencing may result from the introduction of a transgene, it also occurs in wild-type plants. The inheritance of silenced genes is difficult to investigate. In the case of paramutation-like silencing it is known that this feature can be induced by crossing two lines containing fully expressed genes. Separation of the two genes (the paramutator and the paramutant) by segregation (thereby assuming no tight linkage between the genes) has shown that the paramutant trait recovers slowly over several generations. The molecular basis of this process is unknown.

Often the effect of silencing can be detected within the first two generations after the transgenic plant has been created. The level of silencing may also be influenced by environmental circumstances, as has been shown for the chitinase gene in *Nicotiana* (Meins & Kunz 1994). In field experiments silencing might be induced by environmental factors (for example Meyer *et al.* 1992; Brandle *et al.* 1995; Elkind *et al.* 1995).

In some cases silencing is only observed in homozygous and not in hemizygous transgenic plants (De Carvalho *et al.* 1992; Hart *et al.* 1992; Dehio & Schell 1994; Dorlhac De Borne *et al.* 1994; Meins & Kunz 1994; De Carvalho Niebel *et al.* 1995). This form of silencing suggests that the concentration of the transcript is the determinating factor in the regulation, arguing for an autoregulatory model of silencing in these cases (Meins & Kunz 1994). However, post-transcriptional as well as transcriptional inactivation processes have been reported to be involved in gene silencing (for example Mol *et al.* 1991; De Carvalho *et al.* 1992; Lindbo *et al.* 1993; Dehio & Schell 1994; Smith *et al.* 1994; De Carvalho Niebel *et al.* 1995). The existence of post-transcriptional silencing was proven in run-on experiments which showed that in some cases, the initiation of transcription proceeded at the same rate in both silenced and highly expressed genes (Mol *et al.* 1991; De Carvalho *et al.* 1992). Transcriptional silencing has been described as occurring in a variety of species (see Brusslan *et al.* 1993; Meyer & Heidmann 1994; Neuhuber *et al.* 1994), and to cover the paramutation-like phenomena of the maize A1 allele in transgenic *Petunia* (Meyer *et al.* 1993) and the B locus in maize (transcriptional activator of anthocyanin pigment biosynthetic genes) (Patterson *et al.* 1993). Transcriptional silencing is not only restricted to sequence homology in the transcribed region of two loci (Brusslan *et al.* 1993; Meyer *et al.* 1993), but can also be caused by sequence homology in the promoter region (Vaucheret 1993; Neuhuber *et al.* 1994). Recently, a link has been proposed between the transcriptional post-transcriptional silencing process based on virus resistance studies in which non-viral transgenes prevented virus accumulation (English *et al.* 1996). This was supported by the fact that the *de novo* methylation of a gene might be induced and targeted in a sequence specific manner by its own RNA (Wasenegger *et al.* 1994).

Gene silencing may be restricted to a developmental stage. For example, expression of an introduced polygalacturonase gene in tomato was not suppressed in the leaves but only in ripe fruits, where the polygalacturonase gene is highly expressed (Smith *et al.* 1990). Transgene expression has also been observed early in development for β-1,3-glucanase in tobacco, while at later stages it is silenced (De Carvalho *et al.* 1992). In some cases developmental patterns have been observed for transgene expression. High expression levels were found in lower leaves (early in development) while the transgene was co-suppressed in higher leaves (later in development). Examples of developmental patterning include the expression of chitinase in tobacco (Hart *et al.* 1992) and the expression of the *Arabidopsis* S-adenosyl-L-L-methinine synthetase in tobacco (Boerjan *et al.* 1994).

Silencing has been described and studied mainly in T-DNA transformed plants. The introduction of foreign genes by non-T-DNA mediated (direct) gene transfer has shown that silencing is a general phenomenon, as has been demonstrated in maize (Register *et al.* 1994). There is not always a clear correlation between copy number and silencing. Transfer of a DNA construct into tobacco resulted in independent transformants which showed either differential co-suppression or no suppression at all, irrespective of the transgene copy number (Dorlhac De Borne *et al.* 1994). The phenomenon of silencing is not only found in transgenic plants, but has also been observed in non-genetically modified plants. Transcriptionally regulated silencing has been described for the maize B gene (Patterson *et al.* 1993), while post-transcriptional silencing has been observed for the chs (chalcone synthase) gene in *Petunia* (Van Blokland *et al.* 1994). Another form of silencing is the suppression of a chs allele in *trans* described in snapdragon (*Antirrhinum*). Normal chs allele expression is suppressed by a rearranged chs allele (it contains a transposable element Tam3 insertion) in the heterozygous plant. In heterozygotes the inactivated chs allele is semidominant, producing white flowers (as expected) in homozygous plants, whereas in heterozygous plants it produces very pale flowers. The latter was not expected as the normal chs allele is dominant and should give red flowers (Bollmann *et al.* 1991). Thus, silencing may lead to pseudo-instability.

Silencing by antisense technology

Antisense RNA was originally found in bacteria as a mechanism to suppress the expression of genes (Simons & Kleckner 1983; Green *et al.* 1986; Simons 1988). In the antisense strategy the DNA is placed behind a relevant promoter in the antisense orientation resulting in transcription of the antisense RNA. Antisense RNA is thought to hybridize to the sense RNA (coding strand) forming a complex of sense and antisense RNA. This results ultimately in a reduction of specific protein caused by reduced availability of mRNA (sense) to produce that protein. However, this approach to the mechanism of antisense RNA in the cell appears to be too simple (for example see Mol *et al.* 1994). Thus, in a number of transgenic plants the expression of the homologous gene will be down-regulated.

In plants, the use of antisense technology was first published in 1986 by Ecker & Davis who used a transient expression system of carrot protoplasts. For recent reviews on antisense technology in plants see Mol *et al.* (1994); Bourque (1995); and Kuipers *et al.* (1997). The advantage of antisense technology is its gene specificity. This assumes a large degree of homology between the introduced and the endogenous gene. Inhibition of expression of non-homologous genes is possible, as has been demonstrated for the antisense chsA gene, which also inhibited the expression of the chsJ gene (Van der Krol *et al.* 1990).

There are no indications that antisense genes behave differently to other genes with respect to stability and inheritance, as has been shown for the Flavr Savr tomato, the first transgenic crop to be introduced to the US market. In this tomato the delay in fruit softening has been achieved by the introduction of an antisense polygalacturonase gene. Other well-known examples of the antisense gene technique in tomato are the inhibition of fruit softening by antisense pectin methylesterase genes (Tieman *et al.* 1992) and delay in fruit ripening by the introduction of antisense genes for ACC synthase (Oeller *et al.* 1991) or antisense ACC oxidase (Hamilton *et al.* 1990). One has to bear in mind that the inheritance of the introduced antisense gene has to be considered a dominant

characteristic. Consequently, in breeding programmes the trait introduced can be easily followed. In some cases they may lead to aberrant yet predictable segregation numbers, yet they may still be useful.

CONCLUSIONS

In conclusion, a number of factors might influence the stability of a gene or genome of a plant. The contribution of each of these factors, some described and others still enigmatic, is determined by the plant used. Well known factors are B chromosomes, polyploidy, aneuploidy, sex chromosomes and transposable elements. In the case of transposable elements they are expected to be present in all species at a frequency which may differ from element to element and which may be greater than the mutation rate. The factors mentioned are determined by the species or even the plant used. All these factors may occur in both wild-type and cultivated plants and thus should not limit the use of these plants in breeding programmes involving genetically modified plants. Based on the factors discussed, the deviations occur with a predictable incident. Determination of the stability of any introduced trait should relate to the basic level of the deviations occurring in the species or cultivar used. That is, a trait is considered unstable only if the incidence of any deviation is higher than in the host plant. It is in the interest of the breeder to produce stable cultivars.

So far, the data concerning the randomness of transgene integration are too limited to draw definite conclusions. Rearrangements may occur during the integration. There are no indications that the introduced trait is less stable than the non-modified traits.

If the gene of interest is introduced into the extrachromosomal part of the genome, a non-Mendelian type of inheritance will be observed. However, this does not imply that the trait introduced is not stably incorporated.

The insertion of a gene into the genome does not mean that the introduced gene will be expressed at all or the desired level in the transformed plant or in its progeny, since the inactivation of the gene may occur. A way to reduce the variability of expression is to use MAR elements surrounding the gene of interest. In some cases results from experiments with MAR elements have shown a reduction in the variability of the transgene expression. The effect of the MAR elements used may depend on the type of MAR element, the species used and/or the gene of interest. Until now the studies were performed with tobacco as a host, which may limit the results obtained for the use in other species. It is not clear whether the use of MAR elements reduces the variability of the level of expression by demarcating the gene of interest between them or by favouring a specific site of insertion into the genome. The expression levels of genes inserted into a plant genome may be variable. This cannot be explained by improper integration of the transgene in the genome but may be caused by the transgene itself, the promoter used, distinct sequence elements or by environmental factors. Various kinds of variation in expression have been described. As the data published are limited and many results are still preliminary, it is not possible to draw final conclusions about the stability of transgene expression. Moreover, the modes of action of the mechanisms underlying variation in expression remain largely unknown. For transgenes orientated in the antisense direction there are no indications that the inheritance of genes differs from the inheritance of other genes.

However, instability of expression becomes visible within a few generations, and once expression is stable it is expected to remain so under unchanged environmental

conditions. Thus, though stability of expression seems a rather complex phenomenon, mainly it can be diagnosed without any special effort. Any indication of instability during the production of the new cultivar will automatically lead to omission from the breeding programme.

In breeding programmes the inactivation of the expression of the gene of interest is undesirable. Plants showing this state will be discarded, since the expression of the inserted gene will be the determining factor in the selection of the transgenic plants. In addition to the fact that the trait should be inherited, it is also necessary that this trait is inherited in a predictable way in breeding programmes. As instability and non-Mendelian inheritance are undesirable to breeders, plants showing these phenomena are generally discarded. For breeders it is essential to produce lines that are distinct, uniform and stable (the DUS principle according to the UPOV rules). Therefore, unstable traits resulting from unstable expression will be discarded in breeding programmes. The UPOV rules are not based on the genetic, but on the phenotypic stability of distinct traits. The number of off-types highly varies, depending on the lines used and, accordingly, a similar variation in the stability of expression might be acceptable.

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