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Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [*Glycine max* (L.) Merril] plants at a high frequency

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Abstract Imazapyr is a herbicidal molecule that concentrates in the apical meristematic region of the plant. Its mechanism of action is the inhibition of the enzymatic activity of acetohydroxyacid synthase, which catalyses the initial step in the biosynthesis of isoleucine, leucine and valine. The selectable marker gene, *ahas*, was previously isolated from *Arabidopsis thaliana* and contains a mutation at position 653 bp. Combining the use of imazapyr, the *ahas* gene and a multiple shooting induction protocol has allowed us to develop a novel system to select transgenic meristematic cells after the physical introduction of foreign genes. In this study, we describe a protocol to obtain a high frequency of fertile transgenic soybean plants that is variety-independent.

Keywords Agricultural biotechnology · Gene transfer · Soybean · Imazapyr

Introduction

Due to the economic importance of producing soybean-based products through genetic engineering, a great deal of effort has been made to develop a system to recover fertile transgenic soybean plants. The first two successful reports of soybean transformation involved the use of the *Agrobacterium* system to obtain plants regenerated from cotyledonary nodes (Hinchee et al. 1988) and the biolistic system, which is based on acceleration of microparticle coated-DNA into the apical meristematic region of embryonic axes (McCabe et al. 1988). Several groups have been actively involved in the improving these systems through the manipulation of somatic embryogenesis and shoot morphogenesis (Barwale et al. 1986; Finer and McMullen 1991; Hansen et al. 1994; Hooykaas and Schilperooort 1992). However, the protocols published to date have failed to produce a highly efficient system that is both simple to execute and variety-independent. In addition, an efficient selectable marker would allow the regeneration of only transgenic material and increase the frequency of recovery of fertile transgenic plants. A soybean transformation technology that would allow the selection of transgenic meristematic cells and regeneration of fertile plants should facilitate all studies that involve the use of transgenic plants, e.g. detailed studies of gene function, as well as the development of transgenic soybean varieties with improved agricultural characteristics.

We have developed a novel system to select transgenic meristematic cells after the physical introduction of a mutant *ahas* gene by using a selection system based on the use of imazapyr, a herbicidal molecule of the imidazolinone class capable of systemically translocating and concentrating in the apical meristematic region of the plant. The mechanism of action of imazapyr is the inhibition of the enzymatic activity of acetohydroxyacid synthase (AHAS; acetolactate synthase, acetolactate pyruvate-lyase (carboxilating), EC 4.1.3.18), which catalyses the initial step in the biosynthesis of isoleucine, leucine and valine (Shaner et al. 1984). The *ahas* selectable marker gene, previously isolated from *Arabidopsis thaliana*, contains a mutation at position 653 bp resulting in a serine to asparagine substitution that confers imidazolinone-specific resistance (Sathasivan et al. 1990). This selectable marker system, combined with an improved multiple-shooting induction protocol, resulted in a significant increase in the recovery of fertile, transgenic material compared with standard soybean transformation protocols We were also able to demonstrate that this technique is variety-independent. We believe that this

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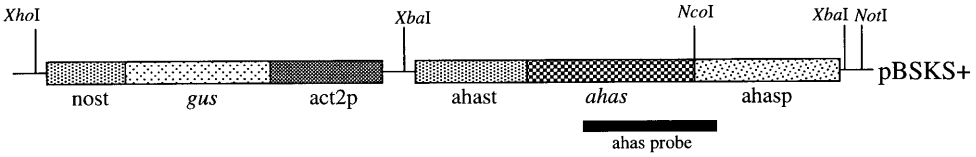

Fig. 1 Expression cassettes utilized for microparticle bombardment. *Bar* indicates the probe for Southern DNA analysis

new technology should have significant applications for studies of gene transfer and expression.

Material and Methods

Plasmid vector

The 3.6-kb fragment containing the act2p (Yong-Qiang et al. 1996), *uid*A and nost, was released from the plasmid pWACT2ÊS (An et al. 1996) by digestion with *Pst*I followed by a partial digestion with *Eco*RI. This fragment was inserted into the sites *Eco*RI/*Pst*I of the plasmid pBS KS+ (Stratagene) to produce the plasmid pBSGUS. Ths plasmid pAC321 (American Cyanamid Co, unpublished results) was digested with *Xba*I to isolate a 5.5-bp fragment containing the *Arabidopsis ahas* promoter, coding sequence and terminator. This fragment was cloned into the *Xba*I site in the plasmid pBSGUS to produce the vector pAG1 (Fig. 1).

Soybean transformation

Mature seeds of commercial Brazilian soybean cultivars (BR-16, Doko RC, BR-91 and Conquista) were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min. and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18–20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium. The bombardment was conducted as previously described by Aragão et al. (1996). Multiple shooting was induced by transferring and completely immersing, the embryonic axes in induction medium immediately after bombardment (IM: MS basal salts medium, supplemented with 22.2 µ*M* benzylaminopurine, 3% sucrose and 0.6% agar, Sigma, pH 5.7) in 10-cm dishes containing 15 ml of the culture medium for 16 h in the dark at 28°C. After this period, the explants were transferred to a baby food jar containing 20 ml of the selection medium (MS basal salts medium, 3% sucrose, 500 n*M* imazapyr and 0.7% agar Sigma, pH 5.7) and cultured at 28°C with 16-h photoperiod (50 µmols m^{-2} s^{-1}). As a control, explants derived from the cultivar BR-16 were also cultured under the same conditions without the selective agent imazapyr. As soon as the embryonic axes-derived shoots were 2–3 cm in length, a 1-mm-long section was removed from the base of each leaf for analysis of GUS (B-glucoronidase) expression (McCabe et al. 1988). The shoots expressing the exogenous DNA were individually transferred to a plastic pot containing 0.2 dm^3 of autoclaved fertilized soil:vermiculite (1:1), covered with a plastic bag sealed with a rubber band and maintained in a greenhouse. After 1 week, the rubber band was removed. After an additional week the plastic bag was also removed. As soon as the acclimatized plantlets reached approximately 10 cm in length they were transferred to a pot containing 5 dm^3 of fertilized soil and allowed to set seeds. The temperature in the greenhouse was 25°C, the relative humidity above 80% and the light intensity was 350 µmols m^{-2} s^{-1}.

Polymerase chain reaction (PCR)

For PCR analysis of transformed plants, DNA was isolated from leaf disks according Edwards *et al.* (1991). Each PCR reaction was carried out in 25 µl aliquots containing 10 m*M* TRIS-HCl (pH 8.4), 50 m*M* KCl, 2 m*M* $MgCl_2$, 160 µ*M* of each dNTP, 200 n*M* of each primer, 2 U of *Taq* polymerase (GIBCO BRL) and about 20 ng of genomic DNA. The primers 5′ ACT AGA GAT TCC AGC GTC AC 3′ (within the *ahas* promoter) and 5′ GTG GCT ATA CAG ATA CCT GG 3′ (within the *ahas* coding sequence) were utilized to amplify a 685 bp sequence. The mixture was overlaid with mineral oil, denatured for 5 min at 95° C in a MJ thermal cycler (USA) and amplified 35 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) with a final cycle of 7 min at 72°C. One half of the reaction was then loaded directly onto a 1% agarose gel and visualized under UV light following ethidium bromide staining.

Southern blot analysis

Genomic DNA was isolated according to Dellaporta et al. (1983). Southern blotting and hybridization were carried out as previously described (Sambrook et al. 1989). Genomic DNA (15 µÊg) was digested with *Xho*I, separated on a 1% agarose gel and transferred to a nylon membrane (Hybond). The hybridization was carried out using the PCR-generated 685 bp *ahas* probe, labelled with α[^{32}P]-dCTP (110 TBq/mol) using a random primer DNA labelling kit (Pharmacia Biotech) according to the manufacturer's instructions.

Progeny analysis

The analysis of the R_0 and R_1 generation transformants was conducted by amplifying the introduced foreign genes (*ahas* and *gus*) by PCR and by GUS histochemical assay analysis of leaves of self-pollinated plants. Chi-square ($\chi 2$) analyses were performed to determine if the observed segregation ratio was consistent with a Mendelian ratio in the R_1 generation.

Test of progeny for resistance to imazapyr

One hundred seeds collected from each of the 35 R_1 generation transgenic lines were sown in 5-dm^3 plastic pots containing autoclaved fertilized soil. Once the plants produced the first trifoliate leaves, they were sprayed with the herbicide (imazapyr) at a concentration of 100 $g.ha^{-1}$.

Results

The apical regions of soybean embryonic axes were bombarded with the plasmid pAG1 (Fig. 1). Following the bombardment the embryonic axes were cultured in a selection and multiple shooting induction medium. Twenty-four hours after bombardment the majority of the embryonic axes showed extensive *gus* gene expression within the apical region (Fig. 2). After 2 weeks in culture, elongated shoots (2–3 cm) were observed. PCR and GUS histochemical assays confirmed the presence of the *ahas* and *gus* transgenes in most of these shoots. After 3–4 weeks additional shoots began to regenerate. A total of 5–7 shoots were typically induced from each bombarded embryonic axis, but only 10% elongated. Table 1 summarizes the re-

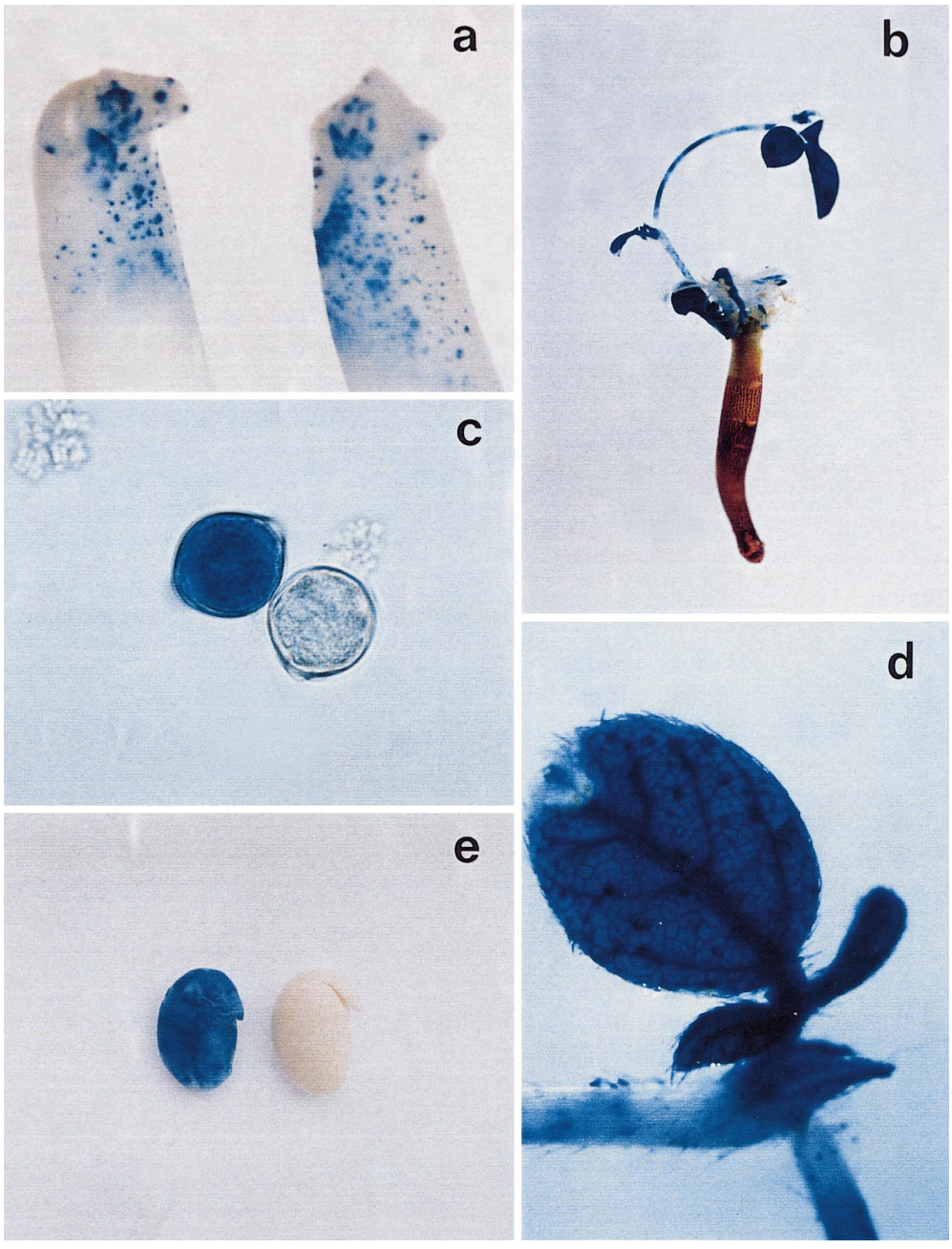

Fig. 2a–e Soybean transformation. **a** Embryonic axes expressing the *gus* gene 24 h after bombardment, **b** transgenic shoot from a multiple-shooting apical meristem, **c** microspores showing *gus* segregation; d) leaf from an acclimatized plant expressing the *gus* gene, **e** seeds from transgenic plants (*left*) and control (*right*)

sults of 22 independent experiments in which a 1343 apical shoots were obtained from the 2843 bombarded embryonic axes (47%, Table 1, experiments 6–22), and 224 potentially transgenic shoots (16.7%) were identified by performing histochemical analysis of the basal stem and leaf tip for the expression of the *gus* gene. The average frequency of transformation (defined as the total number of putative transgenic plants divided by the total number of bombarded embryonic axes) varied from 3.9% to 20.1%.

GUS-positive shoots were transferred to culture medium as described in the experimental protocol. As soon as the plantlets developed vigorous roots they were acclimatized and transferred to soil. All plants presented a normal phenotype, were fertile and set pods and seeds. Histochemical GUS assays performed on leaf tissue from all of the transgenic plants revealed intense enzymatic activity (Fig. 2). All plants expressing the *gus* gene also revealed the presence of the *ahas* gene in the PCR analysis (Fig. 3).

As a control, bombarded embryonic axes from 5 independent experiments were cultivated in a multiple-shooting induction medium without selection. Most of the putative transgenic shoots were chimeric; out of 794 bombarded embryonic axes only 1 showed germline transformation (Table 1, experiments 1–5). The average frequency of transformation was 0.1%.

Southern blot analyses of genomic DNA isolated from the R_0 generation transgenic soybean plants were conducted to evaluate the integration of the introduced *ahas*

Table 1 Summary of 22 independent experiments of transformation with the plasmid pAG1

Experiment	Cultivar	Selection	Bombarded embryonic axes	Elongated shoots	Chimeric plants	Transformed plants	Frequency of transformation (%)
1	**BR-16**	**No**	**111**	**66**	**2**	**0**	
2	**BR-16**	**No**	**224**	**25**	**2**	**0**	
3	**BR-16**	**No**	**249**	**67**	**0**	**0**	
4	**BR-16**	**No**	**110**	**122**	**3**	**1**	
5	**BR-16**	**No**	**100**	**34**	**2**	**0**	
Total			794	314	9	1	0.1
6	**BR-16**	**Yes**	**214**	**106**	**0**	**11**	
7	**BR-16**	**Yes**	**150**	**106**	**0**	**25**	
8	**BR-16**	**Yes**	**166**	**78**	**0**	**9**	
9	**BR-16**	**Yes**	**186**	**70**	**0**	**12**	
10	**BR-16**	**Yes**	**221**	**65**	**0**	**8**	
11	**BR-16**	**Yes**	**150**	**35**	**0**	**11**	
12	**BR-16**	**Yes**	**180**	**41**	**0**	**23**	
Total			1267	501	0	99	7.8
13	**DOKO RC**	**Yes**	**120**	**66**	**0**	**6**	
14	**DOKO RC**	**Yes**	**122**	**79**	**0**	**3**	
15	**DOKO RC**	**Yes**	**140**	**123**	**0**	**7**	
16	**DOKO RC**	**Yes**	**125**	**53**	**0**	**5**	
17	**DOKO RC**	**Yes**	**132**	**74**	**0**	**9**	
Total			639	395	0	30	4.7
18	**BR-91**	**Yes**	**133**	**59**	**0**	**7**	
19	**BR-91**	**Yes**	**144**	**56**	**0**	**3**	
20	**BR-91**	**Yes**	**180**	**72**	**0**	**10**	
21	**BR-91**	**Yes**	**122**	**61**	**0**	**3**	
Total			579	248	0	23	3.9
21	**CONQUISTA**	**Yes**	**178**	**101**	**1**	**30**	
22	**CONQUISTA**	**Yes**	**180**	**98**	**0**	**42**	
Total			358	199	1	72	20.1

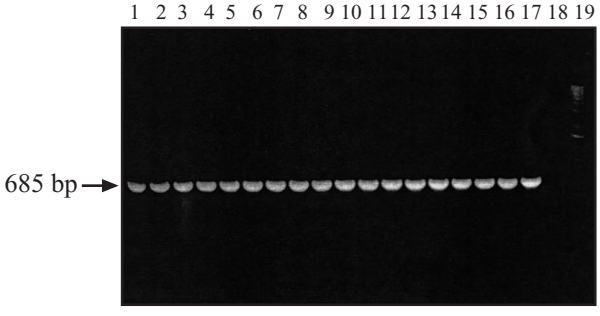

Fig. 3 PCR analysis of transformed soybean plants with the plasmid pAG1. *Lanes 1–16* Transformed lines, *17* positive control (plasmid pAG1), *18* non-transformed plant, *19* molecular size standard (1-kb ladder–GIBCO/BRL). *Arrow* indicates the expected fragments

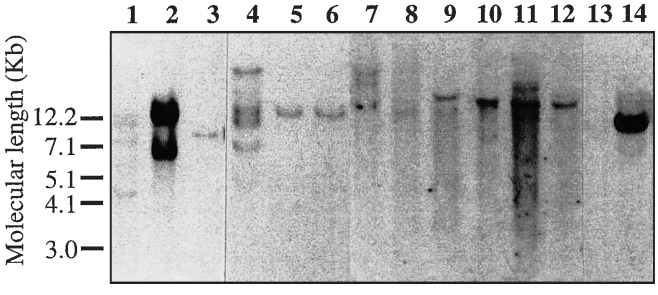

Fig. 4 Southern blot analysis of representative putative transformed lines in the R_0 generation. DNAs were digested with *Not*I, transferred to a nylon membrane and probed with an internal fragment of the *ahas* gene (**Fig. 1**) *Lanes 1–12* Different transformed lines: 9–2 (*lane 1*), 10–9 (*lane 2*), 27–38 (*lane 3*), 27–1 (*lane 4*), 15–61 (*lane 5*), 42–1 (*lane 6*), 37–2 (*lane 7*), 35–11 (*lane 8*), 18–2 (*lane 9*), 18–5 (*lane 10*), 37–2 (*lane 11*) and 8–19 (*lane 12*). *Lane 13*, Non-transformed plant, *lane 14* pAG1 (50 pg). Molecular size markers are indicated on the *left*

gene. The results showed the presence of the *ahas* sequence in all 12 R_0 generation lines analysed (Fig. 4). Since the plasmid pAG1 has a unique *Not*I restriction site (Fig. 1) Southern blot analysis allowed us to confirm integration of the plasmid as well as to estimate the copy number of *ahas* genes (Fig. 4) present in the transgenic soybean plants. Southern analysis performed on 12 transgenic lines revealed the presence of one to two integrated copies of the *ahas* gene, except in the case of transgenic lines 9–2, 27–1 and 37–2, which revealed more than two copies. DNA isolated from non-transformed plants did not hybridize with the *ahas* probe (Fig. 4, line 13).

The progeny of 35 self-fertilized transgenic plants were screened by PCR analysis for the presence of the *ahas* gene. With the exception of 4 lines (11–1, 18–5, 33–3 and 33–8), all R_0 plants transferred the foreign gene to the R_1 generation in a Mendelian fashion (3:1) (Table 2).

Following herbicide treatment of 28 R_1 generation transgenic lines, each line showed differing levels of tolerance to imazapyr (Fig. 5). Red vein symptoms typically seen in imidazolinone-treated plants were observed in all tolerant plants (Fig. 5). However, R_1 generation trans-

Table 2 Segregation analysis of 35 self-fertilized transgenic plants in the R_1 generation

R_0 Lines	R_1 generation [a]		χ^2	P[b]
	Positive	Negative		
3–13	99	32	0.02	0.88
8–19	100	34	0.01	0.92
8–4	105	36	0.02	0.88
9–2	60	21	0.03	0.85
10–9	79	26	0.01	0.96
11–1	0	35	105.0	0.00
12–38	102	32	0.09	0.76
13–24	179	64	0.23	0.63
13–3	70	26	0.22	0.64
14–8	120	38	0.07	0.78
15–61	101	35	0.03	0.84
18–2	73	26	0.08	0.77
18–1	0	94	282.0	0.00
24–1	74	26	0.05	0.82
27–1	69	25	0.12	0.72
27–4	67	20	0.18	0.66
28–13	36	10	0.26	0.61
28–9	73	25	0.01	0.91
33–3	0	85	255.0	0.00
33–8	0	96	288.0	0.00
35–11	32	9	0.20	0.65
37–11	24	10	0.35	0.55
37–14	42	12	0.22	0.64
37–2	134	41	0.23	0.63
37–20	31	10	0.01	0.93
37–57	55	20	0.11	0.74
38–14	68	20	0.24	0.62
38–3	60	21	0.03	0.85
38–6	51	19	0.17	0.68
38–7	36	10	0.26	0.61
38–8	114	36	0.08	0.78
38–14	36	10	0.26	0.61
42–1	90	34	0.38	0.53
49–12	24	10	0.35	0.55
51–1	54	15	0.39	0.53

[a] Data are based on histochemical assay for *gus* gene expression and PCR analysis of *ahas* gene
[b] *P* is the probability that the observed ratios reflect the expected segregation ratio of 3:1

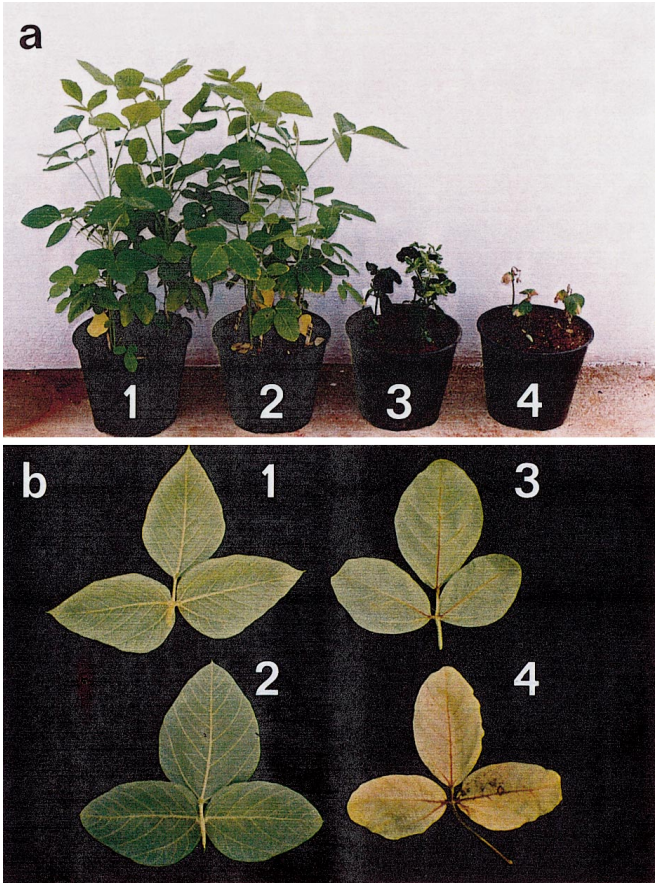

Fig. 5a, b Test of progeny of transgenic soybean lines. **a** Non-transgenic plants (*1* and *4*); transgenic plant, line 8–19 (*2*); transgenic plant, line 9–2 (*3*). Plant 1 was not treated with the herbicide. Plants 2–4 were treated with 100 g.ha^{-1} imazapyr. **b** Details of the abaxial leaves. Non-transgenic leaves (*1* and *4*), transgenic leaves, line 8–19 (*2*), and line 9–2 (*3*). Leaf in *panel 1* was not treated with the herbicide; leaves in panels 2–4 were treated with 100 g ha^{-1} imazapyr

genic lines 8–19, 27–1 and 37–20 showed resistance to the herbicide. No symptoms were observed in these transgenic lines, and growth was comparable to that of the non-treated soybean plants (Fig. 5). Progeny of lines 11–1, 18–5, 33–3 and 33–8 and of control plants died 2 weeks after herbicide treatment.

Discussion

We have developed a technology to select transgenic meristematic cells that enables the recovery of a high frequency of fertile transgenic soybean plants independent of variety. Over the last decade, several molecules have been extensively and effectively utilized to select transgenic cells (Birch 1997), however, none of the compounds select for the growth of transgenic meristematic cells. As previously described by McCabe et al. (1988), we have shown that microparticle bombardment of the soybean meristematic region, followed by a multiple shooting induction, allows the recovery of fertile transgenic soybean plants. Utilization of imazapyr in the culture medium combined with use of the mutant *ahas* gene as a selectable marker introduced by microparticle bombardment into the soybean meristematic region create a highly efficient selection system for meristematic cells. This strategy allowed the recovery of transgenic soybean plants at an increased frequency up to two hundred-fold over the existent non-selective system (Christou 1997; Christou et al. 1989; McCabe et al. 1988).

The integration of the *ahas* gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number, and progeny analysis of self-pollinated R_1 generation transgenic plants showed a Mendelian segregation ratio (3:1). This data suggests that the foreign genes were integrated in the same locus, which is a desirable property for transgenic plants in a breeding programme aimed at product development.

Early studies support the concept that most the foreign genes introduced by the biolistic process are normally transmitted to the progeny (Christou et al. 1989; Hinchee et al. 1988; McCabe and Martinell 1993). In our study, 4 plants did not transfer the introduced foreign gene to the R_1 generation. An insertional mutation of an essential gene required for ovule fecundation and/or development might account for this aberrant inheritance. Of a total of 35 transgenic R_1 generation lines sprayed with the herbicide, 3 transgenic lines showed no symptoms of herbicide injury. Since PCR analysis indicated that all 35 plants contained at least one copy of the *ahas* gene the resistance level in these 3 plants may be due to the site of integration of the gene into the genome. We observed no correlation between the copy number of the integrated *ahas* gene and herbicide resistance. Chromosome *in situ* hybridization and plasmid rescue studies are being carried out to evaluate the correlation between the integration pattern, gene expression and herbicide resistance.

There is nothing intrinsic to this technology that would limit its wide utilization in any transformation system employing the selection of meristematic cells. In principle, it could be utilized to select transgenic meristematic cells in several important crops. The ability to recover a high number of fertile transgenic plants may facilitate studies of gene function in several economically important crops. In addition, it should facilitate the detection of the ”key event(s)” that could be integrated into plant breeding programmes for development of transgenic crops that will be introduced into the market.

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