Theor Appl Genet (1999) 98: 1138–1145

K. Datta · R. Velazhahan · N. Oliva
I. Ona · T. Mew · G. S. Khush
S. Muthukrishnan · S. K. Datta

Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease

Received: 11 August 1998 / Accepted: 26 August 1998

Abstract A 1.1-kb DNA fragment containing the coding region of a thaumatin-like protein (TLP-D34), a member of the PR-5 group, was cloned into the rice transformation vector pGL2, under the control of the CaMV 35S promoter. The Indica rice cultivars, 'Chinsurah Boro II', 'IR72', and 'IR51500' were transformed with the *tlp* gene construct by PEG-mediated direct gene transfer to protoplasts and by biolistic transformation using immature embryos. The presence of the chimeric gene in T_0, T_1, and T_2 transgenic plants was detected by Southern blot analysis. The presence of the expected 23-kDa TLP in transgenic plants was confirmed by Western blot analysis and by staining with Coomassie Brilliant Blue. Bioassays of transgenic plants challenged with the sheath blight pathogen, *Rhizoctonia solani*, indicated that over-expression of TLP resulted in enhanced resistance compared to control plants.

Key words Thaumatin-like protein · PR-5 · *Rhizoctonia solani* · Sheath blight disease · Rice

Introduction

Sheath blight (ShB), caused by the fungus *Rhizoctonia solani* Kühn, is one of the important diseases of rice (*Oryza sativa* L.). It is prevalent in all rice-growing countries, but especially in Asia, and causes significant yield losses. The fungus survives either as sclerotia or mycelia in plant debris, floats to the surface of flood water, germinates, and infects the rice plant (Ou 1985). It forms infection cushions and/or lobate appressoria on the plant surface. After the initial infection, the pathogen moves up the plant by surface hyphae and develops new infection structures over the entire plant. Infection causes significant necrotic damage. Since the introduction of modern, semidwarf, nitrogen-responsive cultivars, sheath blight has increased in importance in the rice-growing countries in Asia. Genetic variability for high levels of resistance is lacking in both cultivated rice and its wild relatives (Bonman et al. 1992). An alternative approach for management of sheath blight disease is to introduce genes which encode proteins with antifungal activity into the genome of rice to enhance tolerance to *R. solani.*

Plants exhibit a variety of responses during infection by pathogens or abiotic stresses, many of which involve the activation of host defense genes. Activation of these genes brings about physical and biochemical changes. Among the major biochemical changes is biosynthesis and the accumulation of a class of proteins termed pathogenesis-related proteins (PR-proteins). These important proteins are encoded by the host plant but are induced in pathological or related stress situations. PR-proteins are of particular interest because they are part of the host plant's defense system. On the basis of serological relationships, amino acid sequence data, and biochemical functions, PR-proteins have been classified into several groups (Van Loon et al. 1994). PR-proteins like chitinases (PR-3 group) and β-1,3-glucanases (PR-2 group) hydrolyze chitin and β-1,3-glucan, respectively, which are major components of fungal cell walls. Hydrolysis of these fungal cell-wall constituents leads to the inhibition of the growth of several fungi in vitro (Schlumbaum et al. 1986; Leah et al. 1991). In addition, it has been

Communicated by G. Wenzel

K. Datta · N. Oliva · G. S. Khush · S. K. Datta (✉)
Plant Breeding, Genetics and Biochemistry Division,
International Rice Research Institute, P.O. Box 3127,
1271 Makati City, Philippines
Fax: +63-2-8911292
E-mail: sdatta@irrl.cgnet.com; sdatta@irrl.cgiar.org

R. Velazhahan · S. Muthukrishnan
Department of Biochemistry, Kansas State University,
Manhattan, KS 66506-3702, USA

I. Ona · T. Mew
Entomology and Plant Pathology Division,
International Rice Research Institute, P.O. Box 933,
Manila 1099, Philippines

demonstrated that constitutive, high-level expression of PR-proteins in transgenic plants can enhance resistance to a variety of pathogens (Broglie et al. 1991; Lin et al. 1995; Zhu et al. 1994).

Thaumatin-like proteins (TLPs) are a group of PR-proteins (PR-5) that are induced in plants in response to infection by plant pathogens, elicitors, stress, and developmental signals (Bryngelsson and Green 1989; Pierpoint et al. 1987; Van Loon et al. 1987; Singh et al. 1987; King et al. 1988). The antifungal activity of TLPs make them attractive candidates for use in genetic engineering to produce disease-resistant crop plants. Recently, Zhu et al. (1996) demonstrated that transgenic potato plants constitutively expressing high levels of osmotin-like protein, a member of the PR-5 group, showed increased tolerance to the late blight fungus, *Phytophthora infestans*. cDNA clones encoding TLP transcripts have been isolated from a number of crop plants including rice (Singh et al. 1987; King et al. 1988; Reimmann and Dudler 1993; Pierpoint et al. 1990).

We are interested in constitutively over-expressing rice TLP in transgenic plants to enhance sheath blight resistance. We recently isolated a full-length clone of TLP cDNA from a rice cDNA library prepared from mRNA isolated from *R. solani*-infected rice plants. The cDNA contains an open reading frame of 693 bp encoding a 231 amino acid protein with a predicted molecular mass of 24.4 kDa (Velazhahan et al. 1998). In this report, we describe the over-expression and function of a TLP in genetically engineered rice under control of the CaMV 35S promoter. The TLP rice gene was stably integrated, and the rice transformants expressed elevated constitutive levels of TLP and had enhanced resistance to sheath blight disease.

Materials and methods

Plasmid construct

A 1.1-kb DNA fragment from a rice cDNA was obtained by digestion of plasmid pBS TLP-D34 with *Bam*HI and *Hin*dIII. The CaMV 35S promoter was excised as a 430-bp *Hin*dIII and *Bam*HI fragment from the plasmid pCaMVNEO. This 430-bp CaMV 35S promoter fragment, the 1.1-kb *Bam*HI-*Hin*dIII fragment of the rice TLP gene (tlp-D34), and the rice transformation vector pGL2, linearized with *Hin*dIII, were mixed together and ligated to yield pGL2CaMVTLPD34. The orientation and correct alignment of the fusion of the fragments in this construct were verified by digestion with restriction enzymes and by direct DNA sequencing with the appropriate primers.

Transformation of protoplasts and regeneration of transgenic rice plants

Transformation was carried out with the Indica rice cultivar, 'Chinsurah Boro II (CBII)'. Embryogenic cell suspensions, protoplast isolation, and transformation procedures have been described previously (Datta et al. 1990; Shimamoto et al. 1989).

Biolistic transformation

Biolistic transformation was done according to the method described earlier (Christou et al. 1991; Vasil et al. 1993). Immature embryos (12 days) of Indica rice cultivars 'IR72' and 'IR51500' were taken as initial explants. Plasmid delivery was conducted with the biolistic PDs-1000 He system (BioRad). The manufacturer's instructions were followed for coating the 10 μm gold microcarriers (BioRad) with plasmid DNA. After bombardment, the immature embryos were transferred to callus induction medium [Murashige and Skoog (1962) medium with 2 mg/l, 2,4-dichlorophenoxyacetic acid (2-4,D)] supplemented with 50 mg/l hygromycin B for selection. Developing calli were subcultured every 2 weeks, on the same medium, for five to seven cycles. Hygromycin-resistant calli were transferred to the medium for regeneration following the standard procedure described previously (Datta et al. 1990).

Genomic DNA isolation and Southern blot analysis

Genomic DNA was isolated from fresh or freeze-dried rice leaves following procedures described earlier (Dellaporta et al. 1983). DNA was treated with RNAse A, and 10 μg of DNA was digested with *Bam*HI and *Hin*dIII to a final volume of 40 μl. The digested DNA samples were electrophoresed in 1% 1XTAE agarose gel. After electrophoresis, DNA fragments were denatured and transferred to Hybond N + membrane (Amersham, UK) following the manufacturer's manual. The 1.1-kb *Bam*HI-*Hin*dIII DNA fragment of the tlp-D34 gene labeled with α-[^{32}P]-dCTP (Rediprime Labeling System from Amersham, Amersham, UK) was used as a hybridization probe. Southern blot analysis was done using standard procedures.

Preparation of protein extract from transgenic rice plants and Western blot analysis

For extraction of protein from both engineered and non-engineered control plants, 0.5–1.9 g of fresh leaf-sheath tissue was ground to a slurry in the presence of 1.0–1.5 ml 0.05 *M* TRIS-HCl (pH 7.0) and 10% (w/v) glycerol containing 0.1 m*M* PMSF at 4°C. After centrifugation at 13,000 rpm for 10 min the supernatant was collected and subjected to a second centrifugation at 13,000 rpm for 5 min. The concentration of total soluble protein was determined by using a BCA protein assay reagent with bovine serum albumin as a standard, according to the manufacturer's instruction (Pierce). Protein extracts from each sample were boiled in buffer (12.5 m*M* TRIS, pH 6.8, 20% v/v glycerol, 2% w/v SDS, 0.001% w/v bromophenol blue, 2% v/v 2-ME) for 5 min. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was performed using a total of 50 μg soluble plant protein per lane (Blackshear 1984). Separated polypeptides were blotted onto nitrocellulose membranes (Towbin et al. 1979), which were then probed with antiserum 23 kDa to bean TLP using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG as the second antibody, and made visible with HRP color reagent (Bio-Rad) as described earlier (Lin et al. 1995).

Coomassie blue staining for relative estimation of TLP from engineered rice plants

Protein (200 μg) from different transgenic plants separated by PAGE electrophoresis in the presence of sodium dodecylsulfate (SDS) (Towbin et al. 1979) was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 (Sigma) in 45% (v/v) methanol and 10% (v/v) acetic acid for 45 min. Bovine serum albumin (BSA) at concentrations of 0.5 μg, 1.0 μg, 2.0 μg and 3.0 μg was loaded onto the gels as

standards. The gels were destained repeatedly with a destaining solution (5% methanol and 7% acetic acid in water) until a clear background was obtained. The gels were then stored in 7% acetic acid. The quantity of TLP in total (200 μg) protein was estimated by visual comparison with a known BSA standard.

Bioassay

A sclerotium of rice sheath blight pathogen, *R. solani*, was inoculated on potato dextrose agar (PDA). After a 3-day incubation in the dark at 30°C, a colonized piece of agar (2 mm^2) was transferred to another PDA plate where it was subcultured for another 3 days under the same conditions. PDA plate contents were halved and transferred into a bottle containing a rice hull-rice grain mixture and incubated at room temperature (28°–30°C) for 7–10 days. Five-gram aliquotes of inoculated rice-hull substrate were placed in the middle of each plant at its maximum tillering stage. The symptoms caused by fungal infection were scored at weekly intervals as described previously.

Results

Transformation and regeneration

A partial diagram of the transformation vector pGL2 (CaMV-TLP-D34) containing rice thaumatin-like protein cDNA under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter is shown in Fig. 1. Five independent transformation experiments were conducted: three using Indica rice cultivar 'Chinsurah Boro II' protoplasts by the PEG-mediated direct DNA uptake method, one using 'IR72', and one using 'IR51500' rice cultivar embryos by biolistic gun delivery (Table 1). From the large number of hygromycin-resistant calli that were obtained from the five experiments, 320 green plants were regenerated, of which 224 healthy plants were transferred to the greenhouse. Of these plants 80% were fertile and produced seeds. For the preliminary screening of transformants, hygromycin phosphotransferase assays (HPT) were carried out and 90% of the plants exhibited hygromycin phosphotransferase activity (data not shown). From the biolistic transformation using immature embryos, 39 'IR72' and 37 'IR51500' putative transformed plants were obtained, which were subsequently transferred to the greenhouse to mature.

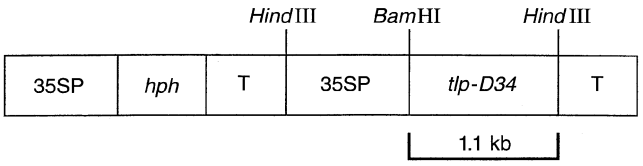

Fig. 1 Partial diagram of the pGL2CaMVTLP-D34 transformation vector showing the structure of the *TLP-D34* gene under control of the expression signals of the 35S transcript of CaMV and NOS terminator T. The hygromycin phosphotransferase gene, *hph* is driven by 35SP as selectable marker gene.

Southern blot analysis

Forty-seven plants from 148 primary transformants (T_0) of 'Chinsurah Boro II' were selected at random for detailed molecular analysis. Southern blot analysis was performed on DNA from leaves of T_0 plants and from the leaves of non-transformed tissue culture derived control plants using the TLP coding region probe (Fig. 2). *Bam*HI/*Hin*dIII digestion of the transformation vector pGL2 (CaMV-TLP D34) released a 1.1-kb fragment of the coding region fragment, as expected (tlp-D34) (lane D34). When genomic DNA from the leaves of transgenic rice plants and non-transformed plants was digested with *Bam*HI/*Hin*dIII and probed with the 1.1-kb tlp D34 fragment, the expected 1.1-kb fragment band was detected in all T_0 plants (transformed) and was absent from the non-transformed plants (Lane NT). Of the 47 plants tested, 35 showed the expected 1.1-kb band. Data from 19 of these plants are shown in Fig. 2. From the Southern blot analysis we could detect 22 independent events of transformation based on integration patterns. Southern blot analysis of 'IR72' showed the integration of the *tlp-D34* gene in the rice genome (data not shown).

Western blot analysis

Leaves from the 35 plants having the 1.1-kb tlp-D34 DNA band diagnostic of the presence of a full-length copy of the tlp-D34 in their genomes were assayed for

Table 1 Summary of transformation experiments with *TLP-D34* construct (*ND* not done)

Experiment	Cultivar	Method[a]	Number of explants used Protoplast/IE[b]	Number of plants regenerated	Number of plants in greenhouse	Number of positive/total	
						Southern	Western
1	CBII	P	3×10^6 protoplsts	80	68	14/20	12/14
2	CBII	P	1.5×10^6 protoplasts	33	26	6/10	5/6
3	CBII	P	1.5×10^6 protoplasts	70	54	15/17	13/15
4	IR72	B	600 IE	70	39	17/19	ND
5	IR51500	B	600 IE	67	37	ND	ND

[a] P, Protoplast; B, biolistic
[b] IE, Immature embryo

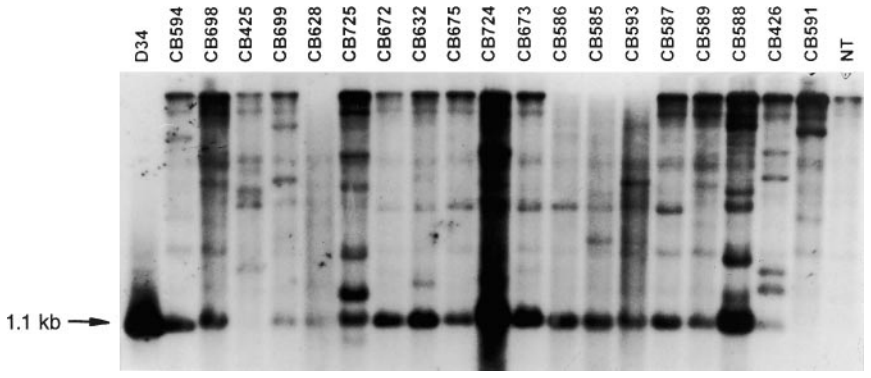

Fig. 2 Southern blot analysis of several independently transformed plants of CBII with *tlp-D34* gene in T_0. Most plants, except for CB245 and CB591, showed the expected 1.1-kb fragment indicative of the *tlp-D34* gene digested by *Bam*HI and *Hin*dIII. *NT* Non-transformed rice DNA, *D34* plasmid DNA used for transformation

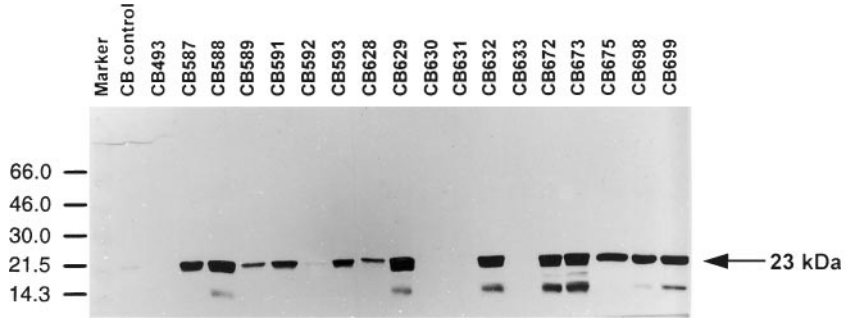

Fig. 3 Western blot analysis showing TLP-D34 protein level in T_0 plants. Molecular weight of the markers is given in kilodaltons (kDa) at the extreme *left*. The TLP-D34 protein of transgenic CBII plants showed a 23-kDa molecular-weight protein. CB493, CB592, CB630, CB631 and CB633 did not show any protein expression. Fifty micrograms of leaf extract protein from freeze-dried samples was analyzed by 10% SDS-PAGE

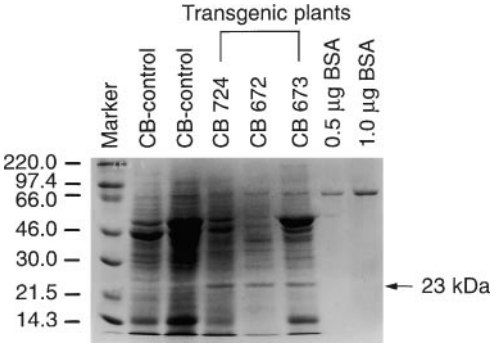

Fig. 4 Coomassie blue-stained SDS-PAGE of total proteins from rice plants (T_0) transformed with *tlp-D34* gene. Three transgenic plants CB724, CB672 and CB673, expressed the 23-kDa protein. Molecular weight of marker given in kiloDaltons (kDa) at the extreme *left*. Bovine serum albumin (*BSA*) was used as standard protein at known concentrations

TLP expression and quantification. Western blots of protein extracts of plants showed variation in their protein expression levels (Fig. 3). Thirty plants had the expected 23-kDa TLP-D34 protein band as detected by the TLP antibody. Coomassie Blue staining of the protein gel was done with a known concentration of BSA standard to estimate the approximate quantity of TLP produced by the transgenic plants (Fig. 4). Three transgenic lines (CB724, CB672, CB673) had a clearly identifiable 23-kDa protein band at apparent concentrations several times higher than background proteins seen in this size range in non-transgenic controls.

T_1 progeny

A detailed Southern blot analysis of DNA from the T_1 plants of transgenic lines was carried out (data not shown). All of the Southern-positive plants showed the same banding pattern of integration as the T_0 plants, and non-transformed plants showed no hybridization at the 1.1-kb level with the tlp-D34 probe. T_1 plant CB629-3 did not show any protein band, like the non-transformed control (Fig. 5). Protein bands of T_0 and T_1 plants of several other transgenic lines showed a high amount of protein (data not shown).

Bioassay

From the T_0 transgenic plants which tested positive for the functional tlp transgene in Southern blot analysis and had a high level of TLP, we selected several lines (CB629, CB632, CB672, CB675 and CB724) for

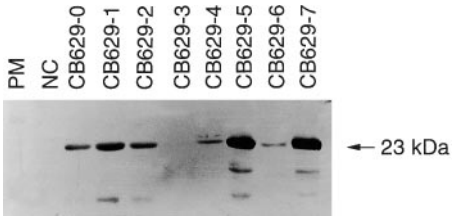

Fig. 5 Western blot analysis showing expression of TLP-D34 protein (23 kDa) in T_0 plants and its T_1 progenies (*CB629*). *PM* Protein marker of known molecular weight, *NC* negative control, *CB629-0* T_0 plant, *CB629-1* to *CB629-7* progenies of CB629-0

bioassay in the T_1 generation. These T_1 plants, which had a high level constitutive expression of rice TLP-D34, were subjected to a bioassay for resistance to fungal pathogen attack. Bioassays were performed using control and T_1 progeny from these five selected lines at the maximum tillering stage (50 days after sowing), and the disease progression was scored as percentage sheath area infected. Lesions appeared within 4–5 days, and their numbers and sizes on transgenic plants and the control were compared. After 6 weeks of infection, control plants showed considerable disease symptoms as compared to transgenic plants (Fig. 6). Different levels of disease progression were observed 2 weeks after inoculation with *R. solani*. The lesion areas in the transformed plants were smaller than those of the control plants. Table 2 shows variation in the infection level in different CB629 T_1 progenies, CB629-7 shows the lowest infection level (7.5%) and CB629-3, which was negative in Western blot analysis (Fig. 5), shows the maximum infection (37.5%). Table 3 shows the bioassay results from progeny of different transgenic lines selected on the basis of Southern and Western data. This result also shows variation in percentage sheath area infected, but in all cases infection was significantly lower in selected transgenic lines than in the controls.

Table 2 Bioassay of transgenic rice[a] for sheath blight (*R. solani*) (transgenic CB629 T_1 progeny)

Line	Total tillers	Infected tillers	% Sheath infection (SI)
CB629-1	9	6	10.8 ± 0.76
CB629-2	11	10	16.6 ± 3.07
CB629-3	16	16	37.5 ± 2.50
CB629-4	18	15	16.6 ± 2.11
CB629-5	9	9	15.8 ± 2.39
CB629-6	10	10	19.1 ± 3.00
CB629-7	8	6	7.5 ± 1.12
CBII-Control-1	14	14	35.8 ± 1.54
CBII-Control-2	18	18	36.6 ± 1.05

[a] T_1 generation plants selected on the basis of Southern and Western data. Scored after 14 days

Table 3 Bioassay of transgenic rice[a] for sheath blight (*R. solani*) (selected progeny analysis)

Line	Total tillers	Infected tillers	% Sheath infection (SI)
CB724-3	7	5	11 ± 0.91
CB675-6	13	13	15.8 ± 2.39
CB675-9	15	15	17.5 ± 2.81
CB672-2	16	16	17.5 ± 2.14
CB672-3	13	12	14.1 ± 1.54
CB672-4	7	5	8.6 ± 2.11
CB632-2	4	4	8.2 ± 2.69
CB632-3	15	15	16.6 ± 2.79
CB632-7	4	4	13.8 ± 5.54
CBII-Control-1	14	14	35.8 ± 1.54
CBII-Control-2	18	18	36.6 ± 1.05

[a] T_1 generation plants selected on the basis of Southern and Western data. Scored after 14 days

Discussion

Transgenic cereals, especially rice, are now routinely available. This allows a better understanding of the manipulation of gene expression and its application. Over-expression of rice genes in rice has an advantage over the use of foreign transgenes with regard to public acceptance. However, gene silencing is a widespread phenomenon in transformed plants. It suppresses endogenous and/or transgene expression and may occur through a wide variety of mechanisms (Flavell 1994; Meyer and Saedler 1996; Itoh et al. 1997). In this study we re-introduced a rice PR-5 gene (thaumatin-like protein) into the rice genome. Molecular characterization revealed the presence of the coding sequence of the introoduced gene and the expression pattern and function of the gene. Bioassay data correlated with over-expession in selected T_1 rice lines.

The PR-5 gene used here has some sequence homologies with the osmotin gene (Singh et al. 1987). Both genes share several common features, such as low molecular weight, the presence of multiple disulfides, a high proportion of proline, etc. The current study only enabled us to demonstrate the antifungal activity of the PR-5 gene in response to pathogen attack. We did not test plants for osmotic stress, but our transformants may show osmotic adaptation. The behavior of our transformants under water stress is now being examined. However, previous studies have indicated that there is no cross-reactivity between thaumatin and anti-osmotin antibodies or between thaumatin anti-PR-5 or anti-PR-5 antibodies (Kauffmann et al. 1990).

Out of 148 CBII plants grown in the greenhouse, 80% were fertile and 90% were confirmed to be carrying multiple copies of the introduced gene; this provided a good understanding of gene functioning. Out of 22 events of transformation, most of the plants were normal in spite of the high expression of TLP.

Fig. 6 Biological assay of control and transgenic rice showing disease symptoms. *Upper left* Control rice plant showing sheath blight disease symptoms caused by *R. solani*, *upper right* transgenic rice showing partial disease symptoms, *lower left* a portion of disease symptoms *lower right* a portion of transgenic plant

It is clear that over-expression of the TLP did not affect other phenotypic characters. A similar phenomenon was found in Bt rice plants having high Bt protein expression (Datta et al. 1998).

A few of the transformed lines did not show enhanced protein expression (CB629-3). This is likely due to co-suppression of the re-introduced gene and has also been reported in earlier studies (Itoh et al. 1997). Co-suppression is considered to be a state of gene expression in which homologous genes suppress the expression of the introduced gene (Van der Krol et al. 1990; Jorgensen 1995; Meins and Kunz 1994; De

Carvaltho et al. 1992). Developmentally regulated co-suppression of the chitinase gene (*Chi11*) has been shown in tobacco (Kunz et al. 1996).

There was no segregation in CB629-T_1, which might suggest the integration of the gene in more than one genomic location (data not shown). A similar observation was noticed in other transgenic lines with *Bt* and chitinase genes (unpublished data).

The bioassay of sheath blight is currently being done under greenhouse conditions. Biomass of the plants, high humidity, optimum temperature, and genotype play important roles in determining the correct evaluation of the bioassay (Cu et al. 1996). Under greenhouse conditions, data were scored based on percentage sheath infection (SI) density occurring on control plants. The mean SI ranged from 8.2% to 19.1% as compared to 36.6% in the control plants. A score of 15% SI or less is considered a good candidate plant for enhanced *ShB* resistance. Some plants showing low tillering showed a high infection index comparable to that of high tillering plants. This suggests that the target gene was not selectively inserted in genomic locations where genes for tillering ability are located. CB629-3 showed an SI comparable to that of the control plants, and this was correlated with protein expression – the gene is cosuppressed or inactivated in this line. It is noteworthy that several transgenic lines were identified as having very limited infection compared to control plants.

As there is no known germplasm available having sheath blight resistance, this has led farmers to depend mainly on the use of fungicides. Our generation of enhanced sheath blight resistance by genetic engineering provides an environmental friendly new germplasm for rice improvement.

Acknowledgments We thank Editha Abrigo and Lina Torrizo for laboratory assistance and discussion. Financial support from the Deutsche Geselschaft für Technische Zusammenarbeit (Germany) and the Rockefeller Foundation, USA are gratefully acknowledged.

References

Blackshear PJ (1984) Systems for polyacrylamide gel electrophoresis. Methods Enzymol 104: 237–257

Bonman JM, Khush GS, Nelson RJ (1992) Breeding rice for resistance to pests. Annu Rev Phytopathol 30: 507–526

Broglie K, Chet I, Holliday M, Cressman R, Biddle Ph, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254: 1194–1197

Bryngelsson T, Green B (1989) Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. Physiol Mol Plant Pathol 35: 45–52

Christou P, Tameria LF, Kofron M (1991) Production of transgenic rice (*Oryza sativa*) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature embryos. Bio/Technology 9: 957–962

Cu RM, Mew TW, Cassman KG, Teng PS (1996) Effect of sheath blight on yield in tropical, intensive rice production system. Plant Dis 1103–1108

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998) Constitutive and tissue-specific differential expression of *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest. Theor Appl Genet 97: 20–30

Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile Indica-rice plants recovered from protoplasts. Bio/Technology 8: 736–740

De Carvaltho F, Gheysen G, Kushnir S, Van Montagu M, Inze D, Castresana C (1992) Suppression of β-1,3-glucanase transgene expression I homozygous plants. EMBO J 11: 2595–2602

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1: 19–21

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci USA 91: 3490–3496

Itoh K, Nakajima M, Shimamoto K (1997) Silencing of waxy genes in rice containing Wx transgenes. Mol Gen Genet 255: 351–358

Jorgensen RA (1995) Cosuppression, flower color patterns and metastable gene expression states. Science 268: 686–691

Kauffmann S, Legrand M, Fritig B (1990) Isolation and characterization of six pathogenesis-related (PR) proteins of Samsun NN tobacco. Plant Mol Biol 14: 381–390

King GJ, Turner VA, Hussey CE, Wurtele ES, Lee M (1988) Isolation and characterization of a tomato cDNA clone which encodes for a salt-induced protein. Plant Mol Biol 10: 401–412

Kunz C, Schob H, Stam M, Kooler J, Meins F Jr (1996) Developmentally regulated silencing and reactivation of tobacco chitinase transgene expression. Plant J 10: 437–450

Leah R, Tommerup H, Svendsen I, Mundy J (1991) Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J Biol Chem 266: 1464–1573

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. Bio/Technology 13: 686–691

Meins F, Kunz C (1994) Silencing of chitinase expression in transgenic plants: an autoregulatory model. In: Paszkowski J (ed) Homologous recombination and gene silencing in plants. Kluwer Academic Publ, Dordrecht, The Netherlands, pp 335–348

Meyer P, Saedler H (1996) Homology dependent gene silencing in plants. Annu Rev Plant Physiol Mol Biol 47: 23–48

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473–497

Ou SH (1985) Rice diseases, 2nd edn. Commonwealth Mycological Institute Pub, Kew, Surrey, UK

Pierpoint WS, Tatham AS, Pappin DJC (1987) Identification of the virus-induced protein of tobacco leaves that resembles the sweet-protein thaumatin. Physiol Mol Plant Pathol 31: 291–298

Pierpoint WS, Jackson PJ, Evans RM (1990) The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). Physiol Mol Plant Pathol 36: 325–338

Reimmann C, Dudler R (1993) cDNA cloning and sequence analysis of a pathogenesis-related thaumatin-like protein from rice (*Oryza sativa*). Plant Physiol 101: 1113–1114

Schlumbaum A, Mauch F, Vogeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365–367

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplast. Nature 337: 274–276

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pflankock E, Regnier FE, Bressan RA (1987) Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol 85: 529–536

Towbin H, Staehelin F, Gordon G (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 76 : 4350–4354

Van der Krol AR, Beld LA, Mol JNM, Straitje AR (1990) Flavonoid gene in *Petussia*: addition of limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2 : 291–299

Van Loon LC, Gerristen YAM, Ritter CE (1987) Identification, purification and characterization of pathogenesis-related proteins from virus-infected 'Samsun' NN tobacco leaves. Plant Mol Biol 9 : 593–609

Van Loon LC, Pierpoint WS, Boller Th, Cornejero V (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Rep 12 : 245–264

Vasil V, Srivastava V, Castillo AM, Fromm ME, Vasil IK (1993) Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology 11 : 1553–1558

Velazhahan R, Chen-Cole K, Anuratha CS, Muthukrishnan S (1998) Induction of thaumatin-like proteins (TLPs) in *Rhizoctonia solani*-infected rice and characterization of two new cDNA clones. Physiol Plant (in press)

Zhu B, Chen THH, Li PH (1996) Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. Planta 198 : 70–77

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb C (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technology 12 : 807–812