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Transgene elimination in genetically modified dry bean and soybean lines

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ABSTRACT. Transgene elimination is a poorly studied phenomenon in plants. We made genetic and molecular studies of a transgenic dry bean line immune to bean golden mosaic geminivirus and a soybean line. In both lines, the transgenes were stable during the vegetative phase but were eliminated during meiosis. Due to its potential biotechnological value, this transgenic line was micropropagated by grafting and the vegetative copies were studied for more than two years. More than 300 plants of progeny were obtained during this period, demonstrating that the phenomenon of elimination was consistently repeated and offering an opportunity for detailed study of transgene elimination, including the characterization of the integration sites. Cloning and sequencing of the transgenic loci, reciprocal crosses to untransformed plants, genomic DNA blots, and GUS assays were performed in the transgenic lines. Based on the molecular and genetic characterization, possible mechanisms involved in transgene elimination include intrachromosomal recombination, ge-

netic instability resulting from the tissue culture manipulations, and co-elimination of transgenes, triggered by a process of genome defense.

Key words: Transgene locus structure, Gene integration, Biolistics, Transgene elimination

INTRODUCTION

Transgene elimination in plants is a poorly understood phenomenon. Normally, when it occurs in the process of going from the primary transformants to the first offspring generation, the transgenic line dies and no transgenic material is available for posterior studies. To be useful to agriculture, genetically modified plants must transmit and express the transgenes to progeny in a Mendelian fashion. Transgene elimination is a phenomenon that has strong implications for the fidelity of transgene transmission; however, it has been little studied in transgenic plants, compared to other phenomena, such as gene silencing (Waterhouse and Helliwell, 2003). Transgene elimination of the bar gene that confers resistance to PPT has been reported in wheat. Of six transgenic wheat lines, five were stably transformed. However, one line with five copies of the bar gene lost gene expression in the R1 generation, and the transgenes were physically eliminated in the R3 generation (Srivastava et al., 1996). Complete physical loss of transgenes has also been reported in *Cyamopsis tetragonoloba* (Joersbo et al., 1999), *Nicotiana tabacum* (Risseeuw et al., 1997), *Nicotiana plumbaginifolia* (Cherdshewasart et al., 1993; Risseeuw et al., 1997), and *Arabidopsis thaliana* (Feldmann et al., 1997; Howden et al., 1998). The mechanisms involved in transgene elimination are poorly understood, and it has been attributed to intrachromosomal recombination (Fladung, 1999), genetic instability resulting from the tissue culture conditions (Risseeuw et al., 1997; Joersbo et al., 1999) or a genomic defense process (Srivastava et al., 1996). In all these studies, several transgenic lines were obtained with the same construct, and transgene elimination was observed only in a few transgenic lines, suggesting that the factor that triggers the elimination process involves the integration sites rather than the transgene sequence. Indeed, it has been suggested that the integration loci of the transgenes are a key factor in the process of elimination (Srivastava et al., 1996; Feldmann et al., 1997; Howden et al., 1998; Joersbo et al., 1999).

We studied a transgenic bean line that presented immunity against bean golden mosaic geminivirus, but the transgenes were not transferred to the progeny. We also studied the same phenomenon in a transgenic soybean line obtained by transformation with a different construct.

MATERIAL AND METHODS

Plant materials

The transgenic bean (*Phaseolus vulgaris* L.) line 158 was obtained by particle bom-

bardment with the plasmid pMD4 (Aragão et al., 1998). The plasmid vector contains the gus gene and the rep-trap-ren genes from bean golden mosaic geminivirus, both under control of the 35S CaMV promoter. The soybean line 33-3 was obtained with the plasmid pAG1 that contains gus gene under control of the act2 promoter, as well as the ahas gene under control of its promoter from *Arabidopsis thaliana* (Aragão et al., 2000).

DNA gel blot hybridizations

Genomic DNA was prepared according to Dellaporta et al. (1983). Fifteen micrograms of genomic DNA from primary transformant and offspring plants was digested with *Eco*RI, separated in 0.8% agarose gel and blotted as described (Sambrook et al., 1989). The gus gene was labeled by random priming (Pharmacia) and used as a probe.

PCR analyses

DNA was isolated from leaf disks by the method of Edwards et al. (1991). PCR reactions were carried out according to Aragão et al. (1998). Primary transformant and offspring plants were analyzed for four different regions of pMD4. The primer pair 5'-TTAT CCGCCTCCATCCAGTCTA-3' and 5'-CAACAGCGGTAAGATCCTTGAG-3' was used to amplify a 491-bp sequence within the amp gene. The primer pair 5'-AGGGATTTTGGT CATGAG-3' and 5'-TAGTTAGGCCACCACTTC-3' was used to amplify a 791-bp sequence within the ori region. The primer pair 5'-TTGGGCAGGCCAGCGTATCGT-3' and 5'-ATCA CGCAGTTCAACGCTGAC-3 was used to amplify a 420-bp sequence within the gus gene. The primer pair 5'-AGGTGGTATACTCTGGTCGTT-3' and 5'-GGAGGTCAACAG ACAGCTAAT-3' was used to amplify a 900-bp sequence within the rep-trap-ren gene sequence.

GUS assays

Explants of all the plant tissues were tested for GUS activity using a histochemical assay, as described by Jefferson et al. (1987).

Cloning of the genomic integration sites by plasmid rescue

Five micrograms of the genomic DNA was digested overnight with *Kpn*I, extracted and precipitated (Sambrook et al., 1989). The digested DNA was self-ligated overnight in a volume of 200 µl at 14°C in the presence of 100 U T4-ligase. Self-ligated fragments were then used to transform *E. coli* XL1 Blue by electroporation using a BioRad system, according to the manufacturer's instructions.

Nucleotide sequencing and data analysis

Nucleotide sequences were determined in an automatic sequencer (ABI 377, Perkin Elmer). A BLAST search was performed against GenBank for analysis of the integration sites of the transgenes.

RESULTS AND DISCUSSION

The transgenic bean line contains at least three copies of the transgenes, which are not transferred to its progeny by self-crossing or reciprocal crosses to untransformed plants

DNA gel blots were made with material from primary transformant (R0) and 20 R1 plants produced by self-pollination. These analyses were carried out using the gus gene as a probe, and genomic DNAs were digested with *Eco*RI, which cuts once within the plasmid vector. Three bands were observed in the primary transformant and none in the progeny (Figure 1). Apparently, at least three copies of the plasmid were integrated into the primary transformant, while the offspring did not present the gus gene.

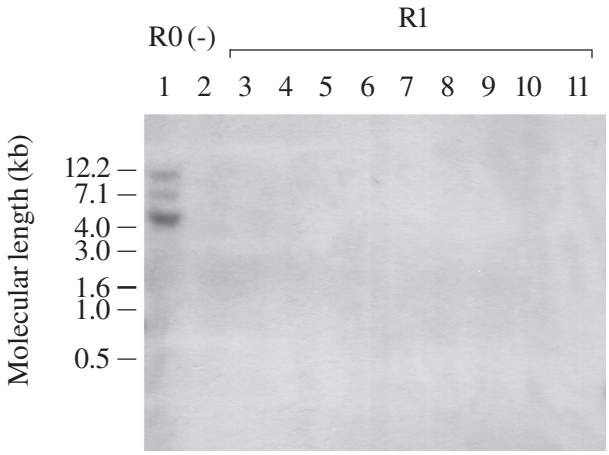

Figure 1. Southern blot analysis showing the gus gene in the primary transgenic line 158 and its absence in the progeny. Genomic DNAs were digested with *Eco*RI and probed with an internal fragment of the gus gene. *Lane 1*: Primary transformant (R0). *Lane 2*: Non-transgenic plant. *Lanes 3* to *11*: Progeny plants (R1). Molecular size markers are indicated on the left.

The phenomenon of transgene loss was systematically repeated for more than two years in plants propagated by grafting

The transgenic line (primary transformant) was maintained by grafting for more than two years. Over this period, 20 progenies (more than 300 plants) resulting from self-pollination and 10 progenies (more than 100 plants) from reciprocal crosses to untransformed plants (five from each cross) were obtained and analyzed by PCR for the presence of four different regions of pMD4 (Figure 2). All reactions were negative for the regions or sequences analyzed (data not shown). GUS assays and genetic analysis were performed to determine if the absence of transgenes in the offspring was a result of DNA elimination, chimerism or insertional mutation of gametophytic genes.

Lack of transgenes in the progeny of the transgenic bean line is not due to chimerism

GUS assays were carried out in the whole primary transformant (R0). All tissues dis-

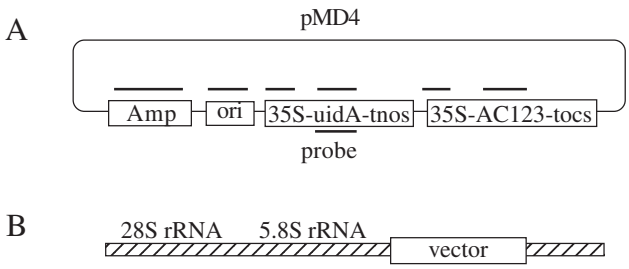

Figure 2. Integration site diagram of the plasmid vector (pMD4) disrupting a ribosomal DNA unit. **A.** Vector pMD4 utilized for bean transformation. Bars indicate the sequence fragments amplified by PCR and the probe utilized for Southern blot analyses. **B.** Scheme of the integration locus. Hatched bars indicate the genomic flanking region.

played strong GUS activity, including the microspores, which presented an approximately 3:1 (positive:negative) ratio (Figure 3). This demonstrated that the transgenic line was not a chimeric plant and that there were two segregating transgenic loci.

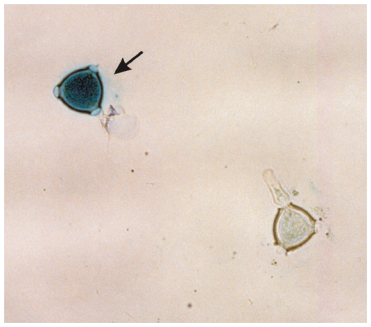

Figure 3. Microspores displaying positive (arrow) and negative GUS activity.

Absence of transgenic plants in the progeny of the bean line is not a consequence of insertional mutation

Another possible explanation for the absence of transgenic plants in the offspring could be a disruption of an essential gametophytic gene due to a plasmid insertional mutation. However, the complete absence of transgenes in the R1 offspring can only be explained by disruption of a gene that is essential for both male and female gametophytes. Therefore, a reduced number of seeds per pod would be expected (Feldmann et al., 1997; Howden et al., 1998), which was also observed with other lines generated in our laboratory (Aragão et al., 1998). However, the bean line that we studied here produced a normal number of seeds per pod. Consequently, insertional mutagenesis of gametophytic genes did not occur, and therefore the absence of transgenes in offspring is a result of DNA elimination.

Cloning and sequencing of transgene integration loci reveal interruption of a ribosomal DNA unit

The integration sites were cloned by plasmid rescue in order to shed light on the phe-

nomenon of transgene elimination from the plant genome. One of the integrated plasmids disrupted a ribosomal RNA gene (rDNA), while another was integrated into a sequence with no significant homology to known sequences (Figure 2). The third integrated sequence could not be isolated, probably due to a lack of sequences essential for plasmid rescue.

Transgene elimination phenomenon was also observed in a transgenic soybean line presenting integration of the transgenes into an rDNA unit

Transgene elimination was also observed in a transgenic soybean line, 33-3, transformed with another construct containing the ahas herbicide resistance gene. Analysis of the integration site revealed that one transgene was integrated into an rDNA, as observed in the transgenic bean line. GUS assays carried out with the microspores produced by the R0 plant revealed positive GUS activity, clearly showing that the transgenic line was not chimeric (data not shown).

Transgene elimination in plants has been attributed to intrachromosomal recombination (Fladung, 1999), genetic instability resulting from tissue culture conditions (Risseeuw et al., 1997; Joersbo et al., 1999) or a genomic defense process against invasive DNA (Srivastava et al., 1996).

Intrachromosomal recombination between two transgene copies integrated in different loci resulting in elimination of transgenes has been demonstrated in transgenic aspen (Fladung, 1999). In support of this hypothesis, recombination of transposons is a well-reported phenomenon that is responsible for the deletion of genome sequences. Therefore, homologous recombination between copies of the transgenes could be responsible for the loss of the transgenes observed in our study.

On the other hand, Kumpatla et al. (1998) suggested that transgene elimination might have a role in a genomic defense system that acts against natural intrusive DNA, as already well described for gene silencing (Matzke et al., 2000; Waterhouse et al., 2001; Voinnet, 2001; Plasterk et al., 2002;). Corroborating with this hypothesis, recently it was demonstrated that elimination of transgenes acts as a genome defense system in *Tetrahymena thermophila* (Yao et al., 2003). Our data also corroborate with this hypothesis. Both the dry bean and soybean transgenic lines presented transgenes integrated in rDNA units, a region subjected to elimination of intrusive elements, such as transposons (Burke et al., 1987). Therefore, it would be expected that a foreign sequence integrated into an rDNA unit would be identified and eliminated. However, this fact does not explain the simultaneous elimination of the two other plasmid copies integrated into different genome regions. The co-elimination of transgenes integrated in different sites could be interpreted as a genome defense, where the process of elimination is trigged by disruption of an rDNA unit and the other homologous sequences (transgenes) would be recognized and eliminated in a defense response against the introduced plasmid. The defense mechanism of transgene elimination described in *T. thermophila* is also mediated by a homology-dependent mechanism. Paramecium cells transformed with high copy number plasmids into the maternal macronucleus were subject to elimination of genomic regions homologous to the plasmids in the developing macronucleus, indicating the involvement of a homology-dependent mechanism for DNA elimination (Madireddi et al., 1996).

Some authors have suggested that the genetic instability imposed by stress conditions during tissue culture is responsible for the loss of transgenes. Risseeuw et al. (1997) demonstrated that T-DNAs in transgenic *Nicotiana tabacum* and *N. plumbaginifolia* that were

stable during plant development, as well as in transmission to the offspring, might become unstable during propagation in callus tissue. It has been suggested that the stress imposed by tissue culture conditions induces genomic instability at particular loci throughout the genome; these loci may be preferential targets for the integration of exogenous DNA (Gould, 1986). It is possible that the plasmids used in our process of transformation have been integrated into these unstable loci; during the meioses the transgenes could be eliminated, as hypothesized by Joersbo et al. (1999) for transgenes eliminated through meiosis in *Cyamopsis tetragonoloba*. Therefore, we cannot exclude the possibility that the tissue culture conditions had a role in the process of transgene elimination in our study of transgenic *P. vulgaris*.

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