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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Expression of a *Bacillus thuringiensis cry1B* synthetic gene protects Mediterranean rice against the striped stem borer

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Abstract We investigated the expression in transgenic rice of a synthetic gene encoding the toxic part of the *Bacillus thuringiensis* Cry1Ba endotoxin, which was shown to exhibit a tenfold lower lethal concentration 50 (LC50) than Cry1Ac in a Striped Stem Borer (SSB) diet incorporation assay. The 1.950-bp synthetic *cry1B* gene, possessing an overall GC content of 58 %, was cloned under the control of the maize ubiquitin promoter first intron and first exon regions. The resulting vector, designated as pUbi-*cry1B,* was transferred to two commercial Mediterranean cultivars of rice, Ariete and Senia, using microprojectile acceleration-mediated transformation. Thirty-two and 47 T0 events were generated in cvs. Ariete and Senia, respectively. Southern blot and immunoblot analyses allowed the identification of 7 Senia and 1 Ariete events harbouring both an intact gene cassette and expressing Cry1B at a level ranging from 0.01 % to 0.4 % of the total soluble proteins. Three Senia and 1 Ariete events were found to be protected against second instar SSB larvae in whole plant feeding assays, exhibiting 90–100 % mortality 7 days after infestation. Spatial and temporal variation in transgene expression was further examined in resistant event 64 of cv. Ariete. Stable accumulation of Cry1B, representing 0.4 % of the total soluble proteins, was observed over the T2 to T4 generations in leaf tissue 20, 40, 70 and 90 days after germination in both young and old leaves and in internodes. Ariete event 64 was found to be fully protected from attacks of third and fourth instar SSB larvae over subsequent generations.

Key words *Bacillus thuringiensis* · *cry1B* · Insect resistance · *Oryza sativa* L. · Synthetic gene · Transgenic plants

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Introduction

The Striped Stem Borer (SSB) (*Chilo suppressalis* Walker) of Asian origin is one of the major constraints affecting rice production in southern European countries, causing yield losses reaching up to 15–20 % in Spain and France. This problem has been addressed by means of chemical treatments with pyrethrenoids and sprays with *Bacillus thuringiensis*(*Bt*) insecticidal formulations. However, the former has proven to be environmentally damaging and the latter of limited efficiency due to their poor persistence and the biological localization of the pest, which develops in the culm. Conventional breeding for SSB tolerance has also proven to be difficult due to the polygenic control of the trait (Khush and Brar 1991), and no gene for host resistance has been mapped (Bennett et al.1997). An alternative is the deployment of engineered rice cultivars adapted to local growth conditions and consumer requirements that harbour one or several -endotoxin genes from the soil bacterium *Bt,* which encodes insecticidal proteins active against the SSB. The production of transgenic plants harbouring synthetic *cry1A* genes driven by constitutive promoters and exhibiting full protection against insect attacks [SSB, yellow stem borer (YSB)-*Scirpophaga incertulas* and leaffolder-*Cnaphalocrocis medinalis*] has been extensively reported in *japonica*(*cry1Ab*: Fujimoto et al. 1993, Cheng et al. 1998; *cry1Ac*: Cheng et al. 1998) and *indica*(*cry1Ab*:Wünn et al. 1996, Datta et al. 1998; *cry1Ac*: Nayak et al. 1997) rices. These studies were aimed at obtaining a high level of expression of the insecticidal genes in the transgenic rice plant. The

delivery of a high dose of toxins combined with the presence of refuges in the *Bt* crops is considered by many entomologists as the most promising strategy to prevent or delay resistance build up in the target pest population (Frutos et al. 1999).

As mentioned above, the expression of genes whose efficacy have been assessed it. in rice has so far been limited to *cry1Ab* and *cry1Ac*, which might not encode the toxins displaying the highest activity against the SSB. A diet incorporation assay involving seven *Bt* toxins has shown that the toxin exhibiting the highest toxicity towards SSB larvae is Cry1Aa, followed by Cry1B and Cry1Ac (Fiuza et al. 1996). As in transgenic plants insect mortality results from both a high dose delivery and specific toxicity of the toxin on the target pest, there is a need to engineer novel synthetic *Bt* genes encoding the most active toxins that have been shown to cause high mortality in SSB diet incorporation assays. This is particularly true for the SSB, which appears to be far less sensitive to *Bt* toxins than YSB and leaffolder (M. Cohen, personal communication). Very recently, the expression of a *cry2A* synthetic gene in transgenic *indica* rice has proven efficient in fully controlling YSB and leaffolder (Bano-Maqbool et al. 1998). However, this toxin has also been reported to be the least effective among those exhibiting toxicity to SSB (Fiuza 1996; Aguda et al. 1997)

The *cry1Ba* gene, which was isolated from *Bt* strain HD2 by Brizzard and Whiteley (1988), encodes an endotoxin whose protoxin and toxic regions share only 55.5 % and 34.8 % amino acid homology with Cry1Ac, respectively. The sequence from nucleotides 1–1944 encoding the active toxin plus 29 amino acids at the N-terminal end of the wild-type *cry1Ba* gene was fully modified to optimize its expression in monocotyledonous crops, as reported previously (Bohorova et al. 1999). The GC content of the resulting 1944-bp modified gene is 58 %, while that of the native *cry1B* is 39.3 %.

The objective of the study reported here was to attain full protection of Mediterranean rice against SSB through the constitutive expression of this novel, monocot codon-optimized *cry1B* synthetic gene. We first reinvestigated the toxicity of Cry1B relative to that of Cry1Aa and Cry1Ac in a diet incorporation assay of a newly field-collected SSB population that will serve for bioassay material. The maize ubiquitin promoter region (Christensen and Quail 1995) was then fused to the *cry1B* coding sequence and the resulting construct transferred to two Mediterranean rice cultivars that are susceptible to SSB attack. We further examined the level of protection afforded by expression of the *cry1B* gene.

Materials and methods

Chilo suppressalis rearings and diet incorporation assays

A field-collected *Chilo suppressalis* population was reared on an artificial diet as previously described (Bordat et al. 1977). Lethal concentration (LC)50 bioassays were performed by spreading 100 µl of various concentrations of Cry1Aa, Cry1B or Cry1Ac *Bt* toxin solutions in PBS buffer onto a 5-mm-deep layer of artificial medium contained in 1-cm^2 individual square wells. Excess liquid was allowed to dry under a laminar flow hood. For each toxin dose, forty 10-day-old L2 larvae were individually placed on the artificial media with a small paintbrush. Percentages of mortality were recorded 7 days after release. Data were analysed using a specific software (WINDL 1998) according to the Probit method (Finney 1971), corrected for the estimation of natural mortality (Dempster et al. 1977; Hasselblad et al. 1980).

Plant material

Transformation was carried out using two japonica (isozyme group VI according to Glaszmann 1987) rice (*Oryza sativa* L.) varieties, Ariete and Senia. Ariete and Senia are two top commercial cultivars in France and Spain and are highly susceptible to SSB (*Chilo suppressalis*) attacks. Mature seed embryos were used to induce callus cultures, which subsequently served as material for particle bombardment according to Chen et al. (1998b).

Transformation vectors

The synthetic *cry1B* gene was cloned under the control of the promoter and the entire 5′ untranslated region of the maize polyubiquitin gene *Ubi-1*(promoter-exon1-intron1) and the *nos* 3′ polyadenylation sequence into the *Bam*HI site of plasmid pAHC25 (Christensen and Quail 1995) deleted for the *uidA* coding sequence. A *Hin*dIII fragment containing the Ubi-*cry1B*-nos gene cassette was inserted into the *Hin*dIII restriction site of pBKS (Stratagene), resulting in the 7.19-kbp pUbi-*cry1B* plasmid. The 5.1-kbp plasmid pILTAB227 consisting of the CaMV35S promoter with a duplicated enhancer sequence (e35S) controlling the hygromycin phosphotransferase *hph* gene and followed by the *nos* 3′ terminator (kindly supplied by Dr. C. Fauquet, ILTAB, La Jolla, Calif.) was used as the selectable construct in the microprojectile bombardment experiment. Schematic representation of the pUbi-*cry1B* plasmid is provided in Fig. 1. All plasmids were prepared according to Birnboim and Doly (1979), purified over a CsCl gradient and resuspended in water at a concentration of 1 µg/µl.

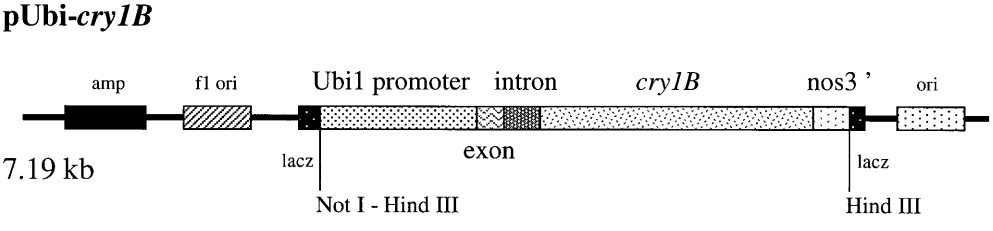

Fig. 1 Diagrammatic representation of the pUbi-*cry1B* plasmid

Production of transgenic rice plants

The pUbi-*cry1B* construct was introduced into embryogenic calli by microprojectile bombardment according to the procedure described in Chen et al. (1998b) and using the PDS1000/He particle gun device (BioRad, USA). The pUbi-*cry1B* and pIL-TAB227 constructs were mixed using a 4:1 molar ratio, and 5 μg of mixed plasmid DNA served for coating 3 mg of gold particles, which was enough for bombarding two bombardment plates twice. A total of 164 and 718 seed embryo primary callus-derived embryogenic units, representing 2 and 8 plates, were bombarded in cvs. Ariete and Senia, respectively. Thirty-two and 75 (19.5 % and 10.4 %) embryogenic units yielded resistant calli that regenerated transgenic plants in cvs. Ariete and Senia, respectively. The procedures followed for evolving transgenic rice plants based on resistance to hygromycin were those of Chen et al. 1998b.

Southern blot analysis

Total genomic DNA was extracted from leaf tissue of transgenic and control plants using the CTAB method (Hoisington 1992). Five μg of DNA was digested with the appropriate restriction endonucleases, and DNA fragments were separated on 0.8 % agarose gels and then transferred to nylon membranes (Hybond N+, Amersham) according to Southern (1975). For the hybridizations, the 1.9-kbp fragment of the *cry1B* coding sequence served as template for synthesizing α-[^{32}P] labelled probes through random priming. Following hybridization, the membrane was washed twice for 10 min at 65 °C (first in 2× SSC and 1 % SDS and then in 0.1× SSC and 0.1 % SDS) and analysed by autoradiography

Western blot analysis

Proteins were extracted from frozen leaf blade, leaf sheath and pith tissues. For pith tissues, grinding was performed in liquid nitrogen for better protein extraction. The extraction buffer consisted of 50 m*M* TRIS-HCl, pH 8.0, 1 m*M* EDTA, 5 % (v/v) glycerol, 1 m*M* DTT and 0.1 % Triton X100. The extract was centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatant was retained, and the protein concentration was determined according to Bradford (1976). Fifty-microgram aliquots of protein, unless otherwise stated, from several plant extracts were subjected to SDS-PAGE on 8 % acrylamide gels. The positive control was obtained by adding 50 ng of trypsinated Cry1B toxin to 50 μg of total soluble leaf protein extracts isolated from an untransformed rice plant. After electrophoresis, the protein was electroblotted onto a nitrocellulose filter (Bio-Rad) and the blots were incubated with immunoaffinity-purified rabbit antibodies specific for the Cry1B protein. Detection was achieved using goat anti-rabbit IgG linked to alkaline phosphatase NBT-BCIP.

Insect feeding assays

T0, T3 and T4 Ariete plants, T1 Senia pUbi-cry1B plants and control plants were assayed for resistance to the SSB. For the T0 generation, the hygromycin-resistant Ariete plants were propagated to make several copies of the same transformation event prior to the transfer to peat pellets and to the phytotron (60 % humidity, light intensity of 600 μmol m^{-2} s^{-1} over 11.5 h) together with control, untransformed regenerated plants. Each individual plant was infested with 5L2 SSB larvae after a growth period of 3 weeks. For T1 Senia and T3 and T4 Ariete plants, bioassays were carried out on adult plants at the tillering stage. Depending on the generation under study, 5–10 second instar (L2) SSB larvae, unless otherwise stated, were applied to a tiller of each plant. Plants were grown under phytotron conditions with pots individually wrapped in a plastic foil cylinder or placed in a nylon mesh cage. After 4 or 7 days, plants were first screened for insect leaf and pith damage, then dissected, starting with the infested tiller, to determine the number of surviving larvae and their developmental stage. Insect antibiosis was assessed by the presence/absence of head capsules and sloughs, recovery of living larvae and developmental stage and body weight of living larvae.

Results and discussion

LC50 determination of Cry1Aa,Cry1B and Cry1Ac toxins

Establishment of LC curves on reared L2 SSB larvae showed that LC50 of Cry1B (0.34 μg/cm^2) and Cry1Aa (0.1 μg/cm^2) were tenfold and 33-fold, respectively, lower than that of Cry1Ac (3.3 μg/cm^2) in diet incorporation assays (Fig. 2). These results are fully consistent with those reported previously using another reared SSB population (Fiuza et al. 1996) and confirms that Cry1Ac is not the most active toxin against SSB.

Characterization and evaluation of Senia transgenic plants

Southern blot analysis of total DNA isolated from 47 Senia T0 plants showed that all the hygromycin-resistant plants had cointegrated the *cry1B* transgene and were independent transformation events. These events exhibited various levels of complexity in the hybridization patterns, ranging from only a few copies of the plasmid integrated at a single insertion site (e.g. events 98, 107, 109 and 117 in Fig. 3a) to multiple sites of integration of both intact and rearranged copies. To determine the range of expression levels of the *cry1B* transgene in the regenerated plant population, we performed Western blot analysis with extracts obtained

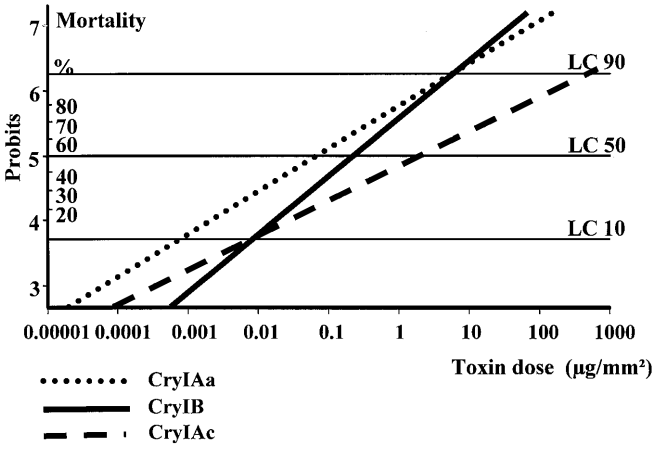

Fig. 2 Lethal concentration (LC) 50 curves of L2 SSB larvae in diet incorporation assay

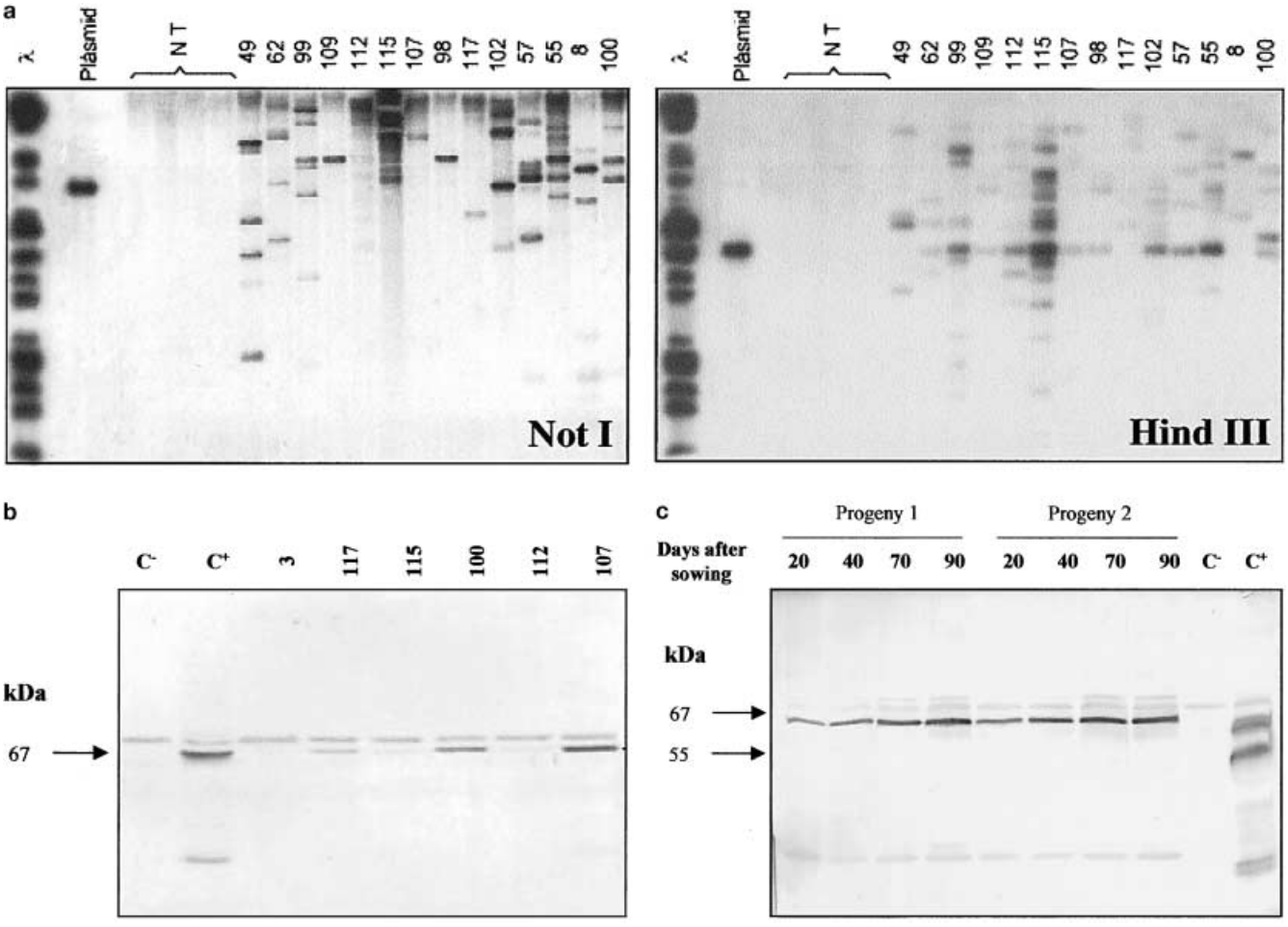

Fig. 3 **a** Southern blot analysis of total genomic DNA of T0 plants of cv. Senia digested by *Not*I (single cut) and *Hin*dIII (double cut releasing the gene cassette of the pUbi-*cry1B* plasmid) and hybridized with the *cry1B* probe. **b** Immunoblot analyses of 6 T0 events of cv. Senia (*C-* protein extract of leaf tissue of an untransformed rice plant, *C+* protein extract of leaf tissue of a transformed rice plant shown to accumulate Cry1B at 0.4 % in a reconstruction assay). **c** Accumulation of the Cry1B toxin in the antepenultimate leaf blade tissue of 2 T3 progeny plants of event A64 of cv. Ariete, 20, 40, 70 and 90 days after sowing (*C+* 0.1 % of Cry1B purified toxin in rice leaf protein extract)

from leaves of T0 Senia plants at the tillering stage. Fourteen events expressed Cry1B above the detection threshold (0.01 %). Based on reconstruction assays, three events (e.g. events 100 and 107 in Fig. 3b) were found to accumulate Cry1B above 0.1 % of the total soluble proteins. Unfortunately, the latter events were either sterile or set very few seeds.

Seven events (1, 57, 62, 98, 109, 112 and 115) accumulating Cry1B at a detectable level (i.e. over 0.01 %) were harvested to challenge their progenies in bioassays. Overall, 122 T1 progeny plants were challenged with 10 L2 larvae per plant and scored at 7 days after infestation (Table 1). A correlation between the accumulation of toxin in leaf tissues and the level of SSB resistance exhibited by the plant in the bioassay was determined based on the observation that the five lines (events 1, 57, 62, 98 and 109) exhibiting 85–100 % toxicity were also those which accumulated Cry1B at the highest levels.

The maize ubiquitin (Christensen and Quail 1995), the rice actin 1 (McElroy et al. 1991) or the CaMV 35S (Odell et al. 1985) promoters have been used to direct constitutive expression of the *cry1Ab*(Fujimoto et al. 1993; Wünn et al. 1996; Cheng et al. 1998; Datta et al. 1998) *cry1Ac*(Nayak et al. 1997; Cheng et al. 1998) and *cry2a*(Bano-Maqbool et al. 1999) genes in transgenic rice plants, often with intron-mediated enhanced expression. The 0.01–0.3 % range of expression levels reported, which provide enhanced to full protection, is consistent with levels of Cry1B accumulation observed in the present study. Expression levels reaching 3 % (Cheng et al. 1998) and even 5 % (Bano-Maqbool et al. 1999) of the total soluble proteins have been occasionally reported, but the plants accumulating toxin at these levels were also phenotypically abnormal. The range of *Bt* toxin expression probably reflects transgene insertion position effects, transcriptional activity of the promoter sequence and transductional efficiency of the transcribed synthetic sequence.

Table 1 Results of the bioassay of hygromycin-resistant T1 progenies of T0 events of cv. Senia transformed with the pUbi-*cry1B* construct and found to accumulate Cry1B, 7 days after releasing ten *Chilo suppressalis* L2 larvae per plant

Plant material	Accumulation of Cry1B in leaves of T0 plants (μg/g fresh weight)	Total number of L2 larvae (10 L2 larvae /plant)	Number of larvae recovered alive (% of dead or missing larvae)	Mean fresh weight of living larvae (mg ± ET) (7 days)
Control	0.0	200	139 (30.5)	6.5 ± 1.2
Line 98	1.9	130	0 (100)	–
Line 109	1.0	200	4 (98.0)	0.9 ± 0.2
Line 62	1.0	300	15 (95.0)	1.1 ± 0.2
Line 57	1.6	60	8 (86.7)	1.6 ± 0.6
Line 1	1.1	270	72 (73.3)	3.7 ± 1.9
Line 112	0.8	90	32 (64.4)	2.0 ± 0.7
Line 115	0.1	170	112 (34.1)	5.7 ± 1.6

Characterization and evaluation of Ariete transgenic plants

Southern blot analyses performed on nine primary transformants of cv. Ariete generated from nodules co-bombarded with the pUbi-*cry1B* plasmid permitted us to compare hybridization patterns with those resulting from the analyses of Senia plants. Integration of the *cry1B* and *hph* transgenes was further characterized in 13 T1 progeny plants of event A64, which exhibited a single insertion site for both *cry1B* and *hph*(not shown). Complete cosegregation of the *cry* and *hph* transgenes was observed, indicating that the transgenes are stably inherited in a linked manner. This is in agreement with the fact that direct co-transfer of unlinked plasmid molecules in plant cells leads to the cointegration of variable numbers of copies of both plasmids at linked insertion sites representing a single genetic locus. This point has been confirmed through the analysis of transgenic rice plants produced by microprojectile acceleration (Cooley et al. 1995; Qu et al. 1996; Chen et al. 1998a).

The 25 T0 events of cv. Ariete were infested with five L2 SSB larvae and scored for damage 4 days after infestation. Twenty-four events were found to exhibit damage comparable to that observed on control plants (extensive tissue browning and feeding damage along the inner surface of the sheath where the larvae were released; penetration at the base of the tiller). Dissection of the infested culms allowed the recovery of nearly all of the released larvae which had advanced to the third instar stage – with individual body weights which did not differ significantly from those recovered on control plants – and of SSB head capsules. One event (A64) exhibited obvious antibiosis with little or no damage on the inner surface of the leaf where the larvae were released. Furthermore, most of the larvae were found to be dead, and those that had survived had not gained weight and no head capsules were recovered.

We selected this event to examine the temporal and spatial variation of transgene expression over subsequent generations. By comparing band intensities with those of a known amount of purified protein, we observed that Cry1B stably accumulated at 0.4 % of the total soluble proteins in T1–T4 progeny plants of event A64. At the T1 generation, segregation of resistance among progeny plants of event 64 correlated with the detection of the CryIB protein as determined by Western analysis and presence of the transgene by Southern blot analysis.

To determine whether toxin delivery varies over plant and organ development, we also examined the accumulation of Cry1B in blade tissue of the antepenultimate leaf collected after 20, 40 (tillering stage), 70 (heading stage) and 90 (grain filling stage) days of plant development (Fig. 3c) and in the blade and sheath of a young and old leaf as well as in young and old internodes (data not shown) of T3 A64 plants. The level of toxin accumulation appeared to be remarkably stable both spatially in the rice plant and temporally over plant development.

The stability of protection over the T1-T4 generations was further investigated. Progeny plants of three

Table 2 Results of the bioassay of three homozygous T2 lines derived from transformation event Ariete 64 harbouring the pUbi-*cryIB* construct 7 days after releasing five *Chilo suppressalis* L2 larvae per plant

Plant material	Total number of L2 larvae (5 L2 larvae plant)	Number of larvae found dead (% of mortality)	Number of larvae found alive (% of survival)	Mean fresh weight of living larvae (mg ± ET) (7 days)
Control	25	0 (0.0)	21 (100)	5.8 ± 0.9
64–4-2	95	59 (100)	0 (0.0)	–
64–2-3	35	15 (100)	0 (0.0)	–
64–2-4	40	25 (100)	0 (0.0)	–

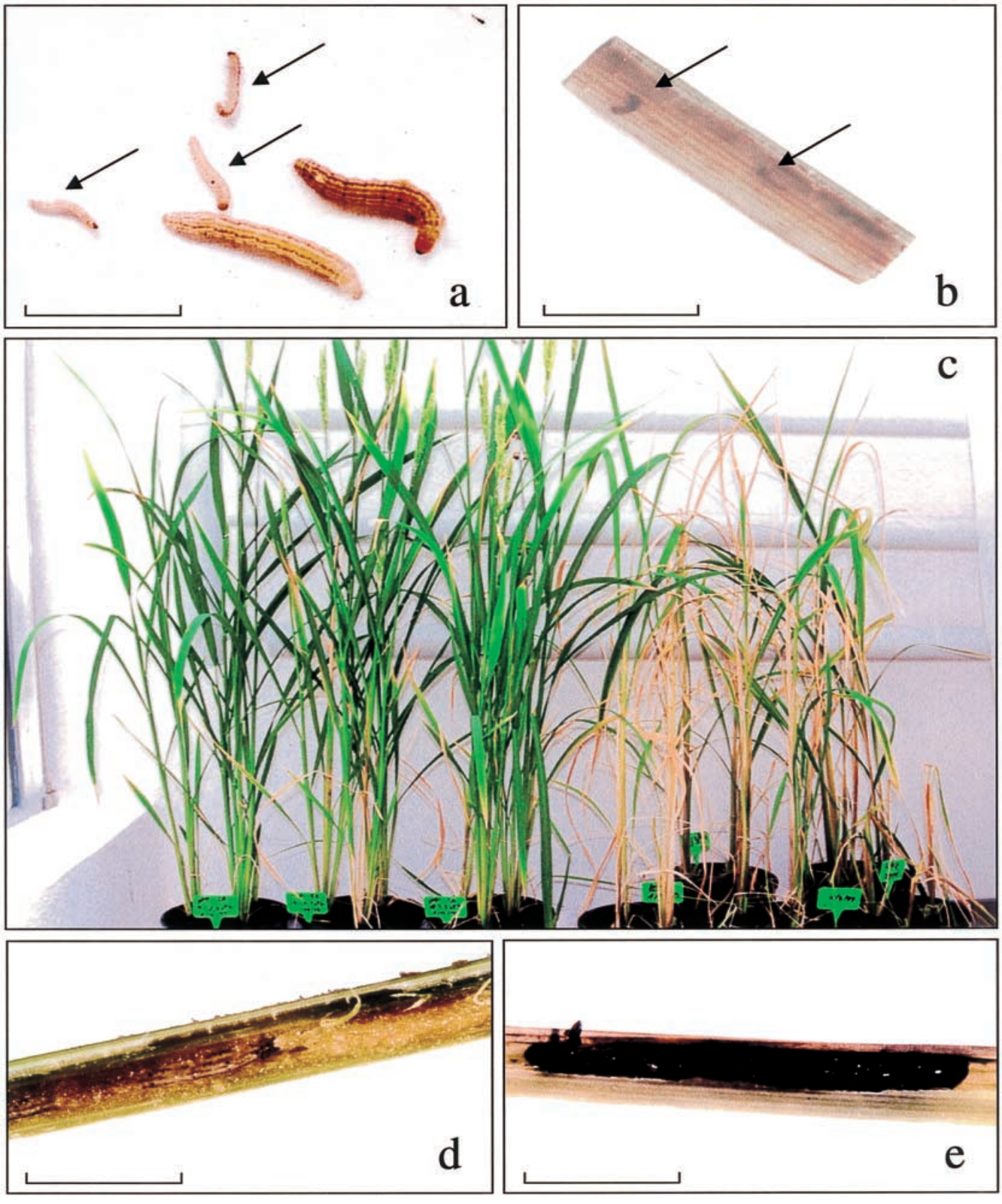

Fig. 4 **a** Detailed view of the few SSB larvae recovered alive on A64 (*arrows*) plants compared to those recovered on control plants, 7 days after release. **b** Detailed view of SSB larvae recovered dead (*arrows*) on a leaf sheath of an A64 plant, 7 days after release. **c** Comparative damage observed on A64 (*left*) and control (*right*) plants 15 days after their infestation with 5L2 larvae. **d** SSB larvae recovered alive in the pith of a control Ariete plant 7 days after release at the L4 stage. ***e*** Dead SSB larvae recovered on the leaf sheath of an A64 plant where it was released at the L4 stage. Bar (**a, b, d, e):**1 cm

Table 3 Results of the bioassay of homozygous line Ariete 64 harbouring the pUbi-*cry1B* construct 7 days after releasing two L3 larvae or one L4 larva per plant

Total number of larvae (2 L3 or 1 L4 per plant)	Number of larvae found dead (% of mortality)	Number of larvae found alive (% survival)	Mean fresh weight of living larvae (mg ± SD) (% weight gain/loss relative to initial fresh weight) (7 days)
14 L3	9	5	10.1 ± 1.9 [–24.1]
4 L4	3	1	15.6 ± 0.0 [–66.0]

T1 homozygous plants were individually challenged with five L2 SSB larvae, and dissections performed 7 days after infestation. The results of these experiments are reported in Table 2. The examination of control plants showed that larvae had advanced to the L3/L4 stage and had penetrated the stem and fed on inner pith tissues. Head capsules were found along the infested sheath or inside the pith. Control plants had started to display extensive feeding damage with infested tillers exhibiting dead hearts. Contrastingly, larvae recovered on the three individual progenies of event A64 were dead and still at the L2 stage (no head capsules found) and had neither gained weight nor penetrated the pith. Tissue damage was limited to 1-cm-long necrotic regions, a result of early feeding browning (Fig 4a–c). This result is consistent with those reported previously on *Bt* rice where the absence of feeding damage and 100 % mortality was generally observed 4–7 days after infestation. In these studies, 1–3 highly expressing events were also generally identified and used to conduct insect feeding assays on leaf or stem tissues, or on entire plants using neonate/second instar larvae.

As plant-to-plant movement of SSB larvae at a more advanced larval stage is reported to occur in paddy fields (Cohen et al. 1996), we also investigated whether larvae which had first developed on a non-transgenic host and later moved to transgenic Cry1B plants could overcome *Bt*-mediated resistance. As a first assessment, either one L4 or two L3 larvae were released on plants of Ariete event 64 . Event A64 was found to exhibit a clear toxicity at these more advanced stages, with 64 % and 75 % mortality 7 days after infestation and a weight loss relative to the initial fresh weight of 24 % and 66 % for the L3 and L4 larvae, respectively (Table 3). Although feeding damage observed on the infested leaf sheath was more extensive than when L2 larvae were released, no L3 nor L4 larvae were found to have penetrated the pith (Fig 4d,e). Therefore, the delivery of the Cry1B toxin to the A64 tissues appears to have been sufficient to prevent damage caused by more advanced larval stages, even though such protection should be ultimately tested under field conditions

Conclusion

In the present study, we used a synthetic *cry1B* coding sequence optimized according to monocot codon usage with a GC content of 58 %. The constitutive expression of that *cry1B* gene directed by the maize ubiquitin promoter proved to afford full SSB control in transgenic *japonica* rice varieties Ariete and Senia, with an accumulation reaching up to 0.4 % of the total soluble proteins. This is the first report of SSB control using the *cry1B* gene. We are currently engaged in experiments to transfer other *cry* genes or other insecticidal genes to commercial Mediterranean cultivars of rice, because it must be borne in mind that developments of resistance to *Bt* toxins in the target insect populations have already been extensively documented (Frutos et al. 1999). The co-expression of multiple insecticidal genes (two *cry* genes or a *cry* gene in combination with a gene encoding a protein having a different mode of action), the so-called gene pyramiding strategy, has been proposed to further prevent the rise of resistance in the insect population. The stacking of *cry* genes encoding toxins binding to different SSB midgut receptors – as reported for *cry1Aa* and *cry1B*(Fiuza et al. 1986) – would be ideal for preventing or delaying resistant phenotype outbreaks in the stemborer populations

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