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Transgenic rice variety 'IR72' with *Xa21* is resistant to bacterial blight

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Abstract An elite indica rice variety, 'IR72', was transformed with a cloned gene, *Xa21*, through particle bombardment. Molecular analysis of transgenic plants revealed the presence of a 3.8-kb *Eco*RV-digested DNA fragment corresponding to most of the *Xa21* coding region and its complete intron sequence, indicating the integration of *Xa21* into the genome of 'IR72'. In the T_1 generation, the transgene was inherited and segregated in a 3:1 ratio. After inoculation with the prevalent races 4 and 6 of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), T_1 plants positive for the transgene were found to be resistant to bacterial blight (BB). We also observed that the level of resistance to race 4 of *Xoo* was higher due to the pyramiding of *Xa21* and *Xa4* present in 'IR72'. Since the inactivation of the transgene *Xa21* occurred in the two transgenic T_1 plants, a larger progeny should be obtained for selecting homozygous line with a consistently higher level of resistance to the BB pathogen.

Key words Indica rice · *Xa21* · Transgenic plants · Disease resistance · *Xanthomonas oryzae* pv. *oryzae*

Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases of rice throughout the world (Mew 1987). Rice yield losses caused by BB in some areas of Asia can be as high as 50% (Adhikari et al. 1995). The use of resistant cultivars is the most economical and effective method to control this disease (Ogawa 1993).

Numerous donors for resistance to *Xoo* have been identified (Kinoshita 1995; Lin et al. 1996). Some of the resistance genes, such as *Xa4*, have been incorporated into improved rice varieties using the traditional breeding approaches, and these varieties are widely grown (Khush et al. 1989). However, the large-scale and long-term cultivation of varieties with *Xa4* has led to significant shifts in the *Xoo* race (Mew et al. 1992). In many areas in Indonesia, India, China, and the Philippines, where rice varieties with *Xa4* have been widely grown, new races of the BB pathogen have emerged. Therefore, new genes for BB resistance are being incorporated into elite rice varieties.

A dominant gene for resistance to BB was transferred from a wild species, *O. longistaminata*, to the cultivated variety 'IR24'. This gene was designated *Xa21* (Khush et al. 1990), and the line with *Xa21* as IRBB21. *Xa21* confers resistance to all the known races of *Xoo* in India and the Philippines (Khush et al. 1990; Ikeda et al. 1990). The molecular structure of *Xa21* represents an uncharacteristic class of plant disease resistance genes. From its deduced amino acid sequence, the gene was found to be translated into a receptor kinase-like protein carrying leucine-rich repeats (LRR) in the putative extracellular domain, a single-pass transmembrane domain, and a serine threonine kinase (STK) intracellular domain (Song et al. 1995). Other previously cloned plant disease resistance genes such as tomato *PTO* (Martin et al. 1993) and *CF-9* (Jones et al. 1994), Arabidopsis *RPS2* (Bent et al. 1994; Mindrinos et al. 1994), tobacco *N* (Whitham et al. 1994), and flax *L6* (Lawrence et al. 1994) encode either STK or LRR motifs (Staskawicz et al. 1995; Michelmore 1995). Hence the molecular structure of *Xa21* supports a role for cellular signaling in plant disease resistance (Song et al. 1995).

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Wang et al. (1996) transformed japonica rice variety 'T309' with cloned *Xa21*, and its resistance spectrum was similar to that of the donor line IRBB21. However, 'T309' is no longer cultivated, and no new commercial rice cultivars with *Xa21* have been released. In this study we transformed elite indica rice variety 'IR72' with *Xa21*.

Materials and methods

Plasmid

Two plasmid DNAs used for rice transformation are shown in Fig. 1. The plasmid pC822 that contains the *Xa21* coding sequence was kindly supplied by Dr. P. Ronald of University of California, Davis, USA. The primer pair U1 and I1, developed to amplify a 1.4-kb DNA fragment of *Xa21* which was polymorphic to fragments amplified from other *Xa* genes (Fig. 1A) (Wang et al. 1996), was used for quick genetic analysis of the transgenic progeny. Plasmid pROB5 contains the selectable marker, the *hph* coding region, flanked by the cauliflower mosaic virus (CaMV) 35S promoter and polyadenylation signals [poly(A)] (Fig. 1B) (Bilang et al. 1991). This plasmid provides a selectable marker that confers resistance to hygromycin for cotransformation with the pC822 plasmid (Datta et al. 1990; Bilang et al. 1991).

Rice transformation

Immature seeds of 'IR72' were collected 12 days after pollination from screenhouse-grown plants and the lemma and palea were removed. Dehulled grains were then immersed in 70% (v/v) ethanol for 1 min and surface-sterilized with 50% (v/v) commercial Clorox for 15–20 min. Immature embryos were isolated and plated on solid MS medium containing 3% (w/v) maltose, 2 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 0.8% (w/v) agarose or 0.3% (w/v) gelrite (MS2). After a 16- to 18-h preculture at 28°C in a dark room, immature embryos (80–100 pieces per petri dish) were bombarded with the PDC-1000/He system (Datta et al. 1996). The manufacturer's instructions were followed for coating 1.0-μm gold microcarriers (Bio-Rad, Hercules, Calif.), with plasmid DNA, prepared using the Magic Maxipreps DNA purification system (Promega, Madison, Wis). After bombardment, target explants were directly transferred to MS2 medium supplemented with 50 mg l^{-1} hygromycin B for selection. Developing calli were subcultured every 2 weeks on the same medium for five to seven cycles. Resistant calli were transferred to 20 ml N6 medium supplemented with 2 mg l^{-1} kinetin, 1 mg l^{-1} NAA, 2 mg l^{-1} glycine, 1 g/l CH, 30 g/l maltose, 3 g/l gelrite, and 50 mg l^{-1} hygromycin B (3N6) in the dark and at 28°C for preregeneration. After 7–10 days, the same calli were transferred to 50-ml flasks containing 20 ml 3N6 medium without hygromycin B for plant regeneration. Two- to three-week-old plantlets were transferred to either Yoshida's culture solution or directly to the soil and placed in the greenhouse under a day : night temperature regime of 29° : 23°C.

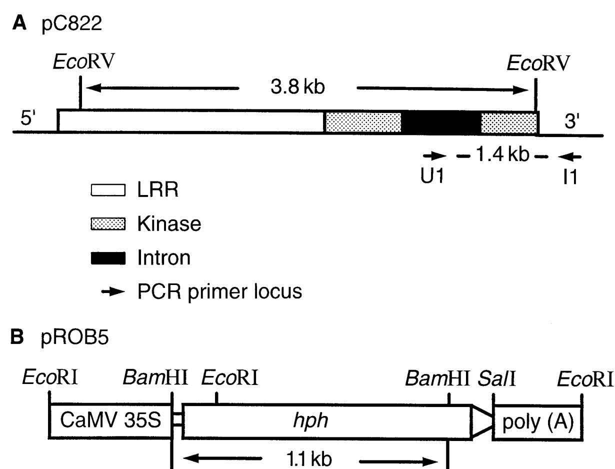

Fig. 1A, B Diagram of the plasmid constructs used in this study. **A** Restriction map and probe/primer combination of *Xa21* in clone pC822. The primer pair U1 and I1 which amplifies a 1.4-kb DNA fragment from the *Xa21* was used in the genotype analysis of the transgenic plants (Fig. 2). The *Eco*RV-digested plasmid DNA, a 3.8-kb fragment, was used as a probe in Southern analysis. **B** Structure of plasmid pROB5, which contains a modified *hph* gene coding region under control of the CaMV 35S promoter and polyadenylation signals [poly(A)]

DNA extraction and Southern blot analysis

Genomic DNA was extracted by an improved CTAB method based on the procedure described by Murray and Thompson (1980). Five micrograms of DNA of each sample, estimated by agarose gel staining and fluorimetry after treatment with RNaseA, was digested with *Eco*RV (Gibco-BRL, Geithersburg, Md.) in a final volume of 50 μl. The digested DNA was electrophoresed on 1% (w/v) agarose gels. After electrophoresis, DNA fragments were denatured and transferred onto a Hybond-N^+ nylon membrane (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions. Most of the *Xa21* coding region, including the intron sequence (3.8-kb fragment), from plasmid digested with the same enzyme of the corresponding blotted DNAs was labeled with α-[^{32}P]-dCTP using the Rediprime Labeling Kit (Amersham, Arlington Heights, Ill.) and used as the hybridization probe.

Polymerase chain reaction (PCR) analysis

The PCR analysis was conducted based on procedures described by Huang et al. (1997). The PCR reaction mixture contained 50 ng template DNA, 50 ng of each primer, 0.16 m*M* dNTPs, 2.1 m*M* $MgCl_2$, 1 × PCR buffer (10 m*M* TRIS pH 8.4, 50 m*M* KCl), and 1 *Taq* DNA polymerase in a volume of 25 ul. Template DNA was initially denatured at 94°C for 5 min, followed by 30 cycles of PCR amplification with the following parameters: a 30-s denaturation at 94°C, a 30-s primer annealing at 55°C, and a 1-min primer extension at 72°C allowed for completion of primer extension. The amplified products were electrophoretically resolved on a 1% agarose gel in 1 × TAE buffer.

Inoculation

PX071 (race 4) and PX099 (race 6) strains of *Xoo* were used to inoculate transgenic T_1 plants. The inoculum of each strain was prepared by incubating the bacteria on Wakimoto's medium for 72 h at 30°C, then suspending each pure culture in sterile distilled water and adjusting the inoculum to about 10^9 cells per milliliter.

The transgenic T_1 plants were grown in an IRRI containment greenhouse under the following conditions: 29°C and 85% humidity

during the day and 25°C and 90% humidity at night. At the maximum tillering stage, each plant was inoculated with the above two strains of *Xoo* using the leaf clipping method (Kauffman et al. 1973). Plant reaction to each race of *Xoo* was scored on six leaves 14 days after inoculation.

Results

Rice transformation and molecular analysis

The indica rice variety 'IR72', was transformed with *Xa21* (Fig. 1) by particle bombardment. A total of 18 plants were obtained through selection. Out of 15 plants analyzed, eight were found to be transgenic and these were regenerated from the same clone. Southern blot analysis of these primary transgenic plants (T_0) demonstrated the presence of a 3.8-kb *Eco*RV-digested DNA fragment corresponding to most of the *Xa21* coding region and its complete intron sequence, indicating the integration of *Xa21* into the genome of 'IR72' (data was combined to Fig. 3 in the next section). In addition to this expected band, several endogenous hybridizing bands with high molecular weight were also detected. After self pollination, one of eight transgenic plants was found to be fully fertile. A T_1 progeny of 20 plants of this fertile plant was grown in the containment greenhouse and used for further analysis.

Inheritance of the cloned *Xa21* in transgenic rice plants

The inheritance of the cloned *Xa21* in T_1 generation was studied by PCR analysis. The primer pair U1 and I1 that specifically amplified a 1.4-kb DNA fragment from the 3′ nontranslated region to the center of intron of *Xa21* was used for this purpose. A DNA fragment of the expected size was amplified from cloned pC822, donor line IRBB21, a primary transgenic plant, and from 15 of the 20 T_1 plants (Fig. 2). This is consistent with a single locus insertion into the rice genome of the primary transgenic line T103 which segregated into a 3 : 1 ratio. In subsequent experiments, these results were further confirmed by Southern blot analysis (Fig. 3).

Fig. 2 PCR analysis of transgenic T_1 *plants*. The *arrow* marks the expected 1.4-kb *Xa21*-specific DNA fragment which was amplified by primer pair U1 and I1 (Fig. 1). *O. longistaminata*, 'IR24', IRBB21, 'IR72'-C, 'IR72'-T_0 and 'IR72' T_1 plants represent *Xa21*'s original donor species, susceptible parental line, susceptible line-derived *Xa21* introgression line, nontransgenic control plant, transgenic primary plant, and transgenic progeny plants, respectively. *pC822* The plasmid containing *Xa21* used to generate transgenic line T103. The PCR products were separated in 1% agarose gel. Markers in the *first* and *last lane* were labeled by a 1-kb ladder.

Resistance reactions of the transgenic T_1 plants to BB pathogen

The reaction of the transgenic T_1 plants to the BB pathogen was evaluated by inoculation with race 6 of *Xoo*. Among 15 PCR/Southern positive plants, 13 were resistant and 2 (T103-2 and T103-19) were susceptible. The lesion length of 13 T_1 resistant plants and donor line IRBB21 was less than 3.1 cm. (Table 1 and Fig. 4). The lesion length of the plants of untransformed 'IR72' and 'IR24' ranged from 13.3 to 20.3 cm. These results indicated that the transgene *Xa21* functioned well in the most of the transgenic T_1 plants, although it was silenced or inactivated in few cases during its inheritance from the primary generation to the next.

'IR72' has *Xa4* and is highly resistant to races 1 and 5 of *Xoo* but only moderately resistant to race 4. However, when inoculated with race 4 of *Xoo*, all of the resistant T_1 plants showed much smaller lesions than those of control plants of 'IR72' and resistant donor line IRBB21. This indicates an increased level of resistance to the BB pathogen.

Discussion

Xa21 from a wild species, *O. longistaminata*, is the first resistance gene to be cloned and transferred in any cereal crop plants. Due to its wide spectrum of resistance (Song et al. 1995; Wang et al. 1996), it is of great

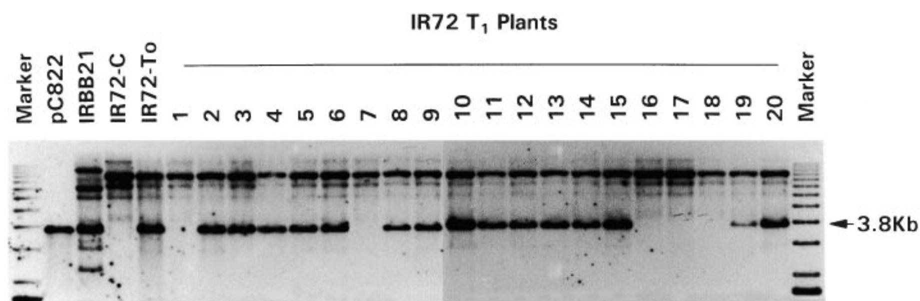

Fig 3 Southern analysis of transgenic T_1 plants. A total of 5 μg plant genome DNA and 30 pg of plasmid DNA were digested with *Eco*RV and hybridized with the same enzyme-digested plasmid DNA fragment. The *arrow* marks the expected 3.8-kb hybridizing band, which appeared in the plasmid, *Xa21*-donor line, and positive transgenic T_0 and T_1 plants. IRBB21, 'IR72'-C, 'IR72'-T_0, and 'IR72' T_1 plants represent *Xa21* introgression line, nontransgenic control plant, transgenic primary plant, and transgenic progeny plants, respectively. *pC822* The plasmid containing *Xa21* used to generate transgenic line T103. Markers in the *first* and *last lane* were labeled by a 1-kb ladder

Table 1 Reaction of control and transgenic 'IR72' plants with *Xa21* to races 4 and 6 of *Xoo*

Control/ transgenic plant	Reaction to race 4 (lesion length in cm)		Reaction to race 6 (lesion length in cm)	
IRBB21 (control)	R[a]	2.3 ± 0.30	R	2.5 ± 0.70
IR24 (control)	S	19.6 ± 2.85	S	20.3 ± 2.86
IR72 (control)	R	3.1 ± 0.65	S	13.3 ± 0.94
T103-1	R	4.8 ± 1.89	S	12.1 ± 0.72
T103-2	R	0.7 ± 0.26	MS	6.3 ± 1.71
T103-3	R	0.6 ± 0.19	R	2.3 ± 0.87
T103-4	R	0.6 ± 0.23	R	2.7 ± 1.43
T103-5	R	0.6 ± 0.13	R	1.9 ± 0.44
T103-6	R	0.7 ± 0.23	R	2.1 ± 0.98
T103-7	R	3.8 ± 1.38	S	14.6 ± 1.95
T103-8	R	0.9 ± 0.41	R	1.9 ± 0.50
T103-9	R	0.7 ± 0.21	R	2.4 ± 0.22
T103-10	R	0.4 ± 0.11	R	1.0 ± 0.40
T103-11	R	0.7 ± 0.25	R	2.9 ± 1.02
T103-12	R	0.5 ± 0.08	R	2.0 ± 0.62
T103-13	R	0.9 ± 0.14	R	1.9 ± 1.38
T103-14	R	0.6 ± 0.22	R	1.9 ± 1.57
T103-15	R	0.4 ± 0.12	R	1.0 ± 0.52
T103-16	R	3.7 ± 0.65	S	13.7 ± 2.68
T103-17	R	4.5 ± 1.46	S	13.2 ± 0.71
T103-18	R	3.4 ± 1.15	S	12.0 ± 2.68
T103-19	R	2.4 ± 0.87	S	12.8 ± 2.63
T103-20	R	0.7 ± 0.28	R	3.1 ± 0.61

[a] R, Resistant; S, susceptible

Fig. 4 Resistant reaction of transgenic plants to strain PX099 of *Xoo* race 6. Photograph taken 15 days after inoculation. *T*: Leaves from transgenic T_1 plant T103-15, *C* leaves from nontransgenic 'IR72' control plant

value in breeding rice for BB resistance. Transformation of cloned *Xa21* into the elite indica rice variety 'IR72' improves the spectrum of resistance of this important variety to *Xoo*. It also provides an opportunity to assess its stability and its function in a new genetic background.

Through artificial inoculation, it was observed that the level of resistance of the transgenic plants to *Xoo* race 4 was higher than that of the control plants of 'IR72' and the donor line IRBB21. This higher level of resistance appears to be result of a pyramiding of *Xa21* and *Xa4* present in 'IR72'. Similar results were also obtained from several rice lines with more than one gene for resistance to *Xoo*, such as *xa5*/*Xa21*, *Xa4*/*xa5*/*xa*13 or *Xa21*, *Xa4* or *xa5*/*xa13*/*Xa21*, and *Xa4*/*xa5*/*xa13*/*Xa21* (Huang et al. 1997). In transgenic japonica rice variety 'T309', however, the higher level of resistance to at least 16 isolates of *Xoo* was attributed to the higher copy number of *Xa21*,

possibly due to having multiple insertions (Wang et al. 1996).

In two Southern/PCR positive T_1 progeny plants (T103-2 and T103-19) different degrees of the transgene inactivation was observed. From Table 1, it can be seen that plant T103-19 (lane 19 in Figs. 2 and 3) exhibited a highly susceptible reaction to *Xoo* race 6 after inoculation, indicating that the expression of the transgene in this plant was completely missing. However, in plant T103-2 (lane 2 in Figs. 2 and 3), the transgene seemed to be partially inactivated since it showed a moderately susceptible reaction to *Xoo* race 6.

The silencing or inactivation of the transgene in plants is a complex phenomenon. It may be explained by the interactions between inserted gene and its DNA sequence homologues (Flavell 1994). The sequence homologues could be endogenous (Jorgensen 1990), multiple inserted, or secondly introduced (Matzke et al. 1993; Flavell 1994). The consequence of the interactions between these loci with DNA sequence homology resulted in chromatin restructuring or DNA sequence modification by methylation of different cytosine residues or inhibition of mRNA processing, transport, export, or translation (Flavell 1994). The expression of the transgene in the transgenic plant was finally cosuppressed and *trans*-inactivated. The same mechanism might have led to inactivation of the *Xa21* transgene because of its origin from a wild species and its DNA sequence homology in the receipient plant as shown by Southern analysis (Fig. 3). Therefore, since inactivation of the transgene *Xa21* in the transgenic T_1 generation occurs at a lower rate, a larger progeny should be obtained for selecting a good homozygous line with a consistently higher level of resistance to the BB pathogen.

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