Cloning and characterization of salicylic acid-induced, intracellular pathogenesis-related gene from tomato (*Lycopersicon esculentum*)

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Intracellular pathogenesis-related gene (IPR) from tomato was cloned from a salicylic acid (SA) induced cDNA library and was designated as *TSI-1* (tomato stress induced-1). The deduced amino acid sequence of *TSI-1* codes for a 178 amino acid polypeptide of molecular weight 20?4 kDa. *TSI-1* is highly homologous to the potato *STH-2* and *STH-21* IPR and tree pollen allergens which cause type I allergic reactions in humans. *TSI-1* lacks a signal peptide like other IPR members. It is organized as a multigene family and is inducible by SA and *Fusarium oxysporum* infection.

1. Introduction

Plant diseases caused by viral, bacterial, fungal and other pathogens are responsible for enormous economic loss. The ability of a plant to stop invasion of a pathogen depends on the presence of preformed barriers. Plants have a natural way of defending against pathogen attack by an array of biochemical responses. Plants express resistance genes against the infection and elicit hypersensitive response (HR). Following HR, a set of genes is expressed, viz., the genes involved in phytoalexin biosynthesis, lignification, proteins involved in cell wall modifications like hydroxyproline rich glycoproteins and glycine rich proteins, oxidative enzymes and pathogenesis related (PR) proteins (Collinge and Slusarenko 1987). Following HR, a local signal spreads throughout the plant to elicit defense genes to acquire systemic acquired resistance (SAR) to resist further attack (Ryals *et al* 1996). These disease response genes are also expressed during abiotic stresses like hormones, elicitors, heavy metals, wounding and UV light. Ethephon, 2,6-dichloroisonicotinic acid (INA) salicylic acid (SA) are the most commonly used abiotic stresses to induce PR genes (Van Kan *et al* 1995). SA plays a key role in mediating disease resistance in plants. The endogenous SA concentration increases at the site of HR and acts as a signal transducer for activation of defense response (Delaney *et al* 1994). Involvement of SA in disease resistance was noticed in transgenic tobacco expressing salicylate hydroxylase (NahG) which converts SA to functionally inactive catechol (Gaffney *et al* 1993). Plants expressing NahG did not accumulate SA following TMV infection and produced larger lesions upon secondary infection as compared to the control plants.. PR genes are well characterized in other dicots and monocots (Ponstein *et al* 1994; Hammond-Kosack and Jones 1996).

A distinct class of PR1 like proteins, called intracellular PR (IPR) proteins is expressed during wounding (Warner *et al* 1992), osmotic stress (Iturriaga *et al* 1994) and pathogen colonization (Chang and Hadwiger 1990). IPR proteins are classified under PR1 since their function is not known but they share a low homology to them. Chemical elicitors like arachidonic acid (Marineau *et al* 1987), probenazole (Midoh and Iwata 1996) and biotic elicitors like fungal spores (Constable and Brisson 1992) induce IPR members. These have been reported in alfalfa (Truesdell and Dickman 1997), potato (Matton and Brisson 1989; Matton *et al* 1990, 1993) asparagus (Warner *et al* 1992), carrot (Yamamoto *et al* 1997), bean (Walter *et al* 1990),

soybean (Crowell *et al* 1992), lily (Huang *et al* 1997), and pea (Chang and Hadwiger 1990; Iturriga *et al* 1994; Mylona *et al* 1994). They are low molecular weight proteins ranging from 17–20 kDa and show high homology to the major pollen allergens
from apple, white birch, hazel and horn bean (Breiteneder *et al* 1993; Larson *et al* 1992) which cause Type I
allergic reactions in humans. IPR proteins lack signal peptide.

We have initiated a programme in our laboratory to clone and overexpress some of the defense related genes that may be involved in giving protection against diseases. Results on the cloning and characterization of a SA and fungal inducible, pathogenesis related, defense responsive, IPR gene in tomato are presented.

2. Materials and methods

2.1 *Plant material*

Seeds of tomato (*Lycopersicon esculentum* var. Pusa ruby) were obtained from Indian Institute of Horticultural Research, Bangalore. They were surface sterilized with sodium hypochlorite solution (5%) and grown aseptically in Murashige and Skoog's (MS) medium. Five to six week old seedlings were used for the experiment. SA was purchased from the Sigma Chemical Company, St Louis, MO, USA.

2.2 *cDNA library construction, screening and sequencing*

Total RNA was isolated from young, cut leaves treated with 5 mM of SA dissolved in sterile water and maintained in tissue culture conditions for 24 h. The leaves were ground to a fine powder in liquid N_2 and extracted using hot phenol-saturated with buffer (50 mM Tris, 5 mM EDTA, 1% SDS and 1% b ME). The aqueous phase was reextracted with chloroform and the RNA was precipitated with 8 M lithium chloride. Poly (A)+ RNA was isolated by oligo d(T) column (Quiagen). First and second strand cDNA was synthesized from 1 m g of poly (A)+ RNA using Amersham cDNA synthesis kit. Double stranded cDNA was ligated with the adapter and cloned into l MOSElox vector under the conditions described by Amersham. The ligation mix was packaged into packaging extract from Amersham. The phages were infected with *Escherichia coli* strain 1647 and plated on LB agar. Recombinant plaques were subcloned into plasmid MOSElox by automatic subcloning. Multiple colonies were screened for the presence of the insert by digesting the adapter with *Eco*RI restriction enzyme. The inserts were released from recombinant plasmids and analysed by sequencing. DNA was isolated by the alkaline lysis method and sequenced by automated sequencing or by the dideoxy chain termination method using a sequence version 2 supplied by USB. Nucleotide search and translated sequences were analysed by BLAST, GAP programme. *TSI-1* protein was compared with the other known proteins using multi align programme to identify the conserved amino acids.

2.3 *Southern analysis*

Genomic DNA was isolated from tomato leaves by the method of Dellaporta *et al* (1983). Ten micrograms of genomic DNA was digested with restriction enzymes *Eco*RI, *Bam*HI and *Hin*dIII,

fractionated on 0?8% agarose gel, transferred to Hybond N membrane and cross-linked with UV light. The blot was probed with *TSI-1* clone. Prehybridization was carried out at 60? C in 5 ×nbsp;Denhardt's, 6 ×nbsp;SSC and 0?5% SDS for 3 h followed by supplementation of 100 m g/ml of salmon sperm DNA and probe to prehybridization solution and hybridized at 60? C for 24 h. The membranes were washed twice with 2 ×nbsp;SSC and 0?5% SDS for 30 min. The probe was prepared by random priming method using redivue random primer kit supplied by Amersham using [a ^{32}P]dCTP.

2.4 *Northern analysis*

Tomato leaves were cut and treated with 1 mM, 5 mM, and 10 mM of SA dissolved in sterile water for 12 h, 24 h and 48 h to analyse the induction of *TSI-1* for increasing time and concentration. As a control, leaves were treated with sterile water for 12 h, 24 h and 48 h. All the treated leaves were maintained under tissue culture conditions till they were taken for RNA isolation. Similarly tomato leaves were inoculated with *Fusarium oxysporum* f.sp. *lycopersici* spores for 24 h. As a control, fresh tomato leaves were taken for RNA isolation. Total RNA (20 m g) isolated from fungal infected leaves and untreated fresh leaves were fractionated on 1?2% agarose formaldehyde gel, transferred to Hybond N membrane and fixed as per manufacturer's instructions. The membrane was probed with full length *TSI-1* cDNA. Probe preparation and hybridization conditions were as described for Southern analysis. The same blot was stripped with 0?1% SDS and 0?2 ×nbsp;SSC in RNase free water and reprobed with tobacco 18S rRNA to normalize the amount of total RNA loaded in each well.

3. Results

Random analysis of the SA induced cDNA library was carried out. Inserts were subcloned and released from plasmid MOSElox by digesting the adapter with *Eco*RI. Inserts of different sizes ranging from 600 bp to 2 kbp fragments were released from the recombinant plasmid. Approximately a 700 bp single fragment was taken for further analysis. Complete sequencing of a recombinant plasmid confirmed the presence of a full length IPR gene (*TSI-1*).

Genomic DNA was completely digested with *Hin*dIII, *Eco*R1 and *Bam*H1. When the entire *TSI-1* was used as a probe for a genomic Southern hybridization, several hybridizing bands were observed in each of the restriction digestion under very high stringent conditions. These enzymes do not cut within the cloned cDNA gene. Southern analysis revealed *TSI-1* organized as multigene family in the tomato genome (figure 1).

The levels of *TSI-1* transcripts increased as the concentration of SA was increased and were maximal at 10 mM SA after 48 h. Expression signals were observed after 24 h exposure of the X-ray films. Longer exposure for 4 days did not show detectable signal in control (figure 2, lane 1). Similar results were observed in tomato leaves treated for 12 h and 48 h (data not shown). *TSI-1* was not expressed in the control and an extremely faint signal was obtained in leaves treated with very low concentration of SA (figure 2, lane 2). The expression was very well correlated with the concentration of SA which indicates that SA induces expression of *TSI-1.* After stripping and reprobing with 18s rRNA, similar signal intensities were obtained which indicates that equal amounts of RNA were loaded in all the lanes. This confirms that the increase in signal intensity was due to SA induction of *TSI-1* expression or reduced turn over of the transcript.

Northern analysis was performed with RNA isolated from *Fusarium oxysporum* treated tomato leaves using fresh tomato leaves as control. High intensity signals were obtained in fungal infected leaves after exposure for 24 h (figure 3, lane 2), but no signal was detected in the control lane after exposure for 2 days (figure 3, lane 1). This shows that *TSI-1* is not expressed constitutively but is induced during fungal infection.

Table 1. Homology at the nucleotide and amino acid level as analysed by GAP programme with the other IPR members and pollen allergens.

Species		Identity nucleotide level (%)	Identity amino acid level (%)	Similarity amino acid level (%)
Potato	STH-2	71	73	65
Potato	STH-21	71	72	63
Pea	ABR-18	52	37	47
Pea	DRR-1	51	34	45
Bean	PR-1	50	36	45
Soybean	SAM-2	52	37	46
Alfalfa	SRG-1	52	38	48
Parsley	PR2	46	34	42
Parsley	PR11	49	38	46
Asparagus	PR3A	50	37	44
White birch	Bet-V1	52	39	46
Apple	Mal-d1	51	41	49
Cherry	PRUA1	52	45	50
Corylus	Cor-a1	53	35	45

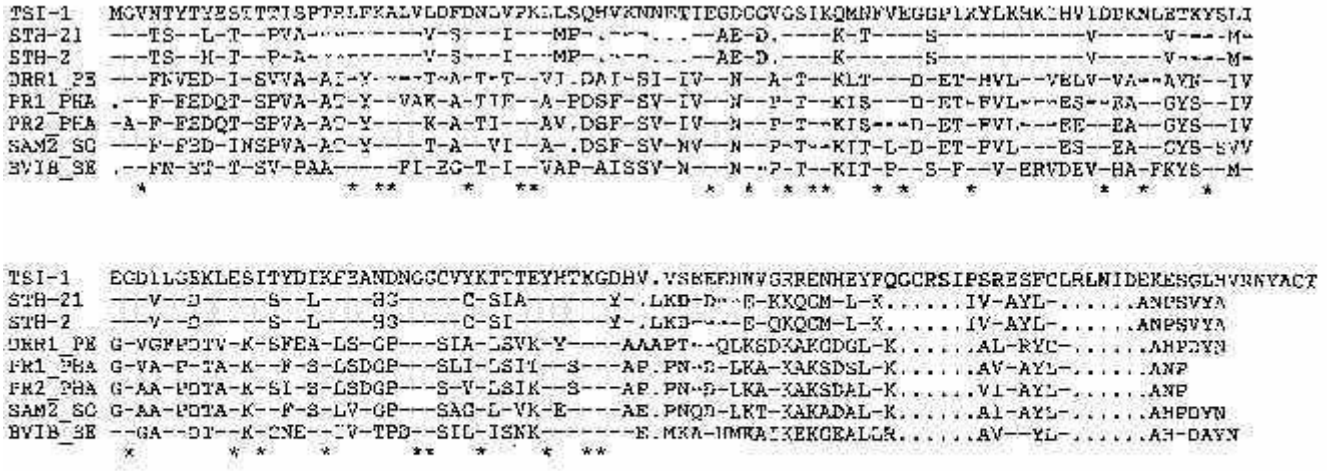

Figure 5. Comparison of the amino acid sequence of *TSI-1* with related proteins. *TSI-1* is compared with potato proteins *STH-2* and *STH-21*, pea DRR1, bean PR1 and PR2, soybean SAM2, and birch Bet V1 allergen. (–) indicates the identical amino acid residues to that of *TSI-1*. Dots in each protein represent the absence of the corresponding amino acid residues after homology search. Asterisks indicate amino acid conserved among all the sequences.

The deduced amino acid sequence of *TSI-1* is a 178 amino acid polypeptide with a 534 bp open reading frame starting with the first translation initiation codon ATG at position 40 and ending with a stop codon TGA at position 574 (figure 4).

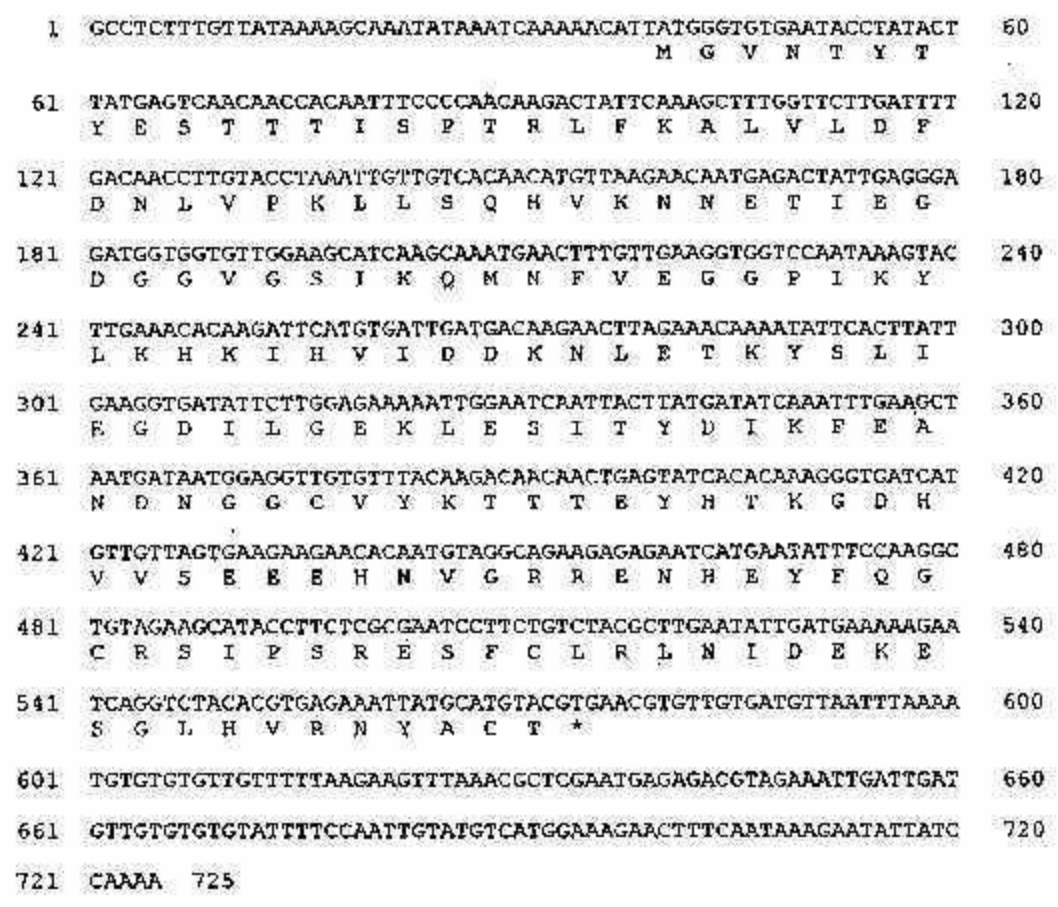

Figure 4. Nucleotide and deduced amino acid sequence of cDNA coding for *TSI-1* protein.

TSI-1 is an acidic protein and the calculated isoelectric pH is 5?8. Its predicted molecular weight is 20?4 kDa. *TSI-1* shows maximum homology to potato IPR genes, *STH-2* and *STH-21* (71%) at the nucleotide level. The identity at the amino acid level is 65% with potato IPR genes, *STH-2* and *STH-21*. Homologies at the nucleotide and amino acid level analysed by the GAP programme with the other IPR members and pollen allergens are shown in table 1. As reported in other plants, *TSI-1* has no signal peptide and is expected to be localized intracellularly. The deduced amino acid sequence of *TSI-1* is aligned with potato *STH-2*, *STH-21*, pea disease resistance response gene, bean PR1 and PR2, soybean stress response gene and white birch major pollen allergen *Bet V1* to show the conserved region (figure 5). *TSI-1* has less homology to tomato p14 and tobacco PR1 proteins.

4. Discussion

Multiple bands of hybridization indicate that *TSI-1* is a member of a multicopy gene family like those in potato (Matton and Brisson 1989; Matton *et al* 1990, 1993),

carrot (Yamamoto *et al* 1997), alfalfa (Truesdell and Dickman 1997) and soybean (Crowell *et al* 1992). These families may have arisen by duplication events as reported in potato and pollen allergens to which it shows considerable homology (Swoboda *et al* 1995). It is expected to have good homology with potato since potato and tomato both belong to the family

Solanaceae and are evolutionarily more related. The IPR genes express during stress conditions like pathogen invasion, wounding and arachidonic acid treatment. These are also induced in response to ABA in pea (Iturriaga *et al* 1994). Exogenous application of SA induces PR genes in many systems and SA acts as a signal to induce PR genes during HR. Potato proteins, *STH-2* and *STH-21* are expressed during *Cladosporium* infection (Constable and Brisson 1992). Tomato accumulates SA after infection with incompatible races of *Cladosporium* to elicit PR genes (Hammond-Kosack *et al* 1992). To the best of our knowledge, this is the first report on the induction of an IPR gene in response to exogenous application of SA. In asparagus IPR is induced both by wounding and pathogen infection (Warner *et al* 1992) and SA provokes the expression of wound induced IPR proteins in asparagus (Mur *et al* 1996). Both the wounding signal, MeJA (Kauss *et al* 1994) and the pathogen signal are known to accumulate H_2O_2. It is possible that IPR genes are controlled by the H_2O_2 signaling pathway.

As given in results, *TSI-1* is not expressed following wounding even after 24 h though expression was observed after treatment with SA for the same time period. A less intense signal could be seen after treatment with 1 mM SA for 12 h (figure 2, lane 2) and the expression increased as the concentration of SA increased for the same time period and clear signals were obtained in all the SA treated leaves (figure 2, lanes 5 and

8). Further analysis on fungal infected leaves showed induction of *TSI-1.* This indicated that *TSI-1* is not expressed constitutively but is induced during pathogen attack. Combined treatment of SA and wounding might lead to overexpression of *TSI-1* transcripts. Xu *et al* (1994) reported that SA hyperinduced PR1b when combined with wounding signal MeJA. Similarly many PR genes and enzymes involved in phenylpropanoid pathway are expressed in response to SA and wounding (Warner *et al* 1994). But *TSI-1* is highly induced with fungal infection which shows that *TSI-1* behaves like any other defense gene, induced during infection. Similar transcripts like potato *STH-2* are expressed during *Phytopthora infestans* infection (Matton *et al* 1990*)*. In addition, it is reported that rice IPR is induced by probenazole derivatives of benzoic acid (Midoh and Iwata 1996) which serve as precursors for SA biosynthesis. It is important to mention that probenazole induced IPR mRNA is not induced by wounding but by the chemical elicitor, N-cyano-methyl-2-chloro-isonicotinamide (NCI) which is known to induce disease resistance genes. It would be interesting to analyse the effect of ABA and methyl jasmonate on the expression of *TSI-1.* The function of *TSI-1* remains unknown. But recent reports on the white birch pollen and grass pollen allergens show that these pollen allergens have RNase activity. RNasin, an inhibitor of RNase, prevents allergic reaction caused by these pollen allergens (Bufe *et al* 1996; Moiseyev *et al* 1994). *TSI-1* has a stretch of amino acids from 83 to 126 which is considered as the motif for structurally related Bet V1 type pollen allergens and other related IPR proteins from plants. *TSI-1* may be involved in degrading the invading pathogenic RNA. Hence we conclude *TSI-1* is a low molecular weight protein expressed against stress, organized as a multigene family in tomato genome and highly inducible by SA and fungal infection.

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