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Transgenic tobacco plants constitutively overexpressing a rice thaumatin-like protein (PR-5) show enhanced resistance to *Alternaria alternata*

R. VELAZHAHAN* and S. MUTHUKRISHNAN**

*Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625 104, Tamil Nadu, India**
*Department of Biochemistry, Kansas State University, Manhattan, Kansas-66506-3702, USA***

Abstract

Overexpression of antifungal pathogenesis-related (PR) proteins in crop plants has the potential for enhancing resistance against fungal pathogens. Thaumatin-like proteins (TLPs) are one group (PR-5, permatins) of antifungal PR-proteins isolated from various plants. In the present study, a plasmid containing a cDNA of rice *tlp* (D34) under the control of the CaMV-35S promoter was introduced into tobacco plants through *Agrobacterium*-mediated transformation system. A considerable overproduction of TLP was observed in transformed tobacco plants by Western blot analysis. There was a large accumulation of *tlp* mRNA in transgenic plants as revealed by Northern blot analysis. Southern blot analysis of the DNA from transgenic tobacco plants confirmed the presence of the rice *tlp* gene in the genomic DNA of transgenic tobacco plants. Immunoblot analysis of intracellular and extracellular proteins of transgenic tobacco leaves using a Pinto bean TLP antibody demonstrated that the 23-kDa TLP was secreted into the extracellular matrix. T_2 progeny of regenerated plants transformed with TLP gene were tested for their disease reaction to *Alternaria alternata*, the brown spot pathogen. Transgenic tobacco plants expressing TLP at high levels showed enhanced tolerance to necrotization caused by the pathogen.

Additional key words: disease resistance, *Nicotiana tabacum*, pathogenesis-related proteins.

Introduction

Fungal diseases of crop plants are of major concern to agricultural production worldwide. Breeding for disease resistance has been complicated by the rapid evolution of pathogens, which results in new strains capable of overcoming host plant resistance. Even though these diseases can be controlled by certain fungicides, much research has been focussed on ecologically safe, non-chemical methods to avoid environmental pollution that is associated with large-scale use of chemical fungicides. Genetic engineering opens the possibilities for alternative strategies to control plant diseases. Single defense gene(s) or specific combinations of genes from the same host or distant species can be directly introduced into crop plants without backcrossing and introgression, allowing preservation of elite genotypes (for review, see Bent and Yu 1999). Introduction and overexpression of genes that encode proteins involved in the synthesis of compounds toxic to fungi and other genes that encode proteins with direct inhibitory effect on the growth of fungi through genetic engineering is an important approach in the management of fungal diseases (Cornelissen and Melchers 1993). Proteins with the ability to inhibit the growth of fungi *in vitro* are present in some plant tissues and in others, they are inducible. These include chitinases (Swegle *et al.* 1992, Velazhahan *et al.* 2000), β-1,3-glucanases (Velazhahan *et al.* 2003), thionins (Carmona *et al.* 1993), thaumatin-like proteins (Roberts and Selitrennikoff 1990, Vigers *et al.* 1991, Velazhahan *et al.* 2002), ribosome-inactivating proteins (Leah *et al.* 1991), defensins (Terras *et al.* 1992) and lipid transfer proteins (Cammue *et al.* 1995,

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Abbreviations: PDA - potato dextrose agar; PMSF - phenylmethylsulphonyl fluoride; PVDF - polyvinylene difluoride; TLP - thaumatin-like protein.
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* Author for correspondence; fax: (+91) 452 2422785, e-mail: velazhahan@hotmail.com

Velazhahan *et al.* 2001). It has been demonstrated that constitutive, high-level expression of these antifungal proteins in transgenic plants can enhance resistance to a variety of pathogens (for review, see Punja 2001).

Thaumatin-like proteins (TLPs) are a group of pathogenesis-related proteins (PR-5) that are induced in plants in response to infection by pathogens, osmotic stress, treatment with ethylene, application of dichloroisonicotinic acid or salicylic acid and wounding (for review, see Velazhahan *et al.* 1999). TLPs have been shown to be antifungal on several types of fungi (Vigers *et al.* 1991, Velazhahan *et al.* 2002). It has been demonstrated that transgenic plants overexpressing TLPs have enhanced resistance against various pathogens (Liu *et al.* 1994, Chen *et al.* 1999, Datta *et al.* 1999). Velazhahan *et al.* (1998) isolated a full-length clone of TLP cDNA (D34) from a rice cDNA library prepared from RNA isolated from *Rhizoctonia solani*-infected rice plants. This cDNA contained an open reading frame of 693 bp encoding a 231 amino acids-long protein with a predicted molecular weight of 24.4 kDa. In the present study, we have regenerated transgenic tobacco plants that express the rice TLP gene constitutively. The transgenic tobacco plants showed enhanced tolerance to necrotization caused by *Alternaria alternata*, the brown spot pathogen.

Materials and methods

Plasmid construction and plant transformation: A 1.1 kb BamHI-HindIII fragment containing the full-length *tlp* cDNA was isolated from the plasmid pBS (*tlp* D34) (Velazhahan *et al.* 1998). The CaMV-35S promoter was excised as a 430-bp HindIII-BamHI fragment from the plasmid pCaMVNEO (Fromm *et al.* 1986). A three fragment ligation was set up using the 430-bp CaMV 35S promoter fragment, the 1.1 kb BamHI-HindIII *tlp* cDNA fragment and the binary vector pMON410 DNA (Rogers *et al.* 1986) linearized by HindIII digestion. The ligation reaction was used to transform *Escherichia coli* JM109. From the transformants, the colony containing pMON-CaMV-*tlp*-D34 with all three fragments was identified. This chimeric gene construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Jefferson *et al.* 1987) via triparental mating using pRK2013 as a helper as described by An (1987). Mobilization was confirmed by Southern hybridization with *tlp*-D34 DNA probe. Plasmid DNA was isolated from putatively transformed *A. tumefaciens* using *Wizard*™ *Plus Maxipreps* DNA purification system (*Promega*, Madison, WI, USA). The plasmid (100 ng) was digested with HindIII or BamHI and HindIII and electrophoresed in a 0.8% agarose gel. The DNA was blotted on to a *Gene Screen Plus*™ nylon membrane (*Du Pont*, Boston, MA, USA) by the alkaline transfer method (Ausubel *et al.* 1987) and probed with *tlp* D34 DNA probe as described below. *A. tumefaciens* harbouring the plasmid with the rice *tlp* gene was used for transformation of tobacco. Leaf-disc transformation and regeneration of transgenic tobacco (cv. Xanthi) were performed as described by Horsch *et al.* (1985). Regenerated plants resistant to 200μg cm^{-3} of kanamycin were transferred to the greenhouse. Leaves of primary transformants were used for Western blot, Northern blot and Southern blot analyses.

Southern blot hybridization: Total genomic DNA was extracted from leaf tissues of regenerants by the CTAB method (Murray and Thompson 1980). Ten μg of total DNA was digested with HindIII (*Promega*, Madison, WI, USA), separated on a 0.8 % agarose gel and transferred on to a Gene Screen Plus™ nylon membrane (*Du Pont*, Boston, MA, USA). The EcoRI insert from the TLP cDNA clone (D34) was labeled with [α^{32}P]dCTP using a multiprime DNA labeling system (*Amersham*, Arlington Heights, IL, USA) and used as the probe. Hybridization was performed following standard protocols (Ausubel *et al.* 1987). The blot was exposed to *Fuji* medical X-ray film with intensifying screens at -70 °C.

Northern blot hybridization: Total RNA was isolated from transgenic and control tobacco leaves using *Trizol* reagent (*Gibco-BRL*, Grand Island, NY, USA) according to the manufacturer's instructions. Twenty μg of RNA was separated on a denaturing 1.2 % agarose formaldehyde gel and transferred to *Gene Screen Plus*™ nylon membrane (*Du Pont*, Boston, MA, USA) according to Sambrook *et al.* (1989). The α^{32}P- labeled *tlp* cDNA probe prepared as in Southern blot analysis was used for hybridization. A commercial RNA ladder (*Gibco-BRL*) was used as a size marker.

Western blot analysis: Protein extracts were prepared by grinding 1 g of tobacco leaves with 5 cm^3 of 0.1 M phosphate buffer, pH 6.5 containing 0.5 mM phenylmethyl-sulphonyl fluoride as described by Velazhahan *et al.* (1998). Proteins (100 μg) in aliquots of extracts were separated on 12 % SDS-PAGE (Laemmli 1970) and electroblotted onto polyvinylene difluoride (PVDF) membrane (pore size 0.45 mm, *Bio-Rad*, Hercules, CA, USA) using a *BioRad* semidry transfer cell in accordance with the manufacturer's instructions. Western blotting was carried out as described by Winston *et al.* (1987) with bean TLP antiserum (kindly provided by Dr. O.P. Sehgal, University of Missouri, Columbia, MO, USA). Apparent molecular mass of proteins was determined by comparison with molecular mass

standards (*Rainbow markers, Amersham*, Arlington Heights, IL, USA). Protein concentrations were determined by the Bradford assay (Bradford 1976).

Collection of intercellular fluids: Leaves were collected from 30-day old transgenic tobacco plants and vacuum-infiltrated at 4 °C with a buffer containing 25 mM *Tris* (pH 7.8), 500 mM sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM mercapto-ethanol (Parent and Asselin 1984). After removing excess fluid using paper tissue, intercellular fluid was collected by centrifuging the leaves at 1000 *g* for 30 min at 4 °C. After collection of the intercellular fluid, these leaves were used to extract intracellular proteins by the same procedure that was used to extract total leaf proteins. Protein samples were fractionated by SDS-PAGE on a 12 % (m/v) gel and subjected to Western blot analysis using a Pinto bean TLP antibody as described earlier.

Bioassay: Transgenic tobacco plants were tested for their resistance to *Alternaria alternata*, the causal agent of brown spot or leaf spot disease. Control and T_2 homozygous transgenic tobacco plants were grown in the greenhouse under controlled conditions. Four leaves per plant from 10 plants were inoculated with 0.01 cm^3 of a spore suspension (10^6 spores cm^{-3}) obtained from *A. alternata* grown on potato dextrose agar (PDA) at four inoculation points between the leaf veins and plants were incubated at 90 % relative humidity. Observations were conducted daily up to 15 d for disease reaction (chlorosis, necrosis or both) as described by Lorito *et al.* (1998).

Results

A rice *tlp* (D34) cDNA (Velazhahan *et al.* 1998) was cloned into the binary vector, pMON410 under the control of the constitutive CaMV35S promoter. This construct, designated, pMON410 (CaMV-35S-*tlp* D34) was mobilized from *E. coli* into *Agrobacterium tumefaciens* strain, LBA4404, by triparental mating. Mobilization of the chimeric gene construct was verified by Southern blot analysis of the plasmid DNA isolated from the putatively transformed *A. tumefaciens* using *tlp* D34 DNA probe (Fig. 1). This transgenic *A. tumefaciens* strain with the rice

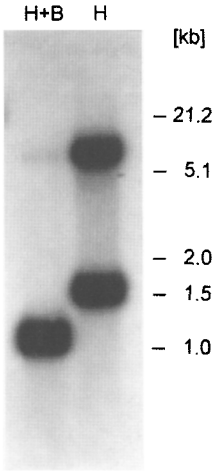

Fig. 1. Southern blot analysis showing mobilization of plasmid, pMON410 (CaMV-35S-*tlp* D34) into *Agrobacterium tumefaciens* strain LBA4404. Plasmid DNA (100 ng) isolated from *A. tumefaciens* after triparental mating was double digested with HindIII and BamHI (H+B) or digested with HindIII alone (H) and resolved in 0.8 % agarose gel before blotting and hybridization with 1.0 kb *tlp* D34 DNA fragment as the probe. Molecular sizes are indicated on the right. The expected 1.0 kb (H + B) and 1.5 kb (H) bands were detected by the probe. The larger band in the HindIII digest is due to undigested plasmid DNA.

tlp gene was used to obtain transgenic tobacco plants. After selection on hygromycin containing medium, several plants were regenerated. These T_0 plants were analyzed for overexpression of TLP by Western blot analysis using a Pinto bean TLP antibody. The results revealed that three plants (TL-7, TL-11 and TL-13) showed overexpression of two closely spaced protein bands with sizes of 22 - 24 kDa that cross-reacted with the TLP antibody. Even though aliquots containing equal amounts of protein were loaded, the intensities of this doublet varied in extracts of the three T_0 plants (data not shown). The extract from the control plant also contained a 22 kDa protein which cross-reacted with the TLP antibody (as did a larger protein of about 40 kDa). However, the intensity of this band was much lower in extracts of control plants. The maximum expression of TLP was observed in TL-13 (Fig. 2). The molecular masses (22 - 24 kDa) are consistent with the values of the processed and unprocessed forms of the rice TLP predicted from the DNA sequence of the rice *tlp* (D34) gene. We could not determine the N-terminal sequences of these two proteins because of the presence of other proteins in this part of the gel.

To examine the accumulation of *tlp* mRNA in leaves, total RNA extracted from untransformed or transgenic tobacco leaves was hybridized with *tlp* DNA probe. An mRNA with the expected size of 1.3 kb was expressed in leaves of two transgenic tobacco plants analyzed (Fig. 3) which indicates the expression of a transcript from the introduced rice *tlp* gene. In non-transformed tobacco leaves, the 1.3 kb transcript was not detected, but a larger transcript of about 4.0 kb was detected. The relationship of this larger transcript to the 40 kDa protein in control and transgenic plants reacting with the TLP antibody remains to be investigated. Consistent with the overproduction of the 22 - 24 kDa TLP in transgenic plants as revealed by

Western blot analysis, a large accumulation of 1.3 kb *tlp* mRNA was observed by Northern blot analysis of RNA from transgenic tobacco plant TL-13 whereas a lower level of transcript was seen in TL-7 and none in the control plant.

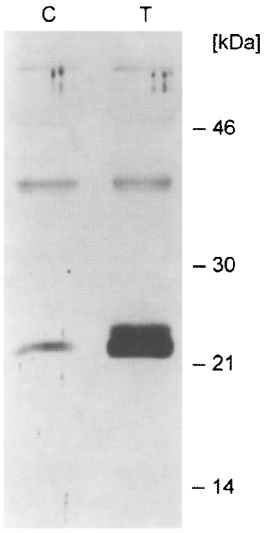

Fig. 2. Overproduction of thaumatin-like protein in primary (T_0) transgenic tobacco plant, TL-13. Total proteins (100 µg) from untransformed control (C) and transgenic tobacco plant, TL-13 (T) were subjected to 12 % SDS-PAGE. Proteins were then electroblotted onto PVDF membrane and subjected to immunoblot analysis with antibody to purified bean TLP. Sizes of marker proteins are indicated on the right.

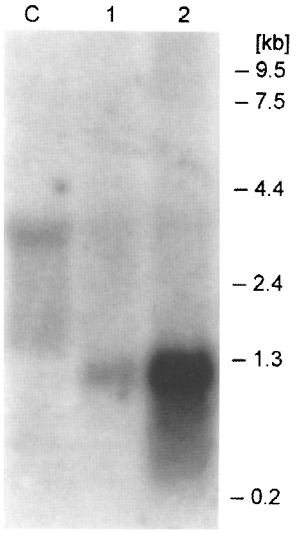

Fig. 3. Accumulation of *tlp* mRNA in transgenic tobacco plants. RNA was isolated from control (C) and transgenic plants (1 and 2) and subjected to electrophoresis on a 1.2 % denaturing gel. The blot was probed with ^{32}P-labelled rice *tlp* cDNA. The sizes of RNA standards are indicated on the right. *Lane 1* - TL-7, *lane 2* - TL-13.

We performed Southern analysis on the DNAs from the two Western-positive plants (TL-7 and TL-13) and a Western-negative plant (TL-5) in order to detect the presence of the *tlp* transgene in genomic DNA of trangenic tobacco plants. When DNA from transformed tobacco plants was digested with HindIII and probed with rice *tlp* (D34) cDNA fragment, the expected 1.5 kb band (corresponding to the fragment containing the CaMV35S promoter and the rice *tlp* D34 cDNA) was detected in DNA from plants TL-7 and TL-13 which gave a positive reaction in Western and Northern analyses. TL-5 that failed to express TLP in Western blot analysis did not have this 1.5 kb band (Fig. 4).

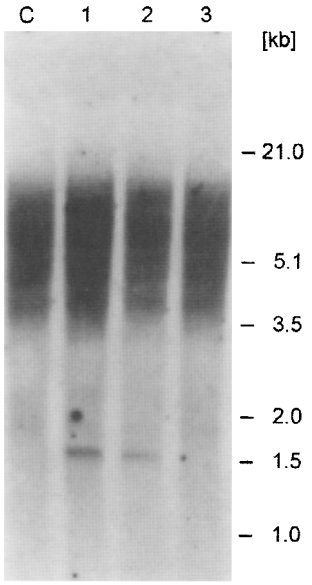

Fig. 4. Southern blot analysis of tobacco plants transformed with pMON-CaMV-*tlp*-D34. Genomic DNA (10 µg) extracted from control (C) and primary transgenic tobacco plants (*lanes 1 - 3*) was digested with HindIII and resolved on a 0.8 % agarose gel. The blot was probed with ^{32}P-labelled rice *tlp* cDNA. Molecular marker sizes of HindIII and EcoRI digested λDNA fragments are indicated on the right. *Lane 1* - TL-13, *lane 2* - TL-7; *lane 3* - TL-5. The band with a size of 1.5 kb (*lanes 1* and *2*) has the size expected from the transforming plasmid. The larger (> 3.5 kb) fuzzy bands presumably represent host DNA bands cross-reacting with the *tlp* probe.

To determine the localization of the TLP expressed in the leaves, intercellular fluid was collected from the leaves of transgenic tobacco plants (TL-13) and analyzed by Western blotting. An extract of leaves recovered after removal of the intercellular fluid was also analyzed. The results indicated that a prominent 22-kDa protein cross-reacting with the TLP antibody was secreted to the intercellular fluid of transgenic tobacco leaves (Fig. 5, *lane 1*). The trace amount of the 22 kDa TLP in the cell extracts (*lane 2*) presumably is due to incomplete removal of the apoplastic fluid.

To determine whether the expression of TLP in T_0 plants was heritable, T_1 progeny derived from the selfed primary transformant (TL-13) was subjected to Western blot analysis. The results proved the stable expression of the

TLP gene in the T_1 generation (data not shown). One of these T_1 plants (TL-13-5) was self-pollinated to create T_2 plants and these T_2 plants were used for further analysis.

To determine whether TLP has any role in resistance against *Alternaria alternata*, the brown spot pathogen, leaves of untransformed control and T_2 homozygous transgenic tobacco plants were inoculated with *A. alternata*. Chlorotic lesions appeared around the infection sites 3 - 4 d after inoculation. After 7 d, some of the lesions became necrotic. The total number of lesions (chlorotic, necrotic, and both) did not differ significantly between the control and transgenic plants. However, the number of necrotic lesions (necrotic and necrotic/chlorotic) was significantly lower in transgenic plants (Table 1). Untransformed control tobacco plants showed typical necrotic symptoms of *A. alternata* infection at 51 % of the inoculation sites whereas the overall value for all transgenic plants was 35 %. The sum of necrotic and chlorotic/necrotic lesions was 92 % for control compared to the sum of 59 % for the transgenic plants.

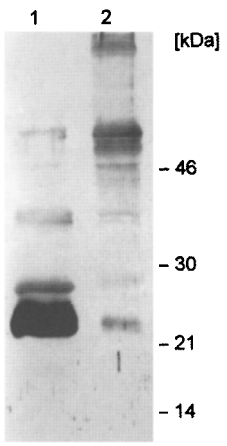

Fig. 5. Immunoblot analysis of intracellular and apoplastic proteins from leaves of primary transgenic tobacco plant, TL-13. Aliquots (100 μg) of apoplastic (1) and intracellular (2) proteins extracted from leaves of transgenic tobacco plant were separated by 12 % SDS-PAGE and subjected to immunoblotting using a Pinto bean TLP antibody. Sizes of marker proteins are indicated on the right.

Table 1. Bioassay of T_2 transgenic tobacco plants for resistance to *Alternaria alternata*. Transgenic and control tobacco leaves (4 leaves/plant) were inoculated with 0.01 cm^3 of a spore suspension (10^6 spores cm^{-3}) of *A. alternata* at 4 inoculation points on each leaf. Disease symptoms were assessed 15 d after inoculation. Means ± SD of 4 leaves per plant (10 plants per line).

Plants		Disease symptoms [% of total lesions] chlorotic only	necrotic only	chlorotic/necrotic
Control		7.5 ± 2.3	51.2 ± 4.1	41.2 ± 5.8
Transgenic	TL-13-5-1	38.1 ± 2.7	29.4 ± 3.8	32.5 ± 4.8
tobacco	TL-13-5-2	13.7 ± 3.4	47.5 ± 4.2	38.7 ± 5.7
plants	TL-13-5-3	41.2 ± 4.9	26.9 ± 5.9	31.9 ± 5.0
	TL-13-5-4	44.4 ± 4.3	22.5 ± 3.7	33.1 ± 5.2
	TL-13-5-5	58.1 ± 7.3	38.1 ± 5.0	3.7 ± 2.1
	TL-13-5-6	38.7 ± 4.9	46.9 ± 3.9	14.4 ± 3.6
	TL-13-5-7	40.0 ± 3.5	49.4 ± 4.0	10.6 ± 3.1
	TL-13-5-8	33.1 ± 5.0	32.5 ± 3.5	34.4 ± 4.8
	TL-13-5-9	33.7 ± 3.7	36.2 ± 4.6	30.0 ± 4.8
	TL-13-5-10	66.9 ± 6.3	22.5 ± 3.7	10.6 ± 2.9
	average	40.8	35.2	24.0

Discussion

TLPs have been shown to have antifungal activities against several types of fungi (Vigers *et al.* 1992, Choi *et al.* 1997, Ye *et al.* 1999, Velazhahan *et al.* 2002). Roberts and Selitrennikoff (1990) observed hyphal rupture of *Neurospora crassa* and *Candida albicans* in suspension cultures when treated with zeamatin and concluded that zeamatin exerts its antifungal activity by a membrane permeabilizing mechanism. There was also a rapid release of ultraviolet light-absorbing material from *C. albicans* and release of preloaded amino [1-^{14}C]isobutyric acid from *N. crassa*. Choi *et al.* (1997) reported that a 27-kDa PR-5 protein (CFTP) purified from flower buds of Chinese cabbage caused a rapid release of cytoplasmic material from the hyphal tips and vicinal regions of septa of *N. crassa*. By using a chitin-binding reagent, calcofluor white, the target site of CFTP was identified as the fungal plasma membrane. Transgenic potato (Liu *et al.* 1994), rice (Datta *et al.* 1999), and wheat (Chen *et al.* 1999) constitutively overexpressing

TLPs have been shown to have enhanced resistance against various fungal pathogens. Datta *et al.* (1999) reported that transgenic rice plants constitutively overexpressing a rice TLP had enhanced resistance to *Rhizoctonia solani*, the rice sheath blight pathogen. Liu *et al.* (1994) reported that expression of a tobacco TLP gene in transgenic potato plants caused a substantial increase in resistance to *Phytophthora infestans*. In the present study, we regenerated transgenic tobacco plants that overexpressed a TLP gene of rice in a constitutive manner. An accumulation of substantial amounts of TLP was observed in some transformed tobacco plants compared to control plants. Corresponding to the overproduction of TLP, there was a large accumulation of TLP mRNA in transgenic plants as observed by Northern blot analysis whereas none was detected in control plants. Southern blot analysis of the DNA from transgenic tobacco plants confirmed the presence of the chimeric rice TLP gene in the genomic DNA of transgenic tobacco plants. Immunoblot analysis of intracellular and extracellular proteins from transgenic tobacco leaves indicated that the 23-kDa TLP was secreted into the apoplastic fluid.

PR-proteins are generally synthesized as preproteins with an N-terminal signal peptide which is responsible for their translocation through the membrane of the endoplasmic reticulum. Bol *et al.* (1990) reported that in tobacco, acidic PR-proteins were secreted to the apoplast as a first line of defense while the basic PR-proteins were sequestered to the vacuoles as a second line of defense. By immunogold-electron microscopy, Dore *et al.* (1991) demonstrated that infection of Samsun NN tobacco with tobacco mosaic virus resulted in accumulation of acidic chitinases, β-1,3-glucanases and thaumatin-like proteins in extracellular "pocket-like" vesicles while the basic chitinases were found in electron-dense inclusion bodies in the vacuoles. It has been shown that in transgenic tobacco transformed with genes encoding the acidic PR-1, PR-3 and PR-5 proteins, the corresponding proteins were found in the intercellular wash fluid (Linthorst *et al.* 1990). The secretion of acidic PR-proteins by the "default pathway" is believed to be due to the absence of specific targetting signals. Velazhahan *et al.* (1998) reported that the calculated isoelectric point of the mature rice TLP(D34) was 6.56. The predicted amino acid sequence of open reading frame of TLP(D34) begins with a region rich in hydrophobic amino acids suggesting that it contains a leader peptide characteristic of secreted proteins. The results of the present study indicated that the 23-kDa TLP was synthesized, properly processed and secreted to the intercellular fluid of transgenic tobacco leaves. Many fungal infections are initiated in the intercellular spaces and subsequently the hyphae penetrate into the host cells. Some fungi grow predominantly in the intercellular spaces. Hence presence of high levels of antifungal proteins in the extracellular matrix may inhibit the infection process more successfully (Cornelissen and Melchers 1993). Liu *et al.* (1996) demonstrated that the basic osmotin that is targeted to the vacuole, could be secreted to the extracellular matrix of transgenic potato after removing the C-terminal 20 amino acids from the gene. The potato plants overexpressing the truncated osmotin protein exhibited resistance to *Phytophthora infestans*. The extracellular location of the rice TLP (D34) studied here is consistent with the absence of a C-terminal vacuolar targeting sequence in this protein (Velazhahan *et al.* 1998).

Bioassays of transgenic tobacco plants indicated that *A. alternata* caused chlorotic lesions around the infection sites after 3-4 days and after 7 days some of the lesions become necrotic. The total number of lesions of the control and transgenic plants did not differ significantly. However, the number of necrotic lesions (including chlorotic/necrotic) was significantly lower in transgenic plants. Our results suggest that overexpression of a rice TLP in transgenic tobacco can restrict the development of necrotic lesions by *A. alternata* but can not completely inhibit the initiation of fungal infection. Overexpression of multiple antifungal protein genes may be needed to retard the fungal infection effectively. Zhu *et al.* (1994) demonstrated that expression of both chitinase (PR-3) and β-1,3-glucanase (PR-2) in transgenic tobacco substantially enhanced resistance to the fungal pathogen, *Cercospora nicotianae*. Jongedijk *et al.* (1995) reported that expression of chitinase and β-1,3-glucanase in combination enhances fungal resistance in transgenic tomato plants. We are currently evaluating the strategy of overexpression of PR-proteins for enhancing disease resistance in cereals, including wheat and sorghum.

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