The transgenes are expressed with different level in plants

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Summary

Variable expression for the same transgene construct has been documented in various plant species, regardless of the type of transgene. This phenomenon and the factors influencing it are reviewed. A variability in the transgene expression level was found to exist between and/or within independently derived lines, different lines of clonal replicates, siblings of the same line, a single plant, a single leaf as well as the same cell. Both the transgene dependent and the recipient dependent factors have been proposed to account for this phenomenon, the transgene dependent factors of which include transgene construct fidelity, T-DNA integration pattern, T-DNA copy number, promoter activity and the effect of nuclear matrix attachment region. In addition, different forms of epigenetic, homology-dependent gene silencing also contribute to the unstable expression of the identical transgene. The recipient dependent factors include position effect, ploidy level, genetic background, homozygosity, and developmental stage. Furthermore, environmental factors such as light intensity, temperature, field growth conditions and the season have been shown to modulate the levels of transgene expression. The approaches to stabilize the transgene expression were also discussed.

Key words:
transgenic plant, transgene expression, variability.

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1. Introduction

The foreseeable transgene expression is the main requirement for its application for plant improvement. However, incre-

asing evidence suggests that the variability in transgene expression levels, including complete silencing of the transgene, is a ubiquitous phenomenon (1-4). The variability (variation) in the expression levels for the same transgene has been documented in various plant species, regardless of type of transgene (Tab. 1). Such variability could be detected on mRNA, protein or phenotypic levels. It was found to exist between and/or within independent transgenic calli (inter-transformant variability), individually transformed clonal callus lines (inter/intra-clonal variability), independent transformants (inter-transformant variability), transgenic lines (including homozygous lines) derived from either sexual or vegetative propagation, plants, organs, tissues as well as cells (Tab. 1). The extent of this variability is extremely large ranging from zero to few hundreds. This raises many questions among scientists and in some cases, it is used as the arguments against introduction of genetically modified organisms (GMO) into agriculture. This paper will mainly focuse on the nature of the variability in the expression levels of the same transgene in plants, the factors influencing it and the approaches to stabilize the transgene expression. The terminology that the authors used for a description of the same phenomenon is so diverse that it makes it difficult to uniform the notions used in this paper. Therefore, we will use the same descriptions which the authors used in their original articles.

2. Factors influencing transgene expression – transgene dependent factors

The early findings suggested that the instability of the endogenous genes was associated with the so called 'genetic flux' such as transposon activity, position effect, paramutation, gene conversion, unequal crossing-over, chromosomal rearrangements and epigenetic changes (22). Transgene instability and silencing in plants were initially considered as anomalies or a quirk in transformation procedures. However, they are now recognized as a facet of vitally important gene regulatory systems present in all organisms (23-27). Many factors have been proposed to account for the variable transgene expression, including transgene dependent and recipient dependent factors. The transgene dependent factors involve T-DNA configuration, transgene construct fidelity, the effect of nuclear matrix attachment region (MARs), the influence of the binary vector sequence, transgene integration pattern and copy number (28,29). In addition, different forms of epigenetic, homology-dependent gene silencing (HDGS) also contribute to unstable transgene expression (27,30-33).

2.1. T-DNA copy number

The correlation between T-DNA copy number and transgene expression level seems to be extremely controversial. Some reports described a positive correlation.

Table 1

Selected instances on the variability of the transgene expression levels in plants

Plant species	Analyzed transgene elements	Type of tissue	Variability in transgene expression levels					Correlation between the expression levels of different transgenes within the T-DNA	Reference
			between independently derived lines	within the same line	within single plant	within the same tissue	within single cell		
1	2	3	4	5	6	7	8	9	10
Arabidopsis thaliana	*nos-nptII*	seedlings	62 (56%) lines show *nptII* silencing	Yes				No	(1)
Barley	β-glucanase	grains	class 1-3 (0.04-1.2 unit)						(5)
Cucumber	35S-thaumatin II cDNA	fruits	Yes	Yes					(6)
	PR-2d-*uidA*	leaves	16-fold	5 to 256 -fold					(7)
Lettuce	35S-*gus nos-nptII*	leaves	50-fold 16-fold	8-fold				Yes	(8)
Lotus corniculatus	35S-*uidA*	embryo	Yes		32-63% show GUS activity				(9)
		radicles			10-35% show GUS activity				
Maize	*act1-uidA*	roots				Yes			(10)
Petunia	35S-antisense CHS	floral tissue	different pigmentation		variable pigmentation				(11)
Potato	35S-GNA 35S-BCH	leaves stem tuber	Yes	Yes	8-fold	Yes		Yes	(12)
Rice	Ubi-*gusA* 35S-*OC-I*ΔD86	leaves	0-28958 unit 0.012-0.25% total protein					No	(13)
	35S-*bar*	leaves	7.5-96.7 unit	Yes				Yes	(14)
	35S-*gusA*		class 0-3	class 0-3					
	35S-*gusA*	leaves	3-fold	No				Yes	(15)

1	2	3	4	5	6	7	8	9	10
	35S-*bar* Ubi1-*gna* RSs1-*gna* Ubi1-*cry1Ac* 35S-*cry2A* Ubi1-*cry1Ac* 35S-*cry2A* Ubi1-*gna*	plants	9-fold 0.2-2% total protein 0.01-2.5% of total protein	Yes				Yes	(16)
Tobacco	*nos-cat* *nos-nptII* *mas-cat* *mas-uidA* *nos-gusA* *Cp-gus* 35S-*luc*	calli clonal callus line calli leaves leaves	200-fold 140-2641 unit 136-fold 175-fold 90-130% 10-fold 4.62-8.62 unit	3-4 fold		15-fold	Yes	No No	(1) (2) (17) (18) (19)
Wheat	Cal-*nptII* Act1F-*nptII* RTsh1-*nptII*	clonal cell lines	50% lines reduction or loss NPTII activity						(20)
White clover	35S-*uidA*	Leaf	No	4-fold					(21)

Promoters

act1-rice actin 1 promoter
Act1F-a truncated *actin1* gene promoter of rice
Cal-35S promoter fused to the first intron of the alcohol dehydrogenase gene of maize
Cp-cammelina yellow mottle virus promoter
mas-mannopine synthase promoter
nos-nopaline synthase promoter
PR-2d-tobacco 1,3 glucanase promoter
RSs1-rice sucrose synthase-1 promoter
RTsh1-35S promoter fused to the first intron of the *sbrunken1* gene of maize
35S-Cauliflower Mosaic Virus 35S promoter
Ubi-maize ubiquitin promoter
Ubi1-maize ubiquitin-1 promoter

Coding sequence

bar-Bialaphos resistance gene
BCH-bean chitinase gene
cat-chloramphenicol acetyltransferase gene
CHS-chalcone synthase gene
cry1Ac / cry2A-Bacillus thuringiensis (Bt) δ-endotoxin gene
GNA-snowdrop lectin (*Galanthus nivalis* agglutinin) gene
gusA / uidA/gus-β-glucuronidase (GUS) gene
luc-firefly luciferase gene
npt-neomycin phosphotransferase gene
nptII-neomycin phosphotransferase II gene
*OC-I*ΔD86-an engineered cysteine proteinase inhibitor (oryzacystatin- IΔD86, *OC-I*ΔD86) gene

Bhattacharyya et al. (34) suggested a positive correlation between transgene copy number and the levels of GUS activity. Similarly, Kohli et al. (35) demonstrated that multi-copy lines that contain up to five copies of *gusA* and/or the Bialaphos-resistance gene (*bar*) express the gene stability at high levels up to the R_3 generation. Comparable or in some cases higher expression levels were detected in plants with multiple copies of *gusA*, compared to plants containing one or two copies. However, others reported a negative correlation. The copy number of the integrated *A1* transgene was found to correlate inversely with the stability of coloration in the primary petunia transformants (36). In transgenic tobacco plants, the high-expressing types contain one copy of the T-DNA and the low-expressing types are composed of inverted repeats (IRs) of the T-DNA (37). Bucherna et al. (38) demonstrated that GUS activity was lost at week 2, 5 or 13 in plants containing 2-6 copies, whereas plants with one copy continuously expressed GUS. Elomaa et al. (39) reported that *A1* transformants with multiple transgenes showed instability in pigmentation. Those having only a single transgene copy showed the most stable pigmentation. Some studies suggested both a positive and a negative correlation (37,40,41). Vain et al. (42) reported that in the presence of MARs, GUS activity increased in proportion to transgene copy number up to 20 copies, but was generally reduced in lines carrying a higher copy numbers. Some studies indicated that there is no distinct correlation between the copy number and the transgene expression level (8). Others have reported that variation in frequency of silencing between progeny of siblings does not depend on loci or copy number (43). No link between β-glucuronidase gene (*uidA*) copy number and GUS expression was detected (44).

2.2. T-DNA configuration

Breyne et al. (17) demonstrated that the configuration of a reporter gene within the T-DNA could significantly affect the overall pattern of its activity (Fig. 1). Cloning the reporter gene with its promoter close to the right border of the T-DNA and with a 3' end in between resulted in 3 to 4 fold higher mean expression without increasing the inter-transformant variability compared to constructions carrying the gene in the middle of the T-DNA. Gidoni et al. (45) also suggested that the degree of co-ordinate expression of the genes was influenced by their location within the T-DNA. Reduced variability in GUS expression was obtained by using a binary vector in which the two selection markers were placed next to the right and left borders, respectively, and a CaMV 35S-*uidA* gene was placed between these markers (34). The placement of the marker genes in this vector was thought not only to ensure the complete integration of the T-DNA and faithful expression of the reporter gene, but also to reduce the potential transcriptional interference from transcriptionally active endogenous plant promoter to which the T-DNA was fused. Other reports suggest that the antisense inhibition of granule-bound starch synthase I (GBSSI) in potato is more efficient with the most homologous coding sequence (potato), cDNA

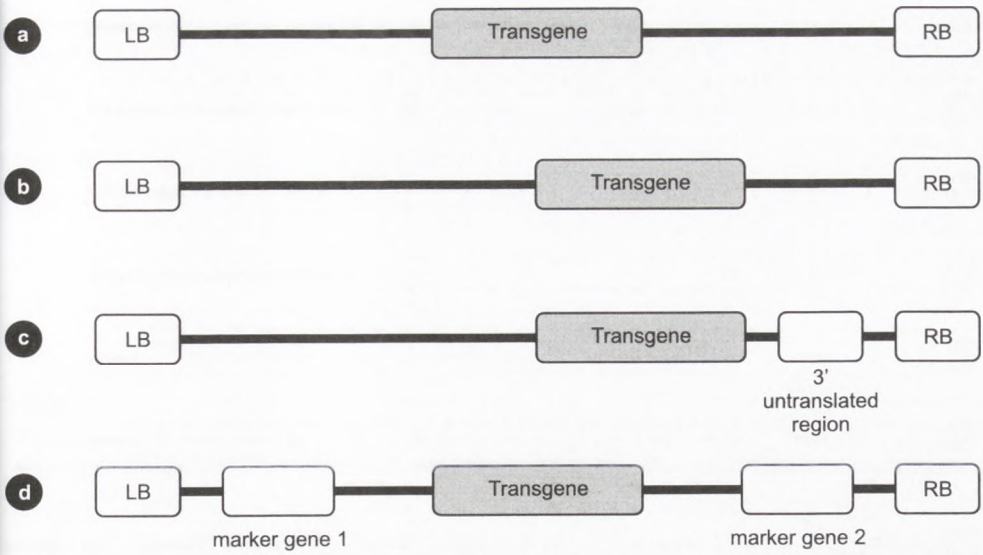

Fig. 1. T-DNA configuration and transgene expression. (a) Placing the gene in the middle of T-DNA results in low level of expression and limited variability. (b) Placing the gene with its promoter next to right border (RB) results in increased expression level as well as variability. (c) Separating the gene from the RB by a 3' untranslated region (3' UT) results in increased expression level and reduced variability. (d) Placing the gene in the middle of two marker genes, where the marker genes were placed next to the right and left borders respectively, results in increased expression level and reduced variability. LB-left border. This figure is adapted and modified from 17 and 34.

construct and 35S promoter than using the least homologous sequence (maize), genomic construct and GBSSI promoter, respectively (46,47).

2.3. T-DNA integration pattern

Most of the T-DNA integrations are nearly perfect, from the right border of the T-DNA to the left border (48). Occasionally, complex T-DNA integrations are created consisting of multiple T-DNAs at the same chromosomal site, and the transforming plasmid may undergo rearrangments prior to or during integration into the genome (49-52). Some documented data on T-DNA integration pattern were illustrated in Figure 2. These rearrangements could occur in the promoter or in the coding region of the gene, which leads to a loss of the integrity of the construct and give rise to an abnormal transcript. Such abnormal transcripts (aberrant RNA) were thought to mediate DNA methylation (51). The tandem linked T-DNAs can be arranged in several configurations: direct repeats (DRs or R_D), right border inverted repeats (R_R), left-border inverted repeats (R_L) or a combination of the three (48,49,53). Transgenes of T-DNA that are organized as inverted repeats (IRs) often show low expression (54,55) and the IRs are

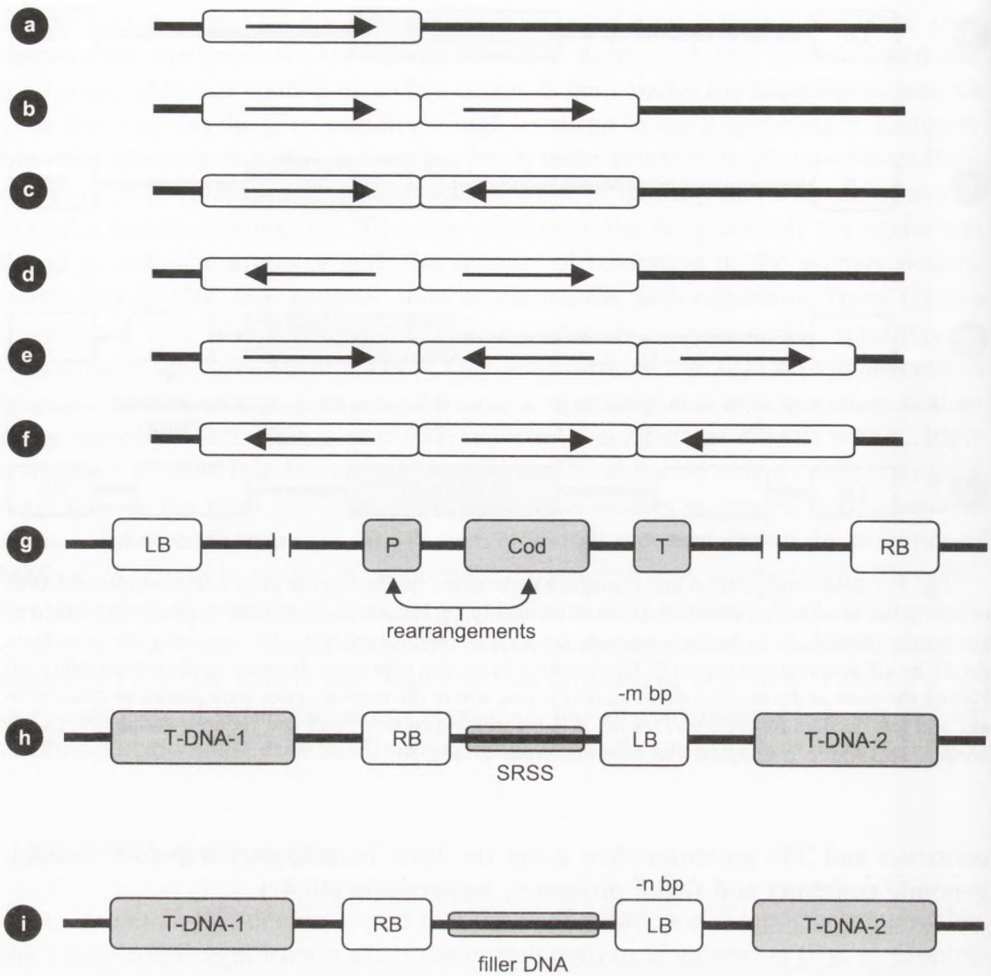

Fig. 2. T-DNA integration pattern. T-DNA may be integrated as single copy (a), direct repeats (b), inverted repeats (c, d, e and f). The T-DNA repeats might be composed of precise junctions (h) or imprecise junctions (i). SRSS means short regions of sequence similarity between recombining strands without any filler DNA in between. The m or n bp below the left border (LB) indicates the number of nucleotide of T-DNA sequence deleted during recombination. P – promoter. T – terminator. Cod – coding region. RB – right border. This figure is adapted and modified from 48-50, 56,57.

known to induce transcriptional and post-transcriptional gene silencing (31,48). The analysis of a transgenic petunia population (53) revealed that R_R were three times more frequent than R_L or R_D. Some multi-copy transformants contain only dispersed repeats only with contiguous repeats and some contain both. The authors conclude that T-DNA organisation pattern, but not position effect, explain most of the observed phenotypic diversity. The studies on transgene repeats in aspen indicated that the

transgene repeats were composed of precise or imprecise junctions and a mechanistic model for transgene rearrangement and filler formation is suggested (56). Molecular analyses of hybrid and wild transgenic lines revealed that the variable *rolC* (root locus C from *Agrobacterium rhizogenes*) expression were always the consequences of transgene repeats (57). However, alternative instance is also documented. Many repeated transgenes are normally expressed, including those residing in IRs (48).

2.4. Binary vector sequences

Besides the T-DNA region, binary vector (non-T-DNA) sequences were found to transfer frequently into the recipient plant genome (49,51,53,58-61). It is possible that the vector sequences and the corresponding changes in nucleotide composition make the transgene particularly susceptible to the conversion of different epigenetic states (28,62). In unsteadily expressed transgenic tobacco lines, the binary vector sequences were directly contiguous with a right T-DNA border (62).

2.5. The effect of promoters

The promoter activity strongly influences the expression levels and patterns of the same transgene. In *Arabidopsis thaliana* plants transformed steadily, the organ specificity and strength of different promoters were compared (63). Using the *uidA* reporter gene, the CaMV-35S promoter give the highest expression level. The barley leaf thionin BTH6 promoter was almost inactive in the majority of lines, whereas the *Arabidopsis* ubiquitin UBQ1 promoter exhibited an intermediate strength. The soybean heat-shock promoter Gmhsp 17.3 was inducible up to 18-fold, but absolute levels were lower than in the case of the ubiquitin promoter. In transgenic apple plants containing the CaMV 35S promoter, the mean GUS activity in leave tissues were approximately twice that of plants containing the SSU (the heterologous ribulose-1,5-bisphosphate carboxylase/oxygenase Rubisco small-subunit) promoter (64). In tomato, when sucrose-phosphate synthase (SPS) is expressed from a Cauliflower Mosaic Virus 35S promoter, yield is enhanced up to 80% compared with that of a ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) small subunit (rbcS) promoter (65). In tobacco, the activities of three different promoters: Cauliflower Mosaic Virus 35SS, modified CaMV 35S and promoter of an *Arabidopsis thaliana* Lipid Transfer Protein gene were shown to vary not only among independent transformants, but also between leaves on the same plant and within a leaf (19).

The enhancer elements in the promoter increase the levels of transgene expression. In transgenic maize, the enhancer elements located approximately 1 and 5 kb 5' of the transcription start site of a maize P gene, increased the levels of GUS activity in floral tissues (66). In transgenic tobacco, 3' deletion of the leader sequence up

to 17bp of the transcription start of an *A. thaliana* single housekeeping gene (ENR-A) greatly impaired GUS activity suggesting that the deleted sequence either functions as an enhancer for transcription initiation or stabilizes the mRNA (67). In transgenic wheat plants, the level of beta-glucuronidase activity declines due to the deletion of the promoter of granule-bound starch synthase 1 gene (gbss1) to -1.9kb or to -1.0 kb (68). It suggests that enhancer elements and cis-acting elements are involved in gbss1 transcription during the grain filling process.

The introns and the 3' non-coding region in the promoter also influence the transgene expression. Chaubet-Gigot et al. (69) demonstrated that the introns located within the 5'-untranslated regions (5'-UTR) of two *Arabidopsis* replacement H3 gene, functionally combined with their endogenous promoters, could produce the high and constitutive expression of the replacement H3 genes in planta. These introns strongly increase gene expression independent of the promoter used. The quantitative extent of reporter gene enhancement in different parts of developing transgenic plantlets ranges from 2-fold to 70-fold. Ali and Taylor (70) demonstrated that the 3' non-coding region of the Mel gene of the dicot Flaveria bidentis increased the expression of the *gusA* reporter gene several-fold in leaves of both transgenic C4 Flaveria plant and C3 tobacco in combination with either the constitutive S4 promoter from subterranean clover stunt virus or a highly expressed, leaf-specific promoter, the lightharvesting chlorophyll a/b-binding protein gene 3 (Lhcb 3) promoter of *A. thaliana*.

2.6. The effect of MARs

Matrix attachment regions (MARs) are defined as DNA sequences that mediate binding of chromatin to the nuclear matrix, a network of proteinaceous fibrils that permeates the nucleus and presumably functions to organize chromatin into a series of topologically isolated loop domains (71). The MARs from different origins including soybean, human, yeast, chicken, bean, tobacco and *Arabidopsis* have been well studied by different laboratories (71). Most of the documented data indicated that MARs enhance transgene expression to various extents in different plant species (71,72). Flanking the chicken lysozyme A element at the borders of the T-DNA significantly reduced inter-transformant variation and position independent expression of the GUS reporter gene in transformed tobacco plants (73-75). Recently, the same group presented the first direct experimental evidence that transgenes within the same chromatin domain exhibit coordinated regulation (76). Two reporter genes, the *E. coli* β-glucuronidase gene and the firefly luciferase gene, were placed between the copies of the chicken lysozyme A element, a member of MAR of chromatin boundary elements, and introduced into tobacco. Comparing the mean GUS activity values suggested that the gene present in the middle of the MAR-delimited loop tends to have a higher activity than when it is closer to the A element. For the *luc* gene, this trend is opposite. The MAR-containing population showed 4.3- and

3.5-fold reduction of variance in GUS activity compared to the control population. In contrast, no significant differences in variation were observed for the LUC activity data compared to the control population. In the control population, there is no apparent correlation between the activities of both genes (R = 0.24), whereas the relationship between both activities is markedly increased in MAR-containing populations (R = 0.83, R = 0.81 respectively). For the MAR containing populations, plants with high GUS activity also show high LUC activity and *vice versa*.

2.7. Homology-dependent gene silencing

Homology-dependent gene silencing (HDGS) is a form of epigenetic modification that results from interaction between transgene and host plant genes of similar sequence (32,77). According to nuclear run-on transcription experiments, HDGS can be classified into two categories: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (29). Gene silencing phenomena were thought to contribute to the overall transgene expression variability in higher plants (3,77-83). Models of the mechanism for the elucidation of transgene silencing phenomena have appeared in many recent reviews (17,32,33,48,55,84). Analyses of transgene silencing phenomena in plants and other organisms have revealed the existence of epigenetic silencing mechanisms that are based on recognition of nucleic acid sequence homology at either the DNA or RNA level (84). These silencing mechanisms can be regarded as host defense strategies to foreign or invasive nucleic acids which are ideally suited for countering natural parasitic sequences such as transposable elements and viruses. Common triggers of HDGS include IRs and double-stranded RNA, a versatile silencing molecule that can induce both degradation of homologous RNA in the cytoplasm and methylation of homologous DNA sequences in the nucleus. IRs might be frequently associated with silencing because they can potentially interact in *cis* and in *trans* to trigger DNA methylation *via* homologous DNA pairing, or they can be transcribed to produce double-stranded RNA. Additionally, the DNA methylation, hetrochromatinization and chromatin component play important roles in the transgene silencing phenomena. The details are referred to in the original articles noted above.

3. Factors influencing transgene expression – recipient dependent factors

3.1. Position effect

The inter-transformant variability often referred to as being caused by "the position effect" (Fig. 3). This is based on the assumption that the expression levels of

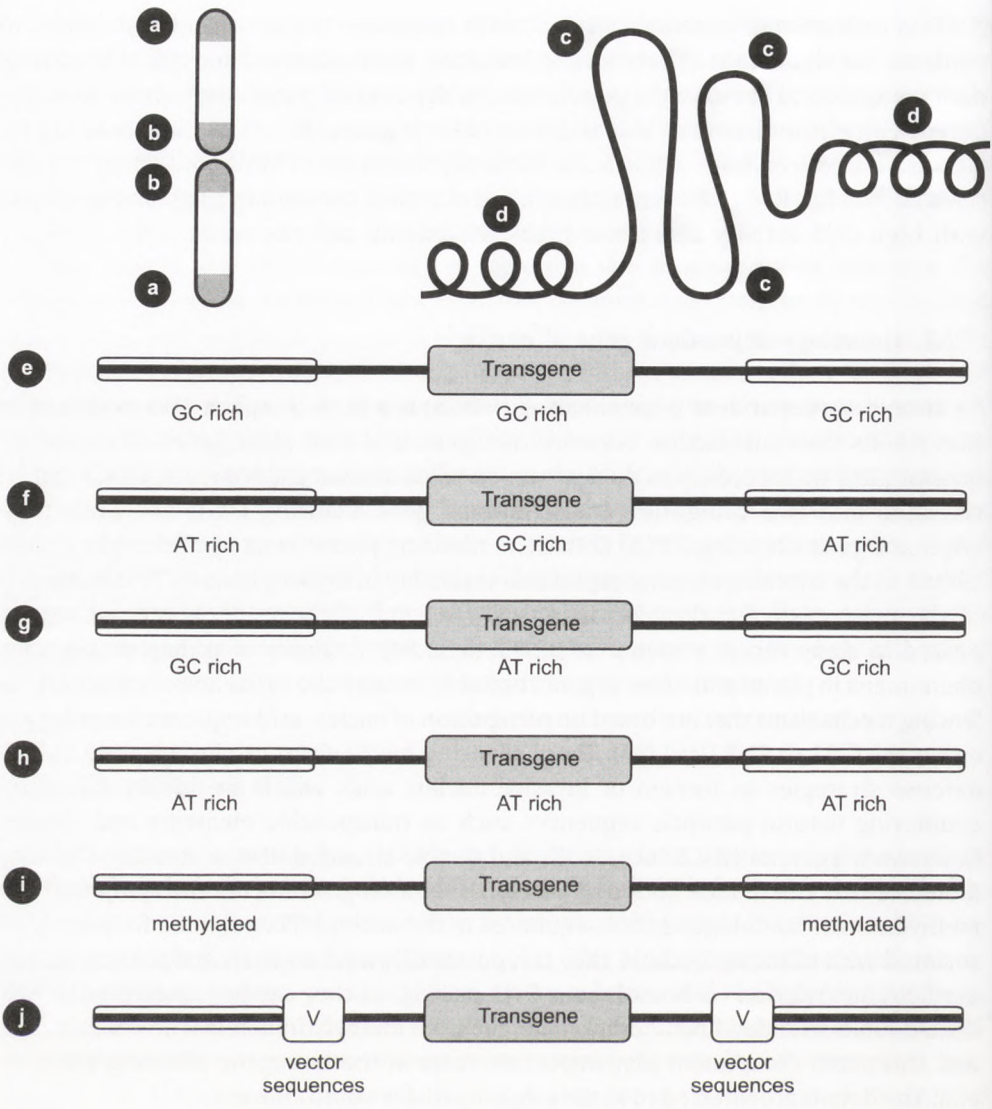

Fig. 3. Position effect and transgene expression. The stable expressed transgene locus usually presented at telomeres (a), euchromatin region (c), isochore compatible region (e and h). The unstable expressed locus occupied intercalary and paracentromeric sites (b), heterochromatin region (d), isochore incompatible region (f and g), methylated region (i) or integrated together with non-T-DNA (vector) sequence (j). Thick bars in e to j represent the flanking plant DNA of the transgenic host. This figure is adapted and modified from 39, 53, 59, 62, 89, 97, 104 and 107.

the introduced genes are directly influenced by the host DNA sequences or chromosomal structure/composition at or near to the site of integration (2).

3.1.1. Chromosomal location

In eukaryotes, genes were thought to be organized in chromatin loops that form an independently regulated functional unit (85-88). An inserted transgene would become a part of a particular domain in which it would integrate and its expression would be influenced by the overall functional properties of that domain (89). Both the local chromatin conformation (90) and the high order chromatin organization are thought to influence transgene expression (89). Heterochromatin differs from euchromatin in both cytological appearance and sequence organization (91). When an euchromatic endogenous gene or a transgene is moved near a heterochromatin region, it shows mosaic expression or position effect variegation (PEV) (92-94,80,95). Either reduction in transgene expression or variable expression pattern is mediated by heterochromatin complex spreading into adjacent chromosomal area (96) which is reminiscent of PEV. Similarly, when transgene is integrated into hypermethylated chromosomal region, methylated pattern can spread into transgene region, inactivating transgene transcription (97). Furthermore, T-DNA might be preferentially integrated close to telomeres (98,99), which usually contain high concentrations of genes in some plant species such as wheat (100) and maize (101). In transgenic tobacco, the stable active inserts were found adjacent to telomeres, the unstable expressed loci occupied intercalary and paracentromeric sites, remote from the gene--rich domains close to telomeres (62).

3.1.2. Isochore compatibility

The nuclear genomes of angiosperm are mosaics of compositionally homogeneous DNA segments which contain defined GC contents of functional gene and their chromosomal environment called isochores (102,103). The transgene with a different AT content relative to that of flanking recipient DNA may be inactivated or methylated as a result of the internal mechanism response for similarity of this region. Silencing was observed when a transgene derived from a monocotyledonous plant was introduced into a dicotyledonous plant, but it was not observed with the corresponding dicot gene (39). The maize *A1* gene (gene that encodes dihydroflavonol reductase), which is inactivated in some of derivatives of the transgenic petunia line R101-17, differs as far as its base composition (47.5% AT) goes from the highly AT rich recipient DNA flanking both 5' (74% AT) and 3' (77% AT) of the integration site (104).

3.1.3. Flanking recipient DNA

T-DNA often integrates element adjacent to enhancer, endogenous matrix attachment region, AT rich domains, highly repetitive regions and retrotranspons or their

degenerate remains (62,97,105,106). The studies on transgenic *Arabidopsis* suggest that transgene integration by particle bombardment tends to occur in AT rich regions carrying S/MAR motifs and/or near regions that have the potential for curvature rather than at random regions in the genome (107). AT rich regions in plant seem to be hot spots for transgene integration (107-109). The T-DNA in transgenic tobacco (108) and the junction region found in transgenic rice by the calcium phosphate method (110) suggest that AT-rich sequences are preferred target site for integration. In transgenic tobacco, the stably expressed inserts were flanked on the left by AT-rich regions which behave as nuclear matrix attachment region *in vitro*, whereas the unstably expressed locus was present, adjacent to an $(AAT)_{12}$ microsatellite which might have resulted from the presence of a second microsatellite sequence $(AAAG)_5$ (62).

3.2. Ploidy level

Early findings suggest a strong link between ploidy level and transgene expression. In transgenic tobacco (44), homozygous double haploid plants expressed β-glucuronidase (GUS) at 2.9-fold the level of the corresponding parental haploid plants. This reflects the influence of increase in chromosome number on transgene expression, suggesting a ploidy dosage effect. It was reported that changing in ploidy resulted in the differences in gene expression pattern in *Arabidopsis* and polyploidy resulted in higher levels of transgene expression in tobacco (111). In transgenic potato, silencing of the endogenous granule-bound starch synthase I (GBSSI) by antisense construct is more efficient in diploid than in tetraploid genotypes (47). A previous study on transgenic potato showed the similar results that 79% of 58 transgenic clones derived from diploid genotype showed inhibition, while only 50% of 66 transformants from the tetraploid genotype showed silencing (112, 113). It was proposed that the expression levels play a role in silencing of GBSSI by antisense transgenes (47). When one T-DNA integrates in a diploid potato, the ratio of transgene to endogenous alleles is 1:2, whereas for tetraploid potato it is 1:4. Thus, the inhibition of GBSSI activity is more efficiently achieved in diploid than in tetraploid potato transformants.

3.3. Homozygosity of the transgene

The presence of allelic copies of the transgene often results in higher levels and higher variability for transgene expression in homozygous *versus* the corresponding hemizygous plants. Mlynárová et al. (75) demonstrated that the GUS activity of the homozygous plants was approximately twice as that of the corresponding hemizygous plants, showing simple additivity of GUS expression. The additive gene activities indicated that the two allelic copies experienced the same (micro) nuclear environment.

On the other hand, the homozygous populations had a two-fold higher variation in GUS activity than the corresponding hemizygous population had, which indicates that the transgene alleles are not only additive, but also act as fully independent from each other. The allelic copies of the transgene behave differently from the ectopic ones where the GUS activity is similar for the transformants irrespective of gene copies integrated (74,75). Beaujean et al. (44) observed that homozygous double haploid transgenic tobacco lines display a 50% increase in GUS activity compared to their corresponding diploid heterozygous parents. This may have resulted from the presence of two GUS inserts, one on each of the homologous chromosomes. In other diploid plant species transformed with sense or antisense GBSSI construct, homozygous plants showed strong or complete inhibition of gene expression, whereas hemizygous plants showed no or low level of inhibition (114,115). However, in transgenic white clover plants, no difference was found between the level of β-glucuronidase gene (*uidA*) expression for F_2 plants homozygous and heterozygous for the transgene (21). In contrast, other reports suggested that the homozygous transgenic plants tended to be susceptible to gene silencing (116-118). Elmayan and Vaucheret (119) describe gene silencing affecting the entire homozygous progeny carrying a CaMV 35S (Cauliflower Mosaic Virus 35S promoter)-*uidA* transgene.

3.4. Genetic background

In out breeding species such as white clover (21) and heterozygous vegetatively propagated species such as potato (47), the genetic background strongly influences the levels of transgene expression. In white clover considerable genetic variation exists between even closely related individuals, each population is a heterogeneous mixture of heterozygous individuals. Therefore, such different genetic backgrounds lead to the large variation in GUS expression level within the same population. In transgenic potato, different genotypes and different clones show variable degrees of antisense inhibition of the GBSSI gene. Another report indicated that various genotypes of petunia also differ in their ability to silence transgenes. The presence of a petunia *dfrA* gene (gene encoding dihydroflavonol 4-reductase isolated from An6 locus of *Petunia hybrida*) in two different petunia lines, W80 and W85, resulted in a color difference of the flowers (120). Variegated flowers were more often detected in W85 transformants, probably owing to inactivation of the *dfrA* gene in this line.

3.5. Developmental stage

Transgene expression levels also varied depending on the developmental stage of the transgenic plant. Leeuwen et al. (19) observed that the *35S-luc* (luciferase gene driven by CaMV 35S promoter) activity decreased in older tobacco leaves in an

orderly manner. In transgenic potato, the accumulation of GNA and BCH was found to increase as the potato plant developed, with maximum levels found in mature plants (12). Developmentally regulated transgene silencing has also been documented for the GUS gene (38,119) and the capsid nucleoprotein of tomato spotted wilt virus (121). In these cases, silencing was induced during development, but the timing varied between individual transformants. Co-suppression of endogenous S-adenosyl-L-methionine synthetase (122), chitinase (123), nitrate reductase (*Nia*) and nitrite reductase (*Nii*) genes (124) was also developmentally regulated.

3.6. Environmental factors

Environmental factors such as high light intensity, high temperature, *in vitro* tissue culture procedure, seedling transplantation and field growth conditions have been shown to modulate transgene expression. When silencing is modulated by environmental factors, the transgene has been integrated into genomic regions that experience epigenetic alterations during stress treatment, such as changes in methylation pattern and/or chromatin conformation (125-128). Krol et al. (11) reported that the variable pigmentation within a single petunia plant carrying an antisense chalcone synthase (CHS) gene is caused by variable physiological conditions, such as internal hormone concentration and external light intensity during flower development. Spraying with gibberellic acid (GA) results in an increase in the pigmented sector area, while spraying with B9 (a growth retardant inhibits the endogenous GA synthesis) resulted in a decrease. High light conditions at an early stage of flower development resulted in large white sectors, while low light intensity at that stage resulted in large pigmented sectors. High light intensity and temperature also induce the silencing of the maize *A1* gene in petunia in the field (4,128). In another plant species, potato, non-uniform conditions and high daytime temperature within the glasshouse resulted in an increased variability in GNA (snowdrop lectin *Galanthus nivalis agglutinin*) and BCH (bean chitinase) accumulation and decreased the overall expression levels (12). In *A. thaliana*, high temperature influences the frequency of silencing positively or negatively (43). The report from Brandle et al. (118) suggested that seedling transplantation could trigger co-suppression of the *csr1-1* (the mutant *A. thaliana* acetohydroxyacid synthase gene) transgene and the endogenous tobacco AHAS (acetohydroxyacid synthase) genes. The young tobacco plants are transplanted into the field following initial germination and growth in the greenhouse. Experiments in controlled environmental chambers were conducted to determine if transplantation had any effect on herbicide susceptibility. 18 of 32 plants gently transplanted from plastic cells into large pots were sensitive to chlorsulfuron. Where the roots were severely pruned prior to transfer, 9 of 32 plants were sensitive. The untransplanted controls showed no sign of herbicide damage. Gatehouse et al. (129) also observed that the levels of GNA declined

during serial propagation in tissue culture. It was suggested that the low expression level in young plants could be attributed to transgene inactivation as the result of environmental stress imposed during tissue culture (130).

4. Explanations proposed for the variability of transgene expression within the same line, plant, tissue or cell

Unlike the inter-transformant variability, the intra-transformant variability is often thought be dependend on the stochastic event or random influence resulting from physiological or biochemical micro-heterogeneity, environmental effects or mutations upon integration rather than position of integration or transgene copy number.

Krol et al. (11) observed variable pigmentation within a single transgenic petunia plant carrying an antisense CHS gene. The author concluded that the changes in the expression of the antisense transgene relative to the expression of the endogenous CHS genes resulted in the extremely sensitive response to physiological changes in these plants. This extreme sensitivity of the plants may account for the variable degree of floral pigmentation. The authors suggest that this sensitivity is caused by DNA sequences flanking the site of integration of the antisense CHS gene construct in these transformants. On the other hand, the changes in the physiological constitution during plant development which may, in turn, influence relative antisense to sense CHS gene expression in this transformant, may also account for the belated phenotypic effect of flower pigmentation. Furthermore, the effects on flower pigmentation in antisense CHS transgenic plants are easily scored by eyes and reveal great variability in the expression of a transgene. One should be aware that a transgene not allowing for such visual scoring might have a similarly variable expression in different parts of the same organ within a plant.

Peach and Velten (2) suggested that the observed intra-clonal variability is more likely to reflect the physiological or biochemical micro-heterogeneity within the callus lines. The localized differences in transcription, mRNA stability, translation, protein stability or overall cellular protein concentration within the callus also contribute to this intra-clonal variability (since the enzyme activities were normalized to total soluble protein within a common extract).

Maqbool and Christou (16) proposed that the variation in the expression levels of δ-endotoxins (Cry1Ac, Cry2A) and GNA proteins in the same lines is caused by a considerable random influence on transgene expression resulting from such effects as mutation upon integration, orientation of adjacent transgenes and promoter occlusion.

Environmental effects, in particular variations in temperature, are thought to introduce a degree of variability in the levels of accumulation of transgene products (GNA and BCH) between clonal replicates within a given line of transgenic potato

(12). Chimeric plants, possessing regions of transformed and untransformed tissues, can arise during transformation procedures, notably when using *Agrobacterium tumefaciens* or if somatic hybridisation occurs. However, this is unlikely to have occurred in all lines tested. Alternatively, these variations between clonal replicates may be a consequence of the variation in the methylation states, which is known to increase as the plant ages (4). Higher degrees of methylation may also render transgene expression more receptive to environmental stimuli (4).

Meza and co-workers (43) suggested that the variation in the frequency of silencing between progeny of siblings derived from single-copy *Arabidopsis* lines is not dependent on locus or copy number. The authors assume that the epigenetic changes leading to *npt* (gene encodes neomycin phosphotransferase) silencing in seedlings are stochastic events taking place in cells of the sibling plants, and that these changes are transmitted to daughter cells generated by mitosis and later meiosis (131). Silencing is thereby transmitted to embryos resulting from self-pollination. The fraction of siblings displaying silencing and the frequency of silencing in progeny from each sibling are likely to be dependent on the position and the number of cells in which the silencing event occurs. Other reports indicated that the variable expression of the neomycin phosphotransferase II gene within lines is believed to be due to constraints on transcription imposed by structural features in the recipient plant DNA or the T-DNA itself. These features include chromatin configuration and/or DNA methylation (57,80,132).

In summary, for a particular transgenic system the expression levels and variability of the integrated transgene are considered to be a consequence resulting from a combination of both the transgene dependent and the recipient dependent factors discussed above. On the other hand, the same factor may function differently in various transgenic systems carrying the same or different transgene expression units.

5. Approaches to stabilize transgene expression

Of great importance for the application of transgenic plants is the foreseeable and stable expression of the integrated transgenes. Multiple factors have shown to control the transgene expression, including some factors beyond the recognition for the practical use. It seems that the complexity of the problem will not hinder the use of the transgenic plants in agriculture. Different approaches towards stabilizing transgene expression have been suggested in many previous works (3,4,27,82, 128,133). These approaches may be classified into several groups and are briefly summarized below.

The first group contains many aspects of transgene quality. As noted earlier, most transgene silencing mechanism are involved in normal endogenous gene regulation processes and host defense systems which protect themselves from foreign

or invasive nucleic acid (27,84). The foreign genetic information could be detected by such surveillance processes as alien and is likely to be functionally inactivated or eliminated (27). Therefore, to create small sequence differences between transgene construct and endogenous recipient genes or within the same transgene construct may be very important in avoiding the detection by such genome surveillance. The transgene should contain base substitutions, so that they contain little or no sequence similarity to putative endogenous sequences, or to similar sequences in the same construct. Introns may also be used to create sequence diversity. The elimination of repeated elements from transgene constructs should alleviate problems with DNA-DNA pairing and *de novo* methylation (3). Multiple transgene constructs should be driven by different promoters and polyadenylation sequences and not be linked to the same selectable marker (82,134). Construct should carry appropriate 5'-leader sequences and polyadenylation regions, as both elements can influence the efficiency of transgene expression (135,136). Because the 35S promoter was frequently associated with silencing effects, it might be wise to avoid using it altogether (3). Since plasmid or phage vector sequence may also be regarded as alien to the recipient genome and serve as targeting elements for surveillance, these sequences should be eliminated from the transgene construct (27). The addition of transcriptional terminators on either side of the transgene construct may prevent transcriptional read through from promoters present in the flanking recipient genomic region, thereby preventing collision of transcription complexes (27). Constructs should not be located adjacent to each other on the vector, and should all be read in the same direction to avoid the formation of aberrant read through transcripts or antisense RNA. In addition, moderate transcription rates might help to avoid RNA turnover induced by excess RNA production (134).

Secondly, other approaches attempt to reduce the negative position effect on transgene expression. The inclusion of some specific elements, such as MARs, enhancers or CpG sequences, to the transgene construct may help to stabilize the transgene expression. Flanking transgene inserts with MARs, derived from either animals or plants, have been shown to increase levels of transgene expression and decrease expression variability in different species (71). Genes are attached to the proteinaceous nuclear matrix at the locations known as MARs, forming the boundaries of DNA loops and insulating genes encoded within these loops from the influence of surrounding chromatin (88). Thus, flanking a transgene with MAR elements may create a discrete transcriptional domain and overcome the potential problem of foreign DNA recognition by virtue of its base composition, therefore insulate the transgene from the potentially deleterious effects of the chromatin surrounding its site of integration. On the other hand, the use of enhancers from tissue specific or developmentally regulated genes may ensure that the expression of a linked transgene occurs in the appropriately regulated manner. The identification and utilization of similar sequences from plant genes might help to obviate those naturally occurring processes that inactivate gene expression in a temporal- or spatial-specific

fashion (82). Positioning the transgene on extrachromosomally replicating vectors and/or transferring the recombinant genes with suitable chromosomal flanking regions also protect the transgene from negative position effects (4). The AT content of the transgenes should match the isochore composition of the host genome as closely as possible. The codon usage should be optimized for most abundant tRNAs of the host plant (134). Flanking each end of the gene construct with CpG sequences may establish a GC rich isochore-like environment (27). Since the integration region has an important effect on the methylation pattern and expression of the integrated transgene, the methylation state of the integration region or of its repetitiveness should therefore be examined (4). The integration close to the hypermethylated or repetitive genomic regions should be avoided. The protection of the transgene against neighbouring genomic regions may be achieved by transferring them into large stretches of 5' and 3' regions of endogenous genes that contain matrix-attachment sites and other sequences that favor the formation of chromatin-loop domains (17). The integration of T-DNA into chromosomal loci that are potentially transcribed may reduce the probability for a negative influence of the integration. Certain transgenes that are free from inactivation influences of the integration region can be selected, e.g. stable *rolA* (root locus A from *Agrobacterium rhizogenes*) expression in *Arabidopsis* (137). Additionally, site-directed targeting system allows targeting transgene into chromosomal region that provide an optimal sequence environment for stable expression. Targeting transgenes into compatible isochores might dampen the "foreign DNA response" (134). Different site-directed targeting systems have been established in plants (138).

Thirdly, an appropriate transformation method should be employed in order to produce transformants with simple integration event. A desirable characteristic for any transgenic plant is single copy transgene integration (4). However, different transformation procedures may generate transformants with rearranged, multiple copy T-DNA inserts, which may often lead to a later inactivation of the transgene. Considering the possible expression-instability generated by multiple copies, any method aiming at an enhancement of transformation frequencies should also be evaluated for the conservation of a high proportion of single copy integration events (4). Extending *Agrobacterium*-mediated transformation technologies to monocotyledonous plants may reduce the copy number of the introduced gene (134). Stress mediated induction of hypermethylation should also be monitored in tissue culture by culturing on propionic acid in order to decrease the number of transformants that lose transgene expression (134).

Furthermore, stable expressed transgenes can be selected from groups of transformants, or stabilized in breeding programs, though it may be time consuming and expensive (134,139). Transgene inactivation is frequently associated with the integration of multiple copies of the introduced DNA. In order to avoid unintended co-suppression effects, simple integration events should be selected and examined in more detail for major rearrangements and short target site duplications (134). For

Agrobacterium mediated transformation, on the average only one in ten transformants will contain a single integrated T-DNA copy (4). Moreover, single copy transgenic lines can be generated from multi-copy parental lines. For example, in wheat, a transgene may be flanked by *lox* recombination sites in an inverted orientation: by crossing lines transgenic for this construct with a line expressing the CRE recombinase, the progeny carrying a single-copy insert can be obtained (133). Transgenic tobacco lines harbouring a single copy of a *gus* gene were produced by using a similar system, *Cre-lox* mediated site-specific recombination (18).

Finally, the stability of expression should be tested in plants that are homozygous for the transgene (134). It should be suggested to use homozygous plants other than hemizygous, since the former give higher and more predictable expression. Other practical indices include the care of the transformant under appropriate environmental conditions in order to avoid stress induced transgene silencing and expression variability.

However, there exist some other factors not to be controlled to increase the stability of the transgene expression so far, owing to the limited knowledge regarding the nature of its mechanism. For instance, how to omit the transfer of binary vector sequence is still a problem to be resolved. The exclusion of the transgene aberration after the integration could be achieved by large screening of the transformant, but exclusion of the transgene aberration prior to or during integration remains to be unresolved.

6. Conclusions

The variability in the transgene expression levels in plants is a common phenomenon rather than exception. This variability could be detected between and/or within the lines, a single plant, organ or tissues. The variability of transgene expression levels in plants was attributed to the influence resulting from both the transgene dependent and the recipient dependent factors, the transgene dependent factors of which strongly affect the transgene expression level. The T-DNA copy number could be positively or negatively correlated with the levels of transgene expression. The configuration of the transgene within the T-DNA could affect the overall pattern of its activity. The complex T-DNA integration patterns such as IRs often show low expression but with exceptions. Most of the documented data indicated that MARs enhance transgene expression. The position effect referred to as the expression levels of the introduced genes are directly influenced by the host DNA sequences or chromosomal structure/composition at or near the site of integration. The expression of an inserted transgene would be influenced by the overall functional properties of the domain it integrated. When a transgene is moved near a heterochromatin region or a hypermethylated chromosomal region, it shows a reduction or a variable expression pattern and in some cases an inactivation of the transgene transcription.

The transgene with a different AT content relative to that of flanking plant DNA may be inactivated or methylated. When the transgene integrated into the hot spots for transgene integration such as AT rich regions in plants, its expression is relatively stable. In some cases, the binary vector sequences were transferred into the plant genome, which led to the unstable expression of the transgene. The recipient dependent factors involve position effect, ploidy level, genetical background, homozygosity and developmental stage. The expression of an inserted transgene is strongly influenced by the functional properties of the chromosomal domain it integrated. When the transgene was integrated into a hypermethylated chromosomal region or was moved near a heterochromatin region, it showed either a reduction or a variable expression pattern. On the other hand, when the transgene is integrated into the chromosomal region containing high concentration of genes, such as the telomeres, it shows active and stable expression. Similarly, the transgene that is integrated into the isochore compatible region or the hot spot such as AT rich region, it is also steadily expressed. Generally, polyploidy resulted in higher levels of transgene expression in some plant species, for instance, tobacco and potato. Homozygous plants often give higher levels and higher variability for transgene expression *versus* the corresponding hemizygous plants. In out breeding species or heterozygous vegetatively propagated species, genetical background strongly influences the levels of transgene expression. Unlike the inter-transformant variability, the intra--transformant variability is often thought to be dependent on the stochastic event or random influence resulting from physiological or biochemical micro-heterogeneity, environmental effects or mutation upon integration. Furthermore, transgene expression levels also varied depending on the developmental stage of the transgenic plant. Stress conditions, such as high light intensity, high temperature, tissue culture procedure, seedling transplantation and field growing condition, usually result in increased variability and decreased overall expression level of the transgene.

Despite the common existance of variability of the transgene expression level in different plant species, it does seem not to hinder the use of transgenic plant in agriculture. The stable expression of a transgene could be achieved by the precise construction of the transgene, using specific elements to reduce the negative position effect and the appropriate transformation method to obtain the simple integration transformation event, selection of the stable expressed transgene from group of transformants and their progenies or stablization of the transgenes in the breeding programs. However, there also exist many factors remaining to be recognized for the practical use, such as how to omit the transfer of binary vector sequence or how to exclude the transgene aberration prior or during integration. Nevertheless, the possible approaches summarized here will help to stabilize the transgene expression in plants to various extents.

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