Transgenic expression of trypsin inhibitor CMe from barley in *indica* and *japonica* rice, confers resistance to the rice weevil *Sitophilus oryzae*

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Indica and *japonica* rice (*Oryza sativa* L.) plants were transformed by particle bombardment with the *Itr1* gene encoding the barley trypsin inhibitor BTI-CMe, under the control of its own promoter that confers endosperm specificity, and the maize ubiquitin promoter. From 38 independent transgenic lines of *indica* (breeding line IR58) and 15 of the *japonica* (cv Senia) selected, 22 and 11, respectively, expressed the barley inhibitor at detectable levels. The transgene was correctly translated as indicated by western blot analysis with a level of expression in R₃ seeds up to 0.31% (IR58) and 0.43% (Senia) of the total extracted protein. The functional integrity of BTI-CMe was confirmed by trypsin activity assays in liquid media and by activity staining gels, performed with seed extracts. The significant reduction of the survival rate of the rice weevil (*Sitophilus oryzae*, Coleoptera: Curculionidae) reared on homozygous transgenic *indica* and *japonica* rice seeds expressing the BTI-CMe, compared to non-transformed controls, and the decrease in the trypsin-like activity of insect crude midgut extracts, confirmed the utility of this proteinase inhibitor gene for the control of important storage pests.

Introduction

Rice (*Oryza sativa* L.) is the second major crop worldwide in planted hectares with a production of about 597 million metric tons (IRRI, 2000), but every year losses due to herbivorous pests, mainly insects, are estimated to be about 27% of the total yield, despite insecticide usage (Krattiger, 1997). The damage is even greater in stored grain, which if not completely lost, is severely reduced in quality and germination rate. One important goal of plant breeders has been the search for insect resistance genes, but a limited number of rice cultivars have natural resistance to the major rice pests and genetic engineering offers a new

strategy for crop protection. Several transfer protocols have been accomplished successfully for the introduction of foreign genes into *indica*, *japonica*, *javanica* and *elite* African rice cultivars (reviewed by Giri & Laxmi, 2000; Tyagi & Mohanty, 2000). As insecticidal genes, the *Cry* genes encoding several variants of the toxic Bt crystal protein of *Bacillus thuringiensis* have been widely used (Maagd et al., 1999, 2001), although the need for identifying new protective genes has been emphasized recently in order to deal with future resistance problems (Jouanin et al., 1998; Schuler et al., 1998; Hilder & Boulter, 1999; Maqbool et al., 2001). Among those explored, hydrolase inhibitors derived from plants are of particular interest because of their putative involvement in the natural defence system against insect pests. The over-expression of several proteinase inhibitors of different plant origin

in transgenic rice plants has resulted in an increase of resistance against insects feeding in vegetative tissues (Duan et al., 1996; Xu et al., 1996; Lee et al., 1999; Mochizuki et al., 1999; Maqbool et al., 2001). No reports have been found related to transgenic protection towards insect storage pest in rice mediated by hydrolase inhibitors, with the exception of Irie et al. (1996) where a corn cystatin encoding gene expressed in rice seeds was able to inhibit *Sitophilus zeamais* gut proteases.

Barley trypsin inhibitor BTI-CMe, encoded by the *Itr1* gene, is one of the best characterised members of a multigene family of protease/ α -amylase inhibitors isolated from cereals (Carbonero et al., 1999), and is putatively involved in plant defence. Both cDNA and genomic clones for the *Itr1* gene have been characterised (Rodríguez-Palenzuela et al., 1989; Royo et al., 1996). This gene is specifically expressed in the barley endosperm, and the purified protein BTI-CMe has been shown to be active *in vitro* against trypsin and trypsin-like proteases of the gut extracts of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae; Alfonso et al., 1997). Transgenic expression of the *Itr1* gene in wheat, significantly reduced the survival rate of the grain moth *Sitotroga cerealella* (Lepidoptera: Gelechiidae) reared on transgenic wheat seeds (Altpeter et al., 1999). However, the decrease in the trypsin-like activity detected in larvae midgut of *S. exigua* (Lepidoptera: Noctuidae) fed on transgenic tobacco plants stably expressing this barley gene, was partially compensated by increases in non target midgut proteases (Lara et al., 2000).

The rice weevil *S. oryzae* (Coleoptera: Curculionidae) is one of the most serious pests of stored rice worldwide. Both larvae and adults are destructive, feeding voraciously on rice grain. The adult females lay eggs in holes chewed in the grain kernel and the larvae feed within the rice grain, hollowing out the endosperm and reducing it to powder. Protein digestion in larvae and adults of *S. oryzae* is provided by a complex proteolytic system that includes serine-, cysteine-, and aspartyl-proteinases, aminopeptidases and carboxypeptidases (Baker, 1982; Liang et al., 1991; unpublished data).

The present paper reports the transformation of *indica* and *japonica* rice plants using the barley trypsin inhibitor gene as an insecticidal transgene, its stable inheritance in three successive generations and the evaluation of its potential for increasing resistance against the important rice storage pest *S. oryzae*.

Materials and methods

Plant material

Rice seeds (*O. sativa*) of *indica* (breeding line IR58) and *japonica* (cv Senia) were provided by the International Rice Research Institute (IRRI, Manila, The Philippines) and the Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain), respectively. All rice plants were grown at $27 \pm 2^\circ\text{C}$ under 18h/6h light/dark photoperiod until mature seed development. Spikelets from barley plants (*Hordeum vulgare* cv Bomi) were collected at approximately 15 days after flowering (daf), hand-dissected, frozen in liquid nitrogen and stored at -70°C until needed. Rice leaves for nucleic acid and protein extraction were similarly frozen and stored.

Immature embryos of *indica* rice breeding line IR58 were aseptically isolated at ≈ 15 daf, plated scutellum side up, as described by Wünn et al. (1996), and subsequently used for particle bombardment. Mature seeds from *japonica* rice cv Senia were used to induce embryogenic callus according to Alfonso-Rubí et al. (1999), which then served as material for bombardment.

Plasmid constructs and transformation

The plasmid pUPM-BTI67 is a construct derived from the pUPM-BTI66 plasmid (Altpeter et al., 1999) obtained by isolation of a ≈ 3 kbp *HindIII*-fragment followed by subcloning into the pBSK vector. It contains the entire coding region of the barley *Itr1* gene encoding the trypsin inhibitor BTI-CMe and the 83 bp upstream of the ATG translation initiation codon (Royo et al., 1996), under the control of the maize ubiquitin-promoter plus its first intron and followed at the 3' end by the nopaline synthase gene (NOS) 3'-terminator (Figure 1). Hygromycin (50–100 $\mu\text{g/ml}$) was used for selection of transgenic rice plants and the pUPM-Hm^R plasmid, containing the hygromycin phosphotransferase (*Hpt*) gene controlled by the CaMV35S promoter and the 3'-NOS terminator (Figure 1), was used in the co-bombardment experiments.

The described plasmids were introduced into immature embryos or into embryogenic callus, by microprojectile bombardment according to the procedure described by Chen et al. (1998), using the DuPont PDS1000/He Biolistic Delivery System. Plant regeneration from bombarded tissues was done following methods described by Chen et al. (1998) for *indica* rice (IR58) and by Alfonso-Rubí et al. (1999) for *japonica*.

Figure 1. Schematic representation of the two plasmids used for rice transformation. On plasmid pUPM-BTI67, the barley trypsin inhibitor (*Itr1*) gene is flanked by the ubiquitin promoter and its first intron followed by the -83 bp of the *Itr1* promoter from the ATG translation initiation codon and the nopaline synthase gene (*nos*) 3' terminator. On plasmid pUPM-Hm^R, the hygromycin B phosphotransferase (*Hpt*) gene is flanked by the CaMV35S promoter and the *nos* 3' terminator. Molecular sizes are expressed in kbp and sites of restriction endonucleases are indicated.

ica rice, respectively, using hygromycin as selective agent.

Nucleic acid analysis

Total DNA isolated from leaves of rice, essentially as described by Taylor and Powell (1988) was tested for the presence of the *Itr1* gene by PCR using the oligonucleotide primers and conditions previously described by Altpeter et al. (1999). The reaction products were electrophoresed in 1% (w/v) agarose gels and blotted onto Hybond-N membranes. Hybridisation and washings were performed under stringent conditions, according to standard procedures (Sambrook et al., 1989), using the complete ³²P-labelled cDNA for the *Itr1* gene as a probe (Royo et al., 1996).

Purified DNA (10 µg) was restricted with *Eco*RI, electrophoresed in 0.8% (v/v) agarose gels, blotted onto Hybond-N membranes and hybridised using the same probe and conditions described above.

Western blot analysis

Plant proteins were prepared by grinding seeds directly into the Laemmli (1970) buffer (1:3, w/v) for 1 h, followed by centrifugation for 10 min at 4°C. The protein content of the supernatant was estimated using a protein assay kit (BioRad) and BSA as a standard. After separation on SDS-PAGE polyacrylamide gradient gels (4–20% w/v), proteins were transferred onto PVDF membranes (Millipore). Immunological staining was done with anti-BTI-CMe antibody produced in rabbits by Eurogentec (Brussels) at a dilution 1:100 (v/v), followed by a second alkaline phosphatase-antibody conjugated anti-rabbit IgG (1/2000, v/v). Colour was developed by the reaction of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. For semiquantitative BTI-CMe evaluation in transgenic rice seeds, standards with purified protein from

barley seeds were prepared at different concentrations (Altpeter et al., 1999).

Trypsin inhibitory activity assays

Proteins were extracted from defatted rice flour, after incubation in petroleum-ether for 1 h at room temperature. Two hundred milligrams of seed material was extracted with 500 µl ice-cold 0.15 M NaCl solution followed by centrifugation for 10 min at 4°C. Trypsin inhibition activities were assayed by the incubation of various amounts (50–150 µg) of soluble protein extracts with 2 µg of bovine trypsin (Sigma) for 30 min at 37°C, followed by the addition of BANA (N-benzoyl-DL-arginine-β-naphthylamide) as substrate and the reaction conditions used by Lara et al. (2000). Inhibition was measured by absorbance at 540 nm and expressed as percentage of the remaining enzymatic activity over controls. Trypsin inhibition assays were also performed by electrophoretic analysis using mildly denaturing gelatin/polyacrylamide gels (Michaud et al., 1993), following the procedure described by Lara et al. (2000). In both assays, negative controls were carried out with protein extracts from non-transformed rice seeds.

Insect bioassays

A laboratory population of *S. oryzae* (Coleoptera: Curculionidae) was reared in a growth chamber for several generations on commercial rice seeds under a 16h/8h light/dark photoperiod, at $25 \pm 2^\circ\text{C}$. Five adults of 30–45 days of age, by which time the oviposition rate of female rice weevils reached a maximum (Balachowsky, 1963), were placed in each of the compartments of a 6-well plate containing a filter paper and 12 rice seeds from the *indica* (7 and 25) and *japonica* (3 and 5) lines and their corresponding non-transformed rice lines (NT). The six lines were arranged in the 6-well plates in a completely random-

ized block design, and the test was replicated 12 times. The plates were maintained under the same conditions described above, and the survival rate of the adults examined twice a week for a period of 6 weeks.

Gut proteolytic activity and inhibition assays

Adults of *S. oryzae* actively feeding on commercial rice seeds were dissected and a pool of about 200 midguts homogenized in 5 ml of 0.15 M NaCl. After centrifugation, the supernatant was frozen to provide samples for enzymatic determinations. Trypsin-like activity was assayed with 1 mM BApNa (N_α -benzoyl-DL-arginine-p-nitroanilide), using 40 μ l of midgut extract in 1 ml of reaction mixture, incubating for 24 h at 30°C, at their optimum pH (10.5). All enzymatic assays were carried out in triplicate and blanks were used to account for autolysis of substrates.

The inhibition assays were performed by incubation of *S. oryzae* midgut extracts, containing approximately 65 μ g of protein, with protein extracts from transformed and non-transformed rice seeds (25 μ g), for 30 min at 30°C. Rice seed extracts were enriched in proteinase inhibitors and inhibited in proteolytic activity, by incubation at 60°C for 1 h. After keeping on ice for 5 min, samples were centrifuged at 10,000 g for 5 min, to remove the precipitated proteins. The trypsin activity assays were carried out as previously described.

Results and discussion

Production of rice transgenic lines

A total of 21 plates containing immature embryos of the *indica* rice breeding line IR58 (30 embryos/plate) and 16 plates of callus derived from the *japonica* rice seeds of cv Senia (250–300 mg cells/plate), were co-bombarded with both plasmids obtaining 329 and 148 resistant calli, respectively, using 50 μ g/ml of hygromycin as selective agent. From those calli, 38 and 16 independent lines, belonging to the *indica* and *japonica* rice, respectively, were regenerated.

The presence of the barley *Itr1* gene in the R_0 transgenic rice lines was determined by PCR and Southern blot analysis. Twenty-two plants among the 38 hygromycin resistant *indica* lines, and 11 among the 15 *japonica* rice lines, exhibited the expected 0.9 kbp band after electrophoresis of the PCR-amplified products and its identity was confirmed by hybridisation with the entire coding region of the *Itr1*

Figure 2. Hybridisation of PCR products of transformed *indica* and *japonica* rice plants with *Itr1* gene from barley. (A) PCR reaction products obtained by amplification of DNA (200 ng) isolated from R_0 transformed leaves of *indica* IR58 (lines 1–38) were hybridised with the 32 P-labelled entire *Itr1* gene. (B) PCR reaction products obtained by amplification of DNA (200 ng) isolated from R_0 transformed leaves of *japonica* Senia (lines 1–15) were hybridised with the 32 P-labelled entire *Itr1* gene. DNA isolated from non-transformed rice leaves (NT: 200 ng) and the plasmid pUPM-BTI67 (PI: 20 ng) were used as negative and positive controls, respectively.

gene as a probe (Figures 2(A) and (B)). Southern blot analysis performed after restriction of the total DNA with *Eco*RI, from plants previously shown to be positive by PCR assays, corroborated the stable integration from one to three copies of the barley *Itr1* gene in the genome of the transformed rice lines (data not shown). The PCR-positive lines were transferred to the greenhouse, where about 80% of the self-pollinated plants produced seeds. Mature embryos excised from transgenic seeds were germinated on MS medium containing 100 μ g/ml of hygromycin for several generations, to study segregation rates. Germination frequencies in hygromycin media of 18 *indica* and 9 *japonica* R_1 lines were consistent with the expected (3:1) Mendelian segregation rate for a single integration site. In the R_3 generation, seven *indica* lines (7, 25, 26, 31, 33, 34 and 38) and five *japonica* lines (1, 2, 3, 4, 5, 6 and 7) were considered homozygous for *Hpt*-expression due to 100% germination on selective medium, and were used for further analysis.

BTI-CMe protein expression

The *Itr1* gene construct, in plasmid pUPM-BTI67, used here for rice transformation, was identical to

Figure 3. Western blot analysis of transgenic rice seeds expressing the *Itr1* gene in SDS-PAGE gels. (A) Protein extracts (20 μ g) of a mixture of 15 seeds of *R₁ indica* IR58 (lines 7, 25, 26, 31, 33, 34 and 38). (B) Protein extracts (20 μ g) of individual seeds (a, b, c and d) of homozygous *R₃ indica* IR58 (lines 7, 25, 31 and 33). (C) Protein extracts (20 μ g) of a mixture of 15 seeds of *R₁ japonica* Senia (lines 1, 2, 3, 4, 5, 6 and 7). (D) Protein extracts (20 μ g) of individual seeds (a, b, c and d) of homozygous *R₃ japonica* Senia (lines 1, 3, 4 and 7). Non-transformed rice seeds (NT: 20 μ g) and developing barley endosperm (Hv1: 20 μ g, Hv2: 10 μ g) protein extracts were used as negative and positive controls, respectively.

that in plasmid pUPM-BTI66 that had been previously used in wheat transgenesis (Altpeter et al., 1999) and shown to produce strong expression in endosperm. The difference being that the selection marker (*Bar* gene) in plasmid pUPM-BTI66, has been deleted in pUPM-BTI67.

We analysed the transgene expression by immunoblotting (western) of protein extracts from de-embryonated seeds (Figure 3). The presence of the BTI-CMe protein in transgenic seeds was further corroborated by enzyme activity gels (Figure 4). Protein extracts from a mixture of 15 de-embryonated seeds of each transformed *R₁* line were separated using SDS-PAGE and the immunoblotting revealed two bands of different intensity in extracts of some transgenic lines, as well as in extracts from developing barley endosperm used as positive control. The upper band, specific of BTI-CMe protein, of 14 kDa was absent in extracts from non-transformed rice (Figures 3(A) and (C)). The presence of the smaller extra band in all tested tissue extracts was also described in wheat and tobacco plants transformed with this *Itr1* gene (Altpeter et al., 1999; Lara et al., 2000), and seems to be neither a degradation nor a cross-reaction product with endogenous inhibitors. The amount of the tryp-

sin inhibitor protein found in transgenic *R₁* seeds was estimated to be in the range of 0.05% (line 26) to 0.32% (line 7) of the total soluble protein in the *indica* transformed rice IR58 and ranged from 0.08% (line 1) to 0.24% (line 5) in *japonica* rice cv Senia. In comparison, approximately 0.86% of total soluble protein from barley endosperm correspond to the BTI-CMe trypsin inhibitor (Altpeter et al., 1999). These values were higher than those quantified in transgenic tobacco leaves (from 0.03 to 0.14% of the total soluble protein) transformed with the same barley gene under the control of the 35S promoter (Lara et al., 2000). However, the content of the barley inhibitor protein in rice seeds was lower than that found in transformed wheat seeds (up to 1.1 % of total protein) with the same barley gene under the control of the same promoter (Altpeter et al., 1999). These differences may be due to the different genetic backgrounds.

Four individual seeds (a, b, c and d) from each *R₃* homozygous *indica* (7, 25, 31 and 33) and *japonica* (1, 3, 4 and 5) lines of rice were assayed for the presence of the trypsin inhibitor (Figures 3(B) and (D)). The highest expression levels were detected in seeds of line 7 (*indica* type) and line 5 (*japonica* type) with BTI-CMe contents of 0.31 and 0.43% of total soluble

Figure 4. Trypsin inhibitory activity of protein extracts from transgenic R₃ rice seeds *indica* breeding line IR58. (A) Zymogram of the gelatinolytic activity of bovine trypsin (2 µg) alone (C) and after pre-incubation with 150 µg of protein extracts from transgenic lines (7, 25, 31, 33 and 38) and from non-transformed rice (NT). (B) Remaining proteolytic activity after incubation of 2 µg of bovine trypsin with different amounts of protein extracts from transgenic lines (7, 25, 31, 33, 38) and from non-transformed rice seeds (NT), using BANA as substrate. Standard errors of the mean of four duplicated samples were less than 10%. The arrow indicates the band corresponding to the bovine trypsin activity.

Figure 5. Trypsin inhibitory activity of protein extracts from transgenic R₃ rice seeds *japonica* cv Senia. (A) Zymogram of the gelatinolytic activity of bovine trypsin (2 µg) alone (C) and after pre-incubation with 150 µg of protein extracts from transgenic lines (1, 3, 4, 5 and 6) and from non-transformed rice (NT). (B) Remaining proteolytic activity after incubation of 2 µg of bovine trypsin with different amounts of protein extracts from transgenic lines (1, 3, 4, 5 and 6) and from non-transformed rice seeds (NT), using BANA as substrate. Standard errors of the mean of four duplicated samples were less than 10%. The arrow indicates the band corresponding to the bovine trypsin activity.

protein, respectively. The higher size band was not observed in non-transformed rice seeds (Figures 3(B) and (D)). No BTI-CMe protein was detected in leaf extracts of *indica* and *japonica* transgenic lines and the corresponding mRNA in transgenic rice leaves was also undetectable (data not shown). This result might indicate that the presence of 83 bp of the *Itr1* promoter acts as an enhancer of the endosperm expression both in transgenic wheat (Altpeter et al., 1999) and rice.

Total protein extracted from the flour of 15 seeds per transgenic R₃ line of *indica* and *japonica* type and from non-transformed rice seeds were used to detect

the inhibitory activity against commercial bovine trypsin (Figures 4 and 5). Gelatin-polyacrylamide electrophoretic gels showed a clear high-mobility band of hydrolytic activity corresponding to trypsin (arrows in Figures 4(A) and 5(A)) and some low mobility bands of protease activity associated with protein extracts from seeds. The gelatin hydrolysis band produced by the commercial trypsin activity was inhibited by protein extracts from transgenic *indica* lines (7, 25, 31, 33 and 38; Figure 4(A)) as well as by *japonica* lines (1, 3, 4, 5 and 6; Figure 5(A)). Those lines showed the higher protein accumulation in western blot analysis (Figures 3(A) and (C)). No trypsin inhibition was

detected in non-transformed seeds. When the trypsin was pre-incubated with 1 μ g of Kunitz trypsin inhibitor from soybean, the corresponding hydrolytic band in the zymogram was completely abolished (data not shown).

The inhibitory activity of homozygous R₃ transgenic rice lines was quantified by the decreased amount of BANA-hydrolyzing activity of trypsin with increased amounts of protein extracts from transgenic rice seeds and was expressed as percentage of remaining activity (Figures 4(B) and 5(B)). The incubation of commercial trypsin with protein extracted from the *indica* transgenic rice lines showed a significant inhibitory activity in lines 7 and 25 (*indica*) over the values obtained with protein extracts from non-transformed controls (Figure 4(B)). At the highest protein concentration tested (150 mg) the remaining trypsin activity was reduced to 49% (line 7) and 63% (line 25) respectively, while an 86% of the activity was detected with protein extracts in non-transformed controls. Similarly, protein extracts from transgenic *japonica* lines 3 and 5 reduced the remaining trypsin activity up to 52 and 41%, respectively; 78% of the activity was maintained after incubation of the trypsin with protein extracts from non-transformed rice seeds (Figure 5(B)). These results corroborate and extend the inhibition data obtained in the zymograms (Figures 4(A) and 5(A)) and highly correlate with the content of BTI-CMe protein detected in these transgenic rice seeds by western blot analysis (Figures 3(A) and (C)).

The presence of endogenous trypsin inhibitors in rice has been reported by Lee and Lin (1995) and Rakwal et al. (2001), but their expression has been restricted to vegetative tissues, where they increased in response to abiotic stimuli such as anaerobiosis, wounding, ethylene and jasmonic acid. However, their amino acid sequences do not have homology with BTI-CMe and do not belong to the same protein family. Nevertheless, their inhibitory activity must be absent in seeds or below the detection limits of the inhibition assay used in this study.

Insect resistance test

Trypsin inhibitor genes of different plant origin, such as those isolated from cowpea, soybean, winged bean and potato have been stably expressed in vegetative rice tissues conferring resistance against two lepidopteran insects, the stem borers *Sesamia inferens* and *Chilo suppressalis* and one homopteran insect, the planthopper *Nilaparvata lugens* (Duan et al., 1996; Xu et al., 1996; Lee et al., 1999; Mochizuki et al., 1999).

However, to the best of our knowledge, there are no reports whether this type of inhibitors could confer protection against insect storage pests when expressed in rice seeds.

To assess the insecticidal activity of transgenic rice seeds stably expressing the barley *Itr1* gene, rice weevil adults were fed with homozygous R₃ seeds (*indica* lines 7 and 25, and *japonica* lines 3 and 5) and with non-transformed seeds and their mortality examined for a period of 6 weeks (Figure 6). Differences on the survival rate of the rice weevil began to be significant after feeding for 10 days on seeds of transgenic *indica* line 7. In this transgenic line, a survival rate of $60 \pm 9\%$ was recorded, whereas this value was $88.3 \pm 5.9\%$ with weevils fed on non-transformed rice seeds. A significant reduction in survival rate was maintained for 2 more weeks. Moreover, after 42 days only $3.3 \pm 2.3\%$ of the insects reared with seeds of *indica* line 7 were alive, compared to $20 \pm 4.9\%$ of

Figure 6. Percentage survival rate of *S. oryzae* adults reared on transgenic rice seeds for a period of 6 weeks. (A) Seeds of transgenic *indica* rice breeding line IR58, lines 7 and 25; (B) Seeds of transgenic *japonica* rice cv Senia, lines 3 and 5. NT: non-transformed rice seeds used as a control. * Indicate values significantly different to control (Dunnett two tailed test, $P \leq 0.05$).

survivors maintained on non-transformed seeds (Figure 6(A)). In contrast, the effect of the barley trypsin inhibitor expressed in *japonica* transgenic seeds on *S. oryzae* adults was only observed at the end of the bioassay. After 6 weeks of feeding on transgenic line 5, the survival rate ($3.3 \pm 2.3\%$) was significantly different from that obtained after feeding on non-transformed rice ($33.3 \pm 7.9\%$, Figure 6(B)). No significant differences were observed in insects fed on seeds of transgenic *indica* line 25 and of *japonica* line 3 during the entire feeding assay. These results are in agreement with the inhibitory activity against bovine trypsin detected in protein extracts from the highest expressing *indica* (line 7) and *japonica* (line 5) rice seeds, with BTI-CMe protein content of 0.31 and 0.43% of total protein (Figures 4 and 5).

Our results indicate a remarkable reduction in the survival rate of insects reared on seeds of *indica* line 7 after only a few days. Since the bioassays were initiated with adults of 30–45 days of age, coincident with the time when the oviposition rate reaches a maximum (Balachowsky, 1963), the transgenic seeds would limit the potential number of the rice weevil progeny and restrain the levels of infestation. On the other hand, the reduction in the survival rate of insects reared on seeds of *japonica* line 5 was only observed at the end of the bioassay, when the females have laid most of their eggs, and its effect on reducing the levels of infestation would be less significant.

To investigate whether the reduction on the survival rate of insects reared with transgenic seeds might be due to an inhibition of trypsin-like activity, one of the major types of serin-proteases in *S. oryzae* gut (Baker, 1982; Liang et al., 1991; data not shown), proteins extracted from transformed and non-transformed rice seeds were incubated with crude gut protein extracts. The remaining trypsin-like activity of gut extracts decreased after being incubated with protein extracts of transgenic *indica* line 7 ($62 \pm 3\%$) and *japonica* line 5 ($78 \pm 2\%$), in comparison to the respective non-transformed controls ($90 \pm 2\%$ and $92 \pm 1\%$ for *indica* and *japonica* seeds, respectively). These results suggest that, despite a digestive proteolytic system based on proteases of different mechanistic classes, the reduction of trypsin-like activity has been sufficient to increase the mortality rate. Complex proteolytic systems for protein digestion appear to be widespread among curculionids (Ortego et al., 1998; Bonadé-Bottino et al., 1999). It has been reported that ingestion of cysteine proteinase inhibitors in natural or artificial diets increased mortality and delayed

developmental time in larvae of the alfalfa weevil *Hypera postica* (Elden, 1995) and the rice weevil (Pittendrigh et al., 1997). Likewise, larvae of the weevil *Aubeonymus mariaefrancisciae* fed on diets containing soybean and lima bean trypsin inhibitors endure lower survival rates and display significant delays in the developmental time to pupation and to adult emergence (Ortego et al., 1998). However, Bonadé-Bottino et al. (1999) showed that larvae of the weevil *Baris coerulescens* were insensitive to oil-seed rape transgenic plants expressing oryzacystatin, because the inhibition of cysteine proteinase activity was compensated by insensitive serine proteinases.

This report demonstrates that the barley trypsin inhibitor expressed in transgenic rice seeds provides significant protection against the coleopteran rice weevil *S. oryzae*, one of the most important insect storage pests of rice. For future strategies to improve resistance towards *S. oryzae* using transgenic cereal seeds, it would be an advantage to increase expression by choosing a more efficient promoter and to transfer combinations of genes encoding hydrolase inhibitors able to inhibit different enzyme activities in the rice weevil gut.

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