SHORT COMMUNICATION

A leaf-section bioassay for evaluating rice stem borer resistance in transgenic rice containing a synthetic *cry1Ab* gene from *Bacillus thuringiensis* Berliner

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Introduction

In Asia, lepidopterous stem borers, such as the yellow stem borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae) are economically damaging to rice, *Oryza sativa* L. (Poaceae). Average annual yield losses are often estimated at 5–10%, and occasionally as much as 60–95% (Pathak & Khan, 1994). Transgenic plants expressing insecticidal toxins offer a completely new approach to stem borer control. Several synthetic *cry1Ab* or *cry1Ac* genes from *Bacillus thuringiensis* Berliner (Bt) have been transferred into *indica* and *japonica* cultivars and proven effective against several lepidopterous species (Fujimoto *et al*., 1993; Wünn *et al*., 1996; Ghareyzaie *et al*., 1997; Cheng *et al*., 1998). With the availability of transgenic germplasms with insect resistance, simple and effective methods for evaluating insect resistance are urgently needed for large-scale screening to assess incorporation of the transgenic insect resistant traits in breeding programmes. Currently two methods are used, cut stem bioassay and whole plant bioassay. Both two methods have shortcomings, notably: (i) small neonate larvae often disperse from test plant material and are not recoverable (Ghareyzaie *et al*., 1997); (ii) manpower required to dissect test plant material and score mortality is often limiting; (iii) the seeds from test plant material cannot be collected from partially damaged plants and used in further breeding programmes. A leaf-section bioassay has been reported by Wünn *et al*. (1996), which required leaf material to be replaced three days after the start of the bioassay. However, this method used larval mortality as the only criterion for evaluating the resistance of transgenic rice to stem borers, and did not directly analyse the correlation between the results from leaf-section and whole plant bioassays. Here we report a novel bioassay using a single piece of plant material which does not need to be changed during the test. Using this method, we were able to bioassay a large number of transgenic rice lines derived from a Chinese *japonica* cultivar (Xiushui 11), into which the *cry1Ab* gene had been inserted by the *Agrobacterium*-infection method (Cheng *et al*., 1998; Shu *et al*., 1998).

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Materials and methods

Test insects and plants

Adult moths or egg masses of *S. incertulas* were collected from rice fields in Huzhou, China in July 1997, and reared in the Zhejiang University insectary. Moths were fed on 10% honey solution, and allowed to mate and oviposit in nylon cages containing rice plants of an untransformed Chinese *japonica* cultivar (Xiushui 11). Egg masses produced by the moths were collected from the plants and transferred to 20 × 2.5 cm glass tubes containing pieces of moist filter paper. Neonate larvae from these egg masses, as well as from field-collected individuals, were allowed to hatch in the glass tubes prior to bioassay.

Two transgenic lines PR16 (homozygous) and PR18 (segregating) at the R3 generation, derived from a Chinese *japonica* cultivar (Xiushui 11) and an untransformed Xiushui 11 (control) were sown in early May and grown in a greenhouse. Both lines contained the *cry1Ab* gene, controlled by a maize ubiquitin promoter (Cheng *et al*., 1998).

Resistance evaluation with a leaf section bioassay

Two small pieces (3–4 cm length), one per leaf, were cut from separate leaves closest to the flag-leaves of two tillers from each of 30 plants per transgenic line and the control. Each leaf section was kept in a small glass tube (6.5 × 1.5 cm), plugged with non-absorbent cotton and placed on a

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Table 1. The toxicity of leaf sections of two transgenic rice lines containing a synthetic *cry1Ab* gene against neonate larvae of *Scirpophaga incertulas*.

Genotype	No. of plants tested	No. of resistant plants[1]	% mortality[2,3]		Cumulative leaf area eaten (mm^2 per larva)[3] $\bar{x}$ ± S.E.	
			2nd	4th day	2nd	4th day
PR16	30	30	98.3a	100.0a	0.67±0.03b	0.67±0.03b
PR18	30	8	88.9a (0.0b)	100.0a (5.6b)	0.75±0.03b (5.78±0.14a)	0.75±0.03b (12.79±0.24a)
Untransformed	30	0	0.0b	6.7b	6.04±0.11a	13.34±0.21a

[1]A plant was judged resistant if the consumed leaf area per larva was ≤ 1 mm^2 and larval mortality was 100% four days after larval inoculation.
[2]Data in brackets refer to 22 non-resistant plants of PR18.
[3]Means in the same column followed by different letters are significantly different at $P_{0.01}$ based on Duncan' s multiple range test.

piece of filter paper (4–5 cm in length) moistened with distilled water containing 100 ppm benzimidazole to prevent fungal contamination. Each cut end was covered with a small piece of filter paper moistened with the benzimidazole solution. Five *S. incertulas* neonate larvae (0~12 h old) were transferred to each tube. Larval mortality and consumed leaf area were recorded every other day for four days. The leaf sections remained green and fresh, and so did not require renewal.

To determine which leaf was more suitable for bioassay, 3–4 cm pieces of each of the four leaves directly beneath the flag-leaf of the same tiller were cut from 30 plants of each tested line. Leaf samples were treated as above. Ten *S. incertulas* neonate larvae (0~12 h old) were placed in each of 30 tubes for leaf samples from each position, and then mortality and feeding area were recorded after two days.

Resistance evaluation of whole plants

Ten neonate larvae of *S. incertulas* were placed on each test plant. The Xiushui 11 plants were 2.5 months old, and still at the vegetative stage because of photoperiod sensitivity. Each plant was covered with a mylar tube (45 × 12 cm) with two windows of nylon mesh (10 cm in diameter). The tube was covered with dark cloth for one day after larval inoculation to deter any larval escape. Thirty plants of each transgenic line and 28 plants of the control were tested, respectively. Sections of leaf for the leaf-section bioassay were obtained from these same plants. The number of damaged plants with one or more deadhearts was scored after one month.

Results and Discussion

The mortality of *S. incertulas* larvae was significantly higher after two and four days in homozygous PR16 and PR18 resistant plants than in untransformed plants and PR18 non-resistance plants (table 1). Consequently, very low levels of feeding damage to leaves were observed in PR16 and PR18 resistant plants (table 1). These results indicate that the leaf-section bioassay can be used to detect differences in stem borer resistance among untransformed and transgenic rice plants.

Results from the bioassay using leaf samples from different positions on the same plant showed that larval mortality after two days was unaffected by leaf position (table 2). However, the amount of leaf area consumed was slightly higher for the first and second leaves than for the third and fourth leaves, especially for transgenic plants, suggesting that neonate larvae of *S. incertulas* preferred to eat younger leaves (table 2). The lack of leaf age or positional effect on larval mortality was in accordance with the results of a previous Bt insecticidal crystal protein assay (X. Cheng

Table 2. Influence of leaf position on the toxicity of leaf sections of two transgenic rice lines containing a synthetic *cry1Ab* gene against *Scirpophaga incertulas* larvae.

Leaf position[1]	48 h % mortality[2]		Leaf area eaten (mm^2 per larva)[2] $\bar{x}$ ± S.E.	
	PR16	Untransformed	PR16	Untransformed
1st	100.0a	0.0a	0.36±0.01aA	5.01±0.08aA
2nd	99.0a	5.0a	0.29±0.01aA	4.78±0.04abAB
3rd	100.0a	0.0a	0.19±0.01bB	4.56±0.08bAB
4th	100.0a	0.0a	0.18±0.01bB	4.39±0.11bB

[1]Leaves are numbered relative to the flag-leaf, with the first leaf being closest to the flag-leaf.
[2]Means in the same column followed by different capital or lower case letters are significantly different at $P_{0.01}$ or $P_{0.05}$, respectively, based on Duncan's multiple range test.

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Table 3. Comparison of stem borer resistance of transgenic rice lines based on leaf-section and whole plant bioassays.

Genotype	No. of plants tested	% resistant plants			
		Leaf-section bioassay[1]			Whole plant bioassay[2]
		I	II	III	
PRI6	30	100	100	100	100
PRI8	30	23.3	33.3	26.7	26.7
Untransformed	28	0	7.1	0	0

[1]In the leaf-section bioassay, an individual plant was judged resistant according to three criteria: I, the leaf area consumed per larva was ≤ 1 mm^2 four days after larval inoculation; II, larval mortality was 100% four days after larval inoculation; III, criteria I and II combined.
[2]In the whole plant bioassay, an individual plant was judged resistant if no deadhearts were observed.

et al., unpublished data), which found that Bt protein level was very similar in all leaves. Thus a section from any leaf can be used for bioassay purposes.

In the whole plant bioassay, all plants of homozygous PR16 and 26.7% plants of heterozygous PR18 were highly resistant to *S. incertulas*, with no observed deadhearts (table 3). In contrast, 100% plants in the controls and 73.3% plants of heterozygous PR18 were encountered with one or more deadhearts. The percentages of deadhearts among total tillers in the controls and PR18 susceptible plants were 37.4% and 38.2%, respectively.

When the results from the leaf-section bioassay and the whole plant bioassay were compared, it was clear that two methods provided similar results, particularly when both larval mortality and leaf area consumed were used as the criteria for assessing resistance in the leaf-section bioassay (table 3). Our leaf-section bioassay is different from the method of Wünn *et al*. (1996), who used larval mortality as the only criterion for measuring stem borer resistance. We found that the amount of leaf area consumed was a critical factor in evaluating resistance, and the amount per larva per plant was positively correlated with the percentage of deadhearts on each plant based on an analysis of the three genotypes tested (fig. 1). Otherwise, some plants of transgenic heterozygous PR18 and the control were scored as resistant based on larval mortality as the only criterion, because larval mortality can be affected by manipulation injury or leaf death. In contrast to the whole plant bioassay, which lasts at least a month and provides little information about larval mortality and feeding damage, our leaf-section bioassay can generate quantitative information on larval mortality and feeding damage in less than a week. The method has the great advantage of not requiring any changes of leaf material, is simple and rapid, and also avoids damaging whole plants. It is particularly useful for evaluating stem borer resistance of transgenic rice at the early experimental-developmental stages when only small numbers of transgenic plants are available or when the resistance of many transgenic lines needs to be evaluated simultaneously. The method should also be suitable for evaluating the resistance of transgenic rice containing Bt toxins against other rice stem borer species such as the striped stem borer. *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae) and the pink stem borer, *Sesamia inferens* (Walker) (Lepidoptera: Noctuidae) (G.-Y. Ye *et al*., unpublished data).

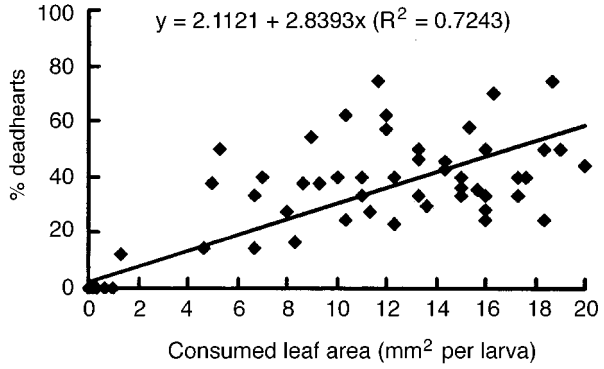

Fig. 1. Correlation between the percentage of deadhearts per rice plant and leaf area per larva consumed by *Scirpophaga incertulas* larvae.

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