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GENETIC TRANSFORMATION AND HYBRIDIZATION

V. Marfà · E. Melé · R. Gabarra · J.M. Vassal
E. Guiderdoni · J. Messeguer

Influence of the developmental stage of transgenic rice plants (cv. Senia) expressing the *cry1B* gene on the level of protection against the striped stem borer (*Chilo suppressalis*)

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Abstract We carried out a comparative assessment of the resistance against the striped stem borer (*Chilo suppressalis*) of transgenic plants of a homozygous line (98-9) of rice (cv. Senia) harboring the *cry1B* endotoxin gene, at a vegetative stage (3–4 leaf stage) and two reproductive developmental stages (booting and heading-ripening). These three developmental stages match those attained by plants in paddy rice fields at successive infestations of striped stem borers in Northeast Spain. Transgenic and non-transgenic plants were infested with L2-stage larvae at these three stages and dissected after 17–21 days of infestation. In non-transformed plants, the larvae grew normally to L4-stage larvae and pupae, but in transgenic-plants the larvae died early in the infestation process. In addition, plant damage was very severe in control plants, while the transgenic plants appeared to be fully protected at all three of the developmental stages tested. Transgenic plants infested at the early vegetative stage developed more tillers than non-infested control-plants. This stimulatory effect was induced by larvae bites at the beginning of the infestation leading to a full recovery of young transgenic plants by compensatory growth. Therefore, transgenic Senia plants expressing the *cry1B* gene are protected against striped stem borer pest throughout plant development.

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V. Marfà (✉) · E. Melé · J. Messeguer
Plant Genetics Department,
Institut de Recerca i Tecnologia Agroalimentaria (IRTA),
Centre de Cabrils, Carretera de Cabrils s/n,
Cabrils 08348, Barcelona, Spain
e-mail: victoria.marfa@irta.es
Tel.: +34-93-7507511, Fax: +34-93-7533954

R. Gabarra
Plant Protection Department,
Institut de Recerca i Tecnologia Agroalimentaria (IRTA),
Centre de Cabrils, Carretera de Cabrils s/n,
Cabrils 08348, Barcelona, Spain

J.M. Vassal · E. Guiderdoni
BIOTROP and Crop Protection Programmes,
CIRAD, TA40/03, 34398 Montpellier Cedex 05, France

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Introduction

To date, transgenic crops have been developed as a means for improving product quality and for controlling disease, weed and insect problems. A large number of these transgenic crops express genes translocated from *Bacillus thuringiensis* (*Bt*) that produce insecticidal crystal proteins in their plant tissues. *Bt* is a gram-positive soil bacterium that expresses a bio-activity against a wide range of insects. On the whole, the various *Bt* strains produce several toxins, each of which shows a rather narrow host range (Frutos et al. 2000). These different *Bt* proteins are highly toxic to lepidopteran, dipteran, and/or coleopteran insects, among which are economically important pests of rice such as the striped stem borer (*Chilo suppressalis*), the yellow stem borer (*Scirpophaga incertulas*) and leaffolders (*Cnaphalocrocis medinalis* and *Marasmia patnalis*).

The advantages of using *Bt* toxin-producing transgenic plants over conventional *Bt* spray application are so significant that several *Bt* plants are already available commercially. The conventional *Bt* sprays lack persistence in the field because of their photosensitivity and their potential to only protect the plant surface. In addition, the commercial growing of *Bt* plants provides some important environmental benefits due to the absence of chemical pesticides and, consequently, the absence of residual pesticide in the soil as well as no effects on non-targeted species.

A long list of more than 30 genes have so far been transferred to japonica and indica varieties of rice, including genes for fungal, viral, bacterial and insect disease resistance as well as genes which affect nutritional qualities. These genes are involved in metabolic pathways, marker genes and abiotic stresses (Khush et al. 1999). Within the group of insect resistance genes, *Bt cry1Ab* and *cry1Ac* (Fujimoto et al. 1993; Wünn et al. 1996;

Ghareyazie et al. 1997; Nayak et al. 1997; Cheng et al. 1998; Datta et al. 1998; Alam et al. 1999), *cry2A* (Maqbool et al. 1998; Kota et al. 1999) and a novel synthetic *Bt* gene, *cry1B* (Breitler et al. 2000), have been successfully introduced and expressed in rice, although *Bt* rice varieties are not yet commercially available to farmers.

The *Bt* transgenic rice plants transformed with the different *cry* genes are protected against lepidopteran pests of rice. The *Bt cry* genes have been synthetically modified to increase their expression in plants. Cheng et al. (1998) reported that the fully modified (plant codon optimized) versions of the two synthetic *cry1Ab* and *cry1Ac* genes were effective against both striped stem borer and yellow stem borer in nine rice cultivars tested. Nayak et al. (1997) reported that transgenic indica rice IR64 *cry1Ac* plants were protected against the yellow stem borer. Wünn et al. (1996) used a synthetic version of the *cry1Ab* gene to transform indica rice line IR58 and demonstrated that these plants were resistant to yellow and striped stem borers as well as against the two leaffolders *Cnaphalocrocis medicinalis* and *Marasmia patnalis*, even though the mortality levels were not as high as in the case of the stem borers. Ghareyazie et al. (1997) transformed aromatic rice Tarom Molaii with the synthetic *cry1Ab* gene and showed that the transgenic plants were resistant against both stem borers, and Alam et al. (1999) reported that transgenic elite IRRI maintainer line IR68899B plants transformed with *cry1Ab* were also resistant against the yellow stem borer.

Breitler et al. (2000) recently reported that a novel synthetic *cry1B* gene is capable of protecting Mediterranean rice (cvs. Senia and Ariete) against the L2- to L4-stage striped stem borer larvae. This *cry1B* endotoxin exhibited a tenfold lower lethal concentration 50 (LC_{50}) than *cry1Ac* in a striped stem borer diet incorporation assay.

In rice fields, striped stem borers complete two to three complete generations per year under Mediterranean climatic conditions. The emerging adults from the first generation lay eggs very rapidly. The larvae feed on young leaves and tend to move from plant to plant in search of good stems where they can stay and grow. The larvae from the second generation do not move from plant to plant but penetrate rapidly into the stems. Some of these larvae hibernate, while others give rise to a third generation.

The plants attacked by striped stem borers in rice fields exhibit different symptoms depending on the developmental stage of the plant. During the first infestation, while the plants are in a vegetative stage, the larvae damage the growing tillers and provoke symptoms called deadhearts. The second natural infestation generally occurs at the reproductive stage of the plants, and the damage produced by the growing larvae blocks the transport of nutrients from the stem to the grain, resulting in whitehead symptoms (formation of panicles but with the grains empty). The third infestation can also provoke damage, especially when susceptible varieties with long crop cycles are cultivated. For these reasons, these two or sometimes three generations of striped stem borer infestations are responsible for yield losses that can reach 15–20% each year. The presence of *Bt* transgenic plants in the field that are able to express their own insecticide in their tissues would considerably decrease these losses. However, the level of acquired resistance against the insect should remain stable throughout plant development in order to protect the rice plant from the three successive infestations.

To address this question, we infested T2 homozygous *cry1B*-resistant plants at one vegetative stage and two reproductive developmental stages that coincide with the three natural field generations of striped stem borer. These infestations were conducted under greenhouse conditions. The transgenic Senia line (line 98) chosen for the experiments has been described as being fully resistant to striped stem borer larvae in insect feeding assays at the tillering stage (Breitler et al. 2000). L2-stage striped stem borer larvae were used for infestation, and the plants were analyzed 17–21 days following infestation to determine the survival and growth of the insects. We report here our investigation on the stability of the resistance of Senia transgenic plants expressing the *cry1B* gene against striped stem borers throughout plant development.

Materials and methods

Plant material

Mature seeds of the japonica cultivar Senia from the Delta de l'Ebre area (Tarragona, Spain) were used as starting material. The mature seeds were dehusked and surface-sterilized first with 70% ethanol for 1 min followed by a commercial bleach-Clorox solution (1.6% active chloral) for 30 min, and then rinsed three times with sterilized water (Pons et al. 2000).

Culture media and transformation procedure

The culture media and the particle gun bombardment transformation procedure used were as described in Breitler et al. (2000).

Plasmid constructs

The *cry1B* gene was synthesized by asymmetric polymerase chain reaction and cloned in a *Hin*dIII site of pAHC17 to make the *pUBIcry1B* plasmid (Breitler et al. 2000). The 5.1-kb pILTAB227 plasmid has the selectable gene *hph* encoding hygromycin phosphotrasferase driven by the CaMV 35S promoter with a duplicated enhancer sequence (Breitler et al. 2000).

Analysis of transgenic plants

Western blots

Fresh leaf material was ground in an extraction buffer (Breitler et al. 2000). Total protein concentration was determined using the method recommended by BioRad (Hercules, Calif.), and 60 mg of the total protein was loaded per lane. The proteins were separated on an 8% acrylamide gel and then transferred to a nitrocellulose membrane. After washing and blocking, the membrane was treated with the rabbit *Bt* kurstaki antibody overnight at 40 C. The *cry1B* protein bound to the membrane was detected by an alkaline phosphatase anti-rabbit *Bt* antibody.

Selection of a striped stem borer homozygous resistant line

Following the transformation of Senia embryogenic nodules by particle bombardment, different transformation events were obtained that expressed the *cry1B* protein (Breitler et al. 2000). The resistance of these plants to striped stem borer (*Chilo suppressalis*) larvae was analyzed by bioassay with T1 plants. Two lines were fully protected against the insect. A homozygous line from event 98 was chosen for the infestation experiments; this line was obtained as follows: T1 seeds from the resistant line 98 were collected, dehusked, surface-sterilized and cultured on MS (Murashige and Skoog l962) standard medium supplemented with hygromycin for germination. The germinated plants were transferred to the greenhouse and grown until harvest, at which time the T2 seeds were collected. The same procedure was followed for germinating the T2 seeds with the difference that the T2 seeds were first germinated on MS medium without antibiotics and then transferred to MS with hygromycin (40 mg/l) when the plantlets reached 2–3 cm in length. Both live and dead plantlets were scored. After analyzing 42 T2 seeds of line 98-9 – all of which germinated and grew in MS with 40 mg/l hygromycin – we concluded that this line was homozygous and subsequently used it for the infestation experiments.

The substrate used for germinating the seeds directly as well as for the plants grown in the greenhouse was composed of peat and vermiculite (2:1; v/v) enriched with osmocote (1 g/l of substrate) and $CaCO_3$ (2 g/l peat) to neutralize the pH of the substrate.

Developmental stage of the plants to be infested

Both T2 homozygous plants from transformed event 98-9, and negative control plants were used for larval infestation at three different developmental stages: (1) young plants with only three to four leaves; (2): plants at the booting stage (development of the panicle); (3): plants at the heading-ripening stage (spikes already formed); these three stages are illustrated in Fig. 1. To be able to infest these plants simultaneously with L2-stage larvae, we planted 20 seeds from each group in the greenhouse 4, 12 and 16 weeks before infestation.

The reasoning for selecting these specific plant stages for the infestation experiments was based on data collected at the Agrupació de Defensa Vegetal of Delta de l'Ebre (Tarragona) during the years 1998–2000 (personal communication). During this time, the periods of natural emergence of *C. suppressalis* adults were very consistent. The first generation starts at the beginning of June; the adults from the second generation appear at the beginning of August and the third generation occurs at the beginning of September. At the time of the three natural infestations, the plants are normally 4, 12 and 16 weeks old, respectively, and therefore at the stages we used to mimic the field conditions.

Insect feeding assays

Transgenic (line 98-9) and control plants were subjected to infestation with three L2 striped stem borer larvae per plant. The three larvae were applied to the same leaf on one tiller of each plant, and the infested plants were kept in the greenhouse. The transgenic plants and the control plants were placed into two different compartments to avoid any cross-larval contamination. After 17–21 days, the plants were dissected and the live larvae and pupae scored. The number of total and damaged tillers per plant was also recorded to evaluate the damage produced by the larvae.

Chilo suppressalis rearings

The *C. suppressalis* population was reared on an artificial diet as described in Breitler et al. (2000). The eggs from *C. suppressalis* were sent to IRTA; Cabrils from CIRAD, and second-instar (L2) SSB larvae were used for insect feeding assays.

Fig. 1 The three different developmental stages of the plants used for infestation with striped stem borer larvae. *1* Plant stage 1, young plants with three to four leaves; *2* plant stage 2, plants displaying panicle formation (booting stage); *3* plant stage 3, plants having spike formation (heading-ripening stage)

Data analysis

The data were analyzed using proc GLM (General Lineal Model procedure) of the SAS version 6.01 (SAS, Raleigh, N.C.) for Windows 98

Results and discussion

Analysis of infested transgenic and control plants

The three different plant developmental stages chosen for investigation – which simulated the stages attained by field-grown plants when pest attack occurs– were analyzed on both non-transformed control and transgenic plants after 17–21 days of insect infestation. The homozygous transgenic line used (98-9) had integrated a single expressed copy of the *Ubicry1B* gene construct (Breitler et al. 2000; Southern analysis), and the expression of the *Ubicry1B* gene could be demonstrated by Western analysis (Fig. 2).

Overall, 90 Senia plants kept in the greenhouse were infested with a total of 270 L2 striped stem borer larvae. Of these, 15 plants of each group (transgenic and non-transgenic) and from each stage of development (Fig. 1) were infested with three L2 larvae per plant. After 17–21 days, the plants were carefully dissected and the larvae or pupae recovered. The results of these infestation experiments indicating the average number of live L2- and L4-stage larvae or pupae recovered per plant are presented in Table 1. L4-stage larvae and pupae were only found alive on non-transformed plants. The average weight of the L4-stage recovered larvae was 67±17 mg. Both the L4-stage larvae and the pupae were kept longer

Table 1 Analysis of plants infested with striped stem borer L2-stage larvae. The plants were infested with three L2-stage larvae per plant, and after 17–21 days the plants were dissected and the number of live L2- and L4-stage larvae and pupae scored. The data represent the mean number of live larvae or pupae recovered per plant with *n*=15 plants infested per treatment

	Plant stage 1[a]		Plant stage 2[a]		Plant stage 3[a]	
	Control[b]	Transgenic[b]	Control	Transgenic	Control	Transgenic
Recovered L2-stage larvae (mean number ± SE)	0	0	0	0.73±0.25	0	0.53±0.24
Recovered L4-stage larvae (mean number ± SE)	0.46±0.13	0	1.80±0.30	0	1.86±0.35	0
Recovered pupae (mean number ± SE)	0.06±0.07	0	0.66±0.13	0	1.06±0.33	0

[a] *Plant stage 1* Young plants with three or four leaves; *plant stage 2* plants displaying panicle formation; *plant stage 3* plants that had already formed spikes

[b] *Control plants* Non-transformed Senia plants; *transgenic plants* T2 homozygous Senia plants transformed with the *pUbicry1B* gene

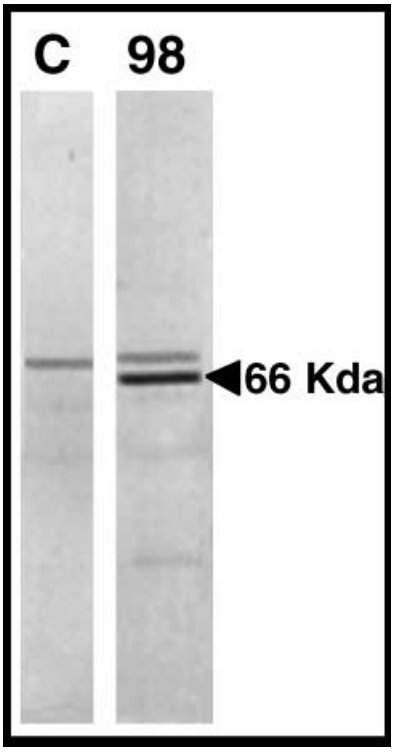

Fig. 2 Western analysis of Senia non-transformed control (*C*) and resistant line (*98*) plants transformed with the *cry1B* gene. The *arrow* shows the size of the cry1B protein

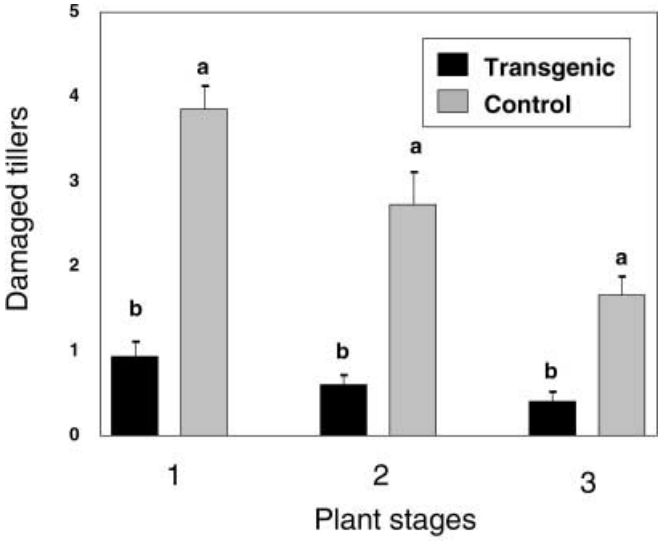

Fig. 3 Number of damaged tillers per plant at the different plant stages. Damaged tillers were considered to be those damaged by the larvae after 17–21 days of infestation with three L2 larvae per plant. *n*=15 plants infested per treatment. Plant stages as defined in Fig. 1. *Control* Non-transformed Senia infested plants, *transgenic* homozygous T2 Senia infested plants transformed with the *cry1B* gene. Within each plant stage, treatments with the *same letter* do not differ significantly at *P*=0.05 using the multiple range Duncan test

in the laboratory until adult emergence. These adults were able to lay fertilized eggs, indicating that they had normal growth and were fertile. A comparison of the three developmental stages of the control plants indicated that the percentage of recovered larvae (L2+L4) and pupae increased as the plant matured, from 17.8% in stage 1 to 83% in stage 2 and 97.8% in stage 3.

Conversely, no L4-stage or pupae were recovered at all during the three different developmental stages of the transgenic Senia plants of event 98. Only a few L2-stage larvae were recovered on plants at developmental stages 2 and 3 (Table 1), but these were very weak and small.

When the scored values were averaged over the three plant stages, the percentage of larval mortality in control plants and transgenic plants was 34% and 85.9%, respectively. The latter value was due to the few, non-viable L2-stage larvae recovered. However, when the recovery of normally developed insects (L4 and pupae) was taken into account, the percentage of larval mortality in transgenic plants reached 100%. It is important to mention that only well-developed larvae will be able to emerge into adults that will lay fertilized eggs.

Damage observed on infested plants at the three developmental stages

To evaluate the deleterious effects of the larvae on the plants, we scored the number of damaged tillers on each infested plant (Fig. 3). The number of damaged tillers in infested control plants was found to decrease as the young plant developed into the adult stage. The frequen-

cies of damaged tillers was 91.2%, 48%, and 18.9% at developmental stages 1, 2 and 3, respectively, and these values were indirectly correlated with the number of L4-stage and pupae recovered on the control plants (Table 1). The leaves of stage-1 plants were too small to serve as an adequate source of food for the three L2-stage larvae over the incubation period of 17–21 days, thereby initially provoking larval movement to the surrounding tillers and, finally, causing the larvae to quit the plants, which led to their death. For this reason, nearly all of the stage-1 control plants were completely destroyed. In the adult plants, fewer tillers were damaged because the larvae tended to stay inside the initial stem with enough food to eat during the whole period of infestation. The typical whitehead symptoms on non-transgenic plants infested with striped stem borer larvae were detected on stage-2 and -3 plants.

Conversely, the proportion of damaged tillers scored on transgenic Senia plants was 75–80% lower than that observed on control plants at the three developmental stages: 16.9% damaged tillers at stage 1, 11.4% at stage 2 and only 5% at stage 3.

In averaging the results observed at the three plant stages, the percentage of damaged tillers in non-transformed plants was 52.7%, while that of transgenic plants was only 11.1%.

The total number of tillers produced per plant was also analyzed at each plant stage (Table 2). To analyze this parameter, we scored the total number of tillers of 15 non-infested Senia control plants from the three stages. As presented in Table 2, an interesting effect of larval attack on young plants became apparent. The total number of tillers in infested transgenic plants appeared to be significantly higher than that of the non-infested plants, meaning that the limited damage inflicted by the L2-stage larvae triggered tiller production in the transgenic plants. The consequences of the larval attacks remained visible in the infested leaves of the transgenic plants, but more tillers were produced to balance this first loss. These plants had recovered quite well after the 17–21 days as the larvae did not survive and probably died early during the infestation process. Interestingly, this stimulating effect was not observed at developmental stages 2 and 3 of the transgenic-plants. More importantly, there were no morpho-physiological differences between control and transgenic plant development (Fig. 4).

Fig. 4 Comparison of non-transformed Senia plants with transgenic Senia plants (line 98) transformed with the *Ubicry1B* gene with infested versus non-infested plants after 17 days of infestation of plants with three L2 larvae per plant

Table 2 Number of total tillers per plant. The number of total tillers per plant of the infested control and transgenic plants was scored after 17–21 days of infestation with three L2-stage larvae per plant and *n*=15 plants per treatment. Plant stage is as defined in Table 1 (*SE* standard error)

	Non-infested[a]	Control[a]	Transgenic[a]
Plant stage1[b] (mean number ± SE)	4.80±0.29b	4.33±0.35b	6.06±0.59a
Plant stage2 (mean number ± SE)	5.93±0.38a	5.86±0.5 a	6.06±0.47a
Plant stage 3 (mean number ± SE)	8.80±0.38a	9.20±0.5 a	8.26±0.75a

[a] *Non-infested plants* Non-infested, non-transformed Senia plants; *control plants* non-transformed, infested Senia plants; *transgenic plants* T2 homozygous infested Senia plants transformed with the *pUbicry1B* gene (line 98)

[b] Within each plant stage, treatments with the same letter do not differ significantly at *P*=0.05 using the multiple range Duncan test

Conclusion

Senia transgenic plants expressing the novel *cry1B Bt* gene are protected against the rice insect pest striped stem borer. A homozygous line expressing the *cry1B* gene in a constitutive manner was fully protected against second-instar striped stem borer larvae even after 17–21 days of infestation and also during early to adult developmental stages. In the three plant developmental stages tested for infestation, the control plants were severely damaged while the transgenic-plants were protected. In addition, live L4-stage larvae and pupae were recovered at all developmental stages of the non-transformed plants, while only few L2-stage larvae were recovered on transgenic-plants at developmental stages 2 and 3. We recorded a compensatory growth effect in transgenic plants at an early developmental stage. The total number of tillers in young transgenic plants with only three to four leaves was higher than that in non-infested control plants. This stimulatory effect was induced by the initial attacks of the L2-stage larvae – the larvae died during the first days of the larval infestation, and the young plants had enough time to balance the damaged tillers by producing new ones. It would be worthwhile to check whether, under field conditions, the increase in tiller number observed in young transgenic plants is still significant at a mature developmental stage

and leads to an overall yield increase, unless a compensatory effect simultaneously decreases the productivity per tiller.

The results presented here with the Senia *cry1B* transformed plants contrast those recently reported by Alinia et al. (2000), who also studied the influence of the age of transgenic aromatic rice expressing a synthetic *cry1Ab* gene on the level of resistance against stem borers. In this latter study, plants expressing the *cry1Ab* gene were found to be more resistant than the control plants only at the vegetative stage and not at the flowering stage. Moreover, the differences observed at the vegetative stage were only significant when neonate larvae were used. When plants at any other developmental stage were infested with 10-day-old larvae, the differences between control and transgenic plants became non-significant. Conversely, in our case, the Senia *cry1B* plants were resistant against L2-stage infestation at all of the developmental stages studied, which included one vegetative stage and two reproductive stages. Alinia et al. (2000) used the *cry1Ab* gene under control of the maize phosphoenolpyruvate carboxylase (PEPC) promoter, while in our case we used the *cyr1B* gene under the maize Ubiquitin (Ubi) promoter. Matsuoka et al. (1994) found that the PEPC promoter was not active in rice stems, and Andow and Hutchinson (1998) observed an important decline of toxicity in mature maize plants transformed with the *PEPCcry1Ab* gene. Also, in recent field assays of maize *Bt* plants carried out in Northeast Spain, Serra and López (2001) observed that commercial maize *Bt* variety Compa CB was less resistant against the borers in the late reproductive stages than in the vegetative stages. The lack of stability of gene expression in transgenic plants can provoke a resistance breakthrough and, as the plant matures, the expression of the transgene can diminish and, consequently, the new character can be lost. In our experiments with the *Ubicry1B* Senia plants, however, expression of the gene seemed to be stable and did not change during the development of the plant. In this sense, Breitler et al. (2000) also demonstrated the stability of expression of the *Ubicry1B* gene over the T2–T4 generations in leaf tissue 20, 40, 70, and 90 days after germination on Ariete rice transgenic plants.

Therefore, the expression of the *cry1B* gene directed by the maize ubiquitin promoter in Mediterranean Senia rice appears to be a good strategy for obtaining a transgenic crop reliably protected from the two to three infestations of the striped stem borer naturally occurring in paddy fields.

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