DOI: 10.1007/s10535-012-0291-z BIOLOGIA PLANTARUM 57 (2): 351-358, 2013

Transgenic rice lines constitutively co-expressing *tlp-D*34 and *chi*11 display enhancement of sheath blight resistance

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Abstract

Transgenic rice (*Oryza sativa* L. subsp. *indica* cv. White Ponni) constitutively expressing the rice thaumatin-like protein gene (*tlp-D*34, PR-5) individually or in combination with the rice chitinase gene (*chi*11, PR-3) was generated using an *Agrobacterium vir* helper strain with multiple copies of pTiBo542 *vir*B and *vir*G. Transformation with the *tlp-D*34 gene alone and *tlp-D*34 + *chi*11 genes yielded five and seven single-copy transgenic lines, respectively. Southern blot analysis with two probes, one flanking the right T-DNA border and the second flanking the left T-DNA border, confirmed that all transgenic plants harboured single and complete T-DNA copies. Homozygous transgenic lines were first identified in the T_1 generation by Southern blot analysis and were subsequently confirmed by segregation analysis of T_2 plants. Accumulation of transcripts encoded by the transgenes was confirmed in T_0 plants and homozygous T_2 plants by Northern blot analysis. The homozygous T_2 plants harbouring *tlp-D*34 + *chi*11 genes showed 2.8- to 4.2-fold higher chitinase activity. Western blot analysis revealed the accumulation of thaumatin-like protein and chitinase in the respective transgenic plants. Upon infection with *Rhizoctonia solani*, the disease index reduced from 100 % in control plants to 65 % in a T_3 homozygous transgenic line T4 expressing the *tlp-D*34 gene alone. In a T_2 homozygous transgenic line CT22 co-expressing *tlp-D*34 and *chi*11 genes, the disease index reduced to 39 %.

Additional key words: *Agrobacterium tumefaciens*, homozygous transgenic lines, *Oryza sativa*, pathogenesis-related protein, *Rhizoctonia solani*, fungal resistance.

Introduction

Plant pathogenesis-related (PR) proteins are defence proteins which prevent or limit pathogen invasion and spread (reviewed in Ferreira *et al*. 2007). PR-proteins with antifungal properties include PR-1, PR-2 (β-1,3-glucanases), PR-3, 4, 8, and 11 (chitinases), PR-5 [thaumatin-like proteins (TLPs)], PR-12 (defensins), PR-13 (thionins), and PR-14 (lipid-transfer proteins) (reviewed in Van Loon *et al*. 2006).

TLPs share high amino acid sequence similarity and structural similarity to thaumatin, a sweet tasting protein from *Thaumatococus danielli* (Perri *et al*. 2008). TLPs are low molecular mass (15 - 30 kDa) acidic or basic proteins which alter the permeability of the fungal cell membrane (Anzlovar *et al*. 1998). Chitinase catalyzes the hydrolytic cleavage of the β-1,4-glycosidic bond present in chitin, the major constituent of fungal cell wall. Transgenic plants constitutively expressing either TLP or chitinase individually exhibit moderate resistance against various fungal pathogens (reviewed in Punja 2006).

Genetic engineering strategies aimed at constitutive high level expression of combinations of two PR-proteins with differing modes of action provided high levels of fungal resistance in many plants (reviewed in Ferreira *et al*. 2007). Synergistic expression of barley chitinase

Received 8 January 2009, *accepted* 15 August 2012.

Abbreviations: *chi*11 - rice chitinase gene, *hph* - hygromycin phosphotransferase gene, Hyg^r - hygromycin-resistant, Hyg^s - hygromycin-sensitive, MS - Murashige and Skoog, PR - pathogenesis-related, P*Ubi*1 - maize ubiquitin promoter intron, *tlp-D*34 - rice thaumatin like protein gene.

Acknowledgements: We gratefully acknowledge Dr. S. Muthukrishnan, Kansas State University, USA, for providing *tlp-D*34 and *chi*11 genes and for TLP and chitinase antibodies. We thank Dr. Toshihiko Komari, Japan Tobacco Inc, Japan, for providing pSB1 and Dr. M. Samiappan, Tamil Nadu Agricultural University, Coimbatore, India, for providing the *Rhizoctonia solani* strain RS7. N. Dhurigai is acknowledged for generating the *tlp-D*34-transgenic plant T7. This research was supported by funds from the Department of Biotechnology (DBT) and University Grants Commission (UGC), Government of India. JMS is thankful to Council of Scientific and Industrial Research (CSIR), New Delhi for Fellowship.

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(*chi*2) and wheat lipid-transfer protein (*ltp*) genes in transgenic carrot plants enhanced the resistance against *Alternaria radicicola* and *Botrytis cinerea* in comparison to individual expression of these genes (Jayaraj and Punja 2007). Transgenic rice plants co-expressing rice chitinase and modified maize ribosome-inactivating protein b-32 genes (Kim *et al*. 2003) and transgenic tobacco plants expressing a combination of barley ribosome-inactivating protein, barley chitinase, and barley β-1,3-glucanase genes (Jach *et al*. 1995) were resistant to *Rhizoctonia solani*. Combined expression of chitinase and β-1,3-glucanase genes proved to be very effective in transgenic tobacco against *Cercospora nicotianae* (Zhu *et al.* 1994), in transgenic tomato against *Fusarium oxysporum* (Jongedijk *et al.* 1995), and in transgenic potato (Chye *et al*. 2004) and transgenic rice (Sridevi *et al*. 2008) against *R. solani*. Pepper basic PR-1 protein and ascorbate peroxidase-like 1 genes, when simultaneously expressed in tomato, enhanced the resistance against *Phytophthora capsici* (Sarowar *et al*. 2006).

Combined expression of the rice TLP gene (*tlp-D*34) and the rice chitinase gene (*chi*11) resulted in increased resistance against *R. solani* in transgenic rice (Kalpana *et al*. 2006, Maruthasalam *et al*. 2007). In both these previous reports of rice transformation, biolistic mode of co-transformation was used. Though the T_0 plant exhibited a higher level of resistance against *R. solani* and *Sarocladium oryzae*, the *chi*11 gene was silenced in the T_1 generation (Kalpana *et al*. 2006). Therefore, there is a need to generate transgenic rice with both chitinase and *tlp* genes through *Agrobacterium*-mediated transformation so that single-copy transgenic plants can be generated with high expression of both genes. In this work, we have transformed an *indica* rice cv. White Ponni with the rice *tlp-D*34 gene alone or in combination with the rice *chi*11 gene. Co-expression of *tlp-D*34 and *chi*11 genes resulted in much higher levels of sheath blight disease resistance. This report further highlights the advantage of *Agrobacterium*-mediated transformation to generate single-copy transgenic plants with full-length transgenes that ensure the inheritance of stably expressed transgenes in subsequent generations.

Materials and methods

The pRS1 and pRS5 are pCAMBIA1300-derived binary vectors harbouring the hygromycin phosphotransferase (*hph*) gene as the plant selectable marker. pRS1 (Fig. 1*A*) harbours the rice *tlp-D*34 gene (Velazhahan *et al*. 1998) under the control of the maize ubiquitin promoter-intron (P*Ubi*1). pRS5 (Fig. 1*B*) harbours in its T-DNA the *tlp-D*34 and rice *chi*11 genes driven by P*Ubi*1 and rice β-actin promoters, respectively. The binary plasmids were mobilized by triparental mating into the *Agrobacterium tumefaciens vir* helper strain LBA4404 (pSB1) (see Sridevi *et al*. 2008). The plasmid pSB1 harbours *vir*B and *vir*G genes of the supervirulent Ti

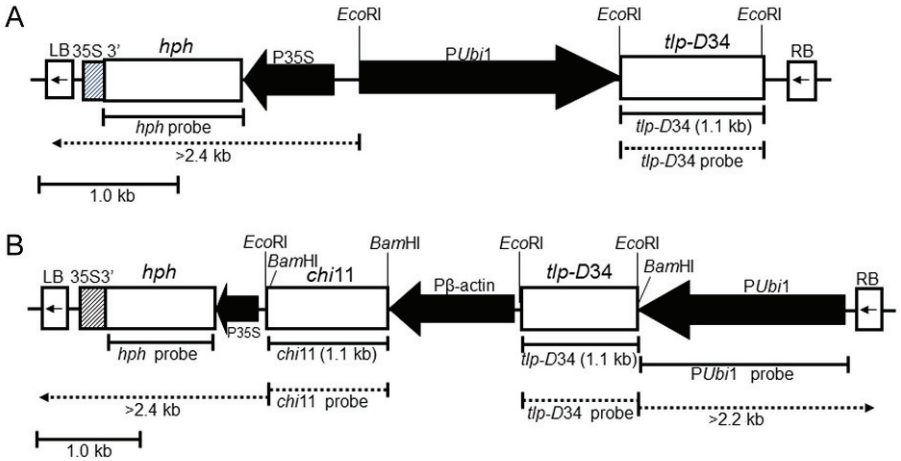

Fig. 1. T-DNA regions of binary plasmids. The T-DNA of pRS1 (*A*) which harbours P*Ubi*1-*tlp-D*34 and P35S-*hph* genes. The left border junction fragment (> 2.4-kb, the distance between the *Eco*RI site and LB), marked with a *dotted arrow*, will hybridize to the *hph* probe (marked with a *bold line*). The 1.1 kb *tlp-D*34 gene (marked with a *bold line*), flanked by *Eco*RI sites on either side, will hybridize to a 1.1 kb *Eco*RI fragment (marked with a *dotted line*). P*Ubi*1 - maize ubiquitin gene promoter; P35S - *Cauliflower mosaic virus* 35S promoter; 35S3' - *Cauliflower mosaic virus* 3' region; *hph* - hygromycin phosphotransferase gene; *tlp-D*34 - rice thaumatin-like protein D34 gene; LB - left border; RB - right border. Scale (1.0 kb) is marked. The T-DNA of pRS5 (*B*) which harbours the P*Ubi*1-*tlp-D*34, Pβ-actin-*chi*11, and P35S-*hph* genes. The LB junction fragment (> 2.4 kb, the distance between the *Eco*RI site and LB), marked with a *dotted arrow*, will hybridize to the *hph* probe (marked with a *bold line*). The RB junction fragment (> 2.2 kb, the distance between the *Bam*HI site and RB), marked with a *dotted arrow*, will hybridize to the P*Ubi*1 probe (marked with a *bold line*). The 1.1 kb *tlp-D*34 and *chi*11 genes (both marked with *bold lines*), flanked on either side by *Eco*RI and *Bam*HI sites, respectively, will hybridize to 1.1-kb *Eco*RI or *Bam*HI fragments (marked with *dotted lines*). Pβ-actin - rice β-actin gene promoter; *chi*11 - rice chitinase gene (for other abbreviations see the section *A* of this figure).

plasmid pTiBo542.

Scutellum-derived calli from mature seeds of *Oryza sativa* L. subsp. *indica* cv. White Ponni were infected with *Agrobacterium tumefaciens* strain LBA4404 (pSB1, pRS1) or LBA4404 (pSB1, pRS5). Callus induction, *Agrobacterium*-mediated transformation, and regeneration of transgenic rice plants were done as described earlier (Sridevi *et al*. 2005).

DNA (2.5 µg), extracted from rice plants (Rogers and Bendich 1988) was digested with *Eco*RI (for analysis with *hph* and *tlp-D*34 probes) or *Bam*HI (for *chi*11 and P*Ubi*1 probes), electrophoresed in 0.8 % (m/v) agarose gels and transferred onto the *Zeta*-probe nylon membrane (*Bio-Rad*, Hercules, CA). The DNA probes (*hph*, *tlp-D*34, *chi*11, and P*Ubi*1) were labeled with [α-^{32}P]dCTP (*Board of Radiation and Isotope Technology*, Hyderabad, India) using the *Megaprime*^{TM} DNA labeling system (*GE Healthcare*, Little Chalfont, UK).

Total RNA from rice plants was extracted (Pawlowski *et al*. 1994), 10 µg RNA was electrophoresed in a denaturing 1.2 % agarose gel containing 1 % (v/v) formaldehyde, transferred onto a positively charged nylon membrane (*Roche Diagnostics*, Mannheim, Germany) and hybridized to [α-^{32}P]dCTP-labeled *tlp-D*34 or *chi*11 gene probes.

Protein extraction and estimation were performed as described earlier (Sridevi *et al*. 2008). Aliquots of protein samples (20 µg) were separated by 10 % SDS-PAGE and subjected to Western blot analysis. For analysis with the rice TLP primary antibody [1:1000 (v/v) dilution], the goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (*Bangalore Genei*, Bangalore, India) was used at 1:2000 (v/v) dilution and finally treated with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium colour reagent until the bands appeared. For Western blot analysis with the barley chitinase antibody [1:2000 (v/v) dilution], *ECL Western Blotting* kit was used for detection (*GE Healthcare*).

Total protein was extracted from rice leaf (Sridevi *et al.* 2008). Chitinase assay (Mauch *et al*. 1984) was performed using colloidal chitin prepared from the crab shell chitin (*Sigma-Aldrich*, St. Louis, USA).

The seeds from selfed T_0 plants were germinated and hygromycin-resistant (Hyg^r) and -sensitive (Hyg^s) T_1 plants were scored on half-strength Murashige and Skoog (MS) medium with 50 mg dm^{-3} hygromycin (Sridevi *et al*. 2008). Validation of data for 3:1 segregation ratio was done by χ^2 test. Homozygous T_1 plants were initially identified by Southern blot analysis on the basis of band intensities with the *hph* probe (Sridevi *et al*. 2006) and then confirmed by segregation analysis of T_2 plants.

Infection assay of the transgenic and control rice for sheath blight resistance was done as described by Sridevi *et al*. (2008). An agar block (5 mm length × 5 mm diameter) with the mycelium of the *R. solani* isolate RS7 was placed between the sheath and the stem of healthy tillers of a 40-day-old plant and wrapped with wet cotton and parafilm. Cotton was wetted with water twice a day. After 7 d, the sheath blight symptom was graded in a scale of 1 to 5. The disease index of each tiller was determined as (grade point per tiller/maximum grade point) × 100.

Results

Transformation of 400 scutellum-derived calli with LBA4404 (pSB1, pRS1), which harboured the rice *tlp-D*34 gene under the control of maize ubiquitin promoter, yielded seven Hyg^r plants. In order to confirm the integration of complete T-DNA and to determine the copy number of T-DNA inserts, Southern blot analyses were performed with *hph* (flanking LB) and *tlp-D*34 (flanking RB) probes. The DNA was digested with *Eco*RI for analysis with both probes. Upon integration of the T-DNA, junction fragments longer than 2.4-kb are expected to hybridize with the *hph* probe (Fig. 1*A*). All seven Hyg^r plants had junction fragments longer than 2.4-kb. Single-copy T-DNA integration was found in five plants (T3, T4, T5, T6, and T7) (Fig. 2*A*) (data not shown for T7 which was generated in an independent experiment). With the *tlp-D*34 probe, the *Eco*RI-digested DNA of all five plants (T3, T4, T5, T6, and T7) and the binary plasmid pRS1 displayed hybridization of the 1.1-kb internal T-DNA band corresponding to the *tlp-D*34 transgene (Fig. 2*B*) (data not shown for T7). The control untransformed plant DNA did not show hybridization of the 1.1-kb band. Since *tlp-D*34 is a rice-derived gene, it hybridized to many genomic fragments corresponding to the endogenous copies of the *tlp* gene in control and transgenic plants (Fig. 2*B*).

Transformation of 550 scutellum-derived calli with LBA4404 (pSB1, pRS5) harbouring both *tlp-D*34 (under the control of P*Ubi*1 promoter) and *chi*11 (under the control of rice β-actin promoter) transgenes in one T-DNA yielded 24 Hyg^r plants. Southern blot analyses were performed with *hph* (flanking LB) and P*Ubi*1 (flanking RB) probes to determine the T-DNA copy number. With the *hph* gene probe, *Eco*RI-digested plant DNA is expected to hybridize to a junction fragment longer than 2.4-kb (Fig. 1*B*). All 24 plants displayed hybridization of one or more copies of T-DNA (results not shown). Eleven plants (CT6, CT8, CT9, CT14, CT21, CT22, CT24, CT10, CT13, CT17, and CT7) displayed single-copy T-DNA integrations (Fig. 2*C*). Single-copy plants identified with the *hph* probe were subjected to analysis with the P*Ubi*1 probe in order to confirm the integration of single copies of complete T-DNAs. With the P*Ubi*1 probe, *Bam*HI-digested DNA is expected to hybridize to junction fragments longer than 2.2-kb (Fig. 1*B*). DNA from seven plants (CT6, CT8, CT9, CT14, CT21, CT22, and CT24) showed hybridization of single junction fragments longer than 2.2-kb to the P*Ubi*1 probe (Fig. 2*D*). Surprisingly, two or more junction

fragments hybridized to the P*Ubi*1 probe in three plants (CT10, CT13 and CT7) (Fig. 2*D*) indicating that they are not single-copy events. One plant (CT17) did not show hybridization with the P*Ubi*1 probe (Fig. 2*D*). These four plants (CT10, CT13, CT7, and CT17) are examples of transgenic plants with truncated T-DNAs. With the *tlp-D*34 and *chi*11 probes, DNA (*Eco*RI- or *Bam*HI-digested for *tlp-D*34 or *chi*11 probes, respectively) of all seven single-copy plants (CT6, CT8, CT9, CT14, CT21, CT22, and CT24) and the binary plasmid pRS5 displayed hybridization of 1.1-kb internal T-DNA bands corresponding to the *tlp-D*34 (Fig. 2*E*) and *chi*11 (Fig. 2*F*) transgenes. The control untransformed plant DNA did not show hybridization of the 1.1-kb band. Since *tlp-D*34 and *chi*11 are rice-derived genes, they hybridized to many genomic fragments corresponding to the endogenous copies of the *tlp* and chitinase genes in control and transgenic plants (Fig. 2*E,F*).

The single-copy T_0 plants transformed with pRS1 and pRS5 were self pollinated and the seeds were germinated on half-strength MS medium containing 50 mg dm^{-3} hygromycin. T_1 plants from all five pRS1-transformed lines (T3, T4, T5, T6, and T7) and seven pRS5-transformed lines (CT6, CT8, CT9, CT14, CT21, CT22, and CT24) showed 3:1 segregation of the *hph* gene as expected for single-copy integrations (data not shown). Ten Hyg^r plants from each single-copy line were subjected to Southern blot analysis with the *hph* probe to study the inheritance of the T-DNA and to identify the homozygous T_1 plants. The intensity of hybridization of a junction fragment from a homozygous plant is expected to be twice higher than that of the corresponding fragment from a hemizygous T_1 plant (Sridevi *et al.* 2006). On that basis, homozygous T_1 plants were identified from all five pRS1-transformed lines (T3, T4, T5, T6, and T7) and seven pRS5-transformed lines (CT6, CT8, CT9, CT14, CT21, CT22, and CT24). To confirm the homozygous status, a homozygous T_1 plant identified by Southern blotting was self pollinated and the T_2 seeds (40 seeds from each T_1 plant) were germinated on half-strength MS medium containing 50 mg dm^{-3} hygromycin. All 40 seedlings from each of the T_1 plants predicted as

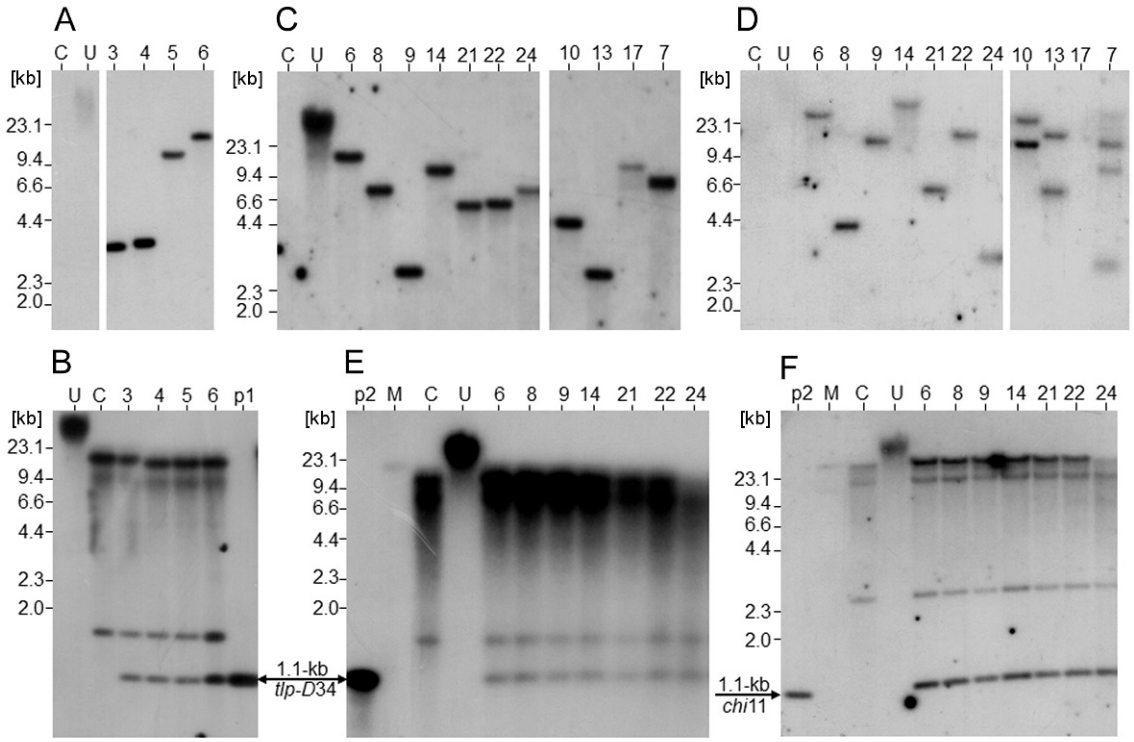

Fig. 2. Southern blot analysis of transgenic rice (T_0) plants. Analysis of LB junction fragments of pRS1-transformed plants (T3, T4, T5, and T6) with the *hph* probe after *Eco*RI digestion (*A*), analysis of the presence of the complete *tlp-D*34 gene in pRS1-transformed plants with the *tlp-D*34 probe after *Eco*RI digestion (*B*), analysis of LB junction fragments of pRS5-transformed plants (CT6, CT8, CT9, CT14, CT21, CT22, CT24, CT10, CT13, CT17, and CT7) with the *hph* probe after *Eco*RI digestion (*C*), RB junction fragment analysis of pRS5-transformed plants with the P*Ubi*1 probe after *Bam*HI digestion (*D*), analysis of the presence of the complete *tlp-D*34 gene in pRS5-transformed plants with the *tlp-D*34 probe after *Eco*RI digestion (*E*), and analysis of the presence of the complete *chi*11 gene in pRS5-transformed plants with the *chi*11 probe after *Bam*HI digestion (*F*). C - untransformed rice plant DNA digested with *Eco*RI/*Bam*HI, U - undigested rice plant DNA, p1 - 100 ng of pRS1 DNA digested with *Eco*RI, p2 - 100 ng of pRS5 DNA digested with *Eco*RI in *E* and with *Bam*HI in *F*, M - λ-*Hin*dIII marker lane, numbers 3, 4, 5, and 6 in *A* and *B* correspond to T_0 plants T3, T4, T5, and T6, respectively, numbers 6, 8, 9, 14, 21, 22, 24, 10, 13, 17 and 7 in *C, D, E*, and *F* correspond to T_0 plants CT6, CT8, CT9, CT14, CT21, CT22, CT24, CT10, CT13, CT17, and CT7, respectively. Numbers on the left indicate the sizes of λ-*Hin*dIII marker. Positions of the 1.1-kb internal T-DNA fragments of *tlp-D*34 and *chi*11 genes are marked with *arrows*.

homozygous were resistant to hygromycin confirming their homozygous status (data not shown).

Expression of *tlp-D*34 (in pRS1- and pRS5-transformed plants) and *chi*11 (in pRS5-transformed plants) was studied by Northern blotting in T_2 homozygous plants. Plants from five T_2 lines T3, T4, T5, T6, and T7 accumulated the *tlp-D*34 transcript (Fig. 3*A*). Low level of *tlp-D*34 transcript accumulation was observed in one line (T6). Northern blot analysis with both *tlp-D*34 (Fig. 3*C*) and *chi*11 (Fig. 3*E*) gene probes revealed accumulation of the corresponding transcripts in plants from all seven single-copy pRS5-transformed T_2 lines. The untransformed control plant RNA in all three Northern blots did not show any hybridization to *tlp-D*34 or *chi*11 probes.

Western blot analysis with the TLP antibody on protein extracts from the homozygous T_3 plants of both pRS1-transformed (T3, T4, T5, T6, and T7) (Fig. 3*B*) and pRS5-transformed (CT6, CT8, CT9, CT14, CT21, CT22, and CT24) (Fig. 3*D*) lines showed the accumulation of the 23-kDa TLP-D34 protein in all analyzed lines. Though the *tlp-D*34 transcript level was low in the transgenic plant T6 (Fig. 3*A*), the TLP-D34 protein level in T6 was comparable to that of other *tlp-D*34 transgenic plants. Homozygous T_2 plants from lines CT6, CT8, CT9, CT14, CT21, CT22, and CT24 exhibited the presence of the 35-kDa chitinase protein (Fig. 3*F*) when subjected to Western blot analysis with the chitinase antibody.

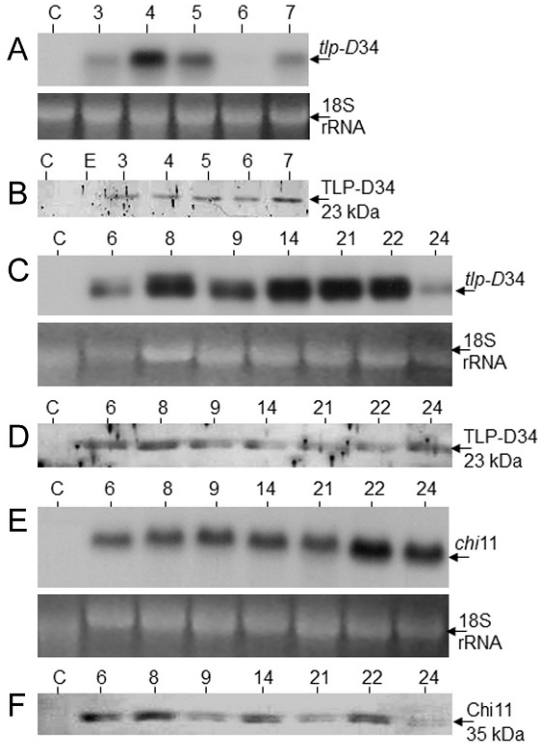

Fig. 3. Northern and Western blot analyses of homozygous transgenic rice plants. Total RNA (10 μg) of T_2 plants was probed with the [α-^{32}P]dCTP-labeled *tlp-D*34 gene (*A* and *C*) and *chi*11 gene (*E*). A portion of the ethidium bromide stained gel indicates equal amounts of 18S-rRNA in all lanes of the corresponding Northern blots. Western blot analysis of 20 μg of total protein samples from the T_3 plants using the TLP antibody (*B* and *D*) and chitinase antibody (*F*). C - control rice plant RNA (in Northern blot analysis) and protein (in Western blot analysis), E - empty lane, numbers 3, 4, 5, 6, and 7 in *A* and *B* indicate plants from the lines T3, T4, T5, T6, and T7, respectively, numbers 6, 8, 9, 14, 21, 22, and 24 in *C* to *F* indicate plants from the lines CT6, CT8, CT9, CT14, CT21, CT22, and CT24, respectively.

Chitinase assay was performed in total protein extracts of seven single-copy homozygous T_2 lines CT6, CT8, CT9, CT14, CT21, CT22, and CT24. The transgenic plants had 2.8- to 4.2-fold higher specific activity of chitinase in comparison to untransformed control plants (Table 1).

Table 1. Chitinase activity in the control and transgenic White Ponni rice plants (CT6 to CT24) transformed with the *tlp-D*34 and *chi*11 genes. Mean ± SE, n = 3 (*nkatal* = nmol of N-acetylglucosamine released s^{-1}).

Plants	Specific activity [nkatal mg^{-1}(protein)]	Fold increase of chitinase activity
Control	2.17 ± 0.11	-
CT6	8.93 ± 0.25	3.1
CT8	8.47 ± 0.38	2.9
CT9	10.20 ± 0.25	3.5
CT14	9.17 ± .011	3.2
CT21	8.86 ± 1.13	3.1
CT22	11.21 ± 0.68	4.2
CT24	8.18 ± 0.50	2.8

Table 2. *Rhizoctonia solani* infection assay of control and transgenic White Ponni (T_2, homozygous) rice plants harbouring the *tlp-D*34 gene alone (T3 to T7) and plants harbouring *tlp-D*34 + *chi*ll genes (CT6 to CT24). Mean ± SE, n = 27 tillers. Means not followed by the same letter in bioassay 1 or bioassay 2 are significantly different at $P < 0.01$ as determined by Student's t-test. *T3, *T4 - generation of homozygous plants.

	Plants	Disease index	[%]
Bioassay 1	control	$73.0^{a} \pm 3.7$	100.0
	T3	$43.7^{c} \pm 1.9$	59.9
	T4	$43.0^{c} \pm 2.3$	58.9
	T5	$54.1^{b} \pm 3.0$	74.1
	T6	$56.3^{b} \pm 4.3$	81.2
	T7	$51.1^{b} \pm 3.1$	70.0
Bioassay 2	control	$78.5^{x} \pm 3.0$	100.0
	CT6	$41.5^{z} \pm 3.2$	52.9
	CT8	$52.6^{y} \pm 3.4$	67.0
	CT9	$37.8^{z} \pm 2.2$	48.2
	CT14	$57.8^{y} \pm 3.1$	73.6
	CT21	$52.6^{y} \pm 3.1$	67.0
	CT22	$30.4^{z} \pm 3.3$	38.7
	CT24	$59.3^{y} \pm 3.6$	75.5
	*T3	$54.7^{y} \pm 5.3$	69.7
	*T4	$50.7^{y} \pm 4.3$	64.6

Infection assay with *R. solani* was performed by inoculating three tillers each of nine control plants and nine T_2 or T_3 homozygous plants of each transgenic line. The disease index was scored seven days post-infection (Table 2). The disease index values in control plants (73.0 in bioassay 1 and 78.5 in bioassay 2) were norma-lized to 100 and disease index in % was calculated for transgenic plants to compare the sheath blight disease levels in two separate experiments. Among the *tlp-D*34 transgenic plants (pRS1-transformed), homozygous T_2 plants of the line T4 exhibited the lowest disease index of 58.9 %.

R. solani infection assay was simultaneously done on two *tlp-D*34 transgenic lines (T3 and T4) and seven *tlp-D*34 + *chi*11 transgenic lines (transformed with pRS5) to compare the effect of co-expression of *tlp-D*34 + *chi*11 genes over the individual expression of *tlp-D*34 (Table 2). Overall, co-expression of *tlp-D*34 + *chi*11 genes brought about a significantly greater reduction in sheath blight disease index in comparison to the transgenic plants expressing the *tlp-D*34 gene alone (Table 2). T_2 plants from the line CT22 exhibited the lowest disease index of 39 %.

Discussion

Thaumatin-like proteins alter the fungal cell membrane permeability and chitinase catalyzes the hydrolysis of chitin (reviewed in Selitrennikoff 2001), both leading to fungal cell lysis. TLPs and chitinases along with β-1,3-glucanase were induced in barley (Zareie *et al*. 2002), grapevine (Jacobs *et al*. 1999), and sugarcane (Ramesh Sundar *et al*. 2008) upon infection with the fungal pathogens *Rhynchosporium secalis* and *Uncinula nectar* and treatment with an elicitor from *Colletotrichum falcatum*, respectively. Apoplastic extracts containing TLPs, chitinases, and β-1,3-glucanases from a lesion-mimic mutant wheat line, as well as a combination of purified TLP and barley chitinase inhibited the growth of *Fusarium graminearum* and *Gaeumannomyces graminis* var. *tritici in vitro* indicating their synergistic role in inhibiting fungal growth (Anand *et al*. 2004). PR-protein genes with different mechanisms of action, when expressed together in transgenic plants, lead to enhanced resistance against fungal pathogens (reviewed in Ferreira *et al*. 2007). We have previously reported that transgenic rice plants over-expressing both rice chitinase (*chi*11) and tobacco β-1,3-glucanase genes exhibited higher resistance against *Rhizoctonia solani* than the transgenic rice plants that expressed only the *chi*11 gene (Sridevi *et al*. 2008). In this work, we generated transgenic rice plants with combined expression of both rice *chi*11 and *tlp-D*34 genes and evaluated their impact on the reduction of sheath blight disease.

Earlier, Datta *et al*. (1999) reported the generation of transgenic rice with the *tlp-D*34 gene alone by polyethylene glycol-mediated transformation of proto-plasts and by biolistic transformation of immature embryos. Transgene copy number was not determined in those plants. Possibly due to integration of multiple transgene copies caused by direct transformation procedures, the *tlp-D*34 gene was silenced in a few transgenic lines in the T_1 generation. In two subsequent reports (Kalpana *et al*. 2006, Maruthasalam *et al*. 2007), co-bombardment of rice with *chi*11and *tlp-D*34 genes was done to achieve co-expression of both genes. One transgenic line was reported to harbour and express both genes in the T_0 generation. In those reports, Southern blot analysis was not designed to determine the copy number of transgenes. Again, possibly due to integration of multiple copies of the transgenes or due to segregation of the two genes, only the *tlp-D*34 gene was expressed in the T_1 generation but the *chi*11 gene was not expressed.

Co-bombardment of wheat with *tlp-D*34 and *chi*11 (Chen *et al*. 1999), bentgrass with rice chitinase and β-1,3-glucanase genes (Wang *et al*. 2003), rice with maize ribosome-inactivating protein b32 and *bar* (Kim *et al*. 1999), wheat with *chi*11 and *bar* (Chen *et al*. 1998), and wheat with *chi*11 and β-1,3-glucanase genes (Anand *et al*. 2003) are other examples where one or both transgenes were silenced in many transgenic lines in subsequent generations. Incorporation of multiple transgene copies, unpredictable segregation patterns, and the consequent lack of inheritance of stable expression of transgenes are major setbacks of transgenic plants generated by biolistic transformation.

Agrobacterium-mediated transformation yields trans-genic plants with low copy number, intact foreign genes, and stable transgene expression in contrast to transgenic plants obtained by particle bombardment (Iyer *et al*. 2000, Dai *et al*. 2001). It is of paramount importance that an introduced transgene in an agronomically relevant crop is integrated as a single copy and expression of the transgene is predictably and stably inherited in subsequent generations (Gelvin 1998). Multiple copies of transgenes, inverted repeats of transgenes, and integration of incomplete genes due to truncated T-DNA transfer will invariably trigger RNA silencing in transgenic plants (reviewed in Eamens *et al*. 2008). We generated transgenic rice plants with rice *chi*11 and *tlp-D*34 genes in the same T-DNA and addressed all above points relevant to transgene silencing by performing detailed Southern, Northern, and Western blot analyses.

All transgenic plants in this report were subjected to junction fragment analysis by Southern blotting to ensure the identification of single-copy transgenic events. Even though *Agrobacterium* accurately transfers the T-DNA region delimited by right and left borders, quite frequently truncated T-DNAs are also integrated (Terada *et al*. 2004). Such events will not be correctly identified if junction fragment analysis is done with only one T-DNA probe. Therefore, we used two T-DNA probes, one flanking the left border and another flanking the right border, to ensure that we select transgenic plants with

single and complete T-DNA copies harbouring full-length transgenes. Such analysis will eliminate transgenic plants with inverted T-DNA repeats and truncated T-DNAs. For example, four transgenic plants in this report (CT10, CT13, CT17, and CT7) were initially identified as single-copy transgenic plants using the LB-flanking *hph* probe. However, hybridization with the P*Ubi*1 probe flanking the RB revealed that CT10, CT13, and CT7 were in fact plants with two or more copies of T-DNA and CT17 harboured a truncated T-DNA. A similar observation of truncated T-DNA integrations was reported in *Nicotiana plumbaginifolia* (Moravčíková *et al*. 2004) and *Brassica napus* (Melander *et al*. 2006) transformed with chitinase and β-1,3-glucanase genes. All single-copy transgenic plants were also checked for the presence of the transgenes *tlp-D*34 and *chi*11. Therefore, all transgenic rice plants reported in this paper were confirmed to harbour single copies of complete T-DNA. As expected, all single-copy transgenic plants inherited the T-DNA as single loci in the T_1 generation. Inheritance of stable expression of the transgenes was studied by Northern blot analysis of T_0 and T_2 plants. Expression studies were supported by Western blot analysis for both chitinase (T_2 plants) and TLP (T_3 plants) and by chitinase enzyme assay (T_2 plants). Homozygous plants for all lines were initially identified by Southern blot analysis in the T_1 generation (Sridevi *et al*. 2006) and were subsequently confirmed by segregation analysis in the T_2 generation.

The disease index of control plants was normalized to 100 % and disease index in % of control was calculated for all transgenic plants to compare their sheath blight disease resistance (Table 2). Two *tlp-D*34 transgenic plants T3 and T4 (T_2 generation) showed the lowest disease index of about 60 % in the first bioassay experiment. They exhibited a similar values of 65 to 70 % in the second bioassay experiment (T_3 generation) indicating the stable inheritance of sheath blight resistance up to the T_3 generation.

A comparison of bioassay results show that the transgenic plants co-expressing *tlp-D*34 and *chi*11 genes exhibit a synergistic reduction in disease index in comparison to those expressing the *tlp-D*34 gene alone. The lines CT22 and CT9 with *tlp-D*34 + *chi*11 genes exhibited significantly lower level of sheath blight disease index in comparison to the lines T3 and T4 which harbour only the *tlp-D*34 gene. Therefore, we conclude that co-expression of *tlp-D*34 and *chi*11 genes was very effective in reducing the sheath blight disease of rice.

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