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Expression and inheritance of hypersensitive resistance to rice hoja blanca virus mediated by the viral nucleocapsid protein gene in transgenic rice

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Abstract Rice hoja blanca virus (RHBV) is a major virus disease of economic importance affecting rice in northern South America, Central America and the Caribbean. This is the first report of transgenic resistance to RHBV and the transformation of an *indica* rice variety from Latin America. Rice transformed with the RHBV nucleocapsid protein (*N*) gene had a significant reduction in disease development. Several reactions were observed that ranged from susceptible to completely resistant plants (immunity). The resistant reactions were characterized by the production of local lesions like a hypersensitive reaction or a recovery phenotype with the emergence of symptom-less new leaves. These transgenic RHBV-resistant rice lines expressed the *N* gene RNA at low levels that were below the detection limit by Northern blots and only resolved by RT-PCR. The nucleocapsid protein could not be detected in any of the transgenic plants either by Western or ELISA tests. These results suggest that the resistance encoded by the *N* gene in these plants appears to be mediated by RNA. When challenged with RHBV, the resistant transgenic lines showed a significant increased performance for important agronomic traits including the number of tillers, the number of grains per plant and the yield as compared to the susceptible control. Furthermore, upon inoculation some of the most-resistant transgenic lines showed agronomic traits similar to the uninoculated non-transgenic Cica 8 control. Using both agronomic traits and disease severity as criteria, several of the most-resistant lines were followed through the R_4 generation and demonstrated that the *N* gene and RHBV resistance was inherited in a stable manner. These transgenic rice lines could become a new genetic resource in developing RHBV-resistant cultivars.

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Introduction

Rice hoja blanca virus (RHBV) is present throughout northern South America, Central America and the Caribbean (Morales and Niessen 1985). When RHBV outbreaks occur, yield losses throughout entire countries can range from 25 to 50% (Jennings 1963). The RHBV vector is the planthopper *Tagosodes orizicolus* (Muir) (Galvez et al. 1961), a serious pest of rice that can cause direct damage. Although many of the rice cultivars grown in Latin America are resistant to the planthopper, most commercial cultivars are not resistant to RHBV. There are active breeding programs developing RHBV-resistant rice, but most of the natural resistance to the virus comes from a single source, which does not confer immunity and does not confer adequate protection to rice seedlings challenged with RHBV (Zeigler et al. 1988). When popular commercial resistant cultivars such as Oryzica Llanos 5 and Fedearroz 50, bred with natural resistance, are inoculated at less than 20 days after planting, significant yield loss may occur. There is a need to incorporate additional sources of resistance into improved germplasm to ensure stable and durable resistance. The development and deployment of new sources of resistance that complement the conventional resistance are objectives of this research.

RHBV is a member of the tenuivirus group, which replicates in the planthopper vector. The genome comprises either four or five species of RNA (Ramirez and Haenni 1994). The lack of enveloped virions in tenuivirus has led to confusion in the terminology for the protein associated with the filamentous particles. In some articles this protein has been referred to as the coat protein and the nucleoprotein, but the tenuiviruses are similar to the Tosopviruses where this protein is referred to as the nucleocapsid protein (N protein). The RHBV nucleocapsid protein gene (*N*) is on the virus complementa-

ry strand of RNA 3. The N protein is predicted to consist of 319 amino acids with a M_r of 35,336 (Miranda et al. 1994). Molecular characterization of RHBV (Calvert et al. 1994; Miranda et al. 1994, 1997; Ramirez et al. 1992, 1993) shows that there are only minor differences between the RHBV strains in Colombia and Costa Rica. There are no reported biological differences for RHBV isolates, nor a is there reported breakdown of conventional sources of resistance throughout the range of RHBV. The lack of virus diversity suggests that single source of transgenic resistance could provide durable resistance to RHBV.

This research included the introduction of the RHBV *N* gene into the RHBV-susceptible *indica* cv Cica 8, and its transfer by crossing with other commercial varieties. As Cica 8 is highly recalcitrant to callus induction and to plant regeneration from immature or mature embryos, which are commonly used for rice (Li et al. 1993; Christou 1995; Cooley et al. 1995), embryogenic callus derived from immature panicles was tested.

Materials and methods

Plasmid construction

A 1.4-kb cDNA clone R3-C8 containing the RHBV *N* gene was derived from the 3′ region of the Colombian isolate of RHBV RNA 3 (Miranda et al. 1997) and was used in vector construction. This clone contains most of the 5′ untranslated region, the *N* gene and about 400 bp of the intergenic untranslated region. Using the restriction sites *Kpn*I and *Sst*I, the entire 1.4-kb R3-C8 clone was inserted in the sense direction between the cauliflower mosaic CaMV 35 S promoter and the NOS polyadenlation signal of the plasmid RT-101 (Yannisch-Perron 1985). The plasmid pJR225 containing a 2.3-kb hygromycin B phosphotransferase (*hmr*) gene cassette (Gritz and Davies 1983), driven by the CaMV 35 S promoter, was inserted into the *Sal*I and *Bam*HI restriction sites in the Stratagene plasmid pBS (+); the derived plasmid was designated pBS hygromycin. The CaMV 35 S promoter-Ngene-NOS cassette was then cut with *Sph*I and transferred to the pBS hygromycin; the plasmid was designated pVR3 (Fig. 1).

Plant material

O. sativa. L. *indica* cv Cica 8, which is highly susceptible to RHBV throughout its whole life cycle, was used for this study. This cultivar is grown by both large- and small-scale farmers in Colombia and Central America, and is a suitable breeding parent given its broad adaptation (from irrigated to water-stress conditions), grain quality and yield. Immature panicles 0.2–2.0 cm in length were harvested immediately after meiosis from about 70-day old glasshouse-grown plants. Panicles were surface sterilized, dissected out and plated on callus-induction medium consisting of MS salts, vitamins, enzymatic casein hydrolysate (300 mg/l), 2,4-D (2 mg/l), NAA (2 mg/l), kinetin (3 mg/l) and 6% sucrose, and then solidified with gelrite (2 g l^{-1}). These were cultured in the dark at 28 °C. The derived embryogenic callus (1–2 mm in diameter) was subcultured 1–2 days before bombardment.

Transformation of rice and regeneration of transgenic rice plants

The direct delivery of genes into the immature-panicle-derived embryogenic callus was done using the particle accelerator system PDS-1000/He (Bio-Rad), driven by helium. The putative transgenic plants were recovered using a step-wise selection on culture medium containing hygromycin (30 mg/l) followed by hygromycin (50 mg/l) throughout plant regeneration. Plants were regenerated on medium consisting of MS salts, sucrose (3%), NAA (1 mg/l), kinetine (4 mg/l) and hygromycin (50 mg/l). Hygromycin-resistant (Hyg^r) plants were transplanted into individual pots and grown in a glasshouse. After a week, plants were maintained under flooded conditions and grown to maturity to obtain seeds.

Southern-blot analysis

Genomic DNA was isolated from 1 g of leaves of primary transgenic plants and their progeny from 30-day old plants (Gilbertson et al. 1991). From 5–20 µg of DNA were digested with restriction endonucleases and electrophorezed on 0.8% agarose gels, treated in 0.25 M HCl for 10 min, neutralized using 0.4 N NaOH and transferred to nylon membranes (Hybond-N+, Amersham)using a Posiblot apparatus (Stratagene). Alpha-[^{32}P]-dATP-labeled hybridization probes specific to the *hmr* gene and the RHBV *N* gene (Fig. 1) were prepared using the multiprime DNA-labeling system (Amersham). The membranes were hybridized with the labeled probes in 5 × SSC, 7 × Denhardts, 0.5% SDS and 100 µg/ml of salmon sperm at 65 °C for 16 h (Sambrook et al. 1989).

Nested PCR amplification

The genomic DNA was amplified by the nested polymerase chain reaction (PCR) (Pooler et al. 1997). The first round of the nested PCR was carried out in 25 µl (total volume) containing 200 ng of DNA, and 0.4 µM of the external primers sk and pr10 which amplify the complete *N* gene sequence (Fig. 1). The 5′ primer sk (5′ CGCTCTAGAACTAGTGGATC) for the *N* gene was in the polylinker, and the 3′ primer pr10 (5′ CTCAAATAGCAAGTACATGG) was in an untranslatable region of the *N* gene at position 1,004–1,024-bp on RHBV RNA3. The reactions were carried out using 1 × PCR buffer, dNTPs (0.133 mM), $MgCl_2$ (2.0 mM) and *Taq* polymerase (2.2 U, Promega) using a programmable thermal controller (MJ Research). The PCR reaction conditions for the first cycle were 94 °C for 2 min, 56 °C for 4 min, 72 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 4 min and 72 °C for 4 min. The final cycle reaction was 8 min at 72 °C. The second round of PCR used the same reaction conditions, and contained 1 µl of the first PCR reaction, 0.4 µM of the nested primers pr3 (5′ GTTGGTCAGATAGAGTACATGC) and pr16 (5′GCAGTCAGAGAGACCAGAGT) (Fig. 1). The products were analyzed by agarose-gel electrophoresis and Southern blots.

Northern-blot analysis

Total RNA was isolated from 2.5 g of leaves of the R_1 and their progeny using LiCl precipitation (Thompson et al. 1983) and resuspended in 20 µl of DEPC-treated water. The equivalent of 20 µg of RNA per sample was run in 0.8% formaldehyde agarose gels and transferred to nylon membranes (Hybond-N+, Amersham) (Sambrook et al. 1989). The hybridization probes were prepared as described for Southern-blot hybridization.

Reverse transcriptase PCR amplification

To extract RNA, 40 mg of leaf tissue were macerated in liquid nitrogen. A solution of 4 M guanidine isothiocyanate (0.8 ml), Tris-HCl (50 mM) pH 8.3, EDTA (10 mM) and β-mercaptoethanol (140 mM) was added to the pulverized tissue. After 30 s of vortexing, 0.6 ml of 100% ethyl alcohol was added, and the samples were centrifuged for 1 min at 10,000 g. The supernatant was collected and 50 µl of Na acetate, pH 5.2, was added. The samples were processed on the GlassMax DNA isolation reagent system

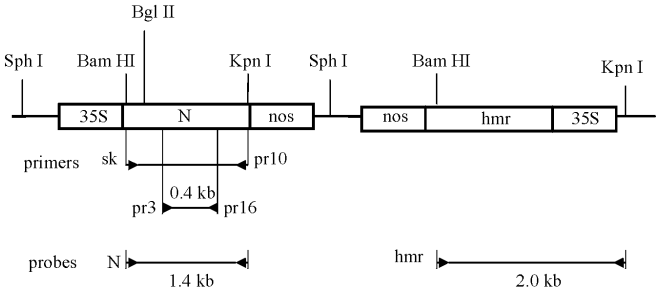

Fig. 1 Schematic representation (not to scale) of pVR3 plasmid used in rice transformation showing a partial restriction map

(Gibco-BRL), following the protocol provided for RNA. The cDNA was produced using 1 µl of total RNA following the protocol for Superscript II reverse transcriptase (Stratagene). Aliquots of these reactions were used in the nested PCR reactions as previously described.

DNA sequencing

Plasmids and PCR products were purified using Wizard purification columns (Promega). Nucleotide sequences were determined using an ABI Prism 377 sequencer (Perkin-Elmer) by the dideoxynucleotide chain-termination procedure using the ABI dye terminator reaction ready kit, and edited with Sequencher (Genecodes, Ann Arbor, Mich.). The sequences were analyzed using the BLAST algorithm (Altschul et al. 1997).

Analysis of RHBV-N protein in transgenic plants

Leaves from 20- to 30-day-old rice plants were tested for the presence of the N protein in a DAS-ELISA (Clark and Adams 1977). The ELISA plates were coated with a 1/4000 dilution of anti-RHBV IgG, and the conjugate was a 1/4000 dilution of alkaline phosphotase-labeled anti-RHBV IgG. The plant samples were made in a 1/100 dilution (w/v) 0.15 M phosphate buffer saline, pH 7.4, containing 0.05% Tween and 2% polyvinyl-pyrrolidine (mol. wt. 40,000). Positive (non-transgenic Cica 8 plants infected with RHBV) and negative (Cica 8 non-infected plants) controls were included in all experiments.

Western-blot analysis

Fresh leaf samples were frozen using liquid nitrogen, homogenized in a 1:1 (w/v) ratio of Laemmli buffer, treated for 2 min at 100 °C and centrifuged for 10 min at 7,000 rpm (Laemmli 1970). Ten microliter samples were electrophorezed in 12.5% polyacrylamide gels and transferred to nitrocellulose using a transblot cell (Bio-Rad). The filters were probed with anti-RHBV IgG (1/1000 dilution). The reactions were detected using ECL Western-blotting detection reagents (Amersham Life Science).

Inheritance analysis of transgenes

Independent primary transgenic lines (R_0) showing integration of the *N* gene by Southern blotting were used to evaluate the inheritance of the *N* and *hmr* genes, and the resistance to RHBV and hygromycin in the following generations. To evaluate the inheritance of the Hyg^r phenotype, 1-cm tall seedlings derived from self pollination of R_0 plants (R_1 generation, 20 R_1 seedlings per R_0 line), and from the non-transgenic control Cica 8 were placed on MS medium containing hygromycin (50 mg/l) and cultured at 80–100 mmol m^{-2} s^{-1}, 16-h photoperiod at 25 °C. The number of surviving plants was scored for 15–21 days until all the control seedlings had died. To determine the inheritance of the *N* and *hmr* genes, another batch of R_1 seedlings derived from the same R_0 lines showing segregation ratios for a hygromycin resistance phenotype of 3:1 or skewed ratios deviating from Mendelian segregation were used. Inheritance of the *N* gene in these plants was determined by Southern blotting and nested-PCR analysis on at least 50 plants per transgenic RHBV-resistant line over each of the next four self-pollination generations. The inheritance of the *N* gene and its expression in different genotype backgrounds was assayed by crossing selected R_3 transgenic plants showing stable inheritance of the *N* gene and RHBV resistance, with three commercial rice varieties of contrasting levels of RHBV resistance. Reciprocal crosses were made between the R_3 plants and the varieties Iniap 12 (susceptible), Oryzica 1 (intermediate resistant) and Fedearroz 50 (intermediate resistant). Inheritance of the *N* gene was determined by Southern blotting, RT-PCR, and RHBV resistance in F_1 plants was inoculated 10 days after germination, the age at which the conventional resistance breeding source does not confer reliable resistance rice against RHBV.

RHBV resistance assays

At least 25 R_1 progeny of each of the R_0 lines 49, 57, 58, 77, 78 and 83 were challenged with RHBV. R_1 plants (initially 35-days old were used to better assure a satisfactory seed set) of the R_0 lines were inoculated with RHBV using two viruliferous 2nd or 3rd instar *T. orizicolus* nymphs per plant. For the RHBV resistance evaluations in the R_2, R_3 and R_4 generations, the plants were inoculated between the ages of 15 to 25 days after planting. At least three replications of 20 plants per line were evaluated. Controls consisted of transgenic Cica 8 plants carrying only the *hmr* gene or non-transgenic Cica 8 plants. Plants were scored for the development of RHBV disease symptoms every 3 days for 25 days, and then evaluated once a week for 5 weeks. Plants were scored for the date of the first appearance of symptoms. The percentages of leaf area with symptoms and plant vigor were scored with a scale from 0 to 3. A score of 0 refers to plants without disease symptoms. A score of 1 refers to vigorous growth and less than 10% leaf area with symptoms. A score of 2 refers to 10–60% leaf area with symptoms, and a minimal effect on the number of tillers and plant height. A score of 3 refers to plants with more than 60% leaf area with symptoms, fewer tillers than normal, stunted growth and, in some cases, necrosis and death.

Agronomic traits of RHBV-resistant rice under greenhouse conditions

Agronomic traits were assayed on plants grown to maturity in individual pots in a glasshouse. The number of days to maturity was determined by scoring the number of days from sowing to flowering of transgenic plants and the Cica 8 control inoculated with RHBV. At harvest time the number of tillers and panicles per plant were counted. Height was measured from the base of the plant to the tip of the youngest fully expanded leaf. Three panicles per plant were sampled, and fertility was determined by the average percentage of filled grains per panicle. The weight of 100 grains and the total seed weight per plant were measured. The uninoculated Cica 8 plants were included as a reference for the agronomic characteristics.

Results

Inheritance of *N* and *hmr* genes, and the Hyg^r phenotype in R_1 progeny plants

After the plant regeneration process, an average of one Hyg^r plant line was recovered per 2 to 33 calluses bombarded initially. Fifty nine primary transformants (R_0) contained both the *N* and *hmr* genes, as indicated by Southern blotting. R_1 progeny derived from 19 of these R_0 lines carrying the *N* and *hmr* genes were analyzed for the presence of both transgenes and the inheritance of the Hyg^r phenotype. For the Hyg^r phenotype, 74% of the lines inherited hygromycin resistance. Of these R_0 lines, 29% had a closely fit 3:1 segregation ratio (χ^2: 0.05–0.69; *p*: 0.41–0.83) in the R_1 generation, whereas 71% showed fewer Hyg^r plants than expected, fitting skewed ratios deviating from Mendelian segregation, as determined by chi-square analysis (χ^2: 2.4–4.9; *p*: 0.03–0.12). A sample of R_1 generation-derived plants from seven R_0 lines was tested by Southern blotting for the presence of the *N* and *hmr* transgenes. In this case, R_1 generation-derived plants from R_0 lines 57 and 77 with 80% and 76% of the plants with hygromycin resistance (3:1 ratio), and those derived from lines 49, 50, 58, 78 and 83 with about half of the Hyg^r plants (skewed ratios) were used. Both R_0 lines with a Mendelian segregation for the Hyg^r phenotype in the R_1 generation also showed a 3:1 (presence:absence) ratio for the *hmr* and *N* genotype (χ^2: 0.01–0.44; *p*: 0.90–0.83). Likewise, R_0 lines 58 and 83 that showed about half of Hyg^r R_1 plants also showed 52–57% of plants with the *hmr* and *N* genes. On the other hand, even though they showed skewed segregation for the Hyg^r phenotype (56% to 60% Hyg^r plants), all of the R_1 progeny plants analyzed from the R_0 lines 49, 50 and 78 had inherited both transgenes as indicated by Southern blots.

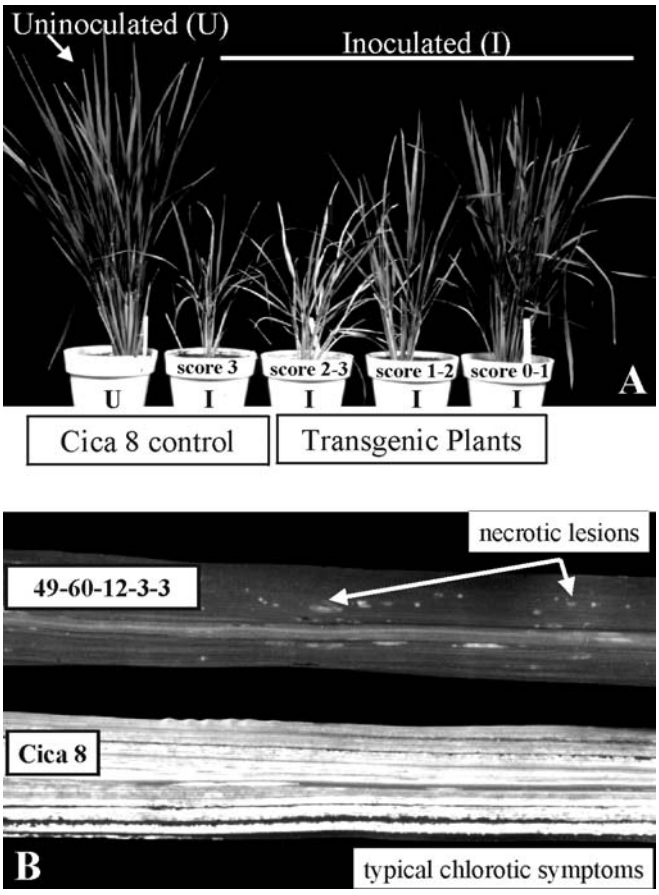

Fig. 2A, B Two-month-old plants showing the reaction to RHBV. **A** Non-transgenic Cica 8 uninoculated (left). Inoculated non-transgenic Cica 8 (second pot from left side). Inoculated transgenic plants (from center to right side). Uninoculated transgenic plants of this line are the same height as the inoculated transgenic plant shown at the far right. The transgenic lines are shorter than the typical Cica 8 plants. **B** Local necrotic lesions resembling a hypersensitive resistance reaction on leaf of 49-60-12-3-3 R_4 transgenic line (top). Typical chlorotic RHBV symptoms on the leaf of inoculated non-transgenic Cica 8 (bottom)

RHBV resistance assays from R_1 to R_4 progeny plants

The type of inheritance pattern obtained for the *N* gene in the R_1 progeny, as resolved by Southern blots, was used as a criterion to select those lines to be evaluated for RHBV resistance. Lines 49, 57, 77 and 78 fitting Mendelian ratios, and line 58 with skewed segregation for the *N* genotype at the R_1 generation, were evaluated. Of these plants only R_0 line 49 showed resistance to RHBV. Although all the R_1 plants derived from R_0 line 49 contained the *N* gene as indicated by Southern blotting, about 1/3 of these plants were RHBV-resistant (score 0–1, Fig. 2A). R_2 plants derived through self-pollination from these resistant R_1 lines were used for testing the inheritance of RHBV resistance.

All R_2 progeny plants derived from the resistant R_1 line 49-37 showed immunity to RHBV (homozygous, score 0). Seventy five percent of the R_2 plants derived from the RHBV-resistant R_1 lines 49-56, 49-60 and 49-101 showed resistance to RHBV (heterozygous, score 0–1, Fig. 2A) (Table 1). At about 3 weeks after inoculation, these lines showed a significant 2 to 5-fold less disease development compared to the inoculated Cica 8 control. These differences increased progressively with time, and at about 2 months all the leaves of the Cica 8 control showed the typical disease symptoms (score 3, Fig. 2A), whereas the new leaves of the transgenic plants showed significantly less disease (score 1) or were symptom-less (score 0) (Table 1). About 50% of the R_2 plants from R_1 lines 49-27 and 49-39 were resistant to RHBV. The advanced R_3 generations derived from R_1 lines 49-56, 49-60 and 49-101 showed a stable significant inhibition of disease progression. Selected homozygous R_4 lines, based on the *N* gene-inheritance Southern profiles, and with the highest stable RHBV resistance are shown in Table 2. Results suggest that the RHBV resistance encoded by the *N* gene is stably inherited through

Table 1 RHBV resistance, yield and tillering of R_2 progeny plants derived from transgenic line R_0 49 inoculated at 20 days after sowing

Line	n[a]	Disease reaction[b]	Grains per plant				Tillers per plant			
			Mean	SE[c]	Min[d]	Max[e]	Mean	SE	Min	Max
49-56	80	0.2 (0.2)	448b[f]	74	100	1,332	15d	1.9	5	19
49-60	80	0.3 (0.3)	302b	46	81	728	14d	1.1	5	20
49-101	80	0.4 (0.3)	364b	73	79	1,240	15d	1.6	5	30
Cica 8[g]	80	2.5 (0.8)	70c	28	26	122	5e	1.3	1	7

[a] n = Total number of plants evaluated, four replicates of 20 plants each
[b] Scale from 1 (low severity, with <10% leaf area with symptoms and healthy plant growth) to 3 (highly susceptible or dead). Plants without symptoms were rated as zero. Number in parenthesis refers to the standard deviation
[c] SE = standard error
[d] Min = minimum value
[e] Max = maximum value
[f] Numbers followed by different letters are significantly different at p: 0.01
[g] Cica 8 = inoculated control

Table 2 RHBV resistance, yield and tillering of R_4 progeny derived from transgenic line R_0 49 inoculated at 20 days after sowing

Line	n[a]	Disease reaction[b]	Tillers per plant	Grains per plant
49-60-4-5	60	1.1(0.3) b	12 (1.1) b	324 (64) b
49-60-4-13	60	0.9 (0.3) b[c]	13 (0.7) b	534 (23) b
49-60-12-3	60	0.9 (0.2) b	14 (0.7) b	437 (26) b
49-101-18-19	60	1.3 (0.3) b	12 (0.9) b	324 (63) b
Cica 8[d]	60	3.0 (0.0) c	5 (1.3) c	9 (0.9) c

[a] n = Total number of plants evaluated, three replicates of 20 plants each
[b] Scale from 1 (low severity, with <10% leaf area with symptoms and healthy plant growth) to 3 (highly susceptible or dead). Plants without symptoms were rated as zero
[c] Mean values followed by the standard error in parenthesis. Numbers followed by different letters are significantly different at p: 0.01
[d] Cica 8 = inoculated control

self-pollination for four generations. In general, the RHBV resistance in these transgenic lines was characterized either by symptoms with a limited distribution or local necrotic lesions resembling a hypersensitive resistance reaction (Fig. 2B). Additionally, in some transgenic plants, the disease rating was more severe at 1 month than at 2 months after inoculation. In this case, the plants exhibited a recovery phenotype characterized by systemic chlorotic symptoms that gradually disappeared, as well as the development of new tillers and leaves free of virus symptoms. The symptom-less area of the plant from a recovery phenotype was similar to immune tissue or the non-inoculated plants. In contrast, the non-transgenic control had systemic chlorotic symptoms or necrosis throughout the old and new leaves, and often the plants died. In order to determine whether resistant plants were infected with the virus, ELISA tests were conducted on leaf extracts. The chlorotic symptoms that developed on transgenic and non-transgenic plants gave positive reactions and similar ELISA values, indicating the presence of RHBV. Tissue from recovery plants that remained symptom-less, the uninoculated control plants and the inoculated plants of transgenic line 49-37, which appeared to be immune to RHBV, were all negative for the presence of the virus using ELISA.

Agronomic performance of RHBV-resistant lines

Inoculated Cica 8 control plants (RHBV diseased) showed a significant 24-day delay in flowering (141 ± 29), stunting (mean of 17 cm shorter, Fig. 2A), three-fold fewer tillers and about half the number of spiklets per panicle when compared to the uninoculated Cica 8 control (healthy, Fig. 2A) plants. In contrast, no significant differences between the inoculated resistant R_1 transgenic plants and the uninoculated Cica 8 controls were noted for the agronomic traits evaluated. There were no significant differences in days from germination to flowering (mean of 124 ± 2 for R_1 plants and a mean of 117 ± 11 for the uninoculated control) or the number of tillers per plant (mean of 17.9 ± 3.8 for R_1 plants and a mean of 15 ± 8 for the uninoculated control). A significant increase in grains per plant was noted in the R_1 lines (mean of 335 ± 55) with respect to the susceptible Cica 8 inoculated control, which did not set seeds. All plants of line 49-37 appeared immune to RHBV but had the lowest fertility when self-pollinated. No progeny were obtained when either backcrossed to Cica 8 or out-crossed to other *indica* cultivars.

The increased level of resistance of these transgenic lines was also reflected in the significant increase in yield potential (grains per plant) as compared to the inoculated Cica 8 control (Table 1). Inoculated R_2 lines derived from 49-56, 49-60 and 49-101 showed a significant five-fold increase in seed set and about three-fold more tillers compared to the inoculated Cica 8 control (Table 1). Some of the infected R_2 transgenic plants showed a yield (range from 728 to 1,332 grains per plant) similar to the uninoculated Cica 8 (range from 825 to 1,465 grains per plant). The number of tillers per plant of these inoculated R_2 plants (Table 1) was similar to the

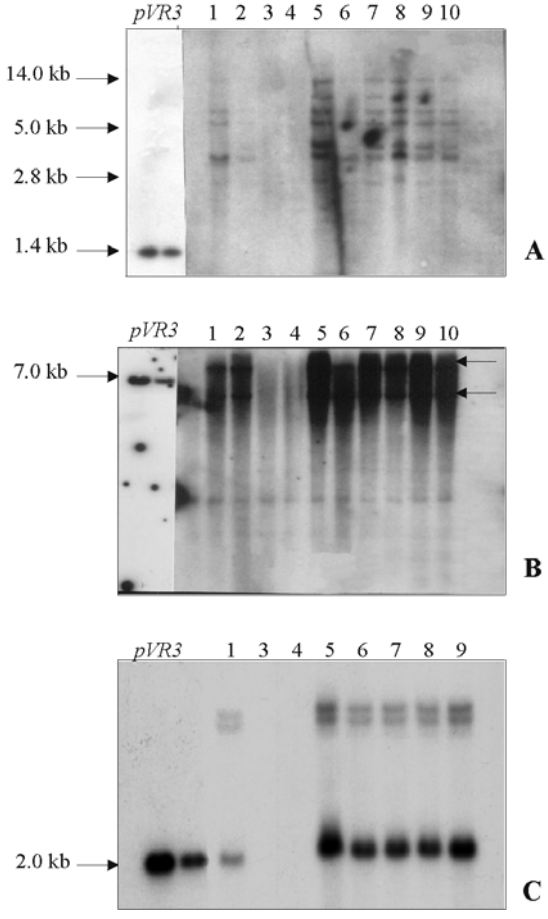

Fig. 3A–C Southern blots of rice transformed using the plasmid pVR3. Genomic DNA from R_1 plants derived from line R_0 49 were digested in: **A** with *Bam*HI and *Kpn*I, and in **B** with *Bgl*II. Both blots were hybridized with the N gene probe. **C** DNA of the same transgenic plants in panel **A** digested with *Bam*HI and *Kpn*I was hybridized with the hmr gene probe. *Lanes 1–2* contain DNA of lines 49-27 and 49-39. *Lanes 3–4* contain non-transgenic Cica 8 control. *Lanes 5–8* correspond to DNA of R_1 lines 49-56, 49-60, 49-101, and 49-37, respectively. *Lanes 9–10* contain DNA of R_0 line 49

uninoculated Cica 8 control (mean of 13 ± 0.6). Inoculated resistant transgenic lines showed a similar height to the uninoculated transgenic plants (control). The uninoculated transgenic lines were shorter than the typical Cica 8 plants (Fig. 2A). Similar differences for these traits have been noted through the R_4 generation (Table 2). When inoculated at 15 days of age, 85% of the R_4 plants derived from line 49-60 had a disease severity rating of 0 or 1 (less than 10% of the leaf area was affected) and a tillering capacity similar to the uninoculated Cica 8 control.

Genotype and *N* gene expression of transgenic lines with a high degree of resistance to RHBV

Genomic DNA was double-digested with *Bam*HI/*Kpn*I to excise the complete *N* sequence and the *35S CaMV promoter-hmr* gene fragment from *pVR3* (Fig. 1). The *Bam*HI/*Kpn*I fragment corresponding to the *N* gene coding region was used as a specific probe to detect the introgression of the *N* gene in the rice genome by Southern blotting. The expected 1.4-kb band corresponding to the *N* sequence was not resolved by Southern blotting. Only multiple DNA bands larger than the expected 1.4-kb *N* sequence were detected in the R_0 line 49 and derived R_1 progeny RHBV resistant plants (Fig. 3A). Although the R_1 progeny lines had similar multiple banding patterns, there were differences between the sister lines, which would be expected in a segregating population. In contrast, using the same digested DNA but probed to detect the *hmr* gene, the expected 2.0-kb DNA band corresponding to the *35S CaMV promoter-hmr* gene was present, as well as two larger fragments in all the plants analyzed (Fig. 3C). Genomic DNA from these same plants was digested with *Bgl*II (it cuts once inside the *N* coding region, Fig. 1). Southern blotting was conducted using the 1.4-kb *N* sequence-specific *Bam*HI/*Kpn*I probe. Two bands were detected in the R_0 line 49 and R_1-derived progeny through self-pollination (Fig. 3B). There was a smaller fragment present, but it was also found in the non-transgenic control. Similar results with *Bgl*II were obtained for the *hmr* gene (data not shown).

Nested PCR analyses were performed to confirm the presence of at least one complete copy of the *N* gene sequence in six of the resistant progeny lines. The amplified 1.4-kb PCR product from the R_1 lines (49-27, 49-37, 49-39, 49-56, 49-60 and 49-101) hybridized with the specific *N* gene hybridization probe. The *N* gene sequence was inherited in the RHBV-resistant lines from R_0 to R_4 generations derived from self-pollination, as well as in the F_1 plants derived from single crosses between resistant R_3 plants and the commercial non-transgenic varieties (Fig. 4A).

Although the complete *N* gene sequence was present in all the RHBV-resistant plants of line 49 and its progeny, the N protein could not be detected using neither ELISA nor Western-blot analyses. When RT-PCR analysis was used the *N* gene RNA could be consistently detected. The DNA products of the expected sizes of 1.4 kb or 0.4 kb were amplified by RT-PCR using the sk/pr10 or pr3/pr16 set of primers, respectively (Fig. 1), and these products hybridized with the *N* gene sequence that was used as the labeled probe (Fig. 4B). R_4 progeny plants derived from self-pollination of line 49-60-4-13, and of the F_1 seedlings from its corresponding crosses with commercial rice varieties, indicate that the segregation of the transgenic RHBV resistance correlates with the low level of RNA expression of the *N* gene that could be detected using RT-PCR (Fig. 4B). The 0.4-kb RT-PCR reaction product from each of these plants was cloned, and in all cases the sequence corresponded to the central part of the *N* gene coding region.

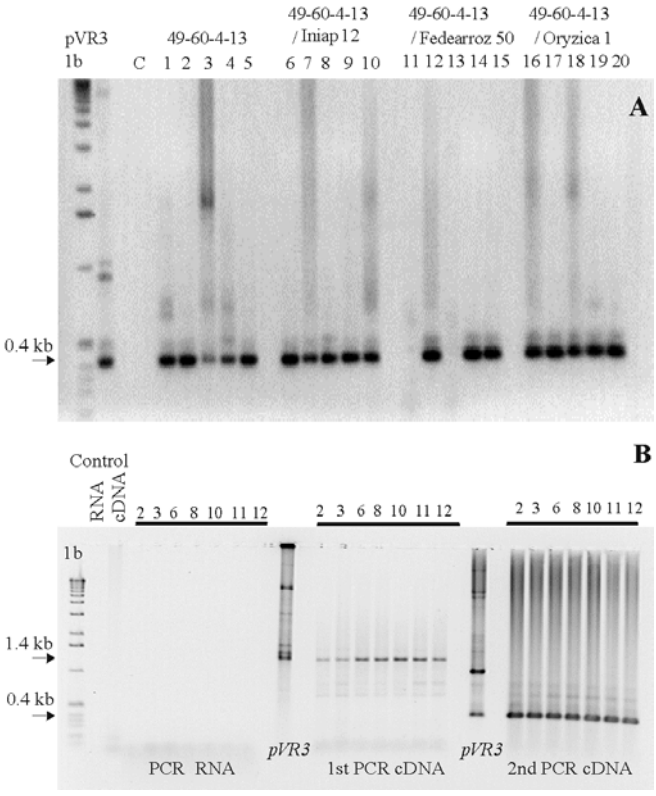

Fig. 4 **A** Southern blots of progeny plants derived from 49-60-4-13 R_3 transgenic line transformed with the plasmid pVR3. Genomic DNA of R_4 plants (*lanes 1–5*) and F_1 crosses R_3/Iniap 12 (*lanes 6–10*), R_3/Fedearroz 50 (*lanes 11–15*) and R_3/Oryzica 1 (*lanes 16–20*), was amplified by nested PCR using the primers sk and pr10 for the first-round amplification, followed by a second, PCR amplification using the internal primers pr3 and pr16. Amplified DNA was hybridized with the N gene probe. Cica 8 non-transgenic control (*C*). **B** RT-PCR of total RNA from R_4 progeny plants derived from RHBV-resistant R_3 line 49-60-4-5 (*lanes 2–12*). Controls consisted of RNA and cDNA from non-transgenic Cica 8 plants. PCR of RNA from transgenic plants (internal control) was done using total RNA treated with DNAases. 1st PCR cDNA was conducted using the sk/pr10 set of primers. 2nd PCR cDNA was performed using the pr3/pr16 set of primers. PCR products were hybridized with the N probe

Discussion

In this study, we have documented that the introgression of the *N* gene can confer RHBV resistance to the susceptible rice cultivar Cica 8. The protection conferred by the *N* gene was noted as a significant reduction in disease progression and severity with respect to the inoculated Cica 8 controls. Several resistant reactions were observed that ranged from susceptible to completely resistant plants (immunity). One unexpected resistant phenotype was characterized by the production of local necrotic lesions resembling a hypersensitive resistance reaction. A second major type of resistance was a recovery phenotype characterized by the development of chlorotic symptoms in inoculated leaves followed by the production of new tillers with no symptoms. The recovery resistant phenotype encoded by the *N* gene is different from the normal disease reaction where the new tillers continue to show severe chlorotic symptoms. The RHBV transgenic resistance is different from the conventional RHBV resistance which is characterized as escape from infection, and the plants do not have either hypersensitive-like reactions nor a recovery phenotype. The conventional RHBV resistance does not confer adequate resistance when the plants are inoculated at 10 days after planting, while the best transgenic derived plants and F1 crosses are resistant when inoculated at 10-day old. These hypersensitive response and recovery phenotypes are typical host responses to pathogens, and the possibility that the *N* gene is activating a host defense pathway needs to be explored as a mechanism of resistance.

The diversity of reactions present in the RHBV-resistant transgenic plants described in this work differed significantly from the coat protein-mediated protection first described by Powell et al. (1990). Coat protein-mediated resistance interferes with or prevents various stages of the viral life cycle, resulting in attenuated disease symptoms or resistance; but the recovery reaction or the hypersensitive-like response were not reported. The recovery phenotype seen in virus-resistant transgenic plants was first described by Lindbo et al. (1993). These plants are infected initially but later produce new leaves that are without symptoms and in which no virus could be detected. The immune and recovery phenotypes are well-documented characteristics of RNA mediated gene-silencing related virus resistance phenomena in transgenic dicots (Lindbo et al. 1993; Smith et al. 1994; Mueller et al. 1995) and transgenic monocots (Ingelbrecht et al. 1999). The time for full recovery in transgenic *N* lines with RHBV resistance was variable. In general, the recovery phenomena started about 1 week after inoculation, and fully expanded leaves without RHBV symptoms occurred about 2 months after initial symptom development. Similar time frames for recovery were noted in transgenic sugarcane resistant to SCMV (Ingelbrecht 1999) and in transgenic tobacco resistant to TVMV, where resistance is RNA mediated by post-transcriptional gene silencing. However, a reaction resembling a hypersensitive-like response related to transgenic virus resistance as described here for RHBV has not been previously reported and deserves a more-detailed analysis.

The RHBV-resistant transgenic lines also had significant increased performance for agronomic traits including the number of tillers and the number of grains per plant, and these traits were inherited through all four generations. Furthermore, upon inoculation some of the most-resistant transgenic plants showed agronomic traits similar to the uninoculated non-transgenic Cica 8 control. These results indicate that the *N* gene appears to protect plants from early developmental stages. The uninoculated transgenic plants were shorter than the typical Cica 8 plants. Uninoculated and inoculated resistant transgenic lines were the same height. These results indicate that the shorter height noted in the transgenic plants

may be due to the in vitro culture and/or genetic transformation process, and not because of virus infection.

Southern analysis of genomic DNA showed multiple *N* gene fragments larger than the 1.4-kb expected size indicating that the transforming DNA did not integrate as a complete unit in the R_0 49 plant. In contrast to the *N* gene, both the expected *hmr* gene fragments of 2.0 kb and larger fragments were detected. The multiple banding patterns for the *N* gene in contrast to the simpler banding for the *hmr* gene suggest integrative fragmentation and rearrangements. Complex transgene integration is associated with variation in the levels of expression of exogenous genes, position effects and co-suppression (Cooley et al. 1995; Kumpatla et al. 1997; Kumpatla and Hall 1998). A variety of integration patterns have been obtained in rice, especially when circular plasmids are used for biolistics (Hayakawa et al. 1992), as in this study.

All the RHBV-resistant transgenic plants evaluated showed at least one complete copy of the *N* sequence as indicated by the presence of the expected 1.4-kb band from the PCR analyses. The detection of corresponding mRNA from RT-PCR also suggests the presence of at least one copy of the full-length *N* gene sequence. The larger *N* gene fragments detected by Southern blotting could be due to rearrangements, but also could be caused by incomplete DNA digestion due to the loss of the restriction site(s) (Christou et al. 1989; Cooley et al. 1995). The detection of the corresponding 1.4-kb *N* sequence by PCR and RT-PCR, and the lack of resolving the corresponding 1.4-kb *N* fragment in the Southern analysis, appears to indicate the loss of a restriction site(s) which could be due to methylation at one or both *Bam*HI/*Kpn*I flanking sites of the *N* gene sequence. *Bam*HI and *Kpn*I are inhibited by methylation of the same cytosine. Additionally, *Kpn*I is also inhibited when methylated at the adenine. *Bam*HI does not cut GGAT^{m4}CC, GGAT^{m5}CC and GGAT^{hm5}C^{hm5}C. *Kpn*I does not cut GGT^{m6}A^{m5}CC and GGTAC^{m4}C. If one assumes that the *Kpn*I and *Bam*HI sites are methylated, a tandem direct repeat is predicted at 5.4 and 5.9 kilobases, and a tandem inverted repeat is predicted at 5.4 and 7.4 kilobases, respectively. The presence of larger and smaller bands than these predicted tandem repeats as resolved by Southern blots of genomic DNA, additionally suggests the presence of other kind of rearrangements.

Single cutter digestion with *Bgl*II suggested that the multiple copies of the *N* and *hmr* genes integrated at one locus. The R_0 and R_1 have the same banding pattern, also corroborating the integration at one locus. The insertion of multiple fragments into a single genomic location is consistent with the organization of transgenes in rice (Kumpatla and Hall 1998). To-date, the studies indicate that the majority of loci involved in multiple integration events are genetically linked (Cooley et al. 1995).

The inheritance of the *N* gene was stable through four generations of progeny derived from self-pollination, but the level of RHBV resistance was variable in some lines. Some transgenic lines showed a 3:1 segregation ratio for the RHBV resistance phenotype, indicating Mendelian inheritance for a single active transgene; however, other lines showed skewed ratios for the phenotype even when the plants contained the *N* gene. Similar results were noted for the inheritance of the *hmr* gene and the Hyg^r phenotype. Earlier reports on the recovery of transgenic rice produced using particle bombardment indicated both Mendelian and skewed segregation ratios (Cooley et al. 1995; Kumpatla et al. 1997; Kohli et al. 1998; Kumpatla and Hall 1998).

RHBV-resistant *N* transgenic plants with the same transgene integration pattern did not necessarily display the same phenotype. The lines analyzed included immune, recovery and susceptible plants. Similar results had recently been reported for transgenic sugarcane resistant to SCMV, where clones derived from the same transformation event or obtained after vegetative propagation could display different levels of virus resistance. The transition from a susceptible to a resistant phenotype was gradual and continuous, indicating that the transgenic resistance to SCMV may involve a quantitative component (Ingelbrecht 1999).

Neither Western-blot analysis nor ELISA could detect the nucleocapsid N protein in any of the transgenic RHBV-resistant plants. This contrasts with transgenic rice resistant to the tenuivirus RSV, where resistance correlates with high levels of expression of the N protein suggesting a coat protein mediated protection (Hayakawa et al. 1992). The transgenic RHBV-resistant rice lines expressed the *N* gene RNA, but only at low levels below the detection limit by Northern blots and only resolved by RT-PCR. The lack of detection of the N protein and the low levels of the N gene RNA in these plants suggest that the RHBV resistance encoded by the *N* gene appears to be RNA mediated.

The inability to detect the RHBV N protein and the low level of RNA transcription indicate that a possible mechanism underlying the transgenic RHBV resistance probably was RNA-mediated rather than protein-mediated. Other studies indicate that gene silencing is a common phenomenon in transgenic rice (Cooley et al. 1995; Kumpatla et al. 1997; Kumpatla and Hall 1998; Chareonpornwattana et al. 1999). However, in order to elucidate the N gene mediated resistance mechanism, nuclear run-off analyses need to be conducted to determine whether transcriptional or post-transcriptional gene silencing is regulating the RHBV resistance in these transgenic plants. The correlation between the level of expression of the *N* gene RNA with the degree of RHBV resistance, and the induction of the local necrotic lesions in these plants also needs further investigation.

In the R_0 generation the principal selection criterion was the presence of the *N* gene. There were very clear differences between resistant and susceptible plants in the R_1 generation. Only line 49-37 was apparently immune to infection, but the progeny were sterile. Using both agronomic traits and disease reaction as criteria, several of the most highly resistant lines were followed through the R_4 generation. This selection produced more

uniformity of both agronomic traits and RHBV resistance, in each of the subsequent four generations.

Several of the RHBV-resistant R_3 transgenic lines showing stable inheritance of the *N* gene were selected and crossed with commercial rice cultivars. The *N* gene RNA and the transgenic RHBV resistance was stably inherited and expressed in young F_1 seedlings (10-day old), independently of the commercial non-transgenic genotype used as parent. Currently, replicated field trials with more advanced generations of transgenic plants and derived crosses are being evaluated for RHBV resistance and various agronomic traits to determine the degree and stability of the RHBV resistance of these plants in different regions. If this resistance proves to be stable under field conditions, it would complement the conventional sources of RHBV and *T. orizicolus* resistance and contribute to the development of new rice cultivars.

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