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Thaumatin gene confers resistance to fungal pathogens as well as tolerance to abiotic stresses in transgenic tobacco plants

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Abstract

We report here the development of transgenic tobacco plants with thaumatin gene of *Thaumatococcus daniellii* under the control of a strong constitutive promoter - CaMV 35S. Both polymerase chain reaction and genomic Southern analysis confirmed the integration of transgene. Transgenic plants exhibited enhanced resistance with delayed disease symptoms against fungal diseases caused by *Pythium aphanidermatum* and *Rhizoctonia solani*. The leaf extract from transgenic plants effectively inhibited the mycelial growth of these pathogenic fungi *in vitro*. The transgenic seeds exhibited higher germination percentage and seedling survival under salinity and PEG-mediated drought stress as compared to the untransformed controls. These observations suggest that thaumatin gene can confer tolerance to both fungal pathogens and abiotic stresses.

Additional key words: disease resistance, *Nicotiana tobacum*, pathogenesis-related proteins, plant transformation.

Introduction

Crop plants often suffer from various kinds of stresses, resulting in significant loss of crop yield and quality. To circumvent these, plants have developed several defense mechanisms that enable them to respond to pathogens and abiotic stresses. The most common resistance exhibited by plants in response to various pathogens is the hypersensitive response (HR). Accompanying the HR is the *de novo* synthesis of a large number of novel proteins with roles in defense. A subset of these proteins is known as pathogenesis-related (PR) proteins (for review see Rao *et al*. 1999, Van Loon and Van Strien 1999, Muthukrishnan *et al*. 2001, Punja 2001).

Thaumatin is a sweet tasting protein isolated from fruits of South African shrub *Thaumatococcus daniellii* (Van der Wel and Loeve 1972). The function of thaumatin is not yet clear, though it has strong homology to thaumatin-like proteins with membrane permeabilizing properties, and thus they have been assumed to play a role in the defense system (Vigers *et al*. 1991). The thaumatin and thaumatin-like-proteins (TLPs) belong to group-5 of PR-proteins. They are induced in plants in response to infection by plant pathogens, elicitors, stress and developmental signals (Pierpoint *et al*. 1987, Bryngelsson and Green 1989, Hu and Reddy 1997). TLPs have been purified from seeds, which are inhibitory to fungal pathogens (Bryngelsson and Green 1989, Roberts and Selitrennikoff 1990, Huynh *et al*. 1992). The application of jasmonic acid and salicylic acid to wheat plants induce accumulation of TLPs and β-1,3-glucanase, and results in significant reduction (up to 56 %) of the incidence of leaf blotch disease incited by *Stagonospora nodorum* as compared with untreated control plants (Jayaraj *et al*. 2004). In addition, TLPs also respond to abiotic stresses in plants (Frendo *et al*. 1992, Rao *et al*.

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Abbreviations: BAP - 6-benzylaminopurine; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; PCR - polymerase chain reaction; PEG - polyethylene glycol; PR-proteins - pathogenesis-related proteins; TLPs - thaumatin-like-proteins.
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1999, Punja 2001) like osmotin and osmotin-like-proteins which are also the members of PR-5 group (Zhu *et al*. 1995).

Although a large number of reports exist on the genetically engineered plants for disease resistance using the various PR genes (for review see Cornelissen and Melchers 1993, Rao *et al*. 1999, Punja 2001), there are very few reports on transgenic plants expressing thaumatin and TLP genes for modulating fruit taste and stress responses, respectively. The expression of thaumatin gene in potato hairy roots has resulted in sweet taste properties in root extracts (Witty and Harvey 1990). Sweeter fruits were produced in transgenic cucumber (Malepszy and Szwacka 2000) and tomato (Bartoszewski *et al*. 2003) following the introduction of thaumatin gene. Liu *et al*. (1994) reported that the expression of a tobacco TLP gene in transgenic potato plants caused a substantial increase in resistance to *Phytophthora infestans*. Recently, the constitutive over-expression of rice TLP gene in transgenic tobacco (Velazhahan and Muthukrishnan 2003), rice (Datta *et al*. 1999) and wheat (Chen *et al*. 1999, Anand *et al*. 2003) have shown the enhanced resistance against *Alternaria alternata*, sheath blight and scab disease, respectively. However, there are no reports of abiotic stress tolerance in transgenic plants expressing thaumatin and TLP genes, although the other PR-5 osmotin-like genes have been shown to confer abiotic stress tolerance in addition to the resistance against late blight fungus *P. infestans* in transgenic potato plants (Zhu *et al*. 1995).

In this communication, we report that the introduction of thaumatin gene (from *T. danielli*) in transgenic tobacco plants can confer resistance against fungal pathogens as well as abiotic stress tolerance.

Materials and methods

Plants and transformation: Tobacco seeds (*Nicotiana tobaccum* var. *xanthi*) were surface- sterilized by treating with 70 % ethanol for 2 min and then rinsed thrice with sterile water. The surface-sterilized seeds were blotted on sterile filter paper and placed on basal Murashige and Skoog (1962; MS) medium and incubated at 16-h photo-period with irradiance of 40 μmol m^{-2} s^{-1} and temperature of 26 ± 1 °C. The tobacco clones were raised by culturing nodal buds and shoot tips on MS basal medium. The leaf explants (about 1 cm long) from about one-month tobacco clones were used for transformation as described earlier (Kumria and Rajam 2002). *Agrobacterium tumefaciens* strain LBA 4404 carrying the binary plasmid pEL103 (obtained from M.L. Choudhary, Indian Agricultural Research Institute, New Delhi) harboring the thaumatin gene and the neomycin phosphotransferase (*npt* II) gene as the plant selection marker under the control of 35S promoter (Fig. 1) within the T-DNA borders, was grown overnight in YEM medium (yeast extract 0.4 g dm^{-3}, mannitol 10 g dm^{-3}, NaCl 0.1 g dm^{-3}, $MgSO_4 . 7 H_2O$ 0.2 g dm^{-3} and K_2HPO_4 0.5 g dm^{-3}, pH 7.0) containing 50 mg dm^{-3} kanamycin. The cells were pelleted at 1000 *g* for 10 min at room temperature and re-suspended in liquid MS basal medium. Leaf explants were infected with bacterial suspension for 10 min, blotted on sterile filter paper and placed on co-cultivation medium [shoot regeneration medium (SRM) containing MS + 1 mg dm^{-3} BAP + 0.1 mg dm^{-3} NAA]. After co-cultivation for 2 d in light at 26 ± 1 °C, the explants were transferred to selection medium (SRM + 500 mg dm^{-3} cefotaxime + 100 mg dm^{-3} kanamycin) and cultured for one month with one sub-culture after 15 d onto a fresh medium. The shoots obtained were transferred to half-strength MS basal medium for rooting. The putative transgenic plants were transferred to pots in greenhouse after 2 weeks of hardening.

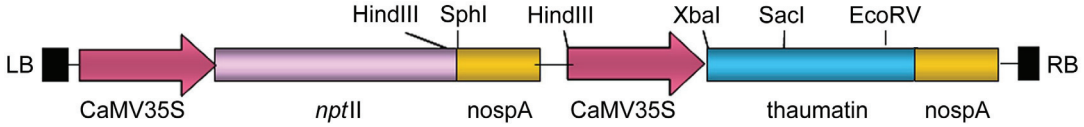

Fig. 1. T-DNA map of binary vector harboring thaumatin and *npt* II gene.

DNA isolation and PCR: Genomic DNA was isolated from the leaves of untransformed control and putative transgenic plants using the protocol developed by Doyle and Doyle (1990). The putative transgenic plants were analyzed by PCR for the presence of the transgene. About 100 ng of the genomic DNA of untransformed control as well as putative transgenic plants was taken, mixed with 200 nM of *npt* II specific primers (Forward primer 5' CGC ATG ATT GAA CAA GAT GGA TTG CAC 3'; Reverse primer 5' GAA GAA CTG CTC AAG AAG ACT AGT 3'), 0.0075 cm^3 of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 μM dNTP mix) and 0.5 U of Taq polymerase (*Fermentas*, Canada) in 0.025 cm^3 of sterile double distilled water. The amplification cycles used were denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and synthesis at 72 °C for 2 min and a final cycle of 10 min at 72 °C. The PCR products were checked on 1 % agarose with a standard DNA size marker.

Southern analysis: The genomic DNA (15 μg) samples of control and transformants digested with *Xba* I (*Fermentas*) were electrophoresed on 0.8 % agarose gel. After electrophoresis, DNA fragments were denatured and transferred to nylon membrane (*Serva*, Heidelberg, Germany) as per the manufacturer's instructions. The 0.9 kb *Xho* I fragment of *npt* II gene labelled with $\alpha(^{32}P)$-dCTP (*BRIT*, Mumbai, India) by nick translation (*Bangalore Genei*, Bangalore, India) was used as probe. Southern blot hybridization was done using the standard protocol (Sambrook *et al*. 1989). Autoradiographs were obtained by exposing the X-ray film (*XK-5 Kodak* film) to the membrane.

Fungal resistance assays: *Pythium aphanidermatum* was grown in liquid Czapek Dox medium (Rajam and Galston 1985) for 4 d. Leaves of the transgenics as well as the control plants were dipped in the fungal culture and placed in Petri plates lined with moist filter paper at 26 ± 1 °C. Leaves were scored for the extent of fungal infection at different time intervals (Liu *et al*. 1994).

Rhizoctonia solani was grown in liquid Czapek Dox medium for 4 d. Spore suspension (10^8 spores cm^{-3}) was inoculated near the root system of transgenics and control plants. Disease scoring was done at different time intervals, which was based on the wilting symptoms of the inoculated plants (Broglie *et al*. 1991).

Effect of leaf extract from transgenic plants on fungal mycelial growth *in vitro*: Leaf tissue (15 g) from a transgenic line (Th1) and control plant was taken, ground in sodium phosphate buffer (pH 7.0) and incubated at room temperature for 30 min. The leaf extract was centrifuged at 1 700 *g* and the supernatant was filter-sterilized. Different aliquots of the extract (5, 10 and 15 cm^3) were added to Czapek Dox medium (100 cm^3) after cooling the medium to about 46 °C. The mycelial discs of *P. aphanidermatum* and *R. solani* were inoculated in the center of the leaf extract-amended medium and the radial growth of the fungi was recorded (Rajam and Galston 1985, Rajam *et al*. 1985, Woloshuk *et al*. 1991).

***In vitro* salinity and drought tolerance assays:** The T_1 seeds of the transgenic lines were tested for their salt and drought tolerance by germinating them on NaCl (150, 200 and 250 mM) or PEG 6000 (10 and 15 %) amended MS medium. Further, salt and drought tolerance was also tested based on the shoot regeneration of the leaf explants on the salt (100, 200 and 300 mM NaCl) or PEG (5 and 7.5 %) amended MS shoot regeneration medium (containing 1.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA), respectively (Prabhavathi *et al*. 2002).

Data analysis: Transgenic lines were scored for their resistance against fungal pathogens and abiotic (salinity and drought) stress tolerance, and each experiment had three replicates and was performed at least thrice. Statistical analysis was done using Student's *t*-test to check the difference between the control and transgenic lines.

Results

The PCR analysis of putative transgenic plants showed the expected 900 bp amplified product of the *npt* II gene (Fig. 2*A*), indicating the presence of the transgene. Southern blot hybridization using *npt* II gene fragment as probe also indicated the stable integration of the transgene, with single as well as multiple transgene copy number (Fig. 2*B*).

The transgenic lines were tested for resistance against *P. aphanidermatum* by using detached leaf method and they showed increased resistance as compared to the control leaves (Fig. 3*A*,*C*). The delayed appearance of disease symptoms and smaller lesions were observed in the transgenic lines as compared to controls as evidenced by line Th1 and Th3, which also varied with the incubation time (Table 1).

Transgenic plants were also tested for resistance against *R. solani* using fungal spore suspension. No apparent disease development was observed in the transgenic lines (Th1 and Th3) within 48 h of infection, however slight infection appeared in the form of wilting and necrosis of the leaves after 4 d. Most of the transgenic plants were healthy even after 6 d with only few leaves showing necrosis while all the control plants were completely wilted and dead (Table 2, Fig. 3*B*).

Table 1. Detached leaf method for testing thaumatin transgenic tobacco lines for resistance against *P. aphanidermatum.* The results were based on three independent experiments and 3 replicates in each experiment. Severity of infection was scored on a comparative scale as compared to the response in the wild plant: - - no infection, + - 20 % infection, ++ - 40 % infection, +++ - 60 % infection, ++++ - 80 % infection, and +++++ - 100 % infection. C - untransformed control plants, Th1 - Th6 - transgenic tobacco lines, DAI - days after inoculation.

DAI	C	Th1	Th2	Th3	Th4	Th5	Th6
1	++	-	-	+	-	-	+
2	++++	-	+	+	+	-	++
3	+++++	+	+++	+	++	+	++

The leaf extracts from transgenic plants of Th1 (this line was considered for subsequent studies as it contained single copy of the transgene) inhibited the growth of the

fungus *P. aphanidermatum*, whereas leaf extract from control plants did not inhibit fungal growth, indicating the antifungal activity of leaf extract from transgenic plants. The inhibition of fungal radial growth was proportional to the volume of the leaf extract added (Table 3; Fig. 3*D*).

The T_1 seeds of various transgenic lines (Th1 - Th4, Th6, Th7 and Th11) were utilized for examining salinity and drought tolerance. The seeds from all the transgenic lines have shown increase in germination and seedling survival on MS agar medium fortified with 150 mM NaCl as compared with control seed (Table 4). At 200 mM NaCl, control seeds did not germinate, whereas transgenic seeds could germinate well (15 - 50 %) with

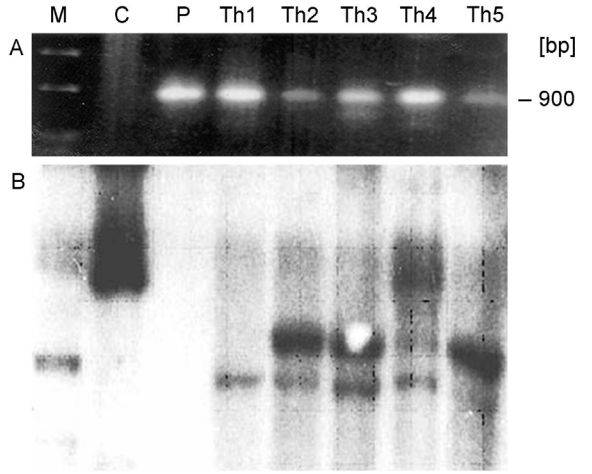

Fig. 2. Molecular analysis of thaumatin tobacco putative transgenic plants. *A* - PCR analysis using *npt* II gene specific primers. M - 1 kb ladder, C - DNA from untransformed control plant, P - Plasmid DNA, Th1 - Th5 - DNA from transformed plants; *B* - Southern analysis of the putative transgenic tobacco plants. Genomic DNA isolated from the leaf tissues of five transformed plants (Th1 - Th5) and from an untransformed control plant (P) was digested with *Xba* I and hybridized with *npt* II probe. Plasmid DNA was also included as positive control (C) and λ Hind III DNA as size marker (M).

Table 2. Response of thaumatin transgenic seedlings of tobacco to *R. solani*. The transgenic seedlings were challenged with the fungal spores by inoculation near the root system. The results were based on three independent experiments and 3 replicates in each experiment. Severity of infection was scored on a comparative scale as compared to the response in the wild plant: - - no infection, + - 20 % infection, ++ - 40 % infection, +++ - 60 % infection, ++++ - 80 % infection, and +++++ - 100 % infection. C - untransformed control plants, Th1 - Th4 - transgenic tobacco lines, DAI - days after inoculation.

DAI	C	Th1	Th2	Th3	Th4
1	-	-	-	-	-
2	++	+	-	+	-
3	+++++	+++	++	+++	++

Table 3. Effect of leaf extract from transgenic (line Th1) and control tobacco plants on the radial growth of fungi, *P. aphanidermatum* (*P.a.*) and *R. solani* (*R.s.*) *in vitro* measured after 6 and 2 d of culture, respectively. The values are mean ± SEM, based on two independent experiments with different volumes of leaf extract and 3 replicates (Petri plates) in each experiment, * - significant at 5 % level.

Fungus	Leaf extract [cm^3]	Colony diameter [mm]	
		control	Th1
P.a.	0	25 ± 0.61	25 ± 0.65
	5	25 ± 0.80	21 ± 0.56*
	10	26 ± 0.57	17 ± 0.47*
	15	25 ± 0.76	9 ± 0.47*
R.s.	0	32 ± 0.30	31 ± 0.57
	5	32 ± 0.33	22 ± 0.33*
	10	30 ± 0.33	18 ± 0.57*

Table 4. Seed germination percentage and seedling survival of thaumatin tobacco transgenic lines on MS basal agar medium supplemented with salt (150 and 200 mM NaCl) and PEG (10 and 15 %), SG - seed germination [%], SS - seedling survival [%].

Transgenic line	Control		150 mM NaCl		200 mM NaCl		10 % PEG		15 % PEG	
	SG	SS	SG	SS	SG	SS	SG	SS	SG	SS
Control	100	100	25	10	6	-	70	45	60	30
Th1	95	95	25	30	15	10	95	90	85	85
Th2	90	90	60	25	45	15	85	85	75	75
Th3	100	100	60	25	40	15	90	90	85	85
Th4	95	90	55	25	45	15	90	80	95	90
Th6	100	100	75	35	45	30	95	90	95	90
7h7	100	100	75	25	50	25	95	90	95	90
Th11	95	95	65	25	45	20	95	90	90	85

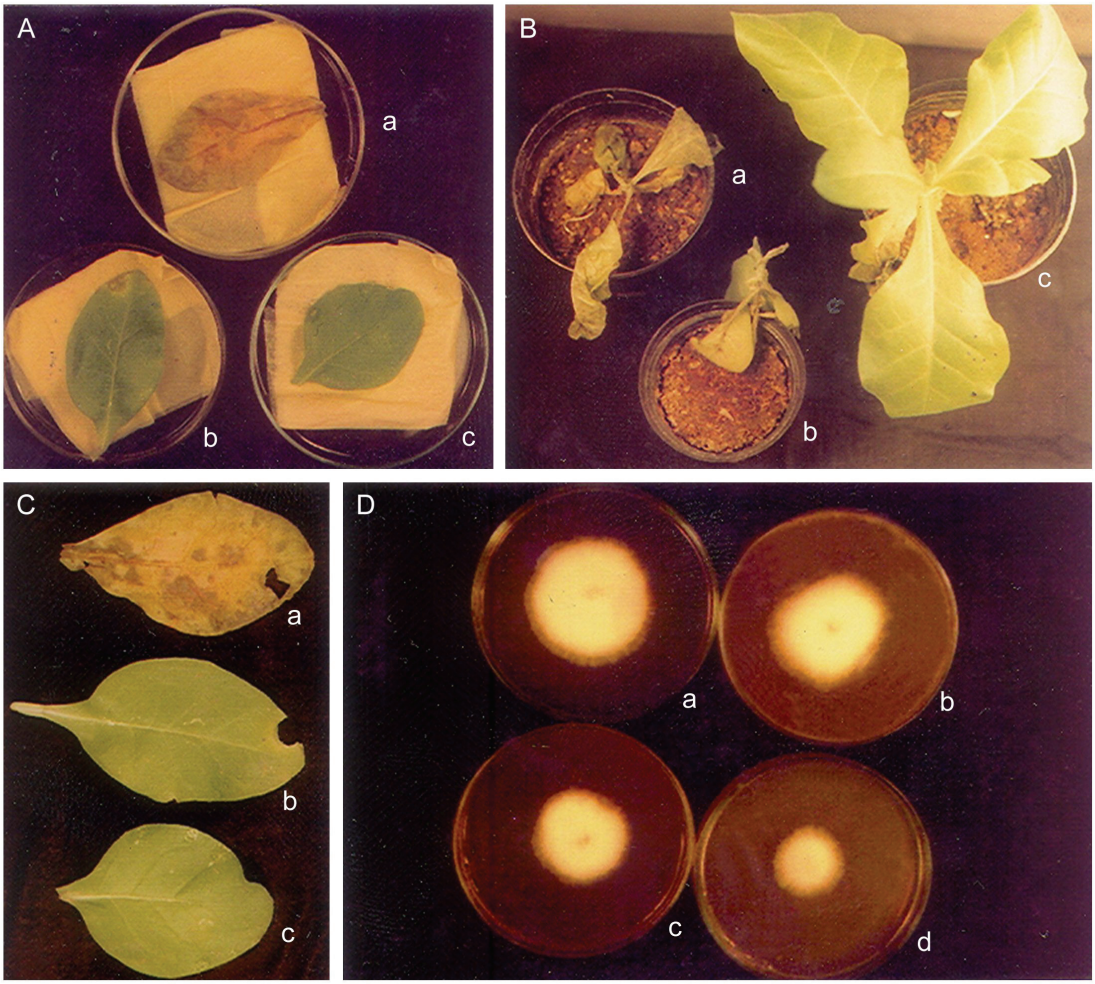

Fig. 3. Assays for fungal resistance in tobacco transgenics. *A*, *C* - Detached leaf method: a - untransformed control; b and c - transgenic lines Th1 and Th3, respectively. *B* - Inoculation of fungal spore suspension in the soil: a - untransformed control; b and c - transgenic lines Th1 and Th3, respectively. D - Effect of leaf extract from transgenic plants on the radial growth of *R. solani in vitro* on Czapek Dox agar medium: a - mycelial growth on medium lacking leaf extract; b - mycelial growth on medium containing the added leaf extract from untransformed control plant; c and d - mycelial growth on medium supplemented with 10 and 15 cm^3 of leaf extract from transgenic line Th1.

Table 5. Salinity and drought tolerance assay based on regeneration ability of leaf explants of transgenic thaumatin tobacco plants (line Th1) on shoot regeneration under abiotic stress conditions. The values are mean ± SEM, based on three independent experiments with 3 replicates (Petri plates) in each experiment (* - significant at 5 % level).

Stress agent	Conc.	Number of explants inoculated	Response [%]	Number of buds [explant^{-1}]
NaCl	0 mM	33	97	14.0 ± 0.57
	100 mM	37	78	10.6 ± 0.88*
	200 mM	32	49	5.3 ± 0.88*
PEG	0 %	32	100	13.33 ± 0.88
	5 %	32	63	7.33 ± 0.88*
	7.5 %	31	33	3.33 ± 0.33*

10 - 30 % seedling survival. However, 250 mM NaCl was lethal for germination of both transgenic and control seeds. Under PEG-mediated drought, the seeds of all the transgenic lines showed increase in germination and seedling survival as compared to control seeds (Table 4).

Further, leaf explants from a transgenic line (Th1) showed a better regeneration on shoot regeneration medium supplemented with 100 mM NaCl as compared to the control explants. However, at 200 mM NaCl, the transgenic explants showed delayed shoot regeneration, whereas it was lethal for control explants; 300 mM NaCl was found to be lethal for both control and transgenic explants. Leaf explants of transgenic line also showed good regeneration at 5 % PEG as compared to the explants of control, whereas at 7.5 % PEG transgenic explants showed some regeneration, but it was lethal for control explants (Table 5).

Discussion

The present study demonstrated, for the first time, the involvement of thaumatin gene in conferring enhanced resistance against fungal pathogens as well as abiotic stress tolerance in transgenic tobacco plants. It has been demonstrated that transgenic plants expressing the various PR-proteins, including chitinase, glucanase and TLPs exhibited resistance against the target fungal pathogens (reviewed by Rao *et al.* 1999, Punja 2001). Curiously, TLPs may induce resistance against fungi whose cell walls do not contain chitin. In fact, it has been suggested that fungi like *Phytophthora cactorum*, *Pythium ultimum* and *P. aphanidermatum*, which do not have chitin were not affected by mixture of chitinase and glucanase (Woloshuk *et al*. 1991). Henceforth, other factors such as TLPs may induce resistance against this class of fungal pathogens. Further, increased resistance against fungal pathogens in the developed transgenic plants with TLP genes might be due to the increased sporangial lysis of fungal hyphae as TLPs have been shown to interact with the plasma membrane because of their hydrophobic nature (Roberts and Selitrennikoff 1990). It was also hypothesized the mechanism of action of TLP zeamatin isolated from maize that it may directly insert into fungal membranes to form transmembrane pores and these pores may lead to the disturbances in the ionic balance across the transmembrane of the fungal hyphae and the spores and thus disrupting the integrity of the membrane (Abad *et al*. 1996).

The regulation of PR-5 genes by multiple abiotic stimuli acts as an alternative plant defense mechanism that is independent of specific chemical elicitors derived from the pathogen (Raghothama *et al.* 1993). It is also possible that these signals can act synergistically in PR gene activation (Xu *et al*. 1994). Osmotin, a member of PR-5 family with sequence homology with thaumatin and share several common features, accumulates during adaptation of cells to high osmotic stress induced by salinity and drought (LaRosa *et al*. 1989). Although neither the function nor the factors regulating the synthesis of thaumatin are known, its homology with stress-induced proteins suggest that its synthesis may also be controlled by stress-related conditions. The enhanced tolerance of the thaumatin tobacco transgenics towards salt and drought stress in the present study supports the above hypothesis.

In conclusion, tolerance against both biotic and abiotic stresses may be successfully engineered in tobacco and other plants for their improvement through the introduction of the gene encoding for sweet protein thaumatin.

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