

Radiation as a tool to remove selective marker genes from transgenic soybean plants

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Abstract

The present study evaluated the use of γ -radiation to physically remove selective marker genes previously introduced into the soybean genome. Homozygous seeds from a transgenic soybean line carrying the *gus* and *ahas* transgenes were irradiated with γ -rays. Six plants presenting a deleted *gus* gene were analyzed by Southern blot to confirm removal of both *ahas* and *gus* genes. Line 1A presented an absence of the *gus* gene cassette and presence of the *ahas* gene cassette.

Additional key words: γ -radiation, genetically modified plant, *Glycine max*, mutation.

Generation of transgenic plants free of these markers is a current challenge for biotechnology (Aragão and Brasileiro 2002). Consequently, several strategies to remove selective marker genes have been developed, such as co-transformation, multi-autotransformation (MAT), intragenomic relocation via transposable elements and site-specific recombination (Aragão *et al.* 1996, Vergunst and Hooykaas 1999, Ebinuma and Komamine 2001, Pavingerová *et al.* 2001, Raizada *et al.* 2001).

Ionizing radiation can generate chromosome breakage whose repair leads to mutations with a large proportion probably involving deletions (Vizir *et al.* 1996). This technique has been used to induce mutations and improve several seed propagated crops, including soybean (Maluszynski *et al.* 1991, Tulmann-Neto and Alves 1997, Ahloowalia and Maluszynski 2001). The objective of the present study was to evaluate the effectiveness of γ -radiation to physically eliminate marker genes introduced into the soybean genome.

Transgenic soybean [*Glycine max* (L.) Merrill], cv. BR-16 line 8-19, was generated in our laboratory (Aragão *et al.* 2000), presented a single locus of the plasmid pAG1 containing the *ahas* gene from *Arabidopsis thaliana* under control of the *ahas* promoter and the *gus* gene (marker gene coding β -glucuronidase) under control of the *actin* promoter from *A. thaliana*

(Aragão *et al.* 2000). Homozygous transgenic seeds (13 % water content) were irradiated with γ -radiation from ⁶⁰Co at doses of 225, 250, 275, 300 and 350 Gy at room temperature. Seeds were then planted in a greenhouse and allowed to set seed. The M₂ generation was analyzed for presence and expression of *gus* and *ahas* genes by polymerase chain reaction (PCR).

PCR analyses were carried out according to Aragão *et al.* (2000).

Primers 5' ACTAGAGATTCCAGCGTCAC 3' (AHASP within the *ahas* promoter) and 5' GTGGCTATACAGATACCTGG 3' (AHAS500C within the *ahas* coding sequence) were applied to amplify a 685 bp sequence in transgenic plants containing the foreign *ahas* gene.

Primers 5' TTGGGCAGGCCAGCGTATCGT 3' (GUS251) and 5' ATCACGCAGTTCAACGCTGAC 3' (GUS671C) were utilized to amplify a 420 bp sequence within the *gus* coding sequence.

Plants were analyzed for *in situ* localization of the *gus* gene expression with a histochemical assay according to Jefferson *et al.* (1987). In order to determine resistance to the dominant selective *ahas* gene, all plants were sprayed during the fourth-trifoliate stage with the herbicide Imazapyr (Arsenal®, BASF, Princeton, USA) at 0.01 g m⁻².

Southern blot analyses were carried out as previously

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described (Sambrook *et al.* 1989). Genomic DNA (10 µg), isolated according to Dellaporta *et al.* (1983), was digested with *Spe*I, separated on a 1 % agarose gel and transferred to nylon *Hybond-N+* membrane. Hybridization was carried out using the *ahas* and *gus* cassettes as probes, labeled with $\alpha^{32}\text{P}$ dCTP (1.1×10^{14} Bq mol⁻¹) using a random primer DNA labeling kit (Pharmacia Biotech, Piscataway, NJ, USA) according to manufacturer's instructions.

Except for one, all irradiated plants analyzed (7 065) revealed an absence of both genes (0.49 %). Plant 1A from the 275 Gy treatment presented absence of the *gus* gene and presence of the *ahas* gene. All 2 499 non-irradiated seeds presented expression of *gus* gene. Six plants presenting *gus* gene deletion (8J from treatment 225 Gy; 1A and 5A from treatment 275 Gy; 3E from treatment 300 Gy; 9D and 10E from treatment 350 Gy) were analyzed by Southern blot for presence of both *gus* and *ahas* genes integrated into the genome. Results showed an absence of both *ahas* and *gus* gene cassettes in the 3E, 5A, 8J, 9D and 10E lines. However, the 1A line presented an absence of the *gus* gene cassette and a presence of the *ahas* gene cassette (Fig. 1, lane 1). Since the plasmid pAG1, used to transform the 8-19 line, has a unique *Spe*I restriction site, Southern blot analysis helped confirm integration of a single copy of the *ahas* gene and two copies of the *gus* cassette. DNA isolated from non-transformed plants did not hybridize with the *ahas* and *gus* probes (Fig. 1A, lane 2; Fig. 1B, lane 2).

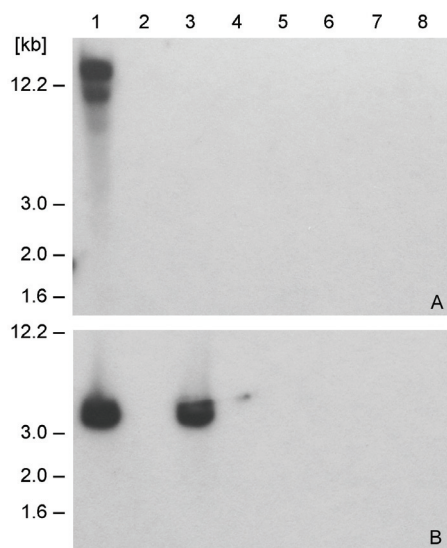


Fig. 1. Southern blot analysis of soybean transgenic line 8-19 (M_2 generation) irradiated with γ -rays. DNAs were digested with *Spe*I, transferred to a nylon membrane and probed with *gus* (A) and *ahas* (B). Lane 1: transgenic line 8-19 (not treated); lane 2: non-transgenic plant; lanes 3 - 8: plants 1A, 3E, 5A, 8J, 9D and 10E, respectively. Size of molecular markers is indicated on the left.

Plants received a treatment of herbicide Imazapyr to evaluate the functionality of the *ahas* gene. Following the

herbicide treatment, no symptoms were observed in plant 1A. As expected, red vein symptoms typically seen in imidazolinone-treated plants were observed in 3E, 5A, 8J, 9D, 10E and control plants (non-transgenic), which died two weeks after herbicide treatment.

The 36 plants analyzed by PCR were allowed to grow to maturity and set pods. Thirty plants, including plant 1A, presented a normal phenotype with normal growth, pods and seed production (Fig. 2A). On the other hand, two plants presented dwarfism as well as wrinkled leaves and pods (Fig. 2B), and four presented a base-branched phenotype.



Fig. 2. Mutated line 1A presenting a normal phenotype (A) and the mutated line 8C (treated with 350 Gy) presenting dwarfism and wrinkled leaves (B).

Results support the hypothesis that γ -radiation can be utilized to physically remove a specific gene (*gus*) from a transgenic soybean line, that contains other genomically integrated transgene. Reports have demonstrated that soybean seeds have been mutagenized with either ethyl methanesulphonate (EMS), γ -radiation or sodium azide (NaN_3) (Carroll *et al.* 1986, Tulmann-Neto and Alves 1997). EMS is notably recognized as the most utilized and efficient agent in generating soybean non-sense mutant plants. It has been applied to generate plants with a deleted gene that remain integrated in the genome rather than physically removed from it. On the other hand, non-sense mutations could be restored and gene expression spontaneously reverted to normal at a frequency ranging from 10^{-7} to 10^{-8} events per base pair (Kovalchuk *et al.* 2000).

Southern blot analyses revealed that plants presenting *gus* gene deletion did not contain the gene integrated into the genome. These results have shown that γ -radiation treatments were efficient in physically removing the transgenes.

Since just one line presented the desired genotype (1A) achieved with radiation dosage 275 Gy (frequency of 0.40 %), this treatment can not be recommended as

optimal. On the other hand, treatments 275, 300 and 350 Gy presented 2 (0.13 %), 10 (0.69 %) and 15 (1.16 %) plants with an abnormal phenotype (Fig. 2). Consequently, treatments above 300 Gy should be avoided. The other treatments did not present detectable

abnormal phenotypes.

Transgenic plants constitute a useful system to study biochemical modifications of DNA bases and strand breaks that can result in point mutations, insertions, deletions or gross rearrangements.

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