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The influence of T-DNA copy numbers on gene expression in primary tranformants *Atropa belladonna* plants

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Abstract

An actual major problem with plant transformation technology is the non-predictability of the integration and expression of transgenes in the host genome. One complication arises due to differences in the copy number of transgenes present in independent transgenic plants. Analyses of transgenes in large number of plant species have revealed that independent transgenics carry one to several insertions of transgenes. This paper is focused on molecular characterization of primary transformants Atropa belladonna plants obtained by Agrobacterium tumefaciens LBA4404 (pBINplus) mediated transformation in order to determinate the number of T-DNA inserts and the way the integrated copies are arranged (single copy, repeats). For this purpose Southern analysis with nptII and gus probes has been used. Also, we compared the T-DNA integration pattern with the corresponding gene expression.

Keywords: T-DNA copy number, gene silencing, medicinal plants, genetic transformation, *Agrobacterium,* GUS expression

Introduction

Plant genetic transformation technology has become important experimental tool for investigation into various aspects of plant biology such as physiology, genetics, developmental biology, molecular biology, etc. The success in this domain represents the culmination of many years of efforts in tissue culture and plant genetic engineering techniques improvement. The introduction of genes into plants via *Agrobacterium-mediated transformation* offers great opportunities to various pharmaceutical and agricultural fields [1].

After transformation foreign DNA is randomly integrated in the plant genome and the transgenic loci frequently harbor multiple copies of the integrated DNA [26, 27]. It is commonly generalized that transgenic plants obtained by direct DNA-transfer methods (e.g. biolistics and electroporation) contain a large number of transgene copies (reportedly even up to 100 copies) whereas *Agrobacterium*-mediated transformation leads to the insertion of fewer transgene copies (<10) and a higher occurrence of single-copy integrants [2, 12, 17, 10, 21].

This multiple copies may change their interaction with the host genome in specific regions and their organization, which may result in truncation, inversion, deletion and other chromosomal rearrangements and also, apparently minor changes such as those linked with DNA methylation. Moreover, these multiple copy insertions are often characterized by complex integration patterns due to sequence homology, including tandem repeat (TR) or inverted repeat (IR) arrays [15, 13, 22, 16, 7, 8, 23, 19, 28].

Also, several studies suggested an inverse relation between transgene copy number and transgene expression level [18]. Theoretically, we expect that the increase in copy number would conduct to an increase in transgene expression, as more copies of the DNA yield more

transcripts. This are sometimes the cases of the posttranscriptional control of gene expression reported by Gendloff et al., (1999) who demonstrated in their run on transcript studies, the direct relationship between the RNA concentration and gene expression, up to the RNA turnover value. However, the multiple copy integration patterns such as TR [22, 16, 28] and IR structures [13, 8, 23, 19] are often associated with low-level transgene expression due to the transcriptional level of gene silencing.

The interaction of transgene with the host gene sequences at the post transcriptional level, could be an important factor leading to co-suppression phenomena observed with the obtaining the GMOs resistant to certain viruses. Also, the literature is abundant with examples of variable expression level of single copy transgene, which are associated with the heterochromatic state of the integration genome sites. The same transcriptional DNA methylation based processes are assumed to be involved in these cases which may be linked with the type of explants used. Based on these conditions, is necessary to generate a large number of independent transgenics to find "true" effects of transgene and to eliminate unrelated, associated effects. Also, it's important to select the plants that contain just one single copy of transgenes, called "elite transgene inserts". Such transgenic plant with a single transgene copy is less prone to the defense mechanism of silencing than plants with multiple copy of transgene. Therefore their segregation pattern is elementary and also the homozygous plants can be easily obtained in these conditions.

This study is concerned on the analysis of primary belladonna transformants (R0) in order to identify the T-DNA inserts pattern (T-DNA copy number) after their transfer into plant genome mediated by *Agrobacterium* in correlation with marker/reporter gene expression. Southern analysis with *nptII* and *gus* probes of the selected primary transformants, which may give us information about T-DNA integration and T-DNA inserts pattern has been correlated with the certain variation of gene expression.

Materials and methods

Obtaining of primary transformant *Atropa belladonna* plants

The techniques for obtaining the *Atropa belladonna* transgenic plants and maintaining as an "in vitro" culture has been performed by an indirect (*Agrobacterium* mediated) method described in a previously published paper [24, 4, 25]. *Atropa belladonna* var. *nigra* plantlets were obtained by in vitro micropropagation of plantlets derived from sterilized seeds, kindly provided by the Cluj Botanical Gardens. The regeneration of putative transformed shoots from the infected tissue has been performed as described earlier [24]. The *Agrobacterium tumefaciens* LBA4404 (pBIN plus) strain, kindly provided by Dr. David Tepfer, INRA, France, has been used. Its binary plasmid vector pBIN plus contained a marker gene, *nptII*, under the control of Pnos promoter and a reporter gene, *gus*, controlled by the P35S promoter.

Characterization of primary transformants (R0)

Molecular characterization of belladonna transgenic plants has been previously done by genotype (DNA level) and phenotype (protein level) [25, 4] analysis.

Determination of NPTII activity (SDS-PAGE assays)

The expression of the *nptII* marker gene as its corresponding NPT II polypeptide has been estimated by the detection of the native 59 kDa dimmer into the total protein extract obtained from the regenerated shoots. Usually this method is applied for different tissue types (shoots, calli, roots or tubers) in order to detect certain monomerisation processes, which may have important effects upon the enzyme activity [25, 4].

Determination of GUS activity y (fluorimetric assays)

In previous studies [25] we have investigated the GUS activity in some belladonna primary transformants. This analysis was based on the ability of β-glucuronidase (GUS) to cleave the substrate 4-methylumbelipheril-β-glucuronidase (4-MUG). Two or three weeks after regeneration plantlets from calli, leaves were harvested and ground in 100μl buffer containing 50mM $Na_2H_2PO_4$, 10mM EDTA, 0,1% Triton-X, 0,1% SDS, 10mM β-mercaptoethanol. The mixture was centrifuged twice at 15,000xg at 4^0C to remove the insoluble material. 1mM MUG in 0,2M Na bicarbonate was added to mixture and the final solution was incubated at 37^0C for 120min. The visualization has been performed directly in tube on transilluminator at 365nm [14]. Also, the GUS activity was express as a U of GUS/mgl^{-1} protein.

Determination of T-DNA pattern by Southern blot analysis

Southern blot analysis was performed to establish the copy number and integration patterns of the T-DNAs inserted in the genome of the primary transgenic plants.

Leaf material of R0 transformants was collected and used for DNA extraction. Total DNA was isolated from 6 transgenic *belladonna* plants (A.b.3.2; A.b.4.7; A.b.9.4; A.b.3.1; A.b.4.9; A.b.10.1) according to method described by Doyle and Doyle, 1990 [9]. Subsequently, 5 μg total DNA was digested with 3U *Eco*RV enzyme subjected to electrophoresis and blotted on a Hybond-N membrane (Roche Diagnostics, Basel, Switzerland). Probe DNA was labeled with fluorescein-dUTP using a random-primed labeling kit (Gene IMAGE Random Prime Labeling Module) and *Taq* DNA polymerase [6]. Prehybridization was carried out at 68°C (1h). After overnight hybridization at 68°C the blots were washed twice at 68°C with two washing solution-containing 25xSSC, 20% SDS and nanopure H_2O. The filters were after words incubated at 22°C in the provided kit blocking solution. A conjugate solution with anti-fluorescein (AP) (Amersham Pharmacia Biotech) was used for detection. Total DNA of non-transgenic belladonna plants was included as a negative control. Restriction enzymes and probes were designed to enable identification of specific T-DNA integration patterns (Figure 2).

Results and discussions

In this study we describe a widely used strategy for molecular characterization and selection of the “elite transgenic insert” based on the level of marker *nptII* and *gus* gene expression and also on the Southern blotting determination on the T-DNA copy number.

Protein assays: NPTII and GUS

A previous paper reported the catalytic activity of the NPTII by both “in vitro” (i.e., its detoxifying ability of a kanamycin containing medium) and molecular (fractionation by electrophoresis) methods (data not show). These results were performed in order to elucidate eventual posttranslational modification of the protein folding which might have had dramatic effects upon its catalytic activity. Both the “in vitro” and the molecular approaches were used for a correct analysis in order to prove a high transformation efficiency [3, 4].

In the other hand, when considering the GUS expression, we observed an inter transformants variation of transgene expression (data not show), within plants transformed with the same transgene construct and the same condition [3]. In present paper, we quantified the GUS activity of 25 primary belladonna transformants and approximately we observed the following pattern of gene expression: 50% (13 out of 25) from these lines exhibited a very week GUS activity (<500 U GUS/mgl^{-1} protein), 16,66% (4 out of 25) demonstrating

moderate activity (>501-2500U GUS/mgl^{-1} protein) and 33,33% (8 out of 25) exhibit higher GUS activity (>2500U GUS/mgl^{-1} protein) (figure 1.).

We suggest that this various gene expression depend on T-DNA copy numbers, which is subsequently analyzed by Southern assays.

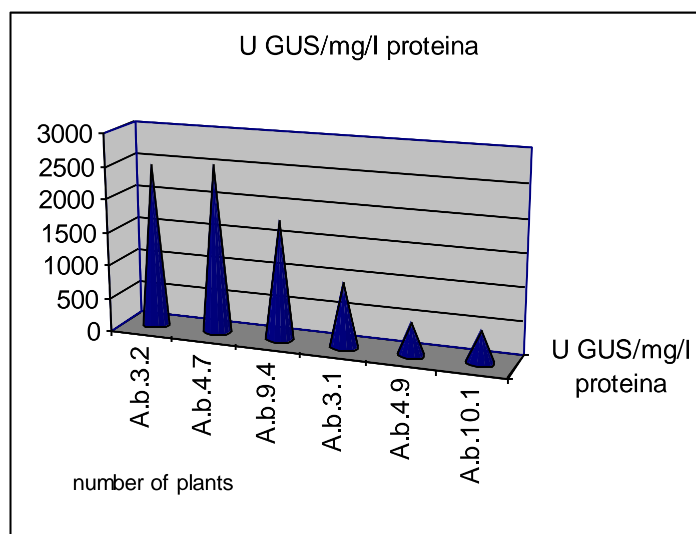

Figure 1. Distribution of GUS expression between selected belladonna primary transformants. Abbreviation: A.b.3.2 A.b.4.7-high GUS expression; A.b.9.4 A.b.3.1-moderate GUS expression; A.b.4.9 A.b.10.1-weak GUS expression

Southern assays

Southern blot assay has been done for determination of the copy T-DNA inserts in belladonna genome and the interpretation of the results is given in Table 1. Any specific band has been verified with non-transformant genomic DNA restricted with the same EcoRV enzyme and hybridized with the same probes.

Proper interpretation of the Southern blots proved difficult due to the high copy number and complexity of the T-DNA integration patterns. As such the results given in Table 1 reflect the probable T-DNA copy number and integration patterns. Southern blot analysis of the A.b.3.2 and A.b.4.7 transformants revealed single copy integration patterns of the T-DNA, which explain the corresponding high GUS gene expression (figure 1). Southern blot analysis of the A.b.9.4, A.b.3.1 and A.b.10.1 transformants revealed multiple copy inserts (figure 2). In similar cases, the literature suggests some numerous factors implicated in gene expression variation, such as the heterochromatic nature of the integration in genome region, the arrangement (TR/IR) of the copies. It is assumed also in many cases that vector DNA was integrated too [6].

Table 1.

Samples (DNA from transgenic belladonna leaves)	**Southern blot Pattern *Gus* probe**	**Southern blot Pattern *nptII* probe**
A.b.3.2	1 fragment: Single copy	1 fragment: Single copy
A.b.4.7	1 fragment: Single copy	1 fragment: Single copy
A.b.9.4	4 fragments: Complex: multiple copy	2 fragment: multiple copy
A.b.3.1	>4 fragments: Complex: multiple copy	2 fragments: Complex: multiple copy
A.b.4.9	>4 fragments: Complex: multiple copy	>4 fragments: Complex: multiple copy
A.b.10.1	>4 fragments: Complex: multiple copy	>4 fragments: Complex: multiple copy

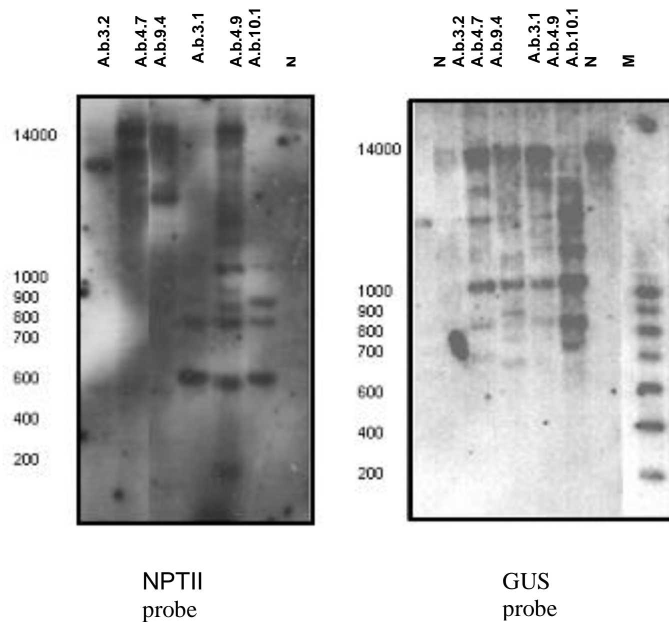

Figure 2. Southern analysis of primary belladonna transformants using *nptII* and *gus* probes

Conclusion

In this study we investigated the impact of the T-DNA copy number on variability of transgene expression in belladonna primary transformants by correlating the results of both protein and DNA (Southern) assays. Our results showed that a simple strategy for transformation *A.belladonna* plants with a simple construct (with marker and reporter genes) using *Agrobacterium* as gene transfer system had influenced the gene expression and T-DNA integration pattern. The transgene copy numbers may be highly variable depending on the transformation method. A high frequency of increased T-DNA copy number is observed with biolistic methods as compared with the indirect, *Agrobacterium* mediated, ones. Our results showed however a variation of such copy number even through a mediated method, which may suggest the involvement of certain other factors, not yet clarified that control the gene

transformation process. Common to all transformation methods in use is that multiple copies tend to integrate in one or a few insertion sites, probably as a result of extra-chromosomal ligation of the transgenic DNA fragments before integration [5, 6, 20].

This factor determines the explanation of the variation of transgene expression by T-DNA copy number of the integrated transgenes. Therefore, in order to obtain more predictable transgene expression levels, single-copy transformants are often preferred and selected as elite transgenes. It is clear that predictable transgene expression level requires further optimization of transformation strategies. Based of knowledge of the molecular mechanisms of the variation of gene expression in these cases, new technologies are being developed to increase the frequency of single-copy transgene integration. The research activities are focused further on targeting of foreign genes in plant genome into a predetermined chromosomal site achieved by homologous recombination using enzymes from another eukaryote systems (such as bacteria and yeasts) or by induction of double-strand breaks in target DNA.

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