

Overexpression of thaumatin-like protein in transgenic tomato plants confers enhanced resistance to *Alternaria solani*

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Abstract

A cDNA encoding thaumatin-like protein (TLP) from rice was cloned into the binary vector pMON410 under the control of the CaMV 35S promoter for *Agrobacterium*-mediated transformation of tomato. All putative transformants were tested for the integration and expression of the chimeric gene by polymerase chain reaction (PCR) for hygromycin resistance gene (*hph*) and enzyme-linked immunosorbent assay (ELISA) for TLP respectively. Constitutive, high-level expression of TLP was observed in transgenic plants. The transgenic lines exhibited increased resistance to *Alternaria solani*, the early blight pathogen compared to non-transgenic tomato plants.

Keywords: Disease resistance, *Lycopersicon esculentum*, pathogenesis-related proteins, thaumatin-like protein, transgenic plants

Introduction

Early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grouet occurs worldwide and is one of the most destructive diseases of tomato (*Lycopersicon esculentum* Mill.) in India. Many commercial varieties of tomato are highly susceptible to early blight. The fungus can cause disease on foliage (leaf blight), stem (collar rot), and fruit and can result in severe damage during all stages of plant development (Barksdale & Stoner 1977; Nash & Gardner 1988). This disease is controlled mainly with agro chemicals such as Chlorothalanyl, Mancozeb, Triphenyltin hydroxide (TPTH), Super Tin and fixed coppers (Stevenson et al. 2001; Timmer et al. 2001). However, the worldwide trend towards environmentally-safe methods of plant disease control in sustainable agriculture calls for reducing the use of these synthetic chemical fungicides.

With the advent of genetic engineering, it is now possible to produce disease-resistant crop plants by the introduction of genes that express proteins and peptides that are directly toxic to

pathogens or that reduce their growth *in situ*; or the gene products that directly or indirectly activate general plant defense responses. Genes coding for antimicrobial proteins and peptides (Epple et al. 1997; Bi et al. 1999; Gao et al. 2000), PR-proteins (Liu et al. 1994; Zhu et al. 1996; Lin et al. 1999; Datta et al. 1999; Datta et al. 2001; Velazhahan & Muthukrishnan 2004), avirulence genes (Honee et al. 1995), phytoalexins (Hain et al. 1993; Hipskind & Paiva 2000), polygalacturonase inhibiting protein (Powell et al. 2000), oxalate decarboxylase (Kesarwani et al. 2000), oxalate oxidase (Liang et al. 2001) are widely used for the development of fungal-resistant crop plants. Among the various defense genes, genes coding for PR proteins are of major interests. Because many PR proteins show direct antifungal action against a broad range of fungal pathogens and are encoded by single genes. Further, it has been well documented that transgenic plants overexpressing PR proteins show enhanced resistance to fungal pathogens (For review, see Punja 2001).

Members of the PR-5 group of PR proteins are called thaumatin-like proteins (TLPs) because their amino acid sequences are homologous to that of the thaumatin, a sweet tasting protein isolated from the West African shrub, *Thaumatococcus danielli* (for review, see Velazhahan et al. 1999). Many TLPs have been shown to have antifungal activity against a broad spectrum of fungal pathogens (Vigers et al. 1992; Choi et al. 1997; Hu & Reddy et al. 1997; Velazhahan et al. 2002). Although several TLPs have been purified and characterized, the mechanism of antifungal action of these proteins remains unclear.

Roberts and Selitrennikoff (1990) suggested that zeamatin, a PR-5 protein isolated from corn seeds, exerts an antifungal activity by a membrane permeabilization mechanism. The crystal structure of tobacco PR-5d, an antifungal TLP isolated from cultured tobacco cells, revealed that the PR-5 proteins only those with antifungal activity had a common motif, a negatively charged surface cleft (Koiwa et al. 1999). Secondary and tertiary structure analysis showed that the amino acid residues that comprise the acidic cleft are conserved among other antifungal PR-5 proteins. It is suggested that the acidic cleft in PR-5 proteins may be the site of interaction between PR-5 proteins and their receptors on the plasma membrane of target microorganisms and may be directly involved in cell permeabilization (Batalia et al. 1996). Velazhahan et al. (1998) isolated a full-length clone of TLP cDNA (*tlp* 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.

Jayaraj et al. (2004) expressed the rice *tlp* D-34 in *Escherichia coli* as a His-tagged fusion protein and purified by Ni-NTA resin column chromatography. The purified 23-kDa rice TLP showed *in vitro* antifungal activity against many agronomically important plant pathogenic fungi. In the present study, we developed transgenic tomato plants that express a rice *tlp* gene (*tlp* D34) constitutively. The transgenic plants exhibited enhanced resistance to *A. solani*.

Materials and methods

Construction of plasmid

A 1.1-kb Hind III fragment containing the rice *tlp* (D34) cDNA and a 430-bp Hind III – Bam HI fragment of the CaMV 35S promoter were inserted into the single Hind III site of the binary vector pMON 410 to produce pMON – CaMV-*tlp*-D34. This plasmid was transferred to *Agrobacterium tumefaciens* LBA4404, by triparental mating using pRK2013 as a helper (Velazhahan et al. 2004). *A. tumefaciens* harbouring the plasmid with the rice *tlp* gene was used for transformation of tomato.

Production of transgenic tomato plants

To produce cotyledonary leaves for transformation, seeds of tomato cv. CO3 were surface sterilized in 0.1% mercuric chloride solution for 5 min, rinsed in sterile distilled water three times and germinated on Murashige and Skoog (Murashige & Skoog 1962) basal salt medium in a controlled environment (16h photoperiod at 25°C) for seven days. Cotyledonary leaves were removed aseptically and used for *Agrobacterium* mediated transformation as described by Sergei et al. (2001).

Plants were regenerated in half-MS basal medium containing 50 µg/ml of kanamycin and 50 µg/ml cefotaxime. Regenerated plants were grown in green house (30°C day/24°C night, with a photoperiod of 14 h). Leaves from the putative transgenic tomato plants were sampled for polymerase chain reaction (PCR) to detect *hph* gene and Western blotting analysis and enzyme-linked immunosorbant assay (ELISA) to detect the expression of TLP and to confirm transformation.

Westernblot analysis

Leaves (1 g) were homogenized in a pre-chilled pestle and mortar in 5 ml of 0.1 M phosphate buffer, pH 6.5 containing 0.5 mM phenylmethyl-sulphonyl fluoride (Velazhahan et al. 1998). The extract was centrifuged at 10,000 × g for 15 min at 4°C and the supernatant was collected. Proteins (100 µg) in aliquots were subjected to 12% SDS-PAGE according to Laemmli (1970). After electrophoresis, the proteins were electrophoretically transferred to PVDF membrane (pore size 0.45 µm, Bio-Rad, Hercules, CA, USA) using a Bio-Rad semidry transfer cell in accordance with the manufacturer's instructions. Western blotting was carried out as described by Winston et al. (1987) using tobacco TLP antiserum (Kindly provided by Dr M. Legrand, Institut De biologie Moleculaire Des Plantes Du C.N.R.S (IBMB), France). Apparent molecular mass of proteins was determined by comparison with molecular mass standards (Rainbow Markers, Amersham, and Arlington Heights, IL, USA). Protein concentrations were determined by the Bradford assay (Bradford 1976).

PCR analysis

Genomic DNA was extracted from tomato leaves as described by Sambrook et al. (1989). The *hph* gene primer set (*hph* forward: 5' GATCTCCAATCTGCGGGAT 3') and (*hph* reverse: 5' ACTCACCGCGCGACGTCTGTG 3') were used for PCR amplification of *hph* gene. PCR analysis was carried out in a 25 µl reaction mixture containing 100 ng of genomic DNA, 1.5 mM MgCl₂, 200 µM of each dNTPs, 20 ng each of *hph* gene primers and two units of *taq* DNA polymerase. The PCR programme was one cycle of 94°C for 3 min (initial denaturation), 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The amplified PCR product was electrophoresed on a 1% agarose gel and viewed under UV light.

ELISA analysis

The expression of TLP in leaves of transgenic plants was also assayed quantitatively by indirect ELISA (Hobbs et al. 1987). Proteins (100 ng) in aliquots were pipetted into wells of a microtitre plate and incubated at 4°C overnight. The antigen was removed from the wells and the walls were washed three times with phosphate buffered saline with Tween (PBST) (0.1 M sodium phosphate buffer, pH 7.0, 0.5 ml/l Tween 20). The plate was then added with 100 µl of polyclonal TLP antiserum raised against tobacco TLP (1:5000 dilution) and incubated at

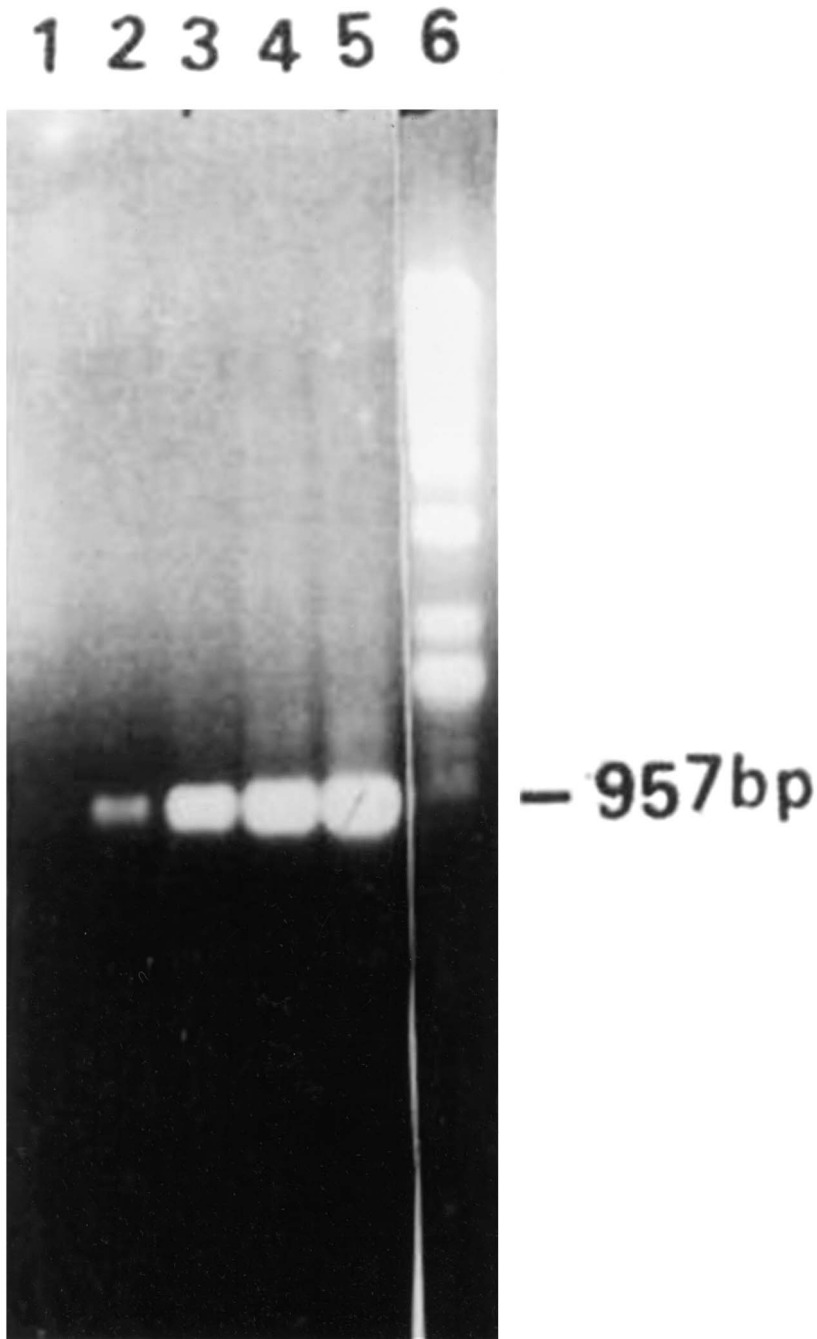


Figure 1. PCR analysis of putative transgenic tomato plants (T_0 generation) for the presence of *hph* gene. Genomic DNA extracted from control (Lane 1) and putative transgenic plants (Lane 2–4), were analyzed by PCR using *hph* gene primers, positive control (pMON410-D34) (Lane 5), 1 kb ladder (Lane 6).

37°C for 4 h. The plate was washed three times with PBST and 100 μ l of goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (ALP) was added. After 2 h of incubation at room temperature ($28 \pm 2^\circ\text{C}$), the plate was washed thrice with PBST and 100 μ l of ALP substrate solution (0.5 mg *p*-nitrophenyl phosphate (PNP) per ml of diethanolamine, pH 9.8) was added and incubated for 35 min at room temperature ($28 \pm 2^\circ\text{C}$). The reaction was stopped by adding 50 μ l of 3M NaOH and the absorbance was read in Multiscan MS ELISA reader (EL 800, Universal Multiple Reader, Bio-Tek Instruments, Inc, USA)

Bioassay

Putative transgenic tomato plants were tested for their resistance to *Alternaria solani*, the early blight pathogen. The fungus, *A. solani* was isolated from naturally infected tomato plants and grown on potato dextrose agar (PDA) medium in a Petri dish at 25°C with 12 h photoperiod. After two weeks, conidia were collected by flooding the Petri dish with sterile distilled water and gently scraping the surface of the medium with a glass rod. The spore density in the suspension was adjusted to 10^6 spores/ml with a hemocytometer (Foolad et al. 2000). Plants were grown under day/night temperatures of 30/26°C with a 16-h photoperiod. Leaves of seven-week-old tomato plants were spray-inoculated with the spore suspension and then incubated at 90% relative humidity. Two weeks after inoculation, plants were evaluated for disease reaction as described by Lorits et al. (1998).

Results and discussion

In recent years, there have been several reports of improved resistance of plants to diseases as a result of overexpression of TLPs (Liu et al. 1994; Chen et al. 1999; Datta et al. 1999;

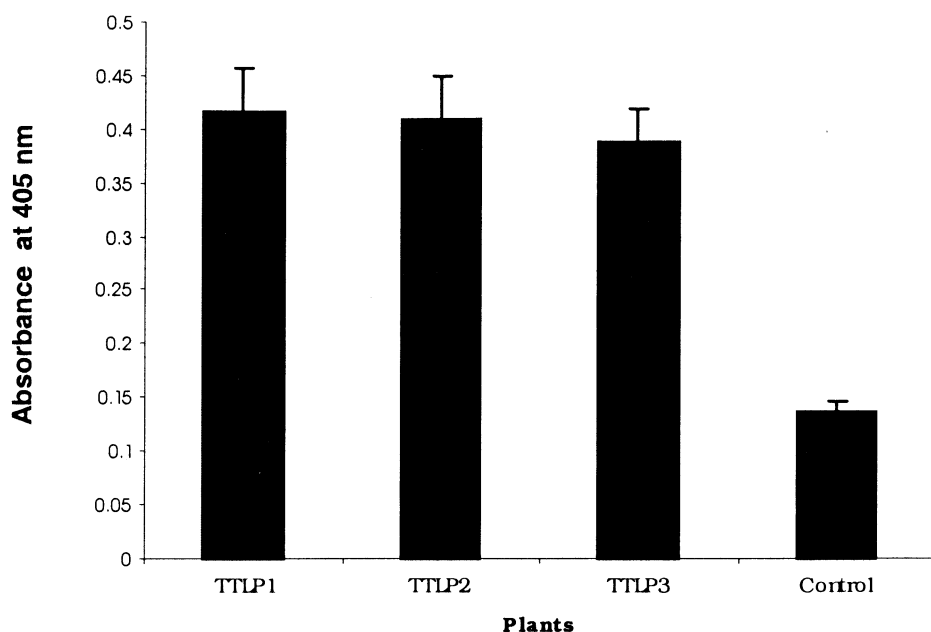


Figure 2. Immunometric assay of TLP expression in transgenic tomato plants. Data are means from three independent samples. Vertical bars indicate the standard deviation.

Velazhahan & Muthukrishnan 2004). The gene encoding TLP has been transferred to tobacco (Velazhahan & Muthukrishnan 2004), rice (Datta et al. 1999), wheat (Chen et al. 1999) potato (Zhu et al. 1996) and carrot (Chen & Punja 2003) to enhance resistance to various phytopathogenic fungi. Liu et al. (1994) demonstrated that constitutive overexpression of a tobacco TLP gene in transgenic potato plants resulted in enhanced resistance against *Phytophthora infestans*, the late blight pathogen.

Datta et al. (1999) reported that transgenic rice plants overexpressing a TLP constitutively had enhanced resistance to *Rhizoctonia solani*, the rice sheath blight fungi. Chen et al. (1999) demonstrated that transgenic wheat plants constitutively overexpressing the rice TLP gene showed delayed development of scab symptoms caused by *Fusarium graminearum*. By sequential N-banding and fluorescent *in situ* hybridization, the authors demonstrated that the transgenes were localized at the telomeric region of the chromosome 6A long arm.

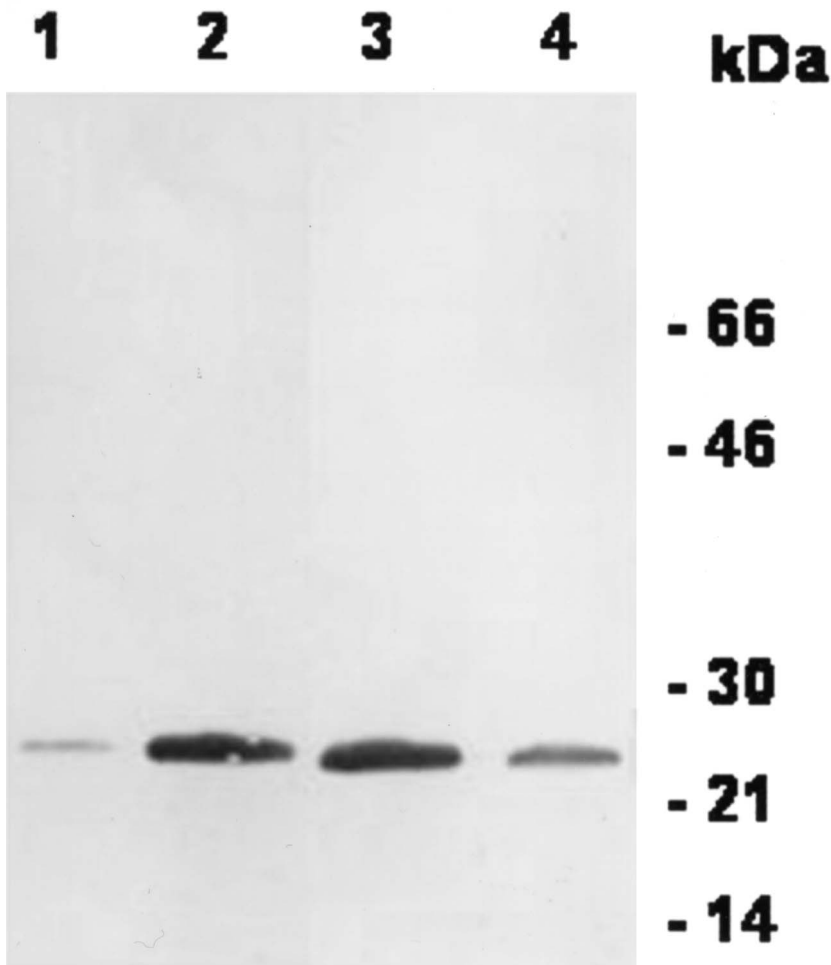


Figure 3. Western blot analysis of transgenic tomato plants. Aliquots (100 µg) of proteins extracted from Untransformed control (Lane 1) and transgenic plants (Lane 2–4) were analyzed by Western blotting after SDS-PAGE using tobacco TLP antiserum. Molecular markers are shown on the right.

Chen and Punja (2002) demonstrated that transgenic carrot plants expressing a rice *tlp* gene showed significantly enhanced tolerance to the foliar and root pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Velazhahan and Muthukrishnan (2004) recently demonstrated that transgenic tobacco plants overexpressing rice TLP constitutively showed enhanced tolerance to necrotization caused by *Alternaria alternata*, the brown spot fungus. Further, the authors demonstrated that 23 kDa TLP was secreted in to the extracellular matrix of the transgenic tobacco plants. In the present study, we introduced a rice TLP gene under the control of the CaMV 35S promoter into tomato plants by *Agrobacterium*-mediated transformation. A large number of independent transgenic tomato lines were produced. These plants were examined for their expression of TLP by western blotting using tobacco TLP antiserum. The results revealed that three of the ten plants showed an expression of a clear 23 kDa protein band (see Figure 1).

The molecular mass of expressed TLP (23 kDa) is consistent with the value predicted from the amino acid sequence of the rice *tlp* D34 gene. Such a band was not detected or at undetectable level in the non-transformed control plants. To detect the level of expression of TLP in leaves of transgenic tomato plants, ELISA was done using tobacco TLP antiserum. The results revealed that the quantity of TLP in transgenic plants was two times higher than that of the non-transformed control plants (see Figure 2).

In order to examine the integration of the chimeric gene into the genome of tomato, we performed PCR analysis on the DNAs from three putative transgenic plants. PCR analysis of putative transformants using primers specific for *hph* gene showed the presence of the marker gene. A 950 bp fragment of *hph* gene got amplified in all the putative transformants (see Figure 3).

No amplification was obtained in the untransformed control. To determine whether constitutive expression of TLP has any role in resistance of tomato against *A. solani*, the leaves of transgenic and control tomato plants were artificially inoculated with *A. solani*,

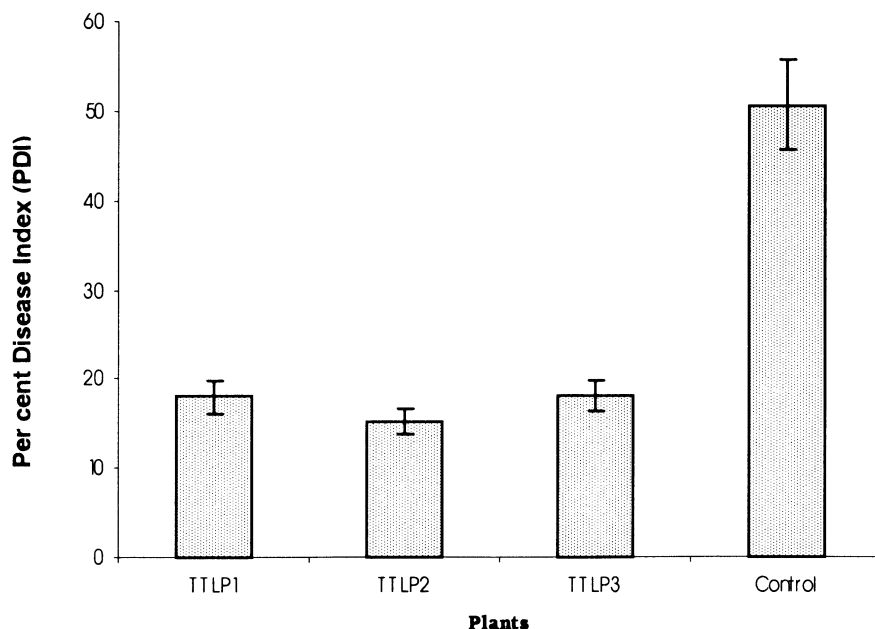


Figure 4. Bioassay of transgenic tomato plants for resistance to early blight. Data are means from three independent samples. Vertical bars indicate the standard deviation.

and observed for disease symptoms. Non-transformed control tomato plants showed typical necrotic symptoms with concentric rings seven days after inoculation and the disease severity was 46%. Whereas in transgenic plants, the disease severity ranged between 15 and 18% (see Figure 4). The results of the present study suggested that introduction and overexpression of TLP in tomato may be a useful strategy in controlling early blight.

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