Molecular Breeding **4:** 551–558, 1998.

Transgenic elite *Indica* rice varieties, resistant to *Xanthomonas oryzae* pv. *oryzae*

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Received 26 March 1998; accepted in revised form 6 July 1998

Key words: Indica rice, cell suspension, transformation, *Xa21*, bacterial leaf blight

Abstract

The agronomically important *Indica* (group 1) rice varieties IR64, IR72, hybrid restorer line Minghui 63, and BG90-2 were co-transformed by microbombardment of embryogenic suspensions with plasmids that contain the *Xa21* gene which confers resistance to *Xanthomonas oryzae* pv. *oryzae* and the *hph* gene for resistance to hygromycin B. Six of the 55 transgenic R0 plant lines containing the *Xa21* gene displayed high levels of resistance to the pathogen, and no partial resistance was observed. The trait was stably inherited in subsequent generations, and transgenic plants are currently in field tests. The ability to transfer agronomically important genes into elite *Indica* rice varieties demonstrates the applicability of genetic engineering for the agronomic improvement of rice.

Introduction

By the year 2050, 90% of the world's projected 11 billion people will reside in the developing countries of the South [13]. Since rice is considered the most important crop in tropical and subtropical regions, increasing its yield is an essential factor in securing sufficient food supplies for the increasing human population. It has been suggested that biotechnology can contribute to the agronomic improvement of rice and, when used in combination with traditional breeding methods, can make it possible to achieve the required increase in crop production [4].

Genetic transformation of rice offers numerous important opportunities for the improvement of existing elite varieties and the production of new cultivars. A major advantage of genetic engineering is that it allows breeders to rapidly develop new varieties by the introduction of cloned genes into commercial varieties. Genetic transformation techniques can also overcome the genetic sterility problems associated with *Indica*/*Japonica* interspecific hybridization, and can bypass non-compatibility problems associated with cultivated/wild specific hybridization. In addition, foreign genes from unrelated plants and other organisms such as bacteria (e.g. the Bt gene from bacterium *Bacillus thuringiensis*) [6, 22] and viruses (e.g. pathogen-derived resistance) [9] may be transferred to the rice genome, as part of completely new strategies for combating disease and improving yield quantity and quality.

When rice genetic transformation programs commenced in the mid 1980s, the goals included genetic modification of the most important varieties for improved resistance to biotic and abiotic stresses [20]. Significant progress has been achieved in developing genetic transformation systems for *Japonica* varieties [2]. However, the vast majority of rice cultivated in Asia and elsewhere in the tropics is based on *Indica* varieties, especially those classified as group 1 *Indica* [8]. Of these, IR72 and IR64 are the most important of the Asia varieties, account for 9.5 million cultivated hectares, or 26% of the cultivated rice in Southeast Asia. Despite considerable research efforts, the *Indica* varieties have remained recalcitrant to the applica-

tion of transgenic technology. The elite *Indica* group 1 rice has been limited to two reports of regeneration of fertile transgenic plants containing selectable marker and reporter genes [3, 24], plus another report in which there was only one plant line of IR58 recovered that expressed a synthetic *cryIA(b)* gene from *B. thuringiensis* [22].

Here we report the regeneration of transgenic fertile plants from four elite *Indica* varieties in group 1, viz. IR64 and IR72, Minghui 63 (restorer line of Chinese *Indica* hybrid rice representing 25% of the rice production in China, Q. Zhang, pers. comm.), and BG90-2 (widely used *Indica* rice variety in West Africa) carrying bacterial leaf blight resistance gene *Xa21*. Bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a devastating rice disease in Asia and resulting in an estimated production loss of USD 250 million annually [10]. The *Xa21* gene, originating from wild rice *Oryza longistaminata* was recently isolated and transferred to the *Japonica* rice Taipei 309 by genetic transformation [19]. In this study, the *Xa21* gene was transferred to four elite *Indica* varieties and found to impart significantly improved resistance to the bacterial blight pathogen, and this resistance was shown to be stably inherited in subsequent generations.

Materials and methods

Plant material

Seeds of *Indica* (group 1) rice varieties IR64 and IR72 were provided by the International Rice Research Institute (IRRI, Manila, Philippines), and those of Chinese hybrid *Indica* rice 'restorer' line Minghui 63 by the Anhui Academy of Agricultural Sciences (AAAS, He Fei, China). The latter results from the crossing of the *Indica* (group 1) rice IR30 and Gui 63 through classical breeding (D. Jin, pers. comm.). Seeds of *Indica* rice variety BG90-2 were obtained from the West African Rice Development Association (WARDA, Bouake, Ivory Coast).

Genetic constructs

Plasmid pC822 contains a 9.6 kb *Kpn*I DNA fragment with the *Xa21* gene that was originally cloned from rice line IRBB21 into plasmid pTA818 [19]. Plasmid pHX4 contains the *hph* gene driven by the CaMV 35S promoter and terminating in the *nos* 3′ end, into plasmid pUC119 (Dr J.J. Finer, Ohio State University).

Callus induction and initiation of embryogenic cell suspension cultures

Callus induction and initiation of embryogenic cell suspension cultures were according to the protocols reported by Zhang [23] with the following modifications. Seeds were cultured on medium that contains Murashige and Skoog basal salts [14] supplemented with Gamborg's B5 vitamins [7], 30 g/l maltose and 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), and solidified with 0.25% (w/v) Phytagel (Sigma) in the dark at 25–26 °C. Three to four weeks later, embryogenic calli were placed into 125 ml Erlenmeyer flasks containing 30 to 40 ml liquid SZ medium, and cultured on a gyratory shaker at 120 rpm. SZ medium consists of the macronutrients of R2 medium [15], the micronutrients and vitamins of Gamborg's B5 medium [7], 300 mg/l casein enzymatic hydrolysate (N-Z-Amine A), 500 mg/l each of proline and glutamine, plus 30 g/l maltose and 2 mg/l 2,4-D. Suspension cells were subcultured with SZ liquid medium every 5 to 7 days.

Particle bombardment

Gene transfer experiments were conducted with the Biolistic PDS-1000 system (Bio-Rad, Hercules, CA). Plasmid pC822 was co-bombarded with plasmid pHX4 in a 4:1 molar ratio. The coating procedures were followed according to Chen *et al.* [2].

Tissues were cultured on modified CC medium throughout the transformation, selection and regeneration procedures. The modified CC medium consisted of CC medium [16] without coconut water or mannitol, but with 300 mg/l casein enzymatic hydrolysate and 30 g/l sucrose, and solidified with 0.25% (w/v) Phytagel. Samples of cell suspensions (0.5 g) were taken 3 to 5 days after previous subculture and plated in the center of a Petri dish containing modified CC medium supplemented with 2 mg/l 2,4-D, 46 g/l each of mannitol and sorbitol, then cultured for 4 h prior to bombardment. The Petri dish was placed ca. 8 cm beneath the stopping plate of the gun with a helium pressure of 1100 psi (7.6 MPa) used to accelerate the particles in a 27 inch Hg vacuum (9.5 kPa absolute pressure). After bombardment, cultures were returned to the dark at 25–26 °C.

Selection and regeneration of putative transgenic plants

Sixteen to twenty hours after bombardment, cell aggregates were transferred from medium containing high osmoticum to selection medium consisting of modified CC medium with the addition of 2 mg/l 2,4-D and 40 mg/l hygromycin B (hyg B, Calbiochem, La Jolla, CA), and cultured in the dark at 25 to 26 °C for 2 to 3 weeks. After which growing cell clusters were transferred to fresh selection medium with 50 mg/l hyg B. After a further 2 to 3 weeks hyg B-resistant (hyg^r) calli were transferred to pre-regeneration medium consisting of modified CC medium with the additional 2 mg/l kinetin, 1 mg/l naphthalene acetic acid (NAA) and 5 mg/l abscisic acid (ABA).

Hyg^r calli, 2 to 3 mm in diameter, were transferred to regeneration medium consisting of modified CC medium without sucrose and Phytagel, but with 30 g/l maltose, 0.5% (w/v) agarose (type 1 Sigma or Gibco-BRL, Rockville, MD), and the addition of 2.5 mg/l kinetin, 0.1 mg/l NAA and 50 mg/l hyg B. Cultures were maintained in darkness for 3 days before transferred to a 16 h photoperiod with a light intensity of 110 to 130 μmol s^{-1} m^{-2} PAR produced by Sylvania F40/CW cool white fluorescent tubes. As small shoots were regenerated, they were transferred to Magenta boxes containing a medium with half-strength MS basal salts, 1% (w/v) sucrose and 0.25% (w/v) Phytagel. Once plants had reached the top of the box, they were transferred to soil in 6 cm pots and placed in a growth chamber at 28 °C, 90% humidity during a 14 h light period (180 μmol s^{-1} m^{-2} PAR), and at 26 °C, during a 10 h dark period for 3 to 4 weeks. After that, plants were transplanted in 13 cm pots to the greenhouse.

Challenge of putative transgenic plants

After 6 weeks' growth in pots, putatively transformed plants were challenged according to Kauffman *et al.* [11]. Three fully expanded leaves from each R0, R1, R2, and R3 plant were cut ca. 4 cm below the tips with scissors dipped in a suspension of *Xanthomonas oryzae* pv. *oryzae* race 6 strain PX099Az. Plants were maintained in a growth chamber and the length of the necrotic lesion on leaves measured 14 days after inoculation.

Molecular analyses of transgenic plants

Genomic DNA was extracted from leaf tissues using the method of Dellaporta *et al.* [5]. PCR (polymerase chain reaction) amplification was carried out using the primers and the conditions as described by Wang *et al.* [21]. For Southern blot analyses, DNAs from each sample were digested separately with *Pst*I (the enzyme cuts pC822 at each end of the *Xa21* gene) and *Xho*I (cuts inside the *Xa21* coding region) restriction endonucleases (Gibco-BRL, Rockville, MD) (see Figure 4, legend). Undigested and digested DNAs (5 μg per well) were loaded on a 0.8% agarose gel and electrophoresed at 3 V/cm for 12 h. DNAs were then transferred onto a Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL) according to the instructions of the manufacturer. The RG103 marker which hybridizes to the promoter and the coding regions of the *Xa21* gene was used as a probe [18] (Figure 4A). The DNA fragment was labeled by random 9-mer priming with Exo-Klenow (Stratagene, La Jolla, CA) and using 5′-[α-^{32}P]dCTP. Hybridization was carried out at 65 °C in hybridization buffer (3× SSC, 2× Denhardts solution, 0.1% SDS, 6% PEG) overnight. The final wash was done at 65 °C with 0.5× SSC, 0.1% SDS, and the membrane was exposed to a X-ray film overnight.

Results

Improvement of culture procedures

Indica (group 1) rice varieties differ from other varieties in their response to *in vitro* culture conditions. In order to obtain numerous transgenic plants, a large quantity of regenerable suspension cells are required as target tissues for transformation. Zhang previously reported that the time required to establish regenerable *Indica* (group 1) rice embryogenic suspension cells was reduced from more than 6 months to 6 to 8 weeks [23]. To further shorten the time for decrease of somaclonal variation and sterility in R0 plants, SZ medium was generated and compared to R2 medium. The results from cultures of IR72, IR64 and Minghui 63 in SZ medium showed that the periods for suspension establishment were reduced to 4 or 5 weeks, instead of 6 to 8 weeks in R2 medium. Using SZ medium, the cell volume doubled each week, and reliable establishment and maintenance of high quality *Indica* suspensions became routine in our experiments.

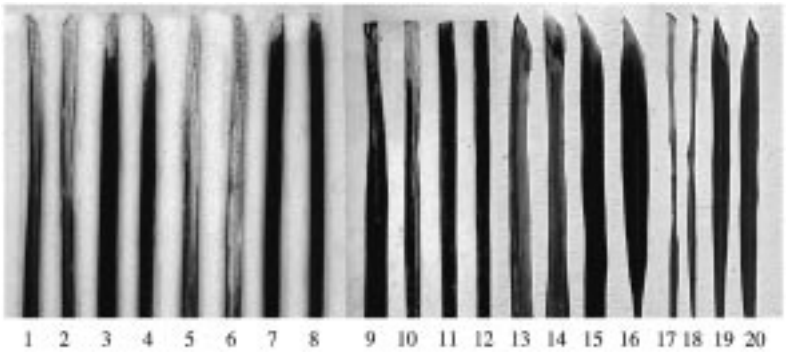

Figure 1. Transgenic R0 rice plants of IR72-82, IR64-65, Minghui 63-12 and BG90-2-27 displaying resistence to *Xanthomonas oryzae* pv. *oryzae.* Photograph taken 14 days after inoculation with *Xoo* race 6 strain PX099Az. Leaves 1 and 2, non-transgenic IR24; leaves 3 and 4, IRBB21, the *Xa21* gene donor line; leaves 5 and 6, non-transgenic IR72; leaves 7 and 8, IR72-82; leaves 9 and 10, non-transgenic IR64; leaves 11 and 12, IR64-65; leaves 13 and 14, non-transgenic Minghui 63; leaves 15 and 16, Minghui 63-12; leaves 17 and 18, non-transgenic BG90-2; Leaves 19 and 20, BG90-2-27.

Table 1. Production of the transgenic *Indica* rice plant lines expressing the *Xa21* gene.

Variety	Number of plates bombarded*	Number of hyg^r lines obtained	Number of PCR-positive lines for the *Xa21* gene	Co-transformation efficiency (%)	Number of resistant to *Xoo*
IR64	40	11	10	91	2
IR72	39	16	16	100	1
Minghui 63	62	26	14	54	2
BG90-2	3	2	2	100	1
Total	144	55	42	76	6

The *Xa21* and the *hph* genes were carried in plasmids pC822 and pHX4, respectively.
*0.5 g suspension cultures per plate.

We previously reported using agarose to solidify the media [24]. An experiment was conducted to compare the media solidified with either agarose or Phytagel for the production of embryogenic calli and hyg^r tissues. No observable difference was observed between tissues cultured on media containing agarose or Phytagel. Therefore, Phytagel, which costs less than agarose, is now routinely used in callus induction and selection media.

Production of transgenic plants

A total of 55 independent hyg^r plant lines were regenerated from 144 bombarded plates of embryogenic suspension cell aggregates (Table 1). PCR analysis confirmed that all hyg^r plant lines were positive for the *hph* gene, and that all the transgenic IR72 and BG90-2 plants contained the *Xa21* gene. Ten of the 11 IR64 and 14 of the 26 Minghui 63 plant lines were PCR positive for the *Xa21* gene. Co-transformation efficiency of the two genes for the four *Indica* cultivars was about 76%, corresponding to our results with a *Japonica* variety [1].)

Bio-assay of transgenic rice plants for bacterial resistance

All R0 transgenic plant lines were challenged with *Xoo* race 6 as described in Materials and methods. Six of the 55 hyg^r R0 plant lines, one each from IR72 and BG90-2, and two each from IR64 and Minghui 63, showed significant reduction in the severity of symptoms, when their lesion length was compared to that of non-transformed plants (Figure 1). Figure 1 illustrates that the transgenic plants showed greater resistance to the bacteria as compared with IRBB21 (Figure 1, leaves 3 and 4). IRBB21 is a resistant line carrying the *Xa21* gene which was introgressed into the IR24 background through 12 years of intensive classical breeding [12, 17]. No partial resistance was observed in any of the transgenic plants that were challenged.

Table 2. Bio-assay of the transgenic *Indica* rice plant lines and their progeny for resistance to *Xanthomonas oryzae* pv. *oryzae.*

Transgenic line	Generation			
	R0	R1*	R2*	R3*
IR64-65[1]	R	nt	nt	nt
IR64-74[2]	R	nt	nt	nt
IR72-82	R	R	R	R
Minghui 63-12	R	R	R	nt
Minghui 63-22	R	R	R	nt
BG90-2-27	R	R	R	nt

[1]R0 plant was sterile. [2]R1 plants are not ready for the bacterial challenge. **Xoo*-resistant individuals included homozygous and heterozygotes plants. R, Resistance to *Xoo.* nt, not tested.

Segregation and inheritance of Xoo *resistance*

After the pathogen challenge, no significant difference was observed in the lesion length among the resistant individuals of R0, R1, R2 and R3 transgenic plant lines (data not shown). Results from Figure 2 indicated that the *Xa21* gene is not only stably inherited, but also stably expressed for at least four generations of IR72, three generations of Minghui 63 and BG90-2 (Table 2).

Segregation of *Xoo* resistance was studied by challenging the R1 progeny of transgenic IR72, Minghui 63 and BG90-2 with the pathogen (Figure 3). A majority of the lines exhibited a 3:1 segregation ratio (resistant plants: susceptible plants) among the offspring (Table 3), indicating Mendelian inheritance from one genetic locus. Line IR72-82, however, showed a 7.5:1 segregation pattern out of 34 R1 plants tested, instead of the 3:1 segregation ratio expected for a single locus insertion. This skewed ratio may be due to the low number of R1 plants inoculated since the R2 generation of heterozygous plants showed a 3:1 segregation ratio. We did not detect differences in the level of resistance to *Xoo* between homozygotes and heterozygotes of transgenic IR72 R2, R3, Minghui 63 and BG90-2 R2 plants in this study.

Southern blot analysis of transgenic plants

R0 plants of Minghui 63-12, Minghui 63-22, R1 and R2 plants of IR72-82 showing resistance to *Xoo* were selected for Southern blot analysis. All plants tested were found to contain *Xa21* gene as reflected by the release of a 5.7 kb DNA fragment when plasmid pC822 was digested with *Pst*I (Figure 4). When

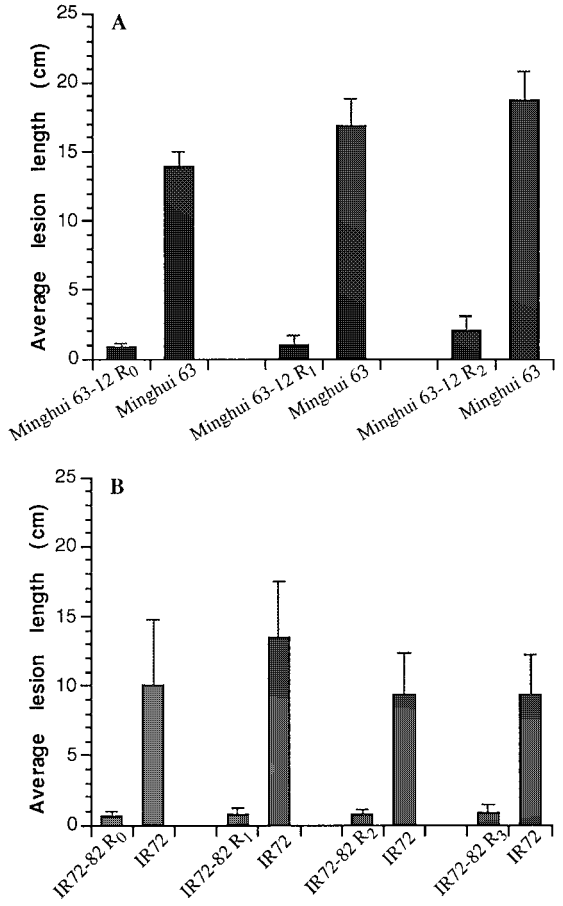

Figure 2. Lesion length data of different generations of transgenic lines Minghui 63-12 (A), IR72-82 (B) and their non-transgenic control plants. Data were collected 14 days after inoculation with *Xoo* race 6 strain PX099Az.

the DNA was digested with *Xho*I (Figure 4B), plant lines of Minghui 63 and IR72 showed different banding patterns, demonstrating that they originated from different primary transformation events. The banding patterns for the R1 and R2 progeny of line IR72-82 were identical, showing that the *Xa21* gene was stably integrated into the genome (Figure 4B, lanes 7, 8, 10, 11). Hybridization of RG103 with *Xho*I digested DNA from IR72-82 (Figure 4B, lanes 8, 11) and the two Minghui 63 lines (Figure 4B, lanes 14, 17), revealed at least two additional bands compared to the non-transgenic controls (Figure 4B, lanes 5, 20), indicating that at least two copies of the *Xa21* gene were inserted. The additional bands observed in the non-transgenic controls are non-active members of the highly polymorphic *Xa21* gene family [19].

Table 3. Segregation of *Xa21* gene expression in IR72-82, Minghui 63-12, Minghui 63-22 and BG90-2-27 R1 plants.

Transgenic line	Number of plants inoculated	Number of plants resistant to *Xoo*	Percentage of resistant plants	χ^2
IR72-82	34	30	88.2	3.176
IR72 (control)	6	0	0	–
Minghui 63-12	52	40	76.9	0.103*
Minghui 63-22	55	42	76.4	0.055*
Minghui 63 (control)	3	0	0	–
BG90-2-27	58	44	75.9	0.023*
BG90-2 (control)	3	0	0	–

* χ^2 tests indicate good agreement with segregation ratios of 3:1.

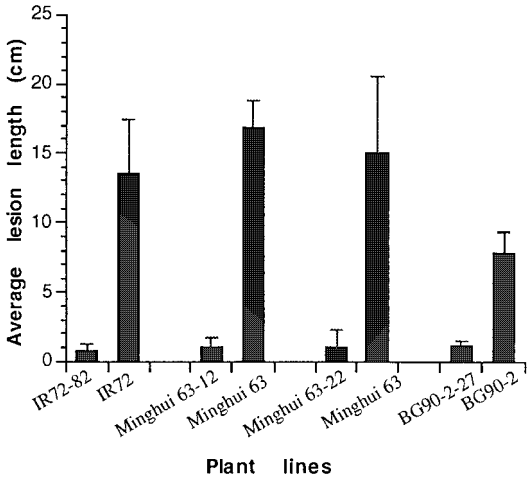

Figure 3. Lesion length data of R1 plants of transgenic lines IR72-82, Minghui 63-12, Minghui 63-22, BG90-2-27 and their non-transgenic control plants. Data were collected 14 days after inoculation with *Xoo* race 6 strain PX099Az. Each data point represents 15 to 20 clonal individuals with three inoculations per individual.

Discussion

Although *Japonica* rice transformation is routine in our laboratory [2], and large numbers of fertile transgenic plants can be obtained from *Indica* (group 5) rice genotypes (e.g. Basmati) and some other unclassified *Indica* varieties (e.g. Chinese variety Chang Li Xian), *Indica* (group 1) rice varieties still are recalcitrant to transformation and the number which can be regenerated is low. Reasons for this could be due to tissue culture techniques and/or difficulties encountered in distinguishing transformed calli from non-transformed calli during selection. High number of escapes may occur because of cross-protection by transgenic calli to non-transformed tissues which results in continual growth of non-transgenic tissue on selective media. These problems are compounded by the low efficiency of regeneration inherent to *Indica* (group 1) transgenic tissues.

Despite these difficulties, transgenic plants were recovered from four different elite *Indica* rice varieties. Over all, 55 independently transformed *Indica* rice lines were obtained by a co-transformation method, and 42 of these were PCR positive for the *Xa21* gene. However, the expression efficiency was much lower than our previous reports using the *uidA* (*gus*) marker gene [24] and the *Japonica* transformation with the *Xa21 gene* [19]. This lower efficiency may be due to the size of the gene being integrated into the plant genome, as the *Xa21* gene containing fragment is 9.6 kb long and the whole cassette of the *uidA* gene is only 3 kb. However since transgenic *Japonica* rice lines showed a higher expression efficiency with the same genes, it is more likely that different rice genotypes have various levels of transgenes rearrangement and/or expression after transgene integration.

One interesting note is that all the *Xoo*-resistant transgenic plants showed a greater level of resistance to the pathogen than IRBB21, the *Xa21* gene donor line (Figure 1). This enhanced resistance is similar to the results obtained previously with the *Xoo*-resistant *Japonica* rice plants [19]. This may be due to more copies of the *Xa21* gene in transgenic rice plants. Southern blot analysis of three *Xoo*-resistant plant lines showed that all the plants have more than one copy of the *Xa21* transgene (Figure 4). This enhanced resistance could provide an advantage for genetic engineering over classical breeding in cases where higher levels of resistance are desirable. Some of our

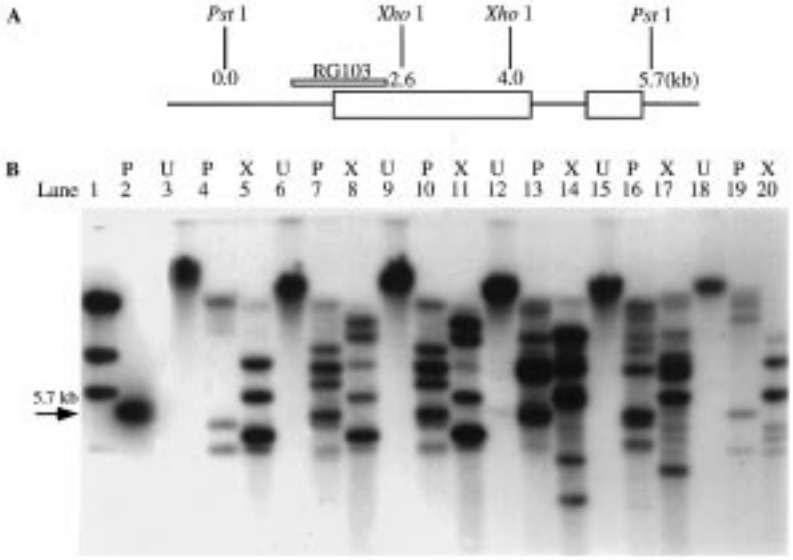

Figure 4. A. Linearized map of the *Xa21* gene. B. Southern blot analysis of transgenic lines IR72-82, Minghui 63-12, Minghui 63-22 and their non-transgenic control plants. RG103 marker was used as the probe. Lane 1, *Hin*dIII DNA marker; lane 2, plasmid pC822 containing the *Xa21* gene; lanes 3–5, non-transgenic IR72; lanes 6–8, R1 progeny of IR72-82; lanes 9–11: R2 progeny of IR72-82; lanes 12–14, R0 Minghui 63-12; lanes 15–17, R0 Minghui 63-22; lanes 18–20, non-transgenic Minghui 63; U, undigested DNA; P, DNA digested with *Pst*I; X, DNA digested with *Xho*I. The bands in lanes 3–5 and 18–20 of non-transgenic controls are non-active members of the highly polymorphic *Xa21* gene family.

transgenic plants are now in the fourth generation, and the resistant phenotype has been stably inherited.

In this report we describe that *Xoo*-resistant *Indica* (group 1) rice plants of IR64, IR72, hybrid *Indica* rice restorer line Minghui 63 and *Indica* cultivar BG90-2, the most widely used commercial varieties in Southeast Asia, China and West Africa, were obtained by microbombardment of cell suspension cultures with the *Xa21* gene. Some of those plants are currently in the process of being field tested, and if these plants display resistance to the pathogen under field conditions and maintained desirable qualities, they can be used as breeding material or directly used for production.

Acknowledgements

We thank the Rockefeller Foundation for their support and S.J. Leitner for maintenance of transgenic plants. C.F was supported by ORSTOM, a French public research organization.

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