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Pyramiding transgenes for multiple resistance in rice against bacterial blight, yellow stem borer and sheath blight

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Abstract Here we describe the development of transgene-pyramided stable elite rice lines resistant to disease and insect pests by conventional crossing of two transgenic parental lines transformed independently with different genes. The *Xa21* gene (resistance to bacterial blight), the *Bt* fusion gene (for insect resistance) and the chitinase gene (for tolerance of sheath blight) were combined in a single rice line by reciprocal crossing of two transgenic homozygous IR72 lines. F_4 plant lines carrying all the genes of interest stably were identified using molecular methods. The identified lines, when exposed to infection caused by *Xanthomonas oryzae* pv *oryzae*, showed resistance to bacterial blight. Neonate larval mortality rates of yellow stem borer (*Scirpophaga incertulas*) in an insect bioassay of the same identified lines were 100%. The identified line pyramided with different genes to protect against yield loss showed high tolerance of sheath blight disease caused by *Rhizoctonia solani*.

Keywords Transgene pyramiding · Bacterial blight resistance · Sheath blight resistance · Stem borer resistance · Stable expression

Introduction

Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is one of the most devastating diseases throughout the world (Mew 1987). Rice yield losses caused by bacterial blight in some cases can reach 50% when plants are infected at the maximum tillering stage. A dominant gene designated as *Xa21* known to confer resistance to most races of BB was transferred from a wild species, *Oryza longistaminata*, to cultivated variety "IR24" by conventional breeding (Khush et al. 1990). This map-based cloned *Xa21* is the first well-characterized disease resistance gene described in rice (Song et al. 1995). Transfer of this gene into the genome of an elite indica rice cultivar (IR72) and field evaluation has been carried out by Tu et al. (2000a).

Stem borer damage is a serious problem in rice, causing a 10–30% loss of total yield (Khush and Toenniessen 1991). *Bacillus thuringiensis* (*Bt*) produces a characteristic crystalline insecticidal protein (Bt protein). The transgenic approach for insect resistance with *Bt* gene expression has been demonstrated in crop plants including rice (Alam et al. 1998; Datta et al. 1998). Bt rice has shown great promise and potential for environment-friendly plants that can be grown in the field without application of pesticides. A hybrid commercial Bt rice, Shan You 63, has now been field-evaluated in China on a large scale and has shown resistance to two insect pests, leaffolder and yellow stem borer (Tu et al. 2000b). Transgenic IR72 with fusion *Bt* also showed very high protection against four lepidopteran insects under Chinese field conditions (Ye et al. 2001).

Sheath blight disease is caused by the soil-borne fungal pathogen *Rhizoctonia solani*. Yield loss ranges from 8% to 50%, particularly when the infection is well distributed and severe in the field (Savary et al. 2000). A large number of pathogenesis-related protein genes are now reported (Datta et al. 1999). It is still a challenge to engineer rice against fungal pathogens, especially *R. solani*. Progress has been made in recent years in understanding plant defense mechanisms against pathogenic fungi (Purkayastha 1998; Sakamoto et al. 1999). Transgenic rice with several pathogenesis-related (PR) genes has been developed, and enhanced resistance against sheath blight disease has been reported (Lin et al. 1995; Datta et al. 1999, 2000, 2001; Nishizawa et al. 1999; Baisakh et al. 2001). One class of

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PR proteins is *chitinase*, which catalyzes the hydrolysis of β-1-4-linkage of the N-acetylglucosamine polymer of chitin, the major component of the fungal cell wall. Lin et al. (1995) introduced a rice chimeral *chitinase* gene, *Chi11*, in rice cultivar CBII. Constitutive expression of another infection-related PR-3 rice *chitinase* gene (*RC7*), isolated from an *R. solani*-infected rice plant, in a different indica rice cultivar showed enhanced resistance to sheath blight disease (Datta et al. 2001).

To obtain cultivars with durable and broad-spectrum resistance, the pyramiding of major genes (multigene strategy) implying a different mode of action with insecticidal and disease resistance genes against target organisms may be a powerful strategy (Wenzel 1985). Gene pyramiding by conventional breeding and marker-assisted selection has been reported for several rice lines with more than one gene for resistance to *Xoo*, such as *Xa4*, *Xa5*, *Xa13* and *Xa21* (Huang et al. 1997; Chen et al. 2000; Singh et al. 2001). Gene pyramiding with the transgenic approach through co-transformation (*gna*, *cry1Ac* and *cry2A*) has been reported (Maqbool et al. 2001). Co-transformation with *gna* and the *Xa21* gene of elite Chinese rice cultivars has been demonstrated (Tang et al. 1999).

In this report, we describe the combination of the *Xa21* gene, the *Bt* gene (*cry*) and *RC7 chitinase* gene in a single elite rice line by conventional crossing of two IR72 transgenic homozygous stable lines, one carrying the *Xa21* gene and the other the *cry* gene and the *RC7 chitinase* gene, as a strategy for multiple resistance.

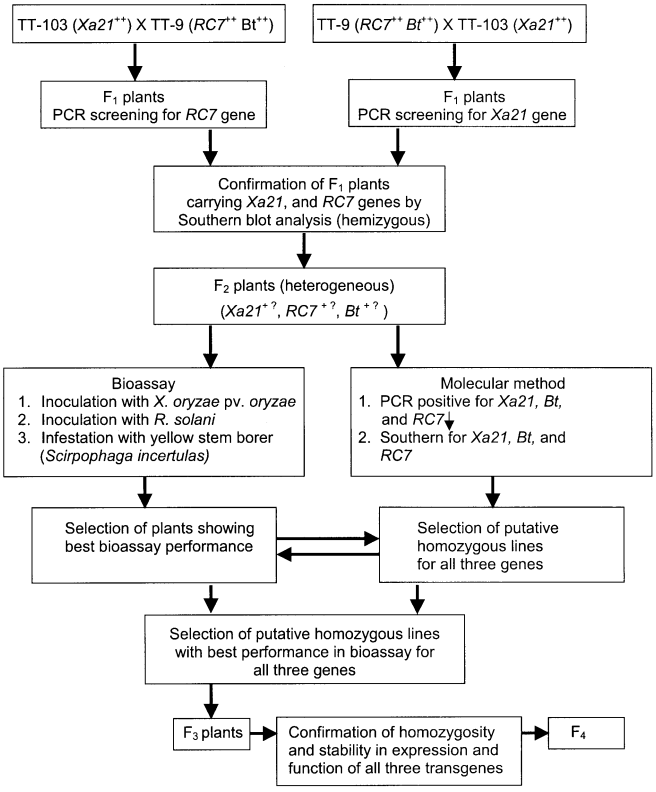

Fig. 1 Flow diagram of the procedure for identification of F_2 plants carrying *Xa21*, *RC7* and *Bt* genes resistant to bacterial blight and yellow stem borer, and enhanced tolerance for sheath blight, respectively

Materials and methods

The parental lines TT-103 and TT-9 were used in this study. They were developed at the tissue culture laboratory of the International Rice Research Institute (IRRI), The Philippines. TT-103 carries the *Xa21* gene in the background of *IR72* in the homozygous stage (Tu et al. 2000a) and TT-9 carries the *Bt* gene, also in a IR72 background (Ye et al. 2001), as well as the *RC7 chitinase* gene, integrated at the same locus, which is homozygous for both genes.

Growing the parental lines and developing introgressed lines with stacked transgenes

At 21 days after seeding, 50 seedlings of each line were transplanted in 6-inch-diameter pots inside the containment greenhouse, which is controlled to obtain full sunshine, sufficient ventilation, and a day/night temperature of 31/23 °C.

When 50% of the panicle emerged from the boot, emasculation was carried out using a vacuum emasculator with 500 mm Hg suction. Pollination was done when maximum anther extrusion from the florets occurred. The pollinated panicles were bagged properly. Crosses were made between TT-103 and TT-9 parental lines, including reciprocals. The collected F_1 seeds were grown to the F_2 generation in the same way, and lines carrying all three desirable genes were selected by molecular methods such as PCR analysis, Southern blot and bioassay, showing maximum resistance to insects, bacteria and fungi.

Figure 1 shows a flow diagram of the procedure for identifying F_2 plants carrying *Xa21*, *RC7* and *Bt* genes, and with the best performance in the bioassay for bacteria, fungi and insects, respectively. In this procedure, through molecular methods and bioassays until the F_4 generation we selected all the desirable genes in a stable homozygous condition in the IR72 line.

Polymerase chain reaction (PCR) analysis

The PCR analysis was done for all three genes as per the procedure of Huang et al. (1997). However, the only modification was the annealing temperature, which was set at 60 °C for the *RC7* primer pair, whereas for *Xa21* it was 55 °C. The primer pairs for *Xa21* are the same as those used by Wang et al. (1998), and for RC7 they are as follows: RC7 F: 5′-TAAGCATGTCGACG-CCGAGAGCGG-3′, RC7 R: 5′-CGTCAGTCCTCATCACTGC-TCCGC-3′.

The PCR reaction mixture contained 50 ng of template DNA, 50 ng of each primer, 0.16 mM of dNTPs, 2.5 mM of $MgCl_2$, PCR buffer and *Taq* polymerase in a volume of 25 µl. The amplified products were electrophoretically resolved on a 1% agarose gel in TAE buffer.

Reverse transcription polymerase chain reaction (RT-PCR)

The total RNA was extracted from freshly collected frozen leaf tissue (approxiimately 100 mg) using the Qiagen RNeasy extraction kit. Two micrograms, of total RNA were used as the template for synthesis and amplification of cDNA of the *Xa21* gene employing the Quiagen single-step RT-PCR kit. The cDNA products were resolved in 1.5% TAE agarose gel.

Southern-blot analysis

Genomic DNA was extracted from the leaf tissues using a procedure described by Datta et al. (1998). Ten micrograms of DNA

was digested with *Eco*RV (for the *Xa21* gene), *Sst*I/*Hind*III (for *Bt* gene) and *Hind*III/*Bam*HI (for the *RC7* gene) restriction endonucleases in a final volume of 40 µl. The digested DNA was separated by electrophoresis on 1% (w/v) agarose gel. DNA fragments were denatured and transferred onto a Hybond-N^+ nylon membrane following the manufacturer's instructions. The coding sequence from plasmid digested with the same enzyme shown above, except for *Xa21* for which the PCR-amplified a 1.4-kb fragment or *Xho*I released a 1.4-kb fragment, was labeled with (α-^{32}p) dCTP employing the rediprime labeling kit (Amersham, Arlington Heights, Ill.) and used as a hybridization probe.

Western-blot analysis

Following the same procedure described by Datta et al. (1998), total protein was extracted from transgenic and non-transgenic control plants. The concentration of protein extract was determined by BCA protein-assay reagents. Electrophoresis was done on a 10% (w/v) SDS-PAGE gel using a total of 50 µg of soluble plant protein per lane. Separated polypeptides were blotted on nitrocellulose membranes. Proteins were probed with rabbit antibody raised against *RC7* and *Bt*, and detected using procedures described by Lin et al. (1995).

Bioassay for bacterial blight

Six-week-old plants and controls were inoculated with PXO99 (race 6) using the leaf-clipping method (Kauffman et al. 1973). Inoculum suspension density was about 10^9 cells per milliliter. Plants were grown in the IRRI containment greenhouse under the following conditions: 31 °C and 85% humidity during daytime, and 23 °C and 90% humidity at night. Plant reaction to bacteria was scored on six leaves by measuring the lesion lengths 14 days after inoculation.

Cut-stem bioassay for yellow stem borer (YSB)

The insect bioassay method is described by Datta et al. (1998). It was conducted using the cut-stem method in a Petri dish. Five cut stems, 8 cm in length, collected at the booting stage were infested separately with six neonate larvae of YSB. Petri dishes were incubated in the dark and the mortality rate of larvae was observed after 4 days.

Bioassay for sheath blight

The bioassay by inoculation with the pathogen *R. solani* is described by Datta et al. (2000). For inoculum preparation, a piece of 4-day old culture of *R. solani* was inoculated with the substrate mixture of rice hull and rice grain (5:1), and incubated at room temperature for 7–10 days. For inoculation of test plants, 5 g of inoculum was placed in the middle of the tillers of each plant at its maximum tillering stage. The symptoms caused by fungal infection were scored after 14 days as described by Ou (1985). The relative integrated infection index was calculated according to the formula described by Datta et al. (2000).

Results

Identification of F_1 plants introgressed with RC7, *Xa21* and *Bt* genes

The crosses between TT-103 and TT-9 transgenic homozygous lines resulted in 80 F_1 (TT-103 × TT-9) and 125 reciprocal (TT-9 × TT-103) seeds. PCR and Southern analysis were done on 50 F_1 plants, chosen at random, to verify the presence of the expected genes. When TT-103 (carrying the *Xa21* gene) was a female parent, PCR analysis of the F_1 plants identified the presence of the *RC7* gene (Fig. 2a). All these PCR-positive F_1 plants showed the *Hind*III/*Bam*HI 1.6-kb *RC7* fragment received from the male parent TT-9 (carrying the *RC7* and *Bt* genes). In the reciprocal crosses in which TT-9 was the female parent and which originally carry the *RC7* and *Bt* genes, the1.4-kb PCR amplicon identified the presence of the *Xa21* gene (Fig. 2b). All the PCR-positive F_1 plants showed the expected 3.8-kb *Eco*RV fragment inherited from the male parent TT-103. PCR-negative lanes in Fig. 2a and b represent the plants from the selfed seeds.

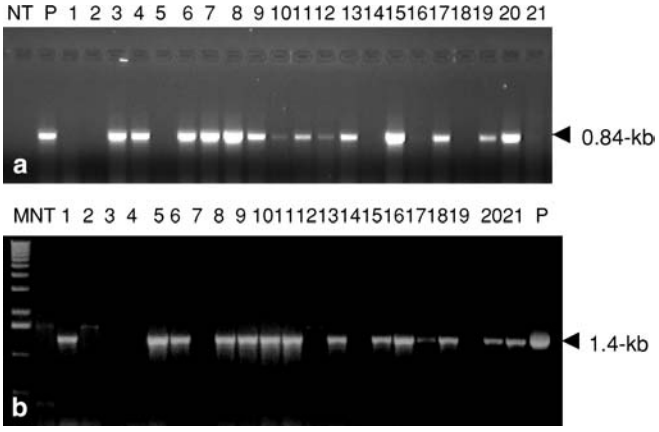

Fig. 2 **a** PCR analysis showing the 0.84-kb amplicon expected for the *RC7* gene. *M* = 1-kb molecular marker, *NT* = non-transformed control, *P* = plasmid pC822 (harboring *Xa21*), numbers *1 to 21* represent the F_1s produced from the cross TT-103 × TT-9. **b** PCR analysis showing the 1.4-kb amplicon expected for *Xa21* gene. *M* = 1-kb molecular marker. *NT* = non-transformed control, numbers 1 to 21 represent the F_1s produced from the cross TT-9 × TT-103, *P* = plasmid pC822 (harboring *Xa21*)

To confirm the presence of the *Bt* gene in F_1 plants, all the F_1 plants were subjected to infestation with YSB and most of them showed 100% mortality of neonate larvae. As the line TT-9 is homozygous for both the *RC7* and *Bt* genes, integrated at the same locus, it is expected that in F_1 plants the presence of the *RC7* gene also implied the presence of the *Bt* gene.

Development and selection of desirable F_4 lines

From the F_1 plants, we selected at random two plants (XYZ-19 and XYZ-21) from TT-103 × TT-9 and two plants (XYZ-7 and XYZ-8) from its reciprocal crosses because of the limited space in the transgenic greenhouse. From each of the four lines, 32 F_2 progenies were grown, considering crossing two characters, and as in TT-9 the *RC7* and *Bt* genes were on the same locus. To obtain the best combination and selection of the lines

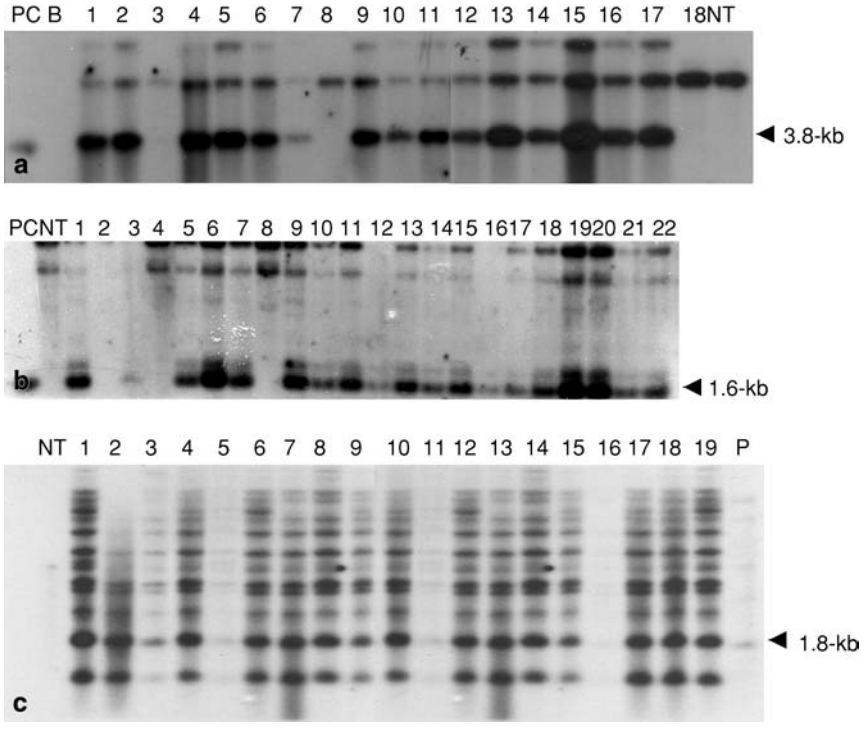

Fig. 3 **a** Southern blot analysis showing the 3.8-kb band expected for most of the intact *Xa21* gene, including its intron. *PC* = positive control (the 3.8-kb fragment released by *Eco*RV digestion of the plasmid), *B* = blank, numbers 1 to 17 represent the F_2s produced from the cross TT-9 × TT-103, 18 = TT9 parent, *NT* = non-transformed control. **b** Southern blot analysis showing the 1.6-kb band expected for the *RC7* expression cassette. *PC* = positive control (the *Bam*HI/*Hind*III-released 1.6-kb *RC7* expression cassette from pGL2RC7), *NT* = non-transformed control, numbers 1 to 22 represent the F_2s produced from the cross TT-103 × TT-9. **c** Southern blot analysis showing the 1.8-kb band expected for the fusion *Bt* gene. *NT* = non-transformed control, *P* = positive control (digested plasmid), numbers 1 to 19 represent the F_2s produced from the cross TT-103 × TT-9

with all three genes, these plants underwent a bioassay with bacterial blight and sheath blight inoculum and infestation with YSB. PCR analysis of all the progenies of four lines was done using three different primer pairs for the respective genes used, followed by Southern analysis for further confirmation and to identify the probable homozygous lines. Southern results for some of the plants for three different genes are shown in Fig. 3a, b and c. Figure 3a represents the Southern blot showing the presence of a 3.8-kb fragment corresponding to most of the coding region, including the intron of the *Xa21* gene in the segregating line. Here, the plants, together with the two positive control plants, show the endogenous band and all *Xa21*-positive plants with a rearranged copy in addition to the 3.8-kb fragment. Southern blots for both *RC7* (1.6 kb, Fig. 3b) and *cry* genes (1.8 kb, Fig. 3c) showed multiple-copy integration of both genes in the segregating population in the F_2 generation.

On the basis of performance in the bioassay and the presence of three genes and band intensity in Southern blot, we chose four lines (XYZ-7-10, XYZ-7-12, XYZ-8-17 and XYZ-21-19). Table 1 represents different lines carrying the *Xa21* and *RC7* genes, showing a variable response to their respective bioassays. We found that XYZ-7-10 showed the lowest lesion length (3.2 cm) with bacterial blight (BB) infection and 15.6 cm when infected with sheath blight (ShB), whereas XYZ-7-12 had a lesion 4.4-cm long with BB and the lowest ShB lesion was 6.6-cm long. XYZ-8-17 showed moderately less (15.0 cm) ShB lesion-length but it showed a slightly higher BB lesion-length (8.2 cm). XYZ-21-19 is another good line, showing a BB lesion length of 6.9 cm and the lowest ShB lesion length of 11.9 cm. For other lines, even though they were positive for the three genes, their bioassay performance was not acceptable. All these lines were *Bt*-positive, showing a 100% mortality rate of neonate larvae of the yellow stem borer.

From each F_2 line, 30 progenies were grown and selected by bioassay and molecular analysis. When a bioassay was done with the infestation of YSB neonate larvae, all the progenies of XYZ-7-10 and XYZ-7-12 showed a 100% mortality rate, whereas the progenies of XYZ-8-17and XYZ-21-19 presented a different level of mortality, ranging from 5.6% to 100%, which was correlated with Southern analysis data for the *Bt* gene. In this F_3 generation, we found a homozygous line in progenies of XYZ-7-10 and XYZ-7-12.

For final evaluation and confirmation, we grew the F_4 generation. Figure 4a, b and c represent the presence of *Xa21*, *RC7* and *cry* genes, respectively, in Southern-blot analysis. All the plants showed multiple rearranged copies of the respective genes in addition to the expected one in the homozygous condition. In Southern analysis of the F_4 plants for *Xa21*, only a 1.4-kb kinase PCR amplicon was used as a hybridization probe (Fig. 4a), whereas, when the *Xho*I-released 1.4-kb fragment was used as a hybridization probe (Fig. 3a), we could see one rearranged copy of the same *Xa21* gene, which shows that the rearranged copy does not have any homology with the kinase fragment.

Expression of the *Xa21* gene was confirmed by RT-PCR, which showed an expected cDNA amplicon of 0.8 kb in size (Fig. 5). This indicated successful splicing of the intron during mRNA transcription and maturation.

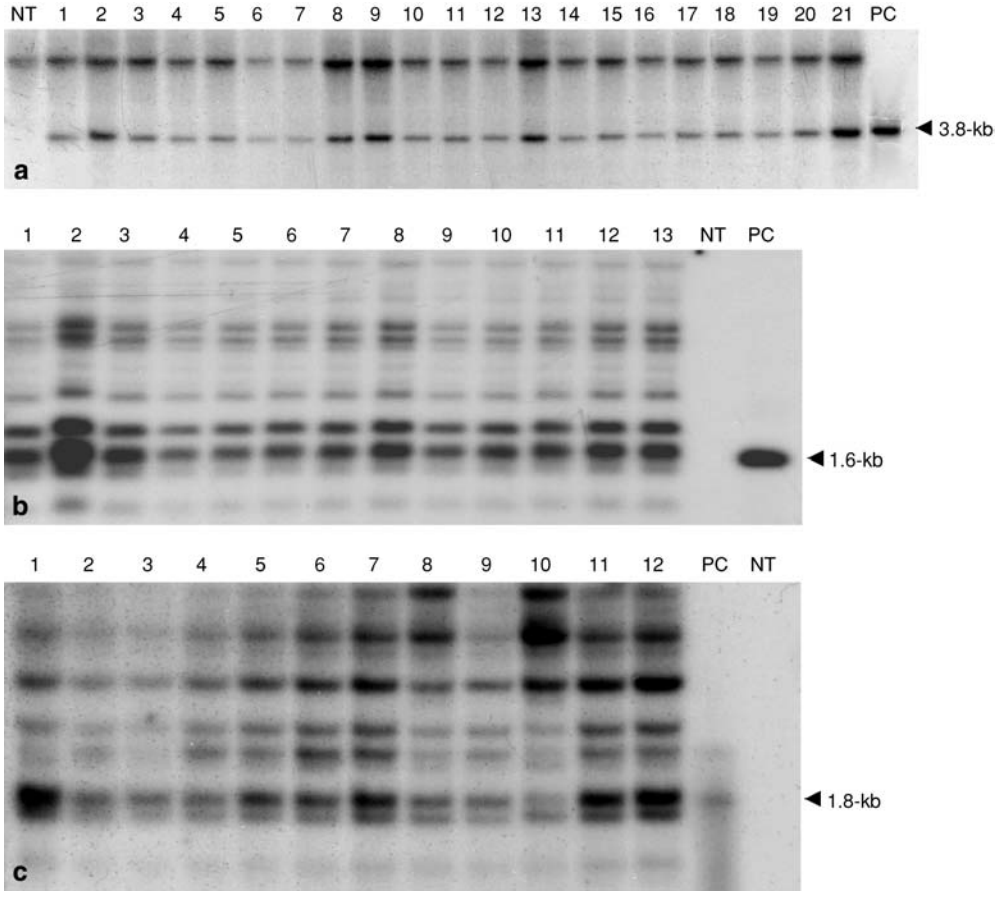

Fig. 4 **a** Southern blot analysis showing the homozygosity of the F_3 progenies by the presence of a 3.8-kb band expected for the *Xa21* gene in all the plants. *NT* = non-transformed control, *PC* = positive control, numbers 1 to 21 represent the progeny plants of an F_3 family. **b** Southern blot analysis showing the 1.6-kb band expected for the *RC7* expression cassette in all the F_3 progenies of a single cross. *Numbers 1 to 13* represent the F_3s of a single family, *NT* = non-transformed control (no expected endogenous band because of the degraded DNA), *PC* = positive control. **c** Southern blot analysis of the F_3 progeny plants exhibiting the fixation of a locus for the fusion *Bt* gene by the presence of a 1.8-kb band in all the plants. *Numbers 1 to 12* represent the F_3 plants, *PC* = positive control (digested plasmid), *NT* = non-transformed control

Table 1 Different lines carrying the *Xa21* and *RC7* genes showing a variable response to their respective bioassays

Plant no.	BB lesion (cm)	PCR result (for *Xa21*)	SB lesion (%)	Southern result (for *RC7*)
XYZ-7-10[a]	3.2	+	15.6	+
XYZ-7-14	3.3	+	20.0	+
XYZ-7-16	3.6	+	25.0	+
XYZ-7-12[a]	4.4	+	6.6	+
XYZ-7-18	4.6	+	14.4	+
XYZ-7-20	7.4	+	5.9	+
XYZ-7-17	7.6	+	11.9	+
XYZ-7-25	8.1	+	15.6	+
XYZ-7-21	9.5	+	5.7	+
XYZ-7-27	12.9	+	25.0	+
XYZ-8-5	7.9	+	22.5	+
XYZ-8-2	8.0	+	25.0	+
XYZ-8-17[a]	8.2	+	15.0	+
XYZ-8-26	10.9	+	23.1	+
XYZ-8-28	12.2	+	35.0	+
XYZ-8-24	12.9	+	13.8	+
XYZ-19-1	7.9	+	24.4	+
XYZ-19-32	8.7	+	29.4	+
XYZ-19-3	10.5	+	26.3	+
XYZ-19-15	15.4	+	8.8	+
XYZ-21-5	5.2	+	41.3	+
XYZ-21-19[a]	6.9	+	11.9	+
XYZ-21-10	8.1	+	14.8	+
XYZ-21-1	10.0	+	33.8	+
XYZ-21-11	10.1	+	23.5	+
XYZ-21-15	10.4	+	22.5	+
XYZ-21-22	10.6	+	25.0	+
XYZ-21-23	10.7	+	16.9	+

[a] Selected for next generation

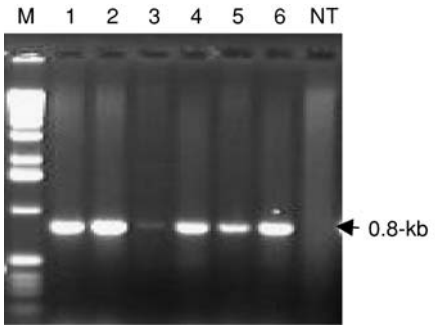

Fig. 5 The reverse transcription polymerase chain reaction (RT-PCR) indicating the expression of the *Xa21* gene by the amplification of about a 0.8-kb cDNA in the F_3 progenies. *M* = 1-kb molecular weight marker, *numbers 1 to 6* = F_3 progeny plants, *NT* = non-transformed control

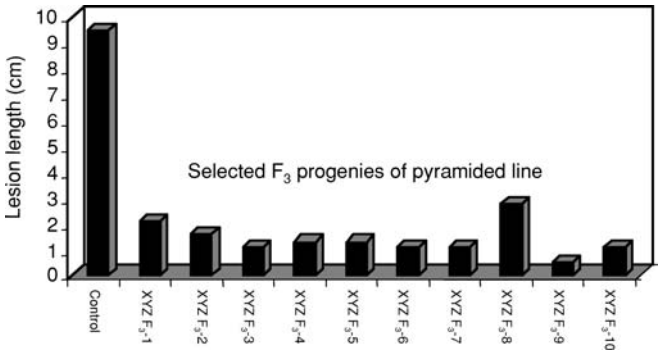

Fig. 7 Bacterial blight (*BB*) bioassay showing the resistant reaction of ten selected F_3 progenies homozygous for the *Xa21* gene. The pyramided plants showed resistance to BB with a lesion length of ≤3 cm versus the susceptibility of the non-transformed control with a lesion length of about 10 cm

Similarly, Western-blot analysis for *RC7* and *Bt* confirmed their expression with 35-kDa (Fig. 6a) and 60-kDa (Fig. 6b) proteins, respectively.

In the bioassay with infection of *X. oryzae* pv *oryzae*, different plants of the homozygous lines showed different levels of infection, but all of them showed a lesion length of less than 3.1 cm, which is considered to be resistant (Fig. 7). On the other hand, when the plants of the homozygous lines were exposed to the infection of *R. solani*, we observed a broad range in levels of infection. In the relative infection index (Fig. 8), average infection was approximately 40%, which represents 60% protection. In the bioassay of our final selected line with infestation with larvae of YSB, we found that all the plants were completely resistant, showing 100% mortality of neonate larvae.

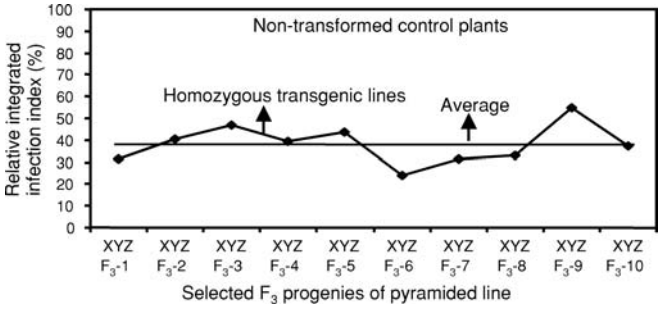

Fig. 8 Reaction of ten selected F_3 progenies homozygous for the *RC7* gene to sheath blight fungus infection. The pyramided plants showed resistance to ShB with an average relative infection index of 38.2%, meaning 62.8% protection versus the control (100%)

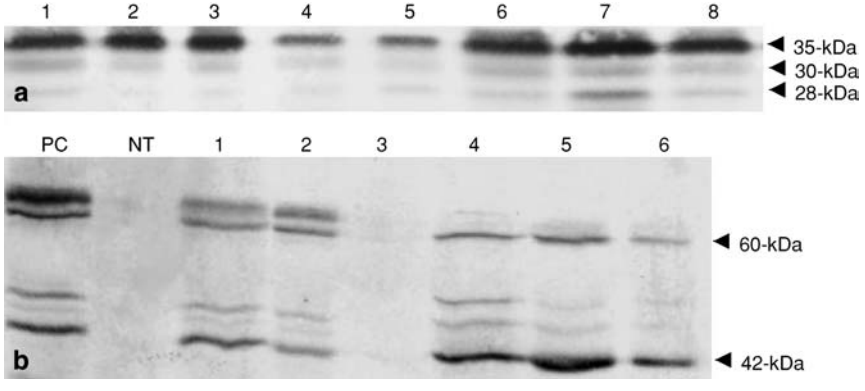

Fig. 6 **a** Immunoblot analysis that confirms the expression and homozygosity of infection-related *RC7* gene by the 35-kDa protein specific to the transgene. The 28-kDa protein in all the plants corresponds to the endogenous chitinase gene expression; the 30-kDa protein could be the proteolytic byproduct of the 35-kDa protein (Datta et al. 2000). Numbers 1 to 8 correspond to F_3 plants. **b** Immunoblot analysis showing the expression of a 60-kDa protein specific to the fusion *Bt* transgene. The additional multiple bands in the western blot could be post-transcriptional and/or post-translational products (Datta et al. 1998). *PC* = positive control (TT-9), *NT* = non-transformed control, *numbers 1 to 6* represent the F_3 plants (note that the faint band in number 3 was due to the very low amount of the protein)

Discussion

Gene pyramiding is emphasized to obtain many complex biochemical pathways in plants for crop improvement and durable resistance. Approaches can involve conventional sexual crossing, re-transformation, co-transformation and the use of linked transgenes. The level of expression of a transgene is variable and is influenced by various factors, such as the site of integration (Matzke and Matzke 1998). Transgene stability also varies among transformants and some plants show a variety of instability even in subsequent generations.

In this attempt, we used two selected stable lines showing a high level of expression for a few generations. Line, TT-103, carrying the *Xa21* gene, has been field-tested in China, and it showed excellent field performance for introduced bacterial blight resistance, and its yield performance was comparable with that of the control under field conditions (Tu et al. 2000a). Another stable line, TT-9, carrying both the *Bt* and *RC7* genes, has also been field-tested for insect resistance and it showed excellent resistance to yellow, striped and pink stem borer and leaffolder (Ye et al. 2001). The *RC7* and *Bt* genes were confirmed earlier to be cointegrated in the same locus of IR72. The three genes of interest were driven by three different promoters: *Xa21* was a cloned gene from wild rice, the *Bt* gene had the rice actin-1 promoter, and *RC7* was driven by the 35-S promoter of cauliflower mosaic virus. This overcomes the problem of gene silencing because of homology in the promoter sequence to drive the expression of different transgenes in the attempt to coordinate expression. As both parents are homozygous for the genes they harbor, all the seeds obtained from the crossing will contain all three genes, as shown in F_1 plants (Fig. 1). But F_2 progenies developed from seeds of F_1 plants contain different pairs of transgenes in a hemizygous condition. In the Southern-blot analysis, we selected lines that showed a Mendelian segregation ratio of 3:1. Seeds for those plants showing the expected intact coding sequence of the transgene and the best bioassay performance were grown for the next generation to expedite the search for the best material, homozygous for the three genes.

The reaction of the pyramided selected plants of the lines to the BB pathogen showed that average lesion length was less than 3.1 cm. A higher level of resistance may be the result of a synergistic effect of *Xa21* and endogenous *Xa4* present in cultivar IR72. All of these selected lines showed 100% mortality of the neonate larvae, which we can expect from gene-for-gene resistance. Multiple-copy integration may lead to high resistance based on a comparison of earlier material with single-copy integration (data not shown).

For the *RC7* gene, our observation is not always consistent. This gene showed different levels of enhanced resistance when challenged with the sheath blight pathogen *R. solani*. The *RC7* gene is a PR protein gene. Environmental stress and developmental regulation probably influenced the endogenous gene regulation, and not all the plants exhibit the desired levels of increase in resistance to the fungal pathogen (Meyer and Heidmann 1994). The successful use of co-transformation as a method for combining multiple transgenes in one plant has been reported (Hadi et al. 1996; Chen et al. 1998; Maqbool and Christou 1999). Only a few groups have looked at the expression of co-transformed transgenes. At the same time, it is very difficult to obtain a stable line with multiple genes if the genes are integrated at multiple sites and in multiple copies.

In this report, we demonstrated the combination of three genes with three different modes of action by sexual crossing of transgenic plants, which provides a multi-mechanism defense for crop protection. The parental transgenic lines were near-isogenic lines (NILs) of IR72 differing in a single locus (TT103 for *Xa21* and TT9 for *Bt* and *RC7*) that reduced the time-consuming recurrent backcrossing for near full recovery of the recipient background. Moreover, the transgenes served as markers for early identification and molecular selection to ultimately achieve stable homozygous lines. These stable homozygous pyramided lines are being grown for seed multiplication and field-testing in the very near future.

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