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Field performance of *Xa21* transgenic indica rice (*Oryza sativa* L.), IR72

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Abstract Based on the characterization of the resistance phenotype and molecular analysis, several homozygous lines carrying *Xa21* against the bacterial blight (BB) pathogen were obtained from previously transformed indica rice, IR72. The homozygous line, T103-10, with the best phenotype and seed-setting, was repeatedly tested under normal field conditions to evaluate its levels of resistance to the BB pathogen in Wuhan, China, in 1998 and 1999. The isolates of *Xanthomonas oryzae* pv *oryzae* (*Xoo*) used in this experiments were PXO61, PXO79, PXO99 and PXO112 isolated from the Philippines, T2 isolated from Japan, and Zhe173 isolated from China. The results demonstrated that the transgenic homozygous line expressed the same resistance spectrum, but with a shorter lesion length to each inoculated isolates as the lesion length of the *Xa21* donor line IRBB21. The non-transformed control IR72 carrying *Xa4* was resistant to PXO61, PXO112, Zhe173 and T2, but susceptible to PXO99 and PXO79. The negative control variety IR24 was susceptible to all isolates under field conditions. The results demonstrated clearly that the *Xa21* transgene led to an excellent field performance of the introduced bacterial blight resistance trait on the recipient plants. The yield performance of this transgenic homozygous line, T103-10, is comparable with that of the control under field conditions.

Key words *Xa21* · IR72 · Bacterial leaf blight · Transformation · Field testing

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Introduction

Recent advances in molecular biology are greatly increasing our ability to characterize and manipulate disease resistance genes in plants. Several resistance genes that commonly occur in tightly linked clusters, such as *Xa21*, *PTO*, *CF9*, *Dm3*, *N*, *L6*, *RP1* and *RPs*, have been cloned from rice, tomato, lettuce, flax, maize and *Arabidopsis* (Martin et al. 1993; Bent et al. 1994; Jones et al. 1994; Lawrence et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994; Song et al. 1995; Witsenboer et al. 1995). Detailed molecular analyses are now beginning to unravel the complexity of these loci and the underlying mechanisms determining their structure (Parniske et al. 1997; Song et al. 1997). We now know that most of those genes encode similar nucleotide-binding sites (NBS) and/or leucine-rich-repeat (LRR) motifs and act early in a signalling pathway in the process of plant disease resistance (reviewed by Baker et al. 1997).

The cloned *Xa21* is the first well-characterized disease resistance gene that has been described in rice (Song et al. 1995). Aside from having the above common molecular features, it possesses a downstream serine-threonine kinase (STK) domain that is similar to *PTO* kinase in tomato (Martin et al. 1993) and is capable of autophosphorylation relative to intracellular signalling at multiple sites (Song et al. 1997; Wang et al. 1998). Thus, the structure of *Xa21* confirms the evolutionary link between different classes of plant disease resistance genes.

Xa21 is known to confer resistance to most known races of *Xoo* in both India and the Philippines (Ikeda et al. 1990; Khush et al. 1990). For better use of this structurally and functionally important bacterial blight (BB) resistance gene, we introduced *Xa21* into the genome of the elite indica rice cultivar IR72 via the biolistic method (Tu et al. 1998b). The transformant obtained was designated as T103. Molecular analysis of T_0 and T_1 plants of T103 demonstrated that the intact coding sequence of *Xa21* was present in the recipient genome, without rearrangement, and that the inheritance of the transgene in

the T_1 generation fits the one-locus integration pattern. The T_1 plants positive for the transgene proved to be highly resistant to two prevalent races, 4 and 6, of *Xoo*.

Although many rice cultivars have been transformed with insect, disease, virus, and herbicide resistance in different laboratories (Datta et al. 1992, 1998; Lin et al. 1995a; Alam et al. 1998; Tu et al. 1998a), only transgenic rice with the agronomically important *bar* gene has been tested under field conditions (Oard et al. 1996). Therefore, we isolated several homozygous lines from the previous transformants based on both a large-scale phenotype bioassay and molecular analysis in the subsequent generations, and one of them was tested under normal field conditions to evaluate the levels of resistance to the BB pathogen. We report here the results obtained from these tests.

Materials and methods

Genetic materials

A T_2 homozygous line of the transgenic IR72 (Tu et al. 1998b), T103-10, was used in the field tests. For its isolation, 15 out of 20 T_2 progenies were subjected to a BB bioassay. In 1997, 30 plants from each T_2 progeny and the IR72 control were grown in the IRRI containment greenhouse under the following conditions: 29°C and 85% humidity at daytime and 25°C and 90% humidity at night. Each plant was inoculated with two prevalent races, 4 (PXO71) and 6 (PXO99), of *Xoo* at the maximum tillering stage using the leaf-clipping method (Kauffman et al. 1973). The plant reaction to each race of *Xoo* was scored on six leaves 16 days after inoculation. The T_2 progenies showing an identical phenotype resistant to both races of *Xoo* (4 and 6) were identified as homozygous and the identified homozygous plants were further confirmed by Southern-blot analysis.

Plot design

Field evaluation of the efficiency of the homozygous line, T103-10, resistant against the BB pathogen, was performed at the Huazhong Agricultural University Research Farm, situated in Wuhan (latitude 30°34′ North; longitude 114°17′ East), near the Yangtze River, China. Transgenic plants were sown in a seedling bed 4 weeks before planting and transplanted to the paddy field in early May, 1998 and 1999. Irrigation was provided as needed during growth. The plot for disease testing in 1998 was divided into three subplots and each subplot consisted of two rows, 1.86-m long and 0.53 m wide, with 11 plants spaced at 13 cm within a row and four replications for each testing material. The plot for 1999 was divided into six subplots and each subplot consistes of two rows, but extended to 3.96-m long and 1.29-m wide, with 15 plants spaced at 19.8 cm within a row and three replications for each testing material. Non-transgenic IR72 and IR24 were planted as susceptible controls and *Xa21* donor line IRBB21 was planted as the resistant control. Normal cultural practices for growing rice were followed during the course of the experiment, except that no chemical treatment was applied to allow for an optimum evaluation of the resistant reaction of *Xa21*. The plots were inoculated at the maximum tillering stage using the leaf-clipping method (Kauffman et al. 1973), with one race per subplot. The races used for inoculation were Philippines race 6, PXO99, Japanese race 2, T2, and Chinese race 4, Zhe 173, in 1998 and the above three plus PXO61, PXO79 and PXO112, which represent Philippines races I, III and V, in 1999. Plant reactions to each race of BB in this experiment were scored on three leaves 16 days after inoculation. Plants with a lesion length of less than 6 cm were considered resistant. The inocula were prepared by incubating the bacteria on Wakimoto's medium for 72 h at 30°C and then suspending each pure culture in sterile distilled water and adjusting the inocula to about 10^9 cells per ml.

The plots for the yield evaluation in 1999 were arranged in a field separated from that used for disease testing and contained three replications. The testing material in each replication was randomly planted in a subplot. The area of the subplot was 12 m^2. The rest of the designs, including the negative and positive controls, and field management were the same as those employed for disease testing, except for the use of 3–5 plants per hill. The field testing was done following the biosafty guidelines set by Chinese Government.

DNA extraction and Southern-blot analysis

Five micrograms of DNA for each sample, estimated by agarose-gel staining and fluorometry after treatment with RNaseA, was digested with the *Eco*RV restriction endonuclease (Gibco-BRL, Geithersburg, Md.) in a final volume of 50 µl. The digested DNA was electrophoresed on a 1% (w/v) agarose gel. After electrophoresis, DNA fragments were denatured and transferred onto a hybond-N^+ nylon membrane (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions. The *Xa21* coding sequence, including the intron part from the plasmid digested with the same enzyme as the corresponding blotted DNAs, was labeled with (α-^{32}P) dCTP using the Rediprime Labeling Kit (Amersham, Arlington Heights, Ill.) and employed as a hybridization probe.

Results

Isolation of the homozygous line from the T_2 generation

In the transgenic greenhouse of IRRI, all 120 plants from 4 of the 13 resistant T_1 generation-derived T_2 progenies exhibited an identical resistant reaction to Philippines *Xoo* races 4 (PXO71) and 6 (PXO99) (Fig. 1). This result is similar to the reaction of the *Xa21* donor line IRBB21 (Fig. 2b), but with a shorter lesion length, indicating that they are homozygous in phenotype. The non-transgenic IR72 and susceptible IR24 are both highly susceptible to race 6, but IR72 is resistant to race 4 because it contains endogenous *Xa4*, whereas the susceptible IR24 does not (Fig. 2a, c). To further confirm the homozygous state, the primarily identified T_2 homozygous lines were then subjected to Southern-blot analysis. The results indicated

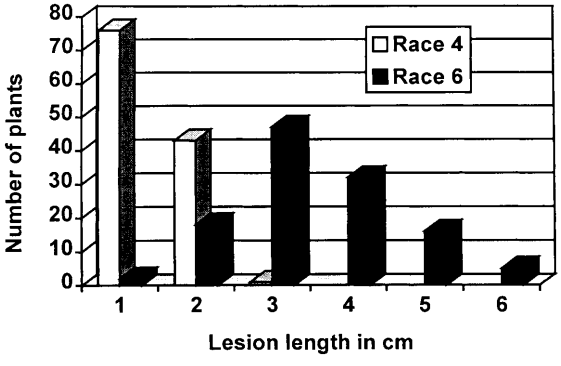

Fig. 1 The resistance reaction of four homozygous lines in the T_2 generation against *Xoo* races 4 and 6. *A* plant with a lesion of less than 6 cm was considered resistant

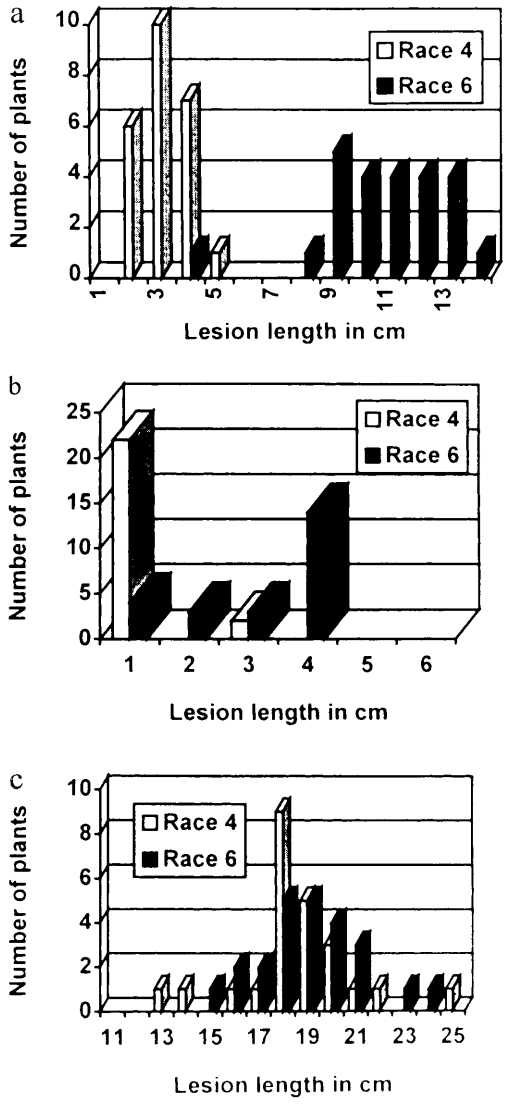

Fig. 2a–c The resistance reaction of three control lines against *Xoo* races 4 and 6. **a** IR72; *b* IRBB21;**c** IR24. A plant with a lesion of less than 6 cm was considered resistant

that all of the plants from these four homozygous lines showed the same band pattern, thus demonstrating that they are also homozygous for the transgene (Fig. 3, the data are presented for up to 15 plants). The plants from the identified homozygous lines were allowed to self and the harvested seeds were stored separately. One line with the best plast phenotype and good seed-setting, designated as T103-10, was then used for field tests.

The other nine resistant T_1 generation-derived T_2 progenies were segregating for their reaction to race 6 of the BB pathogen. Of the 270 plants tested, 195 were resistant and 75 were susceptible (Fig. 4). The segregation followed a 3:1 ratio. This result was identical to the previous genetic analysis in the T_1 generation (Tu et al. 1998b) and further confirmed a one-locus integration pattern. For reaction to race 4, however, 30 out of 270 plants in the nine T_2 preogenies had a lesion length longer than 6 cm (Fig. 4). Their lesion length in the reaction to race 6 was also 5-cm longer than that of the isolated susceptible plants, including the non-transgenic control. These observations seemed to suggest that the co-suppression of both inserted *Xa21* and homologous endogenous *Xa4* might have happened in those 30 plants. From the number of plants that lost their resistance to race 4 of *Xoo*, the frequency of co-suppression was estimated at around 10%. This considerably high frequency of co-suppression incidence seemed not to hamper the isolation of homozygous lines that were obtained at a normal ratio in the T_2 generation, as mentioned above.

Resistance of the homozygous line, T103-10, against multiple races under field conditions

Three prevalent races from the Philippines, Japan, and China, respectively, were used for inoculation in both 1998 and 1999. The results, summarized in Tables 1 and 2, showed that the transgenic homozygous line, T103-10, and the *Xa21* donor line, IRBB21, were resistant to all three races, whereas susceptible variety IR24 was not.

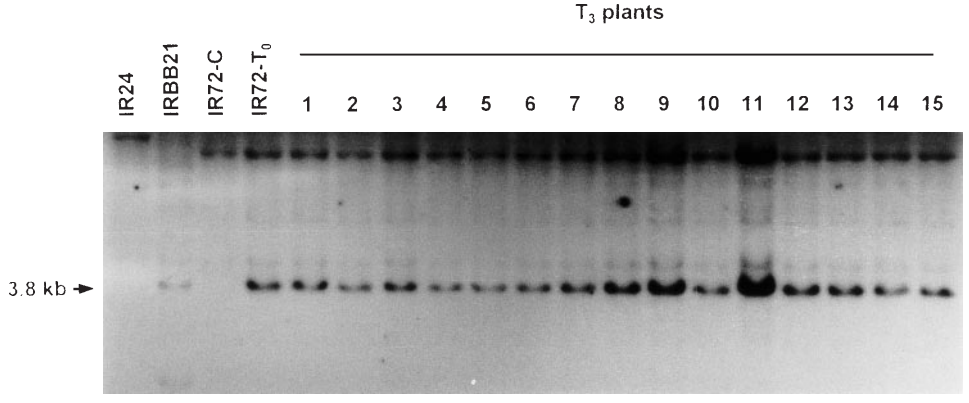

Fig. 3 Southern analysis of transgenic T_3 plants. A total of 5 µg of plant genomic DNA and 30 pg of plasmid DNA was digested with *Eco*RV and hybridized with the same enzyme-digested plasmid DNA fragment. The *arrow* marks the expected 3.8-kb hybridizing band, which appears in the *Xa21* donor line, and positive transgenic T_0 and T_3 plants. IR24, IRBB21, IR72-C, IR72-T_0 and IR72-T_3 plants represent the *Xa21* recipient line, the *Xa21* introgression line, the non-transgenic control plant, the transgenic primary plant, and the transgenic homozygous plants, respectively

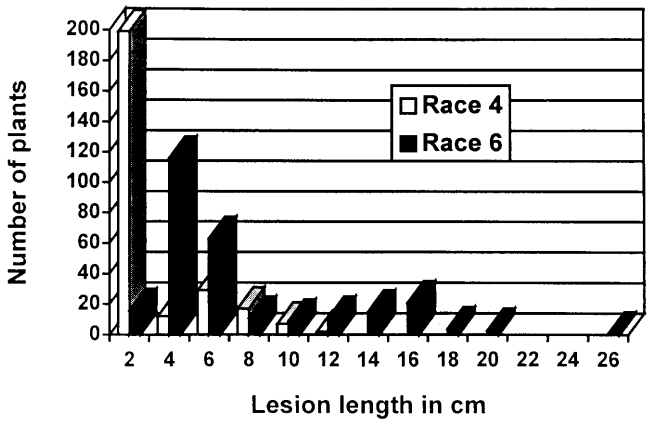

Fig. 4 Segregation reaction of nine T_1 generation-derived T_2 progenies against *Xoo* races 4 and 6. The plant with a lesion length of less than 6 cm was considered as resistant

Fig. 5 Lesion length comparison between transgenic T103-10 plants (*left*) and control IR72 plants (*right*) inoculated with PXO99, Philippines race 6 of *Xoo*, under field conditions

Table 1 Resistance performance of transgenic homozygous line T103-10 against multiple races of *Xoo* under field conditions in 1998 (Huazhong Agricultural University, Wuhan, China)

Isolates	Variety/line	No. of plants tested/line	Average lesion lengths (cm)	R/S
PXO99	IR24	16	19.37±0.81	HS
	IRBB21	7	5.74±0.34	MR
	IR72	16	11.64±0.71	S
	T103	66	2.27±0.08	R
Zhe173	IR24	16	8.52±0.51	S
	IRBB21	8	0.70±0.11	HR
	IR72	18	1.03±0.82	R
	T103	72	0.26±0.01	HR
T2	IR24	15	20.67±0.89	HS
	IRBB21	6	2.07±0.31	R
	IR72	17	1.60±0.49	R
	T103	71	0.89±0.07	HR

[a] R=resistant; S=susceptible; HS=highly susceptible; MR=moderately resistant; HR=highly resistant

Non-transgenic IR72, which carries *Xa4*, was resistant to Chinese race 4, Zhe173 and Japanese race 2, T2, but was susceptible to the Philippine race 6 PXO99. Figure 5 illustrates that the transgenic homozygous plants showed a significant reduction in the severity of symptoms when their lesion length was compared with that of non-transgenic plants. To further verify the resistance spectrum of the transgenic homozygous line, T103, three more races, Philippine were added for the inoculation of field-tested material in 1999 (Table 2). The results revealed that transgenic homozygous line T103-10, as well as donor line IRBB21, were both resistant to all three additional races of *Xoo*, indicating the similar resistance spectrum.

PXO99 is known as the most virulent among the nine races isolated in the Philippines. The lesion length developed by this virulent race on donor line IRBB21 plants, listed in Tables 1 and 2, reached 5.74±0.34 cm in 1998 and 8.00±1.20 cm in 1999, which ranked as moderate resistant to moderately susceptible on the 0–9 IRRI SES scale. The lesion length that appeared on transgenic homozygous line T103-10, however, was 2–3-fold shorter than that of IRBB21 in both testing years. This sharp difference in lesion length between the transgenic line and the non-transgenic control was also observed for the other races (Tables 1 and 2). These differences are due to the complementation of *Xa21* and *Xa4* present in transgenic line as compared to IRBB21 that has only *Xa21*.

Yield performance and agronomic traits of transgenic homozygous line T103-10

The yield performance of transgenic homozygous line T103 was observed in the plot separated from disease testing in 1999. Non-transgenic IR72 was planted as a control for a phenotype comparison. The data showed that T103-10 grew 8.0-cm less in plant height, took 2 days longer to flower, and set 2.9 more filled-seeds per panicle (Table 3). It developed fewer but longer panicles, with 14.1 more spikelets, although some of them were unable to develop into mature seeds. The final seed production of T103-10 as predicted by its yield components, listed in Table 3, however, showed no significant difference from that of the IR72 control, both at about 5 tons per hectare.

Table 2 Resistance performance of transgenic homozygous line T103-10 against multiple races of *Xoo* under field conditions in 1999 (Huazhong Agricultural University, Wuhan, China)

Isolates	Variety	No. of plants tested	Lesion length (cm)	R/S[a]
PXO61	IR72	90	1.04±0.12	R
	T103	90	0.31±0.05	HR
	IR24	90	16.43±1.32	HS
	IRBB21	90	0.97±0.24	HR
PXO79	IR72	90	9.00±0.86	S
	T103	90	0.62±0.24	HR
	IR24	90	14.11±1.46	HS
	IRBB21	90	0.82±0.40	HR
PXO99	IR72	90	9.37±1.21	S
	T103	90	2.43±0.53	R
	IR24	90	15.69±1.24	HS
	IRBB21	90	8.00±1.20	MS
PXO112	IR72	90	0.72±0.13	HR
	T103	90	0.39±0.09	HR
	IR24	90	7.60±1.11	S
	IRBB21	90	1.02±0.62	R
Zhe173	IR72	90	1.41±0.53	R
	T103	90	0.79±0.31	HR
	IR24	90	11.16±1.89	S
	IRBB21	90	1.58±0.73	R
T2	IR72	60	0.71±0.45	HR
	T103	60	0.61±0.31	HR
	IR24	60	20.01±1.89	HS
	IRBB21	60	1.94±0.91	R

[a] R=resistant; S=susceptible; HS=highly susceptible; MS=moderately susceptible; HR=highly resistant

Table 3 Agronomic traits of transgenic homozygous line T103-10 and the IR72 control under normal field conditions (Huazhong Agricultural University, Wuhan, China 1999)

Variety/line	Sowing-heading duration	Plant height (cm)	Panicles/plant	Filling seeds/panicle	Non-filling seeds/plant	Total seeds/panicle	Seed-setting rate (%)	1000-seed weight (g)	Predicted yield (t/ha)	Observed yield (t/ha)
T103-10	98	85.6	16.2	67.9	41.1	108.9	62.4	20.8	4.29	4.89
IR72 control	96	93.6	17.1	65.0	29.8	94.8	68.5	21.3	4.43	4.97

Discussion

Bacterial blight is the most destructive bacterial disease of rice worldwide (Mew 1987; Leach et al. 1995). Yield losses caused by this disease can reach 50% in some areas of Asia (Adhikari et al. 1995). Chemical control is difficult and inefficient because of increased public concern over the environment, and because the pathogen enters the host plant through the hydathodes, multiplies in the epidermis, and moves to the xylem vessels, where the infection becomes systemic and chemicals cannot reach it (Ronald 1997).

Earlier, we demonstrated that the elite transgenic indica rice IR72 with *Xa21* was resistant to the BB pathogen and had the same spectrum of resistance as that of the donor line IRBB21 (Tu et al. 1998b). Similar results were reported using the same rice variety (Zhang et al. 1998). In the present report, we evaluated the resistance levels of transgenic line under field conditions in China for two years and verified earlier results. The present yield-data of transgenic and control plants were comparable. For a more critical evaluation of the usefulness and adaptability of the transgenic homozygous line, however, repeated fields trials will be carried out extensively over the next 1 or 2 years at multiple sites. We hope that the evaluated homozygous line will become available to farmers, in regions where the original IRRI breeding line is adaptable, in the near future.

We also noticed that an increased level of resistance to the BB pathogen persisted in transgenic plants through generations, indicating its stable inheritance. The heritable increased level of resistance to the BB pathogen can, in turn, provide an advantage for genetic engineering over classical breeding in cases where the highest levels of resistance are desirable and can be achieved in a short time. It is also noteworthy that various national agricultural systems in Asia are making efforts to incorporate the other *Xa* genes into popular cultivars through marker-aided selection. The availability of various cultivars with different resistance genes could significantly decrease the yield loss brought about by *Xoo*. Assuming a minimum yield loss of 1% due to this disease, around $32.5 million could be saved over 30 million ha with an average yield of 5.5 t/ha in China, whereas a yield loss of 0.75% covering 132.5 million ha

with an average yield of 3.6 t/ha in Asia translates into $715.5 million. Thus, trangenic rice with BB resistance would have a large economic impact.

This study shows that conventional and molecular breeding techniques could be a powerful combination in rice breeding. Genetic transformation is a one-step process of introducing novel genes into a desirable genetic background of important crops. Because it is a fast and efficient gene-integration tool, it could well be the answer to catching up with the pathogen's ability to mutate fast and render once-resistant plants susceptible. For instance, rice cultivars carrying the *Xa4* gene for resistance, which were widely deployed in the Philippines from the early 1970s, became susceptible to the predominant race of *Xoo* within 5 years (Mew et al. 1992). Transformation techniques could help to develop transgenic plants in less than 2 years to minimize the effects of the breakdown of resistance in the host plant. With the availability of resistance genes from other sources, the strategic deployment of transgenic rice with gene-pyramiding may provide desirable resistance in rice breeding.

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