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BRIEF COMMUNICATION

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.) Merril], 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-glucoronidase) 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 ^{60}Co 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 M_2 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*, Princeston, 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 α^{32}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. 1*A*, *lane 2*; Fig. 1*B*, *lane 2*).

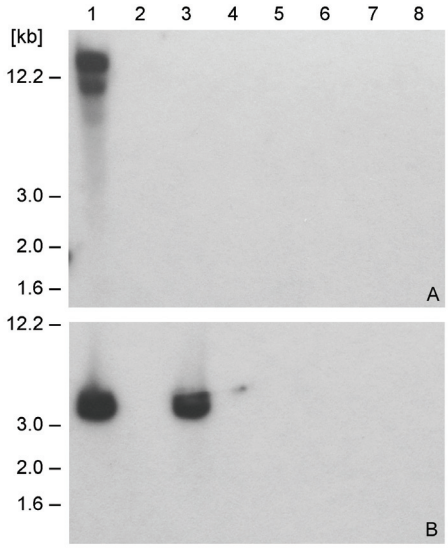

Fig. 1. Southern blot analysis of soybean transgenic line 8-19 (M_2 generation) irradiated with γ-rays. DNAs were digested with *Spe*I, transferred to a nylon membrane and probed with *gus* (*A*) and *ahas* (*B*). *Lane 1*: transgenic line 8-19 (not treated); *lane 2*: non-transgenic plant; *lanes 3 - 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. 2*A*). On the other hand, two plants presented dwarfism as well as wrinkled leaves and pods (Fig. 2*B*), and four presented a base-branched phenotype.

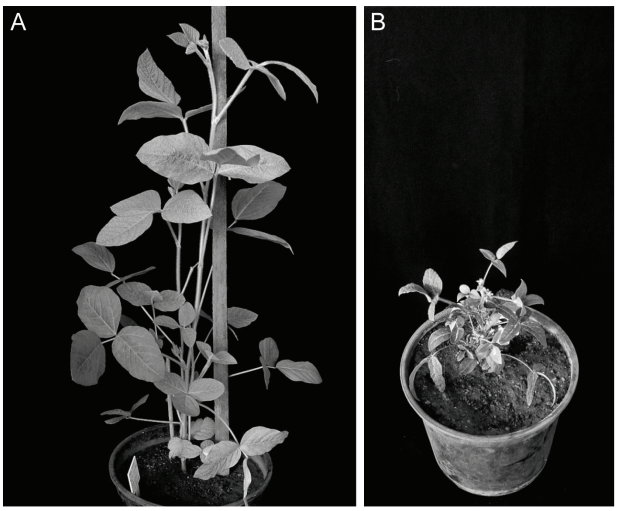

Fig. 2. Mutated line 1A presenting a normal phenotype (*A*) and the mutated line 8C (treated with 350 Gy) presenting dwarfism and wrinkled leaves (*B*).

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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