Effect of Plant Age, Larval Age, and Fertilizer Treatment on Resistance of a *cry1Ab*-Transformed Aromatic Rice to Lepidopterous Stem Borers and Foliage Feeders

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ABSTRACT The resistance of vegetative, booting, and flowering stage plants of a variety of an aromatic rice, *Oryza sativa* L., transformed with a *Bacillus thuringiensis* Berliner *cry1Ab* gene under control of the maize phosphoenolpyruvate carboxylase (PEPC) promoter was evaluated against four lepidopterous rice pests—the stem borers *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) and *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae), and the foliage feeders *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae) and *Naranga aenescens* Moore (Lepidoptera: Noctuidae). Plants of the *cry1Ab*-transformed line (no. 827) were more resistant to young larvae of *S. incertulas, C. suppressalis,* and *C. medinalis* than control plants at the vegetative stage but not at the flowering stage. Survival of 10-d-old stem borer larvae did not differ on *cry1Ab* plants and control plants at either the vegetative or flowering stage, but the development of 10-d-old *C. suppressalis* larvae was retarded on the vegetative stage *cry1Ab* plants. Immunological analysis also showed an apparent decline in Cry1Ab titer in leaf blades and leaf sheaths at the reproductive stage. In experiments comparing three fertilizer treatments (NPK, PK, and none), there was a significant interaction between fertilizer treatment and variety on larval survival only in whole-plant assays at booting stage with *C. suppressalis.* On *cry1Ab* plants, larval survival did not differ significantly among the three fertilizer levels, whereas on control plants survival was highest with the NPK treatment. *cry1Ab* plants tested at the sixth and seventh generations after transformation were more resistant than control plants to *N. aenescens* and *C. suppressalis,* respectively, suggesting that gene silencing will not occur in line 827. The results of the experiments are discussed in terms of resistance management for *B. thuringiensis* toxins in rice.

KEY WORDS *Bacillus thuringiensis, Chilo suppressalis, Scirpophaga incertulas,* transgenic rice, nutrient-pest interaction

STEM BORERS OF the families Pyralidae and Crambidae are among the most important insect pests of rice, *Oryza sativa* L. *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae) is the major stem borer pest of rice in most tropical regions of Asia, whereas *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) is the major pest species in temperate countries such as China, Iran, and Korea. Young stem borer larvae feed within the leaf sheath, and older larvae feed inside the stem and sever vascular tissues. Tillers damaged at the vegetative stage form a structure called a deadheart, and those damaged at the reproductive stage form whiteheads, which are panicles bearing unfilled grains. Rice foliage also is attacked by lepidopterous species in Asia, including *Cnaphalocrosis medinalis* Guenée (Lepidoptera: Pyralidae) (one of several species of rice leaffolders) and *Naranga aenescens* Moore (Lepidoptera: Noctuidae). High levels of resistance to lepidopterous stem borers and foliage feeders have not been identified in rice germplasm (Heinrichs et al. 1985), thus there has been substantial interest in transformation of rice with genes for insecticidal toxins from *Bacillus thuringiensis* Berliner (Bt). There have been numerous reports of transgenic rice lines with enhanced resistance to lepidopterous pests (Fujimoto et al. 1993, Wunn et al. 1996, Ghareyazie et al. 1997, Nayak et al. 1997, Cheng et al. 1998, Datta et al. 1998), although *B. thuringiensis* rice varieties are not yet available to farmers.

In most of the Bt-transformed crop varieties that have been commercialized to date, the toxin gene is under control of the cauliflower mosaic virus 35S promoter, which drives gene expression in most tissues. An exception is Event 176 maize, developed by Novartis Seeds, in which *cry1Ab* genes are under control of a maize pollen-specific promoter and the maize phosphoenolpyruvate carboxylase (PEPC) promoter (Koziel et al. 1993). The PEPC enzyme catalyzes the first step of CO_2 fixation in photosynthesis of C_4 plants, such as maize, and is highly expressed in mesophyll cells. In rice (a C_3 plant) transformed with a reporter

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gene driven by the PEPC promoter, the reporter gene was found to be expressed in the mesophyll cells of the leaf blades and leaf sheaths (Matsuoka et al. 1994). Levels of expression were very low in other tissues. Tissue-specific promoters such as PEPC are potentially of great use in Bt crops, for example to create within-plant refuges of nontoxic tissues (Alstad and Andow 1996, Gould 1998) or to minimize toxin levels in grain or roots, where the presence of toxins may not be necessary or desirable.

We have previously reported on the transformation of Tarom Molaii, a high-quality, aromatic rice, with a synthetic *cry1Ab* gene under control of the PEPC promoter, and the resistance of one transgenic line to *C. suppressalis* and *S. incertulas* (Ghareyazie et al. 1997). We showed that this line (no. 827) has greatly enhanced stem borer resistance at both the vegetative and booting stages of growth (at booting stage, the developing panicle of the rice plant is still enclosed within the leaf sheath.). Here, we test the resistance of line 827 to stem borers and foliage feeders at a later stage of growth, flowering, and compare the resistance of line 827 to neonate versus 10-d-old larvae of *C. suppressalis* and *S. incertulas.* Because it has been found that a lack of nitrogen results in a reduction of PEPC protein in maize leaves (Sugiharto et al. 1990), we also examine the effect of different levels of nitrogen and other nutrients on insect resistance in line 827. The effects of fertilizer levels on the performance of Bt rice plants is of particular importance in Asia, where many rice farmers do not apply sufficient amounts of fertilizer to their fields (Cassman et al. 1998). Finally, because gene silencing (a lack of transgene expression caused by inhibition of transcription or of mRNA accumulation) is a major problem in plant genetic engineering (Kohli et al. 1996, Kumpatla et al. 1998), we tested whether *cry1Ab* gene expression in line 827 had remained stable in the sixth and seventh generations after plant transformation.

Materials and Methods

Insects. Moths of *C. suppressalis*, *S. incertulas*, *C. medinalis*, and *N. aenescens* were collected from rice fields in the vicinity of the International Rice Research Institute, Laguna Province, Philippines, and transferred to cages containing rice seedlings in a greenhouse. Rice is the only cultivated host plant of *S. incertulas*, whereas the other three insect species have been reported to occur on maize and sorghum (Dale 1994). Bt sprays are very rarely used on cereal crops in the Philippines, thus the exposure to Bt toxins of the insect populations sampled for our experiments was presumably limited to natural *B. thuringiensis* populations. Egg masses laid by stem borers were collected from the plants 1 d before hatching and were transferred to vials as described in Ghareyazie et al. (1997). For *C. medinalis* and *N. aenescens*, 5- and 7-d-old larvae, respectively, were collected from the caged plants on the day of infestation of test plants.

Plants. Seeds of *cry1Ab* and control lines were germinated in an incubator at 28°C and transferred to a seed box containing sterilized soil. Ten to 15 d after seed germination, young seedlings were transferred from the seed box to plastic pots (13 by 15 cm) containing sterilized soil. The following fertilizer treatments were used in all experiments except the experiment specifically designed to test the effect of fertilizer level. In the rainy season (June–December), each pot was treated with 0.52 g N added as $(NH_4)_2SO_4$, 0.08 g P added as P_2O_5, and 0.25 g K added as K_2O. In the dry season (January–May), the N was increased to 0.84 g because of greater solar radiation and higher fertilizer absorption. The levels and timing of N application were chosen to provide a nonlimiting N supply that would be comparable to that in a well-fertilized rice field.

Location of Experiments. All plants were grown in a containment greenhouse approved for growth of transgenic plants, with conditions of 24–28°C, 70–80% RH, and a natural photoperiod of ≈12:12 (L:D) h. To control for variation in temperature and light in different parts of the greenhouse, plants in all experiments were arranged in split-plot or randomized complete block designs, as described below. For experiments using whole plants, the infested plants also were maintained in the containment greenhouse. For experiments using isolated leaves or stem pieces cut from plants, petri dishes containing the plant parts were placed in an air-conditioned room maintained at ≈25°C, 70–80% RH, and a natural photoperiod of 12:12 (L:D) h.

Effect of Plant Growth Stage and Larval Age on Insect Resistance. Vegetative (45-d-old) and flowering stage (10 d after first panicle exertion; 75-d-old) plants of the T_2 generation were evaluated against neonates and 10-d-old larvae of *C. suppressalis* and *S. incertulas.* (A plant derived from tissue culture of transformed embryogenic callus is referred to as a T_0 plant, thus T_2 plants are the third plant generation after transformation.) Seeds of plants to be tested at flowering were germinated 25 d earlier so that the two growth stages could be tested simultaneously. The plants were arranged as two separate experiments, to be dissected at 7 or 14 d after infestation. Each experiment was a split-plot design with four replications. Growth stage of plant was the main plot factor and variety, species, and larval age were subplot factors. There were two levels of each factor. The eight subplot treatments were completely randomized within each main plot.

To produce 10-d-old larvae for the bioassays, 3,000 neonates of *S. incertulas* were released on 480 potted 50-d-old nontransgenic Tarom Molaii plants in a greenhouse. At the same time, 1,600 *C. suppressalis* neonates were released on an additional 420 plants. We released more *S. incertulas* than *C. suppressalis* because we expected that there would be greater larval dispersal and lower larval recovery of *S. incertulas.* The plants were dissected 10 d after infestation, and 400 larvae of each stem borer species were collected. The 10-d-old larvae were mostly third instars. To produce neonates for the experiments, additional *S. incertulas* and *C. suppressalis* moths were collected

from the field 8 and 6 d, respectively, before infestation. Eight hundred neonates of each of the two species were collected from egg masses produced by these moths. All treatments were infested on the same day. Whole plants and isolated leaves and stem pieces were tested as described below.

Cut-Stem Assay with Stem Borers. Thirty-two tillers from 16 plants of each plant line (control and *cry1Ab*) and growth stage were cut at soil level. Two 7-cm stem pieces were cut from each tiller and were placed in petri plates lined with moist filter paper. Each stem piece was infested with five neonates or 10-d-old larvae of *C. suppressalis* or *S. incertulas.* The petri plates were arranged in a completely randomized design with four replications. The stems were dissected after 4 d and the numbers of dead, live, and unrecovered larvae were recorded.

Whole-Plant Assays with Stem Borers. Plants of both growth stages were infested with 20 neonates or ten 10-d-old larvae of either *C. suppressalis* or *S. incertulas* and enclosed individually in tubular Mylar cages. Following infestation, the plants were again arranged in the split-plot design as described above, with four plants per treatment. The plants were dissected after 7 or 14 d and the numbers of dead and live larvae and unrecovered insects were recorded. For plants infested with 10-d-old larvae, the number of pupae at 14 d also was recorded.

Cut Leaf Assay with C. medinalis. Leaf blades were cut from vegetative and flowering stage plants, one per plant, and placed in petri plates (100 by 15 mm) lined with moist filter paper. The petri dishes were arranged on shelves in a completely randomized design with four replications. Each leaf was infested with five larvae of *C. medinalis* (5-d-old) and dissected after 3 d. Numbers of dead, live, and unrecovered larvae were recorded.

Effect of Fertilizer on Insect Resistance. Forty-eight plants of the *cry1Ab* and nontransgenic lines were germinated and transplanted into pots as described above. Plants received one of three fertilizer treatments: NPK, PK, and none. One week after transplanting, the NPK fertilizer treatment received 0.31 g N added as $(NH_4)_2SO_4$, 0.08 g P added as P_2O_5, and 0.25 g K added as K_2O. The PK fertilizer treatment received only 0.08 g P added as P_2O_5 and 0.25 g K added as K_2O, and the third treatment received no fertilizer. Two weeks after the first application, an additional 0.21 g of N as $(NH_4)_2SO_4$ was added to plants receiving the NPK fertilizer treatment.

Whole-plant assays were arranged in an randomized complete block design with six replications. Ten and 20 neonates of *C. suppressalis* were released on each vegetative and booting stage plant, respectively. The booting stage plants were ≈60-d-old. Vegetative plants were dissected after 8 and 20 d and booting stage plants after 25 d. Cut-stem and leaf assays (as described above) were arranged in a completely randomized design with six replications at vegetative stage and with eight replications at booting stage. Leaves and stems and were dissected after 3 and 4 d, respectively.

Resistance of T_5 and T_6 Generation Plants to *N. aenescens* and *C. suppressalis*. Booting stage plants of the T_5 and T_6 generations were tested against *N. aenescens* and *C. suppressalis*, respectively, as described above. Cut leaves were used for the assay with 7-d-old larvae of *N. aenescens* and were dissected 4 d after infestation. Cut stems were assayed against neonate larvae of *C. suppressalis* and were dissected 4 d after infestation.

Immunological Analysis of Cry1Ab Titer. Beginning at 6 wk after sowing, leaf samples (including both blade and sheath) were collected at 2-wk intervals from a single *cry1Ab* plant grown from T_5 seed and a single control plant. Leaf samples were obtained from a single tiller except for the first sampling, when multiple leaves were collected because of their small size. The leaf blade and leaf sheath were stored separately in liquid nitrogen. The samples were ground to a fine powder in the presence of liquid nitrogen. To this powder, 1 ml of extraction buffer (1 mM Tris–Cl, pH 8.1, 10 mM ethylenediaminetetraacetic acid, 0.4 mg/ml phenylmethylsulfonyl fluoride, 100 mM 2-mercaptoethanol) was added. The extracts were centrifuged at 12,000 × *g* at 4°C for 10 min and the supernatant was collected. Protein concentration was determined using the method of Bradford (1976). Forty μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a nitrocellulose filter. Three lanes of trypsinated Cry1Ab (60 kDa) were loaded as standards, at quantities of 5, 15, and 30 ng. The filter was probed with an antiserum raised against Cry1Ab protein (Fujimoto et al. 1993). The filter was washed and treated with secondary antibody conjugated to alkaline phosphatase, then washed and incubated with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine for color development. The amount of Cry1Ab per lane was quantified by densitometry (model GS-700, Bio-Rad, Hercules, CA).

Data Analysis. The data from all experiments were converted to percentages, transformed to arcsine-square root values, and subjected to analysis of variance (ANOVA) (SAS Institute 1998). To facilitate the interpretation of the results and comparisons across the three four-factor experiments, we present the ANOVA tables (Table 1) and the untransformed means for the 16 treatments (Figs. 1–3). The least significant difference (LSD) test was used for planned comparisons between the *cry1Ab* and control varieties within each of the eight growth stage by variety by larval age combinations. In the remaining experiments, which included fewer treatments, we compared means by use of the LSD test if interactions or main effects were significant and there were more than two means.

Results

Effect of Plant Growth Stage and Larval Age on Insect Resistance. In the three experiments with stem borers—the cut stem assay (Table 1; Fig. 1) and the

Table 1. ANOVA results for experiments examining the effects of plant growth stage, stem borer species, larval age, and rice variety

Source of variation[a]	Cut-stem assay[b]		Whole-plant assays: Larval survival[c] 7d			Whole-plant assays: Larval survival[c] 14 d			Whole-plant assays: Pupation[d]		
	F	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Block	—	—	0.5	3, 3	0.72	10.6	3, 3	0.04	0.3	3, 3	0.85
G	8.6	<0.01	0.7	1, 3	0.47	43.3	1, 3	<0.01	3.7	1, 3	0.15
S	16.0	<0.01	36.1	1, 42	<0.01	65.0	1, 42	<0.01	51.1	1, 18	<0.01
L	7.9	<0.01	99.6	1, 42	<0.01	95.8	1, 42	<0.01	—	—	—
V	7.6	<0.01	8.9	1, 42	<0.01	9.2	1, 42	<0.01	4.5	1, 18	0.05
VxL	0.6	0.45	4.6	1, 42	0.04	0.5	1, 42	0.49	—	—	—
VxS	1.5	0.23	2.8	1, 42	0.10	4.6	1, 42	0.04	0.0	1, 18	0.91
SxL	1.5	0.22	10.4	1, 42	<0.01	2.0	1, 42	0.17	—	—	—
GxS	1.0	0.33	1.2	1, 42	0.27	2.8	1, 42	0.10	3.7	1, 18	0.07
GxL	0.0	0.88	5.9	1, 42	0.02	4.7	1, 42	0.04	—	—	—
GxV	14.0	<0.01	25.1	1, 42	<0.01	22.2	1, 42	<0.01	10.7	1, 18	<0.01
VxSxL	0.0	0.99	2.6	1, 42	0.11	3.6	1, 42	0.07	—	—	—
GxSxL	0.3	0.58	0.3	1, 42	0.57	6.8	1, 42	0.01	—	—	—
GxVxS	0.1	0.83	0.3	1, 42	0.60	0.2	1, 42	0.63	3.9	1, 18	0.07
GxVxL	0.4	0.54	6.7	1, 42	0.01	13.0	1, 42	<0.01	—	—	—
GxVxSxL	0.3	0.58	0.4	1, 42	0.52	6.0	1, 42	0.02	—	—	—

[a] G, plant growth stage; S, stem borer species; L, larval age class; V, plant variety.
[b] Completely randomized design; df = 1, 48.
[c] Split plot design with G as main plot factor and S, V, and L as subplot factors.
[d] Split plot design with G as main plot factor and S and V as subplot factors.

whole-plant assays dissected after 7 d (Table 1; Fig. 2) and 14 d (Table 1; Fig. 3)—*cry1Ab* plants were more resistant to neonates than control plants at the vegetative stage but not at the flowering stage. In the cut-stem bioassay, the only significant interaction term was growth stage (G) by variety (V). At the flowering stage, survival did not differ between control and *cry1Ab* plants for either larval age class (L) of either stem borer species (S) (Fig. 1). At the vegetative stage, survival of neonates, but not 10-d-old larvae, was significantly lower on *cry1Ab* plants than on control plants. Despite the difference in survival of neonates and 10-d-old larvae, the GxVxL interaction presumably was not significant because both age classes of larvae showed the same trend across the two varieties.

In the whole-plant assay dissected after 7 d, the GxVxL interaction was significant. At the vegetative stage, neonate survival was higher on control plants than on *cry1Ab* plants, but 10-d-old larval survival did not differ on control and *cry1Ab* plants (Fig. 2). At the flowering stage, survival did not differ on control and *cry1Ab* plants for either age class of larvae (Fig. 2). These results were consistent for both species of stem borers, as indicated by the nonsignificant GxVxSxL and GxVxS interactions (Table 1).

In contrast, in the whole-plant assays dissected after 14 d, the GxVxSxL interaction was significant. Larval survival across the treatments showed a similar pattern for both stem borer species at the flowering stage. However, at the vegetative stage, survival of neonate *C. suppressalis* was higher on control than *cry1Ab* plants, whereas neonate *S. incertulas* survival was low and did not differ on control and *cry1Ab* plants. For both species, survival of 10-d-old larvae did not differ

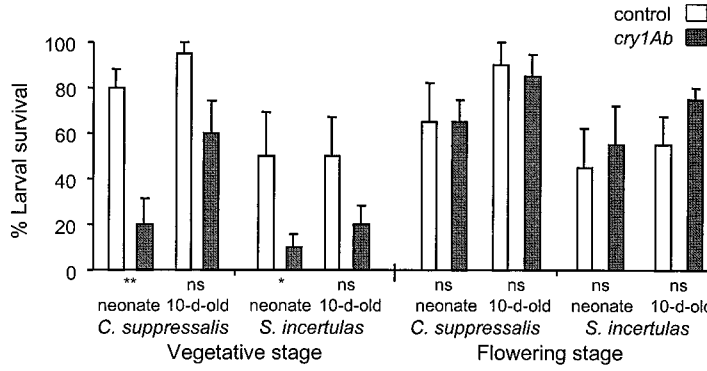

Fig. 1. Mean percentage survival (+SE) of two age classes of *C. suppressalis* and *S. incertulas* larvae on *cry1Ab* and control plants at vegetative and flowering stage in cut-stem assays. For comparisons of means of *cry1Ab* and control plants: ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

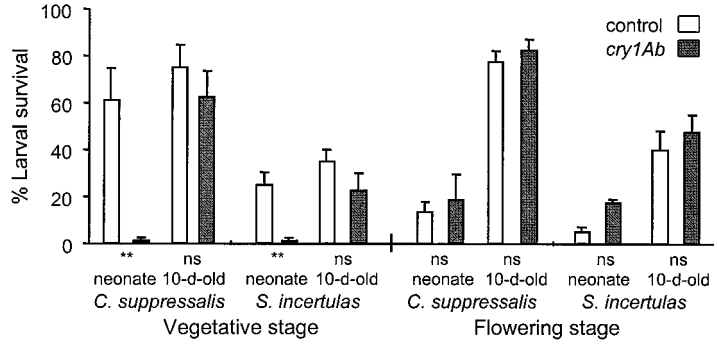

Fig. 2. Mean percentage survival (+SE) of two age classes of *C. suppressalis* and *S. incertulas* larvae on *cry1Ab* and control plants at vegetative and flowering stage after 7 d in whole-plant assays. For comparisons of means of *cry1Ab* and control plants: ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

between control and *cry1Ab* plants at the vegetative stage (Fig. 3A).

None of the neonates had pupated after 14 d in the whole-plant assay, thus neonates were not included in the ANOVA for the percentage of recovered live insects that had pupated (Table 1; Fig. 3B). In this analysis, the GxV interaction was significant. At the flowering stage, pupation of *C. suppressalis* was high on control and *cry1Ab* plants, whereas pupation of *S. incertulas* was low on control and *cry1Ab* plants. At the vegetative stage, pupation of *C. suppressalis*, but not *S. incertulas*, was significantly higher on control than *cry1Ab* plants. Thus, although survival after 14 d of 10-d-old *C. suppressalis* larvae did not differ on *cry1Ab* and control plants (Fig. 3A), larval development was retarded on the *cry1Ab* plants. Larval development of *S. incertulas* is slower than that of *C. suppressalis* (F.A., unpublished data), a difference reflected in the apparently lower pupation rate of *S. incertulas* on control plants at both growth stages (Fig. 3B).

With *C. medinalis*, a foliage-feeding species, the GxV interaction was significant (Fig. 4). *cry1Ab* plants were highly resistant at the vegetative stage, but the *cry1Ab* and control plants did not differ in resistance at the flowering stage (Fig. 4). Larval survival on control plants was lower at the vegetative stage than

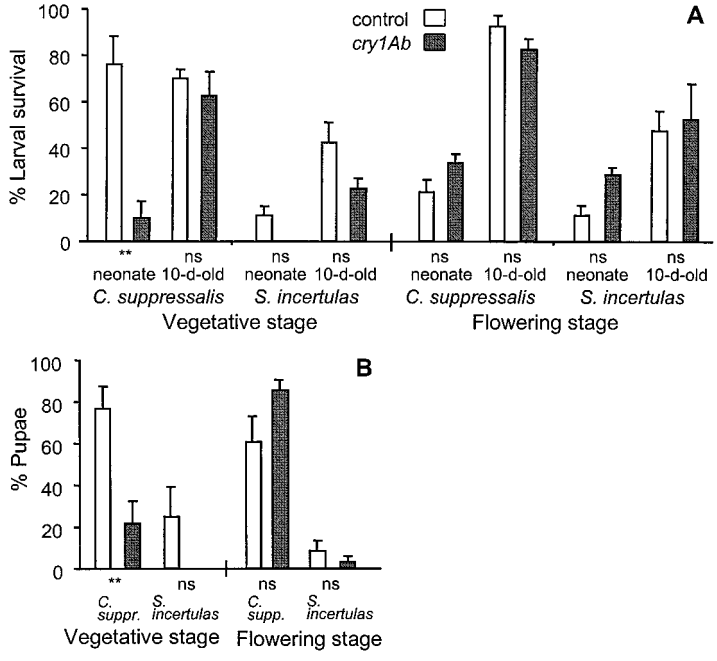

Fig. 3. (A) Mean percentage (+SE) larval survival two age classes of *C. suppressalis* and *S. incertulas* on *cry1Ab* and control plants at vegetative and flowering stage after 14 d in whole-plant assays. (B) Percentage live insects that were pupae on plants infested with 10-d-old larvae. For comparison of means of *cry1Ab* and control plants: ns, not significant; **, significant at $P \leq 0.01$.

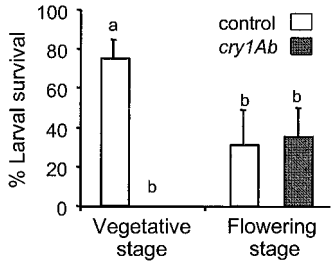

Fig. 4. Mean percentage (+SE) survival of *C. medinalis* on leaves of vegetative and flowering stage plants at 3 d after infestation. The plant growth stage × variety interaction is significant ($F = 10.5$; df = 1, 12; $P < 0.01$). Means sharing the same letter within a growth stage are not significantly different ($P > 0.05$, LSD test).

Table 3. Mean percentage (± SE) larval survival of *C. suppressalis* on booting stage *cry1Ab* and control plants with three fertilizer treatments

Variety (V)	Fertilizer treatment (F)	Cut-stem assay[a]	Whole-plant assay[b]
Control	None	67.5 ± 9.2	37.5 ± 7.5c
	PK	77.5 ± 8.0	68.3 ± 6.7b
	NPK	67.5 ± 10.0	90.8 ± 4.5a
cry1Ab	None	30.0 ± 5.3	30.8 ± 13.5cd
	PK	27.5 ± 6.5	29.2 ± 7.0cd
	NPK	30.0 ± 5.3	10.0 ± 3.4d

[a] Dissected after 4 d. FxV: $F = 0.5$; df = 2, 42; $P = 0.62$. F: $F = 0.2$; df = 2, 42; $P = 0.85$. V: $F = 44.4$; df = 1, 42; $P < 0.01$.
[b] Dissected after 25 d. Means followed by the same letter are not significantly different (LSD test, $P > 0.05$). FxV: $F = 9.9$; df = 2, 25; $P < 0.01$. F: $F = 2.3$; df = 2, 25; $P = 0.12$. V: $F = 38.4$; df = 1, 25; $P < 0.01$.

at the flowering stage, indicating that leaves from older plants are less suitable for *C. medinalis* development.

Effect of Fertilizer on Insect Resistance. Vegetative stage *cry1Ab* plants at all three fertilizer levels were highly resistant to *C. suppressalis* in comparison with control plants (Table 2). In the cut-stem and whole-plant assays with *C. suppressalis*, and the experiment with *C. medinalis*, the fertilizer by variety (FxV) interaction and the effect of fertilizer were not significant, whereas the effect of variety was highly significant. In contrast, in the whole-plant assay with *C. suppressalis* at the booting stage, the FxV interaction was highly significant (Table 3). On control plants, larval survival was lowest on plants receiving no fertilizer and highest on plants receiving the NPK fertilizer treatment. However, larval survival on *cry1Ab* plants did not differ significantly among the three fertilizer levels. In the cut-stem assay with booting stage plants, the FxV interaction and effect of fertilizer were not significant (whereas the effect of variety was highly significant), possibly because the period of exposure of larvae to the plants was limited to 4 d (Table 3). In all experiments, plants that did not receive N had yellowed foliage, a typical sign of nitrogen stress.

Resistance of T_5 and T_6 Generation Plants to *N. aenescens* and *C. suppressalis*. In a bioassay with booting stage plants of the T_5 generation, survival of *N. aenescens* larvae after 4 d was lower on leaves from *cry1Ab* plants (2.5 ± 2.5%) than leaves from control plants (95.0 ± 3.3%) ($F = 441.5$; df = 1, 14; $P < 0.01$). Vegetative plants tested at the T_6 generation were more resistant to *C. suppressalis* than control plants. After 4 d, 90.0 ± 3.8% of larvae were recovered alive on control plants, compared with 45.0 ± 13.0% on *cry1Ab* plants ($F = 10.9$; df = 1, 15; $P < 0.01$), indicating that *cry1Ab* gene expression was stable seven generations after plant transformation.

Immunological Analysis. As observed previously (Ghareyazie et al. 1997), our Cry1Ab antiserum recognizes an abundant 67 kDa protein as well as a series of smaller products that range from 60 to near 67 kDa in size in line 827 (Fig. 5). We analyzed extracts from only one control and one *cry1Ab* plant, thus the levels of Cry1Ab in leaf blades and sheaths at different growth stages (Fig. 5) could not be statistically compared. In the plant that was analyzed, the titer of Cry1Ab was apparently lower at flowering stage than at vegetative stage; a result consistent with our bioassays. The level of Cry1Ab appeared to be higher in leaf blades than in leaf sheaths (Fig. 5), whereas Cry1Ab levels in stems of line 827 were below the level of detection of our immunoblots (not shown). When extrapolations were made from the lanes of the Cry1Ab standards, the estimated titers of Cry1Ab in

Table 2. Mean percentage (± SE) larval survival of *C. medinalis* and *C. suppressalis* on vegetative stage *cry1Ab* and control plants with three fertilizer treatments

Variety (V)	Fertilizer treatment (F)	% larval survival			
		C. medinalis[a]	*C. suppressalis*		
			Cut-stem assay[b]	Whole-plant assay	
				8 d[c]	20 d[d]
Control	None	60.0 ± 10.7	92.5 ± 5.3	78.3 ± 0.09	38.0 ± 10.0
	PK	72.5 ± 7.5	92.5 ± 5.3	93.3 ± 0.03	45.0 ± 8.0
	NPK	67.5 ± 10.0	92.5 ± 3.7	88.3 ± 0.05	56.7 ± 13.3
cry1Ab	None	30.0 ± 5.3	12.5 ± 5.3	0.0 ± 0	3.3 ± 3.3
	PK	32.5 ± 10.6	17.5 ± 8.0	0.0 ± 0	1.7 ± 1.7
	NPK	30.0 ± 5.3	5.0 ± 3.3	0.0 ± 0	3.3 ± 3.3

[a] FxV: $F = 0.2$; df = 2, 42; $P = 0.84$. F: $F = 0.4$; df = 2, 42; $P = 0.69$. V: $F = 25.5$; df = 1, 42; $P < 0.01$.
[b] FxV: $F = 0.7$; df = 2, 42; P = 0.53. F: $F = 0.7$; df = 2, 42; $P = 0.51$. V: $F = 322.6$; df = 1, 42; $P < 0.01$.
[c] FxV: $F = 1.6$; df = 2, 25; $P = 0.23$. F: $F = 1.6$; df = 2, 25; $P = 0.23$. V: $F = 588.3$; df = 1, 25; $P < 0.01$.
[d] FxV: $F = 0.8$; df = 2, 24; $P = 0.45$. F: $F = 0.9$; df = 2, 24; $P = 0.44$. V: $F = 80.4$; df = 1, 24; $P < 0.01$.

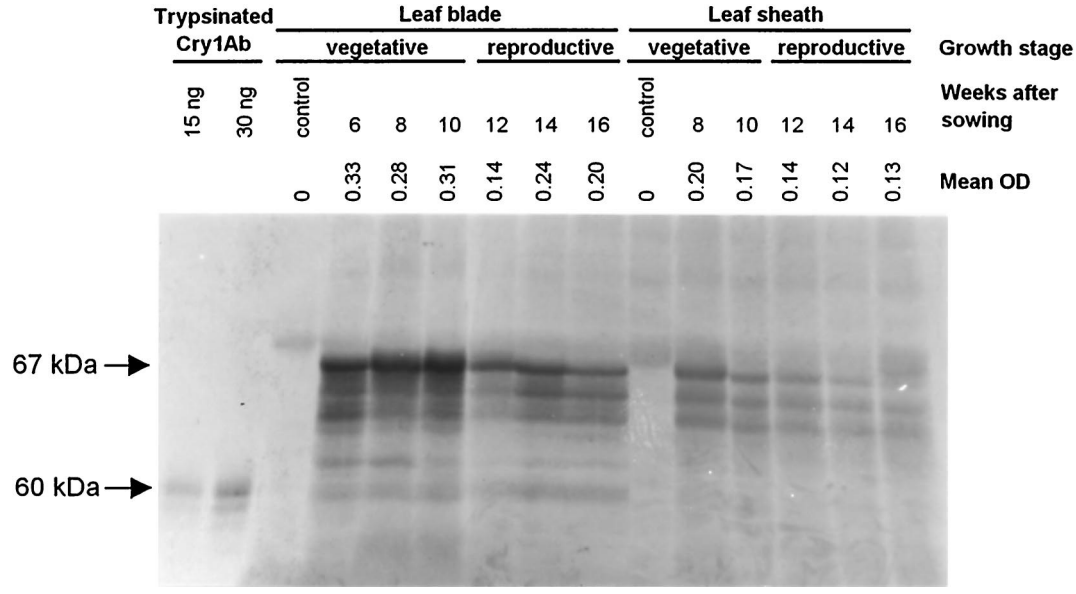

Fig. 5. Immunological detection of Cry1Ab in leaf blades and sheaths of line 827 at different times after sowing. Vegetative stage refers to tillering and booting developmental stages. Reproductive stage refers to flowering and postflowering developmental stages. Mean OD is the mean number of pixels in the area scanned between 60 and 67 kDa, after correction for background staining in the lanes of protein from the control plant, as determined by densitometry.

leaves at the vegetative and reproductive stages were 0.7–0.8 and 0.4–0.6% of soluble protein, respectively. In leaf sheaths, the estimated titers of Cry1Ab at the vegetative and reproductive stages were 0.5 and 0.4% of soluble protein, respectively.

Discussion

At the vegetative stage, plants of line 827 transformed with *cry1Ab* under the control of the PEPC promoter were, in all assays but one (the whole-plant assay with *S. incertulas* dissected after 14 d), significantly more resistant than nontransgenic plants to neonates of *C. suppressalis* and *S. incertulas.* In contrast, survival of 10-d-old larvae of *C. suppressalis* and *S. incertulas* did not differ significantly on *cry1Ab* and control plants at the vegetative stage (Figs. 1 and 2, and 3A), although development of 10-d-old larvae of *C. suppressalis* was retarded (Fig. 3B). Studies with numerous insect species have found that, on plants transformed with *B. thuringiensis* toxin genes, older larvae have higher survival or delayed mortality compared with younger larvae (e.g., Jenkins et al. 1993, Halcomb et al. 1996, Wierenga et al. 1996). However, another important factor explaining the greater survival of 10-d-old stem borer larvae in our experiments may be the feeding behavior of *S. incertulas* and *C. suppressalis* relative to the pattern of *cry1Ab* gene expression among plant tissues when driven by the PEPC promoter. Neonate stem borer larvae feed on the leaf sheath, a tissue in which the PEPC promoter is active. After ≈4–7 d, the larvae move to the lumen of the stem, where they feed on the nodes and internodes (F.A., unpublished data; Pathak and Khan 1994). In contrast, 10-d-old larvae (approximately third instar) that colonize new tillers penetrate the leaf sheath and move directly to the nodes and internodes of the stem. Matsuoka et al. (1994) found that the PEPC promoter is not active in rice stems, and in line 827 the nodes and internodes do not contain detectable levels of Cry1Ab (B.G., unpublished data). Thus, in our experiments, the 10-d-old larvae that were transferred from non-Bt plants to *cry1Ab* plants ingested only a small amount of toxic tissue (while penetrating the leaf sheaths) before continuing development on the toxin-free tissues of the stem.

At the flowering stage, the *cry1Ab* and control plants did not differ in resistance to 5-d-old larvae of *C. medinalis* or to neonates or 10-d-old larvae of *C. suppressalis* and *S. incertulas.* The decline in resistance of line 827 at the flowering stage is consistent with the results of our immunological analysis (Fig. 5), which showed an apparent decline in Cry1Ab titer in both the leaf sheath and blade at the reproductive stage in comparison with the vegetative stage. The decline in toxin titer of line 827 is also consistent with the decline in insect resistance observed after pollen shed in Event 176 maize (Andow and Hutchinson 1998), which also has a *cry1Ab* gene under control of the PEPC promoter (Koziel et al. 1993). The decline of toxin titer in the leaf sheaths of line 827 may be related to morphological changes during development, such as a decline in the proportion of mesophyll cells relative to vascular tissues, in which the PEPC promoter is not active. Leaf senescence and an associated decline in photosynthesis also may be a contributing factor. Our experiments were conducted with only one rice transformation event containing *cry1Ab* un-

der control of the PEPC promoter, thus additional transformation events should be studied to determine whether the decline in toxin titer at reproductive stage is consistent across transformation events.

There are thus two mechanisms that will result in some stem borers on plants of line 827 ingesting relatively reduced amounts of Cry1Ab during the course of larval development. First, larvae that are able to survive long enough to penetrate the stem and feed on the nodes and internodes will no longer ingest significant amounts of toxin. These may be either larvae that hatch on transgenic plants and survive early development in the leaf sheath; or, in the case of a seed mixture, older larvae that disperse from Bt or non-Bt plants. Second, larvae that attack plants at, or slightly before, flowering stage will ingest leaf sheath tissue that contains substantially reduced levels of toxin. This reduced exposure to toxin may result in increased stem borer damage at the reproductive stage of the crop. In tropical irrigated rice, where rice plants are available throughout the year and stem borer generations are overlapping, plants can be attacked by stem borers from shortly after transplanting to several weeks after flowering (Pathak and Khan 1994). From a resistance management perspective, reduced toxin exposure can increase the survival of larvae with low levels of resistance (e.g., larvae that are heterozygous at resistance-associated loci in which alleles for resistance are partially recessive). Population genetics models indicate that the resistance of pest populations to toxins can increase much faster when such insect genotypes survive (e.g., Gould 1998, Onstad and Gould 1998).

Nitrogen is an important regulator of carbon flow and the synthesis of some proteins in higher plants and algae (Yamazaki et al. 1986, Sugiharto et al. 1990). In maize, a lack of nitrogen reduced the levels of PEPC protein, especially in the photosynthetic maturing leaf cells, as a result of reduction in the level of PEPC mRNA (Sugiharto et al. 1990). A similar effect would be expected for a *cry1Ab* gene under control of the PEPC promoter, and we therefore tested the effect of fertilizer on insect resistance in line 827. There was a significant interaction between fertilizer treatment and variety (i.e., *cry1Ab* versus control) only in the whole-plant assay at booting stage with *C. suppressalis* (Table 3). The interaction was caused principally by higher larval survival on control plants that received fertilizer compared with control plants without fertilizer. Numerous studies have found increased survival of insect herbivores on plants with higher nitrogen content (Mattson 1980, Scriber 1984). Larval survival did not differ significantly on *cry1Ab* plants receiving no fertilizer compared with plants treated with NPK (10.0 ± 3.4 and 30.8 ± 13.5%, respectively). However, the effect of nitrogenous fertilizer on insect resistance in Bt rice with the PEPC promoter deserves further study, particularly at reproductive stage and under field conditions.

A major difficulty in whole-plant assays with rice stem borers is the large number of unrecovered neonates (Ghareyazie et al. 1997). The problem is particularly severe with neonate *S. incertulas*, which have a strong tendency to disperse shortly after egg hatch, by ballooning on silk threads (Dirie 1998). The lower survival on control plants of neonate *S. incertulas* compared with *C. suppressalis* (Figs. 2 and 3A) is probably in part attributable to the greater proportion of *S. incertulas* larvae that attempted to disperse, and either succeeded in escaping from the tubular cage enclosing the plants or died and fell into the soil. Another factor that contributes to low recovery of stem borer larvae is that, even on relatively susceptible varieties, many larvae either fail to penetrate the leaf sheaths or die shortly after penetration. Neonate larvae that die shortly after penetrating the leaf sheath are very difficult to detect in plants that are dissected a week or more after infestation. As a consequence of these factors, ≈95% of larvae that we did not recover alive during plant dissection were not found dead within the plant, but instead could not be located.

In experiments examining dispersal behavior of stem borer larvae from *cry1Ab* (line 827) and control plants, Dirie (1998) found that a significantly higher proportion of neonate *S. incertulas* dispersed from *cry1Ab* than control plants, but that the proportion of neonate *C. suppressalis* dispersing from *cry1Ab* and control plants did not differ. It is likely that this higher rate of dispersal from *cry1Ab* plants contributed to the difference in *S. incertulas* larval survival between *cry1Ab* and control plants in our whole-plant assays.

Cropping practices may affect development of resistance to transgenic *cry1Ab* rice. Line 827 is potentially a useful transformation event for future Bt rice development in Iran, where ≈40% of the 650,000 ha of rice are planted to short-duration aromatic varieties such as Tarom Molaii. At the vegetative stage, line 827 is highly resistant to *C. suppressalis* and *N. aenescens*, the two most important insect pests of rice in Iran. In regions of Iran where short-duration varieties predominate, ≈10% of land is planted to long-duration varieties, interspersed among the short-duration varieties. If only the short-duration varieties were transformed with Bt toxin genes, then fields under long-duration varieties would serve as refuges of non-Bt rice. *C. suppressalis* populations in Iran complete two generations per year on short-duration varieties, and two or three generations per year on long-duration varieties. The entire first generation and part of the second generation develop on plants that are at the vegetative stage. Some late instars of the second generation will enter diapause and overwinter in the rice crop stubble. Those second-generation *C. suppressalis* that do not enter diapause must oviposit on long-duration varieties because the short-duration varieties are no longer suitable. Late instars of the third generation overwinter in the stubble of the long-duration varieties. More information is needed to predict how the decline in Cry1Ab titer in line 827 at flowering stage will affect the evolution of *cry1Ab* resistance in *C. suppressalis* and *N. aenescens*, in interaction with local seasonal conditions and cropping practices. Important research topics include the premating dispersal of adults between fields of long-duration and

short-duration rices, and the effect of resistance to Cry1Ab on overwintering mortality and timing of postdiapause development.

Gene silencing is one of the major constraints to the efficient development of transgenic plants (Kohli et al. 1996, Kumpatla et al. 1998). Although gene silencing has been observed as early as the T_0 generation, it can appear gradually during second and third generations (Kohli et al. 1996). We have shown that *cry1Ab* expression in homozygous plants of line 827 has been stable up to the T_6 generation, which suggests that gene silencing is unlikely to occur in this line. Because the expression of the *cry1Ab* gene in line 827 has been stable for seven generations after transformation, this transformation event would appear to be a good candidate for transfer of the *cry1Ab* gene to other elite varieties through conventional breeding.

Because it is active in the leaf sheath and leaf blade, the PEPC promoter is a promising promoter for use in Bt rice targeted against lepidopterous foliage feeders and stem borers. To provide a more consistent high dose of toxin in transgenic plants, the PEPC promoter could be used in combination with a promoter that has a complementary pattern of expression. Use of the PEPC promoter with a pith-specific promoter such as that isolated from maize (Kramer and Koziel 1995) may be a promising combination. Production of rice plants with *cry1Ab* under control of both these promoters is in progress at International Rice Research Institute.

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