

Agronomic performance, chromosomal stability and resistance to velvetbean caterpillar of transgenic soybean expressing *cry1Ac* gene

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Abstract – The objective of this work was to analyze the agronomic performance and chromosomal stability of transgenic homozygous progenies of soybean [*Glycine max* (L.) Merrill.], and to confirm the resistance of these plants against *Anticarsia gemmatilis*. Eleven progenies expressing *cry1Ac*, *hpt* and *gusA* genes were evaluated for agronomic characteristics in relation to the nontransformed parent IAS 5 cultivar. Cytogenetical analysis was carried out on transgenic and nontransgenic plants. Two out of the 11 transgenic progenies were also evaluated, in vitro and in vivo, for resistance to *A. gemmatilis*. Two negative controls were used in resistance bioassays: a transgenic homozygous line, containing only the *gusA* reporter gene, and nontransgenic 'IAS 5' plants. The presence of *cry1Ac* transgene affected neither the development nor the yield of plants. Cytogenetical analysis showed that transgenic plants presented normal karyotype. In detached-leaf bioassay, *cry1Ac* plants exhibited complete efficacy against *A. gemmatilis*, whereas negative controls were significantly damaged. Whole-plant feeding assay confirmed a very high protection of *cry1Ac* against velvetbean caterpillar, while nontransgenic 'IAS 5' plants and homozygous *gusA* line exhibited 56.5 and 71.5% defoliation, respectively. The presence of *cry1Ac* transgene doesn't affect the majority of agronomic traits (including yield) of soybean and grants high protection against *A. gemmatilis*.

Index terms: *Anticarsia gemmatilis*, *Glycine max*, plant resistance to insects, gene transfer.

Performance agronômica, estabilidade cromossômica e resistência à lagarta-da-soja em soja transgênica que expressa o gene *cry1Ac*

Resumo – O objetivo deste trabalho foi analisar a performance agronômica e a estabilidade cromossômica de progênies transgênicas homozigotas de soja [*Glycine max* (L.) Merrill.], e confirmar a resistência dessas plantas a *Anticarsia gemmatilis*. Onze progênies com expressão dos genes *cry1Ac*, *hpt* e *gusA* foram avaliadas quanto às características agronômicas, em relação à cultivar parental IAS 5 não transformada. Análises citogenéticas foram realizadas em plantas transgênicas e não transgênicas. Duas das 11 progênies transgênicas também foram avaliadas quanto à resistência a *A. gemmatilis*, em bioensaios in vitro e in vivo. Dois controles negativos foram utilizados nos bioensaios: uma linha transgênica homozigota, com apenas o gene repórter *gusA*, e plantas de 'IAS 5' não transgênicas. A presença do transgene *cry1Ac* não afetou nem o desenvolvimento nem a produtividade das plantas. As plantas transgênicas apresentaram cariótipo normal. No bioensaio com folhas destacadas, as plantas *cry1Ac* exibiram completa eficácia contra *A. gemmatilis*, enquanto os controles negativos foram significativamente danificados. Ensaios com plantas inteiras confirmaram a elevada proteção do *cry1Ac* contra a lagarta-da-soja, enquanto as plantas 'IAS 5' não transgênicas e a linha homozigota *gusA* exibiram 56.5 e 71.5% de desfolhação, respectivamente. A presença do transgene *cry1Ac* não afeta a performance agronômica da soja (incluindo a produtividade) e confere elevada proteção a *A. gemmatilis*.

Termos para indexação: *Anticarsia gemmatilis*, *Glycine max*, resistência de plantas a insetos, transferência de genes.

Introduction

Velvetbean caterpillar (*Anticarsia gemmatilis* Hübner) is a major pest of soybean, occurring mainly in

growing regions of this crop in North and South Americas (Panizzi & Corrêa-Ferreira, 1997; Macrae et al., 2005). This insect causes extremely high levels of defoliation, when infestation is heavy, and can severely damage

axillary meristems. A single larva can consume up to 110 cm² of soybean foliage (Walker et al., 2000). Consequently, expensive insecticide application or control measures are necessary to prevent yield losses in regions with high occurrence of *A. gemmatilis* (Macrae et al., 2005; Miklos et al., 2007). Chemical insecticides are commonly used for controlling infestations of velvetbean caterpillar in soybean; however, narrow application windows, the emergence of insecticide resistance, and public pressure for reducing pesticide use limit the desirability of this approach to pest management (Thomas & Boethel, 1994). Alternative control strategies, such as biological insecticides, are available but not widely used (Luttrell et al., 1998; Moscardi, 1999), and efforts to develop elite insect-resistant soybean lines – with high yield and desirable agronomic traits, through conventional breeding of germplasms exhibiting endogenous insect resistance – have not been successful (Boethel, 1999). Recent advances in plant biotechnology, such as the use of transgenic cultivars to better exploit host plant resistance, have provided new approaches for the management of insect pests. One of the most promising of these approaches is the development of cultivars that express one of the delta-endotoxin genes from *Bacillus thuringiensis* (*Bt*) subsp. *kurstaki* (Roush, 1997). *B. thuringiensis* has long been used for controlling insect pests as a biological insecticide. The activity of delta-endotoxin from *B. thuringiensis* in controlling the larvae of *A. gemmatilis* has been described (Stewart Junior et al., 1996; Walker et al., 2000; Macrae et al., 2005; Miklos et al., 2007).

In a previous study, Homrich et al. (2008) developed an 'IAS 5' transgenic line containing an intact copy of a truncated synthetic *cryIAc* gene from *B. thuringiensis*. A preliminary in vitro insect feeding assay indicated that transgenic plants were highly toxic to *A. gemmatilis* larvae.

The introduction of a transgene into a recipient genome is a complex event depending on the transgene itself and the host genome (Yin et al., 2004). Agronomic traits of field crops may become adversely affected upon their transformation with foreign genes. According to Filipecki & Malepszy (2006), the unintended effects in transgenic plants can be divided into three main groups: first, pleiotropic effects of integrated DNA on the host plant genome; second, the influence of the integration site and transgene architecture on transgene expression level

and stability; and third, the effect of various stresses related to tissue handling, regeneration and clonal propagation.

Genetic alterations and chromosomal abnormalities of regenerated plants, based on tissue culture process (somaclonal variation), have been described for many plant species, including soybean (Stephens et al., 1991; Singh et al., 1998; Jain, 2001). Many plant characters can be altered, including plant height, yield, number of flowers per plant, grain quality, and resistance to diseases, insects, pests, cold, drought and salt (Jain, 2001). The change in chromosome number is commonly associated with reduced fertility and with altered genetic ratios in the progeny of self-pollinated plants (Singh et al., 1998). Thus, agronomic performance evaluation of transformed plants is necessary to evaluate if nontransgenic parent cultivar traits, including desirable yield components, have been retained along the tissue culture, transformation and regeneration processes. It is also important to ascertain if the obtained transgenic plants present normal karyotype ($2n = 40$).

The objective of this work was to characterize 11 homozygous *cryIAc* progenies, by comparing agronomic characteristics and yields; to establish the chromosomal stability in relation to the nontransformed parent IAS 5 cultivar; and to confirm the resistance of these plants against *A. gemmatilis* larvae.

Materials and Methods

The transgenic progenies of the cultivar IAS 5, analyzed in this study, were developed by Homrich et al. (2008) and carry the synthetic *cryIAc*, *hpt* and *gusA* genes. The *cryIAc* gene is truncated in the C terminus region and harbors the intact N terminus region of the native gene (Sardana et al., 1996). *B. thuringiensis* toxins produced in transgenic plants have often been expressed as protein-truncated at the C terminus, because this has resulted in higher expression levels (Vaeck et al., 1987). In terms of codon usage, the *cryIAc* gene was designed for expression in dicots and has a G+C content of 47.7%. The overall modifications have resulted in higher expression levels of CryIAc in transgenic plants (Sardana et al., 1996).

A transgenic homozygous line harboring only *gusA* reporter gene, obtained in the previous work (Homrich et al., 2008), was used as a negative control in the bioassays. This *gusA* line was likely derived by interchromosomal recombination from transformants,

which contained *cryIAC*, *hpt* and *gusA* genes integrated at a single locus.

The progenies included in the present study were obtained from self-pollination of eleven T₃ homozygous transgenic plants. The T₃ homozygous plants resulted from the same transformation event (Homrich et al., 2008). The progenies identified by the numbers 44, 46, 162, 169, 438, 497, 507, 520, 551, 937, 1274 were evaluated for their agronomic performance. A greenhouse experiment was conducted at Embrapa Trigo, in Passo Fundo, Rio Grande do Sul State, Brazil, to compare the transgenic progenies with nontransgenic 'IAS 5' regarding: date of emergence, initial development, seedling hypocotyl color, flower color, pubescence color, growth habit, plant height, number of nodes, flowering date, maturity date, seed tegument color, seed hilum color, 100-seed weight, number of seeds and total grain yield. Initial development was scored 15 days after planting using a scale of 1 (poorly developed first trifoliolate leaf) to 5 (completely developed first trifoliolate leaf).

Seeds were individually planted in 30-cm plastic pots containing 8 kg of soil fertilized with an equivalent of 500 kg ha⁻¹ of 0-25-25 (N-P-K) and 6 Mg ha⁻¹ of lime, in accordance with soil analysis. Seeds were sown in December 2006 and harvested in April 2007, in a greenhouse, under natural light at 25±5°C, relative humidity of about 50±10%, and photoperiod varying from 14 hours in December to 12 hours in April. Water was daily applied to soil, using a dripping system. Pots were arranged in randomized complete blocks design. Plants grew until physiological seed maturity. Data were recorded from six plants per progenie and six nontransgenic 'IAS 5' plants.

ANOVA was performed, using the GLM procedure of SAS (SAS Institute, 2004). When the assumptions for this test were not met, variable transformations were selected according to Box & Cox (1964). Transformations were performed on initial development (x²), flowering date (1/x⁴), plant height (natural log) and number of nodes (1/x). Multiple comparisons were made using Tukey's HSD test, at 5% probability.

For the cytogenetical analysis, chromosome numbers (mitotic pro-metaphase to metaphase chromosomes) were determined in five transgenic progenies (44, 169, 438, 937 and 1274) and in nontransgenic control plants, with three repetitions (plants) per genotype. Ten cells were analyzed per plant. A total of 180 cells from root tips of five transgenic progenies and nontransgenic control plants were analyzed. Root tips with 1 cm in

length were removed from seedlings of the transgenic plants and the nontransgenic control plants. Root-tip squash and chromosome counts were carried out according to Bodanese-Zanettini et al. (1996). The root tips were pretreated with a saturated solution of paradichlorobenzene for 18–20 hours at 4°C, fixed in 3:1 ethanol:acetic acid for 12–24 hours, and stored in 70% ethanol at 18°C until required. Tips were, then, hydrolyzed in 1N HCl at 60°C for 8 min and subsequently stained with leuco-basic fuchsin. Chromosome counts were made on well-spread metaphases from intact cells, directly on the microscope.

For the insect bioassay, two homozygous *cryIAC*, *hpt* and *gusA* transgenic progenies (162; 438) were evaluated through in vitro and in vivo assays for resistance to *A. gemmatilis*. A homozygous *gusA* line (732) and nontransgenic 'IAS 5' plants were used as negative controls. Velvetbean caterpillar eggs were provided by Embrapa Soja (Londrina, PR, Brazil). In vitro and in vivo assays were conducted at Embrapa Trigo (Passo Fundo, Rio Grande do Sul State, Brazil).

In order to evaluate the in vitro insecticidal activity of transgenic and control plants towards *A. gemmatilis* larvae, leaflets from trifoliolate leaves were placed in 100x20 mm Petri dishes, containing a piece of moistened filter paper, and were incubated for 72 hours at 25°C with 12 hours photoperiod. The leaf tissue was kept as uniform as possible (about 200 mg) from one dish to another, with leaves being replaced as necessary. Leaf samples were infested with 20 second-instar larvae of *A. gemmatilis* per plate. After 24 hours, the percentage of foliage consumption was estimated. Leaf consumption percentages were converted to scores as follow: 1 (very low consumption), 2 (less than 50%), 3 (around 50%) and 4 (almost 100% consumption). The number of alive and dead larvae was determined 24, 48 and 72 hours after infestation. At the end of the experiment, the surviving larvae had their development and weight evaluated.

The whole-plant feeding assay was carried out in a greenhouse. The transgenic and controls seeds were planted in 30-cm diameter plastic pots filled with soil fertilized [500 kg ha⁻¹ of 0-25-25 (N-P-K) and 6 Mg ha⁻¹ lime] and were maintained under natural light at 25±5°C. A randomized complete block design was used with six repetitions (plants) per genotype. Each plant was infested with 50 second-instar larvae, when plants were in the V3 to V4 stage of development. Resistance was evaluated

through visual estimates of defoliation percentages and the insect survivorship 7 and 14 days after infestation. Percent defoliation refers to the percentage of leaves with consumed area higher than 50%.

Results and Discussion

No variation was observed within transgenic progenies and among transgenic progenies and the nontransgenic 'IAS 5' control plants, for the following agronomic traits: date of emergence (6–7 days); seedling hypocotyl color (light green); flower color (white); pubescence color (grey); growth habit (determinate); maturity (155 days); seed tegument color (yellow); seed hilum color (light brown). These traits are in accordance with the 'IAS 5' description.

Significant differences were observed for initial development, flowering date, plant height, number of nodes, number of seeds, 100-seed weight, and total grain weight (Table 1). Ten days after planting, the nontransgenic 'IAS 5' control was at a more advanced development stage (score = 4.67) than those of the transgenic plants. Only two transgenic progenies (937, 520) showed a late flowering, while the other progenies did not differ from nontransgenic 'IAS 5' control. Likewise, the number of nodes and plant height at maturity of most of the transgenic plants did not differ from the control.

Concerning the yield evaluation, five transgenic progenies (169, 438, 497, 520, 1274) had a significant lower 100-seed weight, while the other progenies did not differ from the control. However, for the number of

seeds and total grain yield, no significant differences were detected among the transgenic progenies and the control. Thus, yield components measured for the majority of transgenic progenies, tested in the present study, were comparable to the parental cultivar IAS 5, suggesting retention of desirable market traits by these plant lines. Taking into account the majority of analyzed traits, the presence of *cryIAc* transgene did not affect the yield and the agronomic performance of the transgenic plants. Stewart Junior et al. (1996) reported similar results for soybean transgenic lines expressing a synthetic *cryIAc* gene. According to these authors, plants morphologically normal and fertile were produced in the greenhouse for four generations. Similarly, Miklos et al. (2007) showed no significant differences in agronomic performance (emergence, flowering, plant height, lodging, maturity and yield) of transgenic soybean plants containing a highly expressed *cryIA* gene. Maqbool & Christou (1999) also observed that in most rice transgenic plants, the expression of *cryIAc*, *cry2A*, *gna* (snowdrop lectin) and *hpt* genes did not affect the overall morphological phenotype. However, the authors verified that plants with very high protein levels occasionally showed developmental abnormalities, including sterility and stunted growth.

Cytological examination of cells showed that transgenic plants and controls presented normal karyotype ($2n = 40$) with no apparent chromosomal abnormalities. Singh et al. (1998) provided extensive cytological information on transformed soybean produced by bombardment of embryogenic suspension cultures. Various chromosomal aberrations, such as deletions,

Table 1. Agronomic performance of T₄ transgenic plants for initial development (ID), flowering date (FD), plant height (PH), number of nodes (NN), number of seeds (NS), 100-seed weight (100-SW) and total grain weight (TGW)⁽¹⁾.

Lines	ID	FD	PH	NN	NS	100-SW	TGW
44	2.33c	51.17abc	56.83b	12.33cd	120.83b	16.67ab	20.10ab
46	2.67c	47.17c	53.67b	12.33cd	125.50b	15.93abc	20.01b
162	3.17bc	47.17c	74.33ab	13.33abcd	204.33a	16.22ab	32.00a
169	3.17bc	47.00c	56.33b	11.83d	145.00ab	14.92bc	21.23ab
438	3.17bc	56.17abc	71.83ab	12.83abcd	141.83ab	15.24bc	21.44ab
497	3.00bc	49.00bc	67.67ab	12.67bcd	132.33ab	15.59bc	20.28ab
507	3.17bc	53.00abc	97.67a	15.33abc	164.33ab	16.65ab	26.69ab
520	3.00bc	58.50a	105.50a	15.33ab	140.50ab	14.01c	19.29b
551	3.50bc	57.00ab	107.00a	15.83a	137.33ab	16.20ab	21.74ab
937	3.33bc	59.33a	103.67a	15.00abc	153.17ab	16.21ab	22.96ab
1274	3.83b	50.83abc	68.00ab	12.83bcd	124.67b	15.56bc	19.05b
NT ⁽²⁾	4.67a	49.17bc	96.00a	15.33ab	162.33ab	17.66a	29.05ab

⁽¹⁾Means followed by the same letters, in the columns, do not differ from each other by Tukey's HSD test, at 5% probability. ⁽²⁾Nontransgenic 'IAS 5' control.

duplications, aneuploidy and tetraploidy, were described. That study showed that soybean genotypes differ in their susceptibility to chromosomal instability induced by tissue culture. Therefore, chromosome analysis can help eliminate chromosomally abnormal plants from transformation experiments.

For the *in vitro* detached-leaf feeding assay, isolated leaves were infested with neonate larvae of *A. gemmatilis*. After 24 hours, leaves from the homozygous *gusA* line and from the nontransgenic plants were completely defoliated (Figure 1). In contrast, consumption was significantly reduced in homozygous *cryIAc* transgenic leaves (Table 2 and Figure 1). Larvae fed on *cryIAc* transgenic leaves showed browning and

severe growth retardation, in comparison to larvae fed on control leaves. Considering the larval weight on nontransgenic leaves as 100%, the larvae reared on *cryIAc* leaves had greatly reduced weight (64 and 66% for 162 e 438 progenies, respectively). The fate of the larvae was monitored for two more days. Seventy-two hours after infestation, larvae reared on *cryIAc* leaves exhibited 100% mortality, compared with 1.65% larvae fed on leaves from both nontransgenic and *gusA* plants.

Insect damage in whole-plant feeding assay was similar to the detached-leaf feeding experiments, as control plants suffered a much greater amount of leaf damage than transgenic plants (Table 2 and Figure 2). The percent defoliation of the homozygous *gusA* line (732) and nontransgenic 'IAS 5' plants, fourteen days after the infestation, was, in average, 56.5 and 71.5%, respectively. However, almost no damage was observed on *cryIAc* transgenic progenies (162, 438).

Although experimental lines have been developed, *Bt* soybean cultivars have not yet been commercialized. A transgenic line of soybean Jack cultivar, expressing a synthetic *cryIAc* gene, caused complete *A. gemmatilis* larval mortality, in laboratory bioassays (Stewart Junior et al., 1996) and in artificially infested field cages (Walker et al., 2000). More recently, transgenic lines of soybean (A5547), expressing a synthetic *cryIA* gene, were evaluated for the resistance against several lepidopteran pests in screenhouse and in conventional field trials, in which *Bt* lines exhibited virtually complete efficacy against all pests tested (Macrae et al., 2005). Moreover, Miklos et al. (2007) reported the generation of three transgenic soybean lines via *Agrobacterium*-mediated gene transfer of a *cryIA* gene (*tic107*), which exhibited a high degree of resistance against lepidopteran pests (*Pseudoplusia includens*, *Helicoverpa zea*, and *A. gemmatilis*) in leaf disc and whole-leaf bioassays.

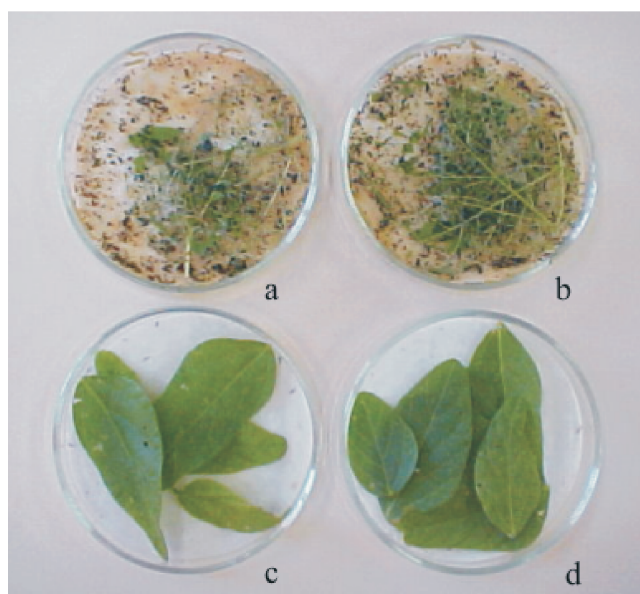


Figure 1. Detached-leaf feeding assay. Defoliation of nontransgenic controls 'IAS 5' (a) and *gusA* isolate (b), and *cryIAc* transgenic 162 isolate (c) and 438 isolate (d) leaves, 24 hours after infestation with *Anticarsia gemmatilis* larvae.

Table 2. Feeding assay of neonate larvae of *Anticarsia gemmatilis* on detached leaves and on whole plants⁽¹⁾.

Genotype	Leaf consumption	Larval weight (mg)	Mortality (%)			Defoliation (%)	
	24 h	24 h	24 h	48 h	72 h	Seven days after infestation (mean)	Fourteen days after infestation (mean)
162	1.0	0.40	90.8	98.3	100.0	0.0	0.0
438	1.0	0.39	85.0	95.0	100.0	0.0	0.0
732	3.7	1.56	0.8	1.6	1.6	60.0	71.5
NT ⁽²⁾	3.8	1.12	1.6	1.6	1.6	37.5	56.5

⁽¹⁾Feeding assay on detached leaves: twenty larvae were applied to detached leaves; leaf consumption scores: 1 (very low consumption), 2 (less than 50%), 3 (around 50%) and 4 (almost 100% consumption); whole-plant feeding assay: each plant was infested with 50 velvetbean caterpillar larvae; the percent defoliation refers to the percentage of leaves with consumed area higher than 50%. ⁽²⁾Nontransgenic 'IAS 5' plants.

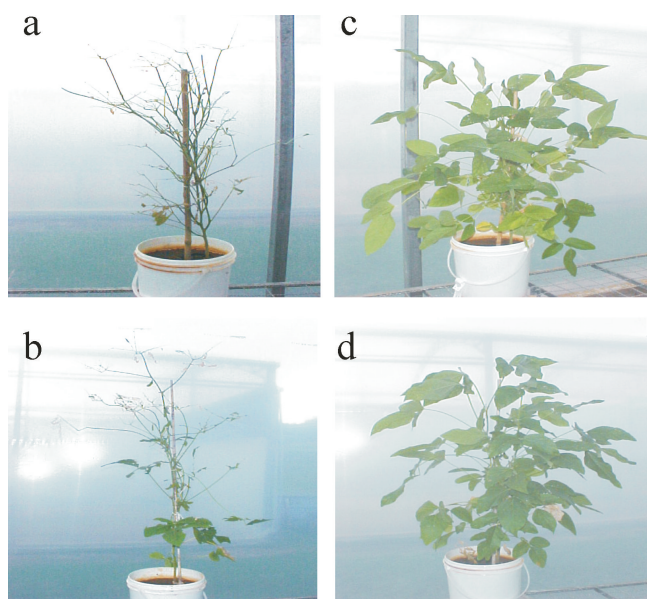


Figure 2. Whole-plant feeding assay. Defoliation of nontransgenic controls 'IAS 5' (a) and *gusA* isolate (b), and *cry1Ac* transgenic 162 isolate (c) and 438 isolate (d) leaves, 14 days after infestation with *Anticarsia gemmatilis* larvae.

The results obtained in the present study showed that the high level of resistance against velvetbean caterpillar is similar or even higher than that reported by Miklos et al. (2007).

Conclusions

1. The presence of the *cry1Ac* transgene doesn't affect the development of the majority of transgenic progenies tested and yield of soybean plants.
2. Transgenic plants present normal karyotype ($2n = 40$) with no apparent chromosomal abnormalities.
3. Progenies with *cry1Ac* are resistant to *Anticarsia gemmatilis*.

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