Phytoparasitica (2011) 39:269–283
DOI 10.1007/s12600-011-0156-6

Biochemical and molecular studies of early blight disease in tomato

Suchita J. Patel · R. B. Subramanian · Yachana S. Jha

Received: 8 October 2010 / Accepted: 8 March 2011 / Published online: 13 April 2011

Abstract Tomato early blight occurs worldwide and it is prevalent wherever tomatoes are grown. *Alternaria solani* Sorauer, the causal agent, has been recognized as a serious foliar pathogen of tomato and there are very few cultivars which possess resistance against early blight. Alternaric acid is the major toxin of *A. solani.* In this study, alternaric acid and fungal culture filtrate were used as an elicitor in NDT-96 (tolerant) and GP-5 (susceptible) tomato varieties in order to study and compare their abilities to induce defense-related enzymes, *viz.,* catalase, peroxidase, β-1,3 glucanase, phenylalanine-ammonia-lyase (PAL), chitinase and polyphenol-oxidase (PPO) along with total phenols, and total soluble proteins. NDT-96 showed a rapid induction of all these pathogenesis-related enzymes except catalase along with total phenols as compared to GP-5 with both the treatments. Differential expression of total soluble proteins revealed higher protein content in NDT-96 as compared with GP-5. A 49.48 kDa protein was observed to be absent in GP-5. In addition, 25 microsatellite markers (SSR) were screened for polymorphisms among the above mentioned two tomato varieties. Of these, SSR 286 revealed a significant polymorphic band of 108 bp in NDT-96.

S. J. Patel (✉) · R. B. Subramanian · Y. S. Jha
B. R. Doshi School of Biosciences, Satellite Campus,
Sardar Patel University,
Vallabh Vidhyanagar 388120, India
e-mail: suchitapatel5@yahoo.com

Keywords *Alternaria solani* · Pathogenesis-related enzymes · Simple sequence repeats · Systemic acquired resistance

Introduction

Early blight, caused by *Alternaria solani* Sorauer, is one of the most common and destructive diseases of the cultivated tomato in areas of heavy dew, rainfall, and high relative humidity (Barksdale 1971; Nash and Gardner 1988). Alternaric acid is one of the major toxins found in the fungal culture filtrates (Brian *et al*. 1952); it causes chlorosis and necrosis and therefore plays a major role in early blight symptoms and defoliation (Pound and Stahmann 1951). Symptoms produced by an aqueous solution of crystalline alternaric acid when introduced into the plants are identical to symptoms produced by crude fungus filtrates. (Pound and Stahmann 1951) Control measures for these diseases include a 3- to 5-year crop rotation, routine applications of fungicides, and the use of disease-free transplants (Madden *et al*. 1978; Sherf and MacNab 1986).

Plants evoke a series of general defense reactions, including the production of phytoalexins and antimicrobial proteins, upon sensing invading microorganisms. (Radhajeyalakshmi *et al*. 2009). During the course of fungal attack, plants are induced to express a number of pathogenesis-related enzymes and proteins. Inoculation of plants with pathogens or treatment

with some chemical compounds can result in the establishment of systemic acquired resistance (SAR) (Ryals *et al*. 1996), which is accompanied by synthesis of pathogenesis-related proteins (PR-proteins) (Linthorst 1991). Defense-related genes encode a variety of proteins including enzymes controlling secondary metabolism, PR-proteins and regulatory proteins that control the expression of other defense-related genes (Dixon *et al*. 1994). In early blight resistant lines a higher and more rapid induction of the PR-proteins, *viz.,* chitinase and β-1, 3-glucanase (Lawrence *et al*. 1996, 2000), peroxidase (Fernandez *et al*. 1996), polyphenol oxidase (Thipyapong and Steffens 1997) and phenyalalanine ammonia lyase (Solorzano *et al*. 1996) are observed during the early infection process compared with those in susceptible lines (Lawrence *et al*. 1996, 2000). Catalase activity was observed to increase in a susceptible variety (Rani and Yasur 2009). Induction of defense proteins makes the plant resistant to pathogen invasion (Van Loon 1997), and has been correlated with defense against pathogen invasion in tomato (Bashan *et al*. 1985). Secondary plant metabolites correlated to early blight resistance include a higher total phenolic content (tannin, flavonol and phenol) in leaves and stems of early blight resistant varieties (Bhatia *et al*. 1972).

Fungicide treatments are generally the most effective control measures, but are not economically feasible in all areas of the world and may not be effective under weather conditions favorable for epidemics (Herriot *et al*. 1986). Resistant cultivars are potentially the most economical control measure because they can extend the intervals between fungicide sprays while maintaining control of the disease (Keinath *et al*. 1996; Madden *et al*. 1978; Shtienberg *et al*. 1995). The development of the polymerase chain reaction (PCR) (Saiki *et al*. 1988) has led to the development of many techniques to detect polymorphism at the DNA level (Powell *et al*. 1995; Rafalski *et al*. 1996). Of these techniques, some require the use of sequence-specific primers, such as microsatellite markers (SSR) or microsatellites (Powell *et al*. 1996). The SSR are short (mostly 2–4 bp) tandem repeats of DNA sequences and are useful due to their high degree of polymorphism and co-dominant character of heredity (He *et al*. 2003). The use of microsatellite polymorphisms to study the genetic diversity and variability was described for a number of plant species especially in tomato (Wang *et al*. 2006). They are mostly used as markers for genomic mapping, variety identification and marker-assisted selection in tomato (He *et al*. 2003).

The objectives of this investigation were to: (1) screen the two selected tomato varieties, *viz.,* NDT-96 and GP-5, by *in vitro* and *in vivo* bioassays using alternaric acid as an elicitor; (2) study and compare the effect of alternaric acid and fungal culture filtrate (FCF) on accumulation of phenolics and activities of catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5), β-1,3 glucanase (EC 3.2.1.39), polyphenol oxidase (PPO; EC 1.14.18.1) and chitinase (EC 3.2.1.14) in tomato varieties; (3) study the induction of total soluble proteins in tomato varieties; and (4) study polymorphism among NDT-96 and GP-5 through SSR markers.

Materials and methods

Fungal culture and its maintenance *Alternaria solani* isolate was grown on potato-dextrose agar (PDA) plates and these plates were incubated at 25°C±1°C. After sporulation these plates were maintained at 4°C until further use.

FCF production and its analysis An 8 mm mycelial mat was cut with a sterile cork borer from 1-month-old *A. solani*, grown on a PDA plate and transferred into a 250 ml Erlenmeyer flask containing 100 ml of autoclaved potato-dextrose broth (PDB). Flasks were incubated from 21 days at 25°C±1°C in static submerged condition. Thereafter, the broth was filtered using Whatman filter paper No. 1.

Isolation of alternaric acid For development of crystals, an 8-mm mycelial mat was cut with a sterile cork borer from 1-month-old *A. solani*, grown on a PDA plate and transferred into a 250 ml Erlenmeyer flask containing 100 ml of PDB. Flasks were incubated for 18 days at 25°±1°C in a static submerged condition. Mycelial mat from an 18-day-old culture grown on PDB was removed and the broth was filtered using Whatman No. 1 filter paper. The pH of the filtrate was adjusted to between 3.0 and 3.5 by 1 N HCl and extracted with an equal volume of ethanol. The colorless solution obtained was dissolved by adding drop by drop boiling carbon

Table 1 Microsatellite marker (SSR) primers used for the screening of two tomato cultivars (F: forward primer; R: reverse primer)

Ser. No.	Name of marker	Sequence (5′–3′)
1	SSR 45	**F:** TGTATCCTGGTGGACCAATG
		R: TCCAAGTATCAGGCACACCA
2	SSR 46	**F:** TGTATCCTGGTGGACCAATG
		R: TCCAAGTATCAGGCACACCA
3	SSR 52	**F:** TGATGGCAGCATCGTAGAAG
		R: GGTGCGAAGGGATTTACAGA
4	SSR 67	**F:** GCACGAGACCAAGCAGATTA
		R: GGGCCTTTCCTCCAGTAGAC
5	SSR 76	**F:** ACGGGTCGTCTTTGAAACAA
		R: CCACCGGATTCTTCTTCGTA
6	SSR 80	**F:** GGCAAATGTCAAAGGATTGG
		R: AGGGTCATGTTCTTGATTGTCA
7	SSR108	**F:** TGTGTTGGATGTTTGGCACT
		R: GCCATTGAAACTTGCAGAGA
8	SSR 136	**F:** GAAACCGCCTCTTTCACTTG
		R: CAGCAATGATTCCAGCGATA
9	SSR 181	**F:** CAATCGAAACCGACGATACA
		R: GGTAGATCTGGATCGAGGAGG
10	SSR 241	**F:** TCAACAGCATAGTGGAGGAGG
		R: TCCTCGGTAATTGATCCACC
11	SSR 276	**F:** CTCCGGCAAGAGTGAACATT
		R: CGACGGAGTACTTCGCATTT
12	SSR 285	**F:** AGTGGCTCTCACCTACTGCG
		R: CAATTCTCAGGCATGAAACG
13	SSR 286	**F:** AGCTATGGAGTTTCAGGACCA
		R: ATTCAGGTAGCATGGAACGC
14	SSR 304	**F:** TCCTCCGGTTGTTACTCCAC
		R: TTAGCACTTCCACCGATTCC
15	SSR 565	**F:** GAGGATGATGAGAACTCGCC
		R: TCAGAGGCTTCTGGGTCAGT
16	SSR 637	**F:** AATGTAACAACGTGTCATGATTC
		R: AAGTCACAAACTAAGTTAGGG
17	Tom 8–9 ATT7	**F:** GCATTGATTGAACTTCATTCTCGTCC
		R: ATTTTTGFCCACCAACTAACCG
18	Tom 31A-32A TA11	**F:** AATGTAATGGTGATGCTCTTCC
		R: CTCGGTTTTAATTTTTGTGTCT
19	Tom 41–42 TCC6	**F:** GAAATCTGTTGAAGCCCTCTC
		R: GACTGTGATAGTAAGAATGAG
20	Tom 43–44 TCC6	**F:** GCAGGAGATAATAACAGAATAAT
		R: GGTAGAAGCCCGAATATCATT
21	Tom 47–48 AT10	**F:** CAAGTTGATTGCATTACCTATTG
		R: TACAACAACATTTCTTCTTCCTT
22	Tom 49–50 AT10	**F:** AAGAAACTTTTTGAATGTTGC
		R: ATTACAATTTAGAGAGTCAAGG
23	Tom 57–58 CT8	**F:** TCTAAGTGGATGACCATTAT
		R: GCAGTGATAGCAAATGAAAAC
24	Tom 144	**F:** CTGTTTACTTCAAGAAGGCTG
		R: ACTTTAACTTTATTATTGCGACG
25	Tom 196	**F:** CCTCCAAATCCCAAAACTCT
		R: TGTTTCATCCACTATCACGA

tetrachloride (2 ml) using a glass dropper. Residual ethanol was removed by evaporation and crystalline alternaric acid appeared on cooling.

Plant materials The following two tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) varieties were included in our studies: NDT-96 and GP-5. Seeds of NDT-96 were obtained from Anand Agriculture University, India, and seeds of GP-5 were obtained from Junagadh Agriculture University, India. The surface of tomato seeds was sterilized in 70% ethanol for 2 min, and thoroughly washed with sterile distilled water (Jung *et al.* 2005). Thereafter, seeds of both varieties were treated with 1% H_2O_2 (Jaskani *et al*. 2006) and allowed to germinate in petri plates at 28°C for 2 days and sown in plastic cups filled with cocopeat (made from coconut husks) initially and after one week the seedlings were transferred to plastic cups filled with soil. The plants were allowed to grow for 4 weeks and watered daily; they were maintained under natural conditions.

Phytotoxicity tests of culture filtrates and toxin on tomato plants

Detached leaf bioassay Tomato leaves were washed in tap water. The leaves were surface sterilized with a cotton swab dipped in 4% sodium hypochlorite solution for 10–15 s. They were then washed thoroughly in sterile water and placed in sterile petri plates containing Whatman No. 1 paper. Thereafter these leaves were treated with toxin and FCF as follows: (*a*) 100 µl of crystals along with water was applied gently on the leaf surface using a micropipette and 100 µl of water was applied on control leaves; (*b*) 100 µl of FCF was applied gently on the leaf surface using a micropipette and 100 µl sterile PDB was applied on control leaves. These plates were allowed to incubate at 25°±1°C for 1 to 2 days and the

Table 2 Resistance of NDT-96 and GP-5 to *Alternaria solani*, using a 0–9 scale disease index (DI) and two types of assay

Evaluation technique	Cultivar	DI for FCF treatment	DI for toxin treatment
Detached-leaf bioassay	NDT-96	2.6	2.5
	GP-5	7.2	7.4
In vivo bioassay	NDT-96	3.5	3.6
	GP-5	8.2	8.3

All the values were significant at $P \leq 0.001$ according to Tukey's Multiple Comparison Test method

moisture in dishes was maintained throughout the experiment. Visible symptoms were recorded after 24 h. The experiment was carried out in triplicate.

In vivo bioassay Tomato plants were grown and maintained in the similar way as described above for about 4 weeks and were used for *in vivo* bioassay. The tomato plants were treated with toxin and FCF as follows: (*a*) 0.5 ml of crystal solution along with water was taken in an injection syringe and this was injected in the petiole region of the plant; sterile water was injected in the control plants; (*b*) 0.5 ml of FCF was taken in an injection syringe and was injected in the petiole region of the plant; sterile PDB was injected in the control plants. The surface of the leaf was slightly pricked with a sterilized syringe in order to enhance the rate of infection. The plants were monitored regularly for the development of visible symptoms. The experiment was carried out in triplicate.

Disease assessment Disease severity was rated as described previously (Gaube *et al*. 2004), by visual assessment based on a 0–9 scale. A disease index (DI), obtained from the average of each leaf value, was used to assess plant resistance. Differences between cultivars were tested using Tukey's Multiple Comparison Test method. The software used was GraphPad Prism Version 3.

Induction of biochemical defense mechanisms To study the induction of catalase, peroxidase, β-1,3-glucanase, PAL, chitinase, PPO and phenolics,

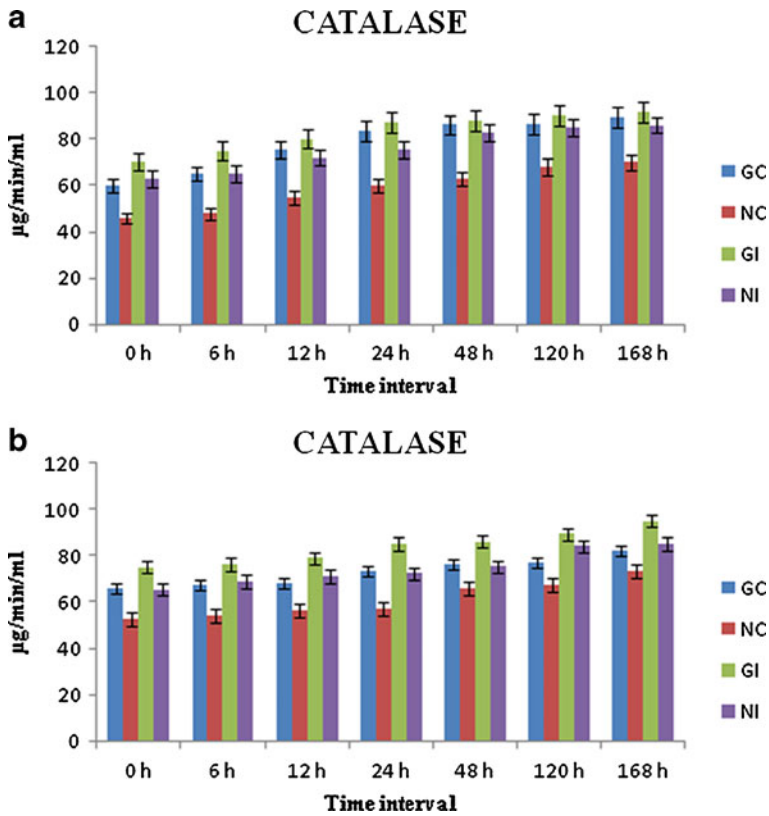

Fig. 1 The enzymatic activity of (**a**) Catalase (FCF) and (**b**) Catalase (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

one-month-old tomato plants were given the treatments by toxin and FCF as described above for *in vivo* bioassay. Four sets of tomato plants (GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control, and NI = NDT-96 Infected) were treated similarly as above. Whole plants from four sets were harvested at various time intervals (0, 6, 12, 24, 48, 120 and 168 h) after treatment and leaf samples were collected and used for enzyme analysis. The experiment was carried out in triplicate.

Catalase (CAT) assay CAT activity was measured using the modified Barber (1980) method. The enzyme activity was expressed as μg/min/ml.

Peroxidase (POX) assay

POX activity was measured using the modified Sumner and Gjessing (1943) method. The enzyme activity was measured at 430 nm and expressed as change in units/min/g/ml.

PAL assay PAL activity was measured using the Dickerson *et al*. (1984) method. The enzyme activity was measured at 290 nm and expressed as nmol trans-cinnamic acid/min/g.

β-1,3 glucanase assay β-1,3 glucanase activity was measured using the Pan *et al*. (1991) method. The enzyme activity was measured at 500 nm and expressed as units/ml/min.

PPO assay PPO activity was measured using the Siriphanich and Kader (1985) method. The change in O.D. was recorded at 30-s intervals up to 3 min at 495 nm. The enzyme activity was expressed as changes in units/min/mg total protein.

Chitinase assay 1% colloidal chitin (pH 6.6) was prepared by Rani and Yasur's method (2009). A 1% solution was prepared from this precipitate and stored at 4°C until use. Chitinase activity was measured using the Reissig *et al*. (1959) method. Enzyme activity was expressed as μmol GlcNAc/h/mg protein.

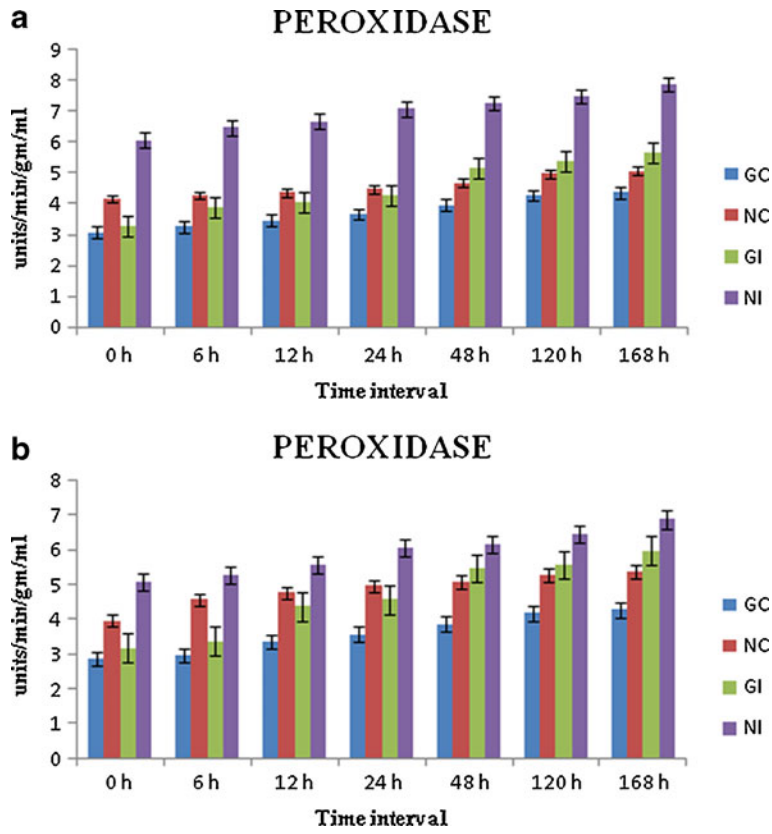

Fig. 2 The enzymatic activity of (**a**) Peroxidase (FCF) and (**b**) Peroxidase (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

Estimation of total phenolic content Total phenolic content was measured using the Zieslin and Ben-Zaken (1993) method. The content of the total soluble phenols was calculated based on a standard curve obtained from a Folin Ciocalteau reaction with phenol and expressed as μg/g f.wt.

Data analyses

Data were subjected to analysis of variance (ANOVA). Multiple mean comparisons were performed using Tukey's Multiple Comparison Test and comparisons between treatments were made using unpaired *t*-test in GraphPad Prism Version 3. One-way ANOVA and unpaired *t*-test were performed between both controls and between both infected tomato varieties.

Analysis of total soluble proteins Four sets of tomato plants (GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control, and NI = NDT-96 Infected) were treated with toxin in a similar way as described above. Whole plants from four sets were harvested at various time intervals (0, 12, 24, 48, 120 and 168 h) after treatment and leaf samples were collected and used for total soluble protein analysis. Tomato leaves (1 g) were homogenized in 10 ml of 0.1 M phosphate buffer (pH 7.0). The mixture was centrifuged at 10,000*g* for 20 min. The supernatant was used as a sample to analyze soluble proteins. The concentration of proteins was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard. For one-dimensional separation, 12% linear sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (1 mm thickness) overlaid with stacking gel (Laemmli 1970) were used. The electrophoresis was carried out at 50 V. Once tracking dye reached the anode, the run was stopped and the gels were carefully removed and placed in 5 volumes of Colloidal Blue Stain by the modified method of Neuhoff *et al*. (1988) for 1 h or overnight. The gels were washed with distilled water and destained using deionized water. The electropherograms were photographed and analyzed using the gel documentation

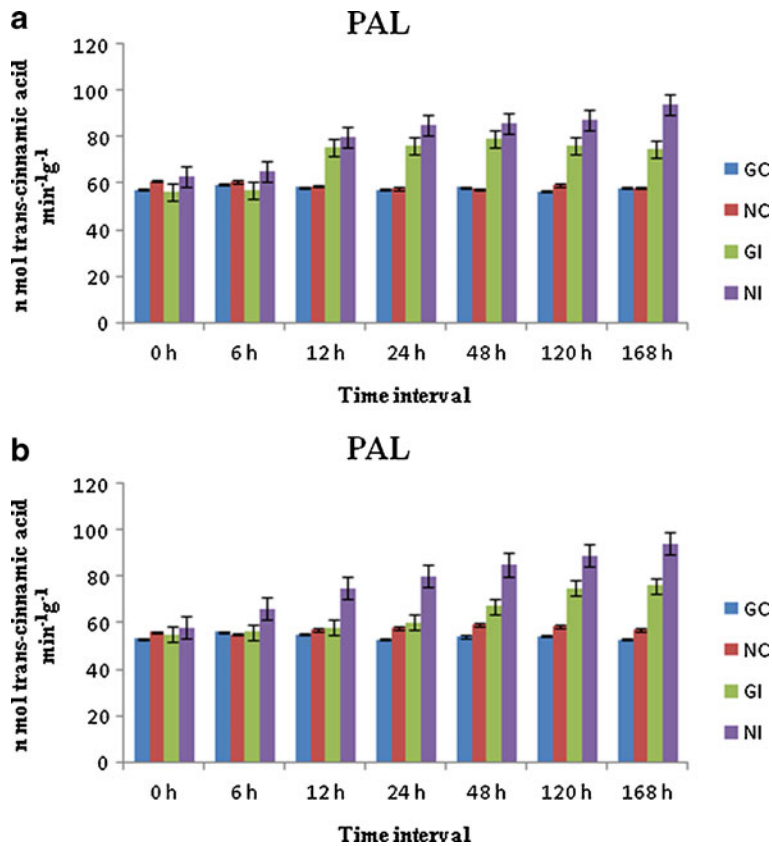

Fig. 3 The enzymatic activity of (**a**) β-1,3-glucanase (FCF) and (**b**) β-1,3-glucanase (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

system (AlphaEase FC 4.0, Alpha Innotech, Minneapolis, MN, USA).

DNA isolation and marker analysis For the SSR analysis, DNA was isolated from young and fresh leaves of NDT-96 and GP-5 according to Doyle and Doyle's method (1990). A total of 25 SSR primers were surveyed (Table 1). Primers for SSR 45 to SSR 637 and Tom 144–Tom 198 were obtained from the Sol Genomics Network at www.sgn.cornell.edu. Primers for Tom 8–9 to Tom 57–58 were obtained from the literature (Rajput *et al*. 2006). PCR was conducted in 12-µl volumes containing the following: 1 µl DNA, 50 ng μl^{-1}; 0.75 µl, 10x assay buffer; 1.5 µl, 2.5 mM dNTPs; 0.5 µl, 15 mM $MgCl_2$; 0.25 µl, 3 Units Taq DNA polymerase (Bangalore Genei, Karnataka, India); 1 µl, 10 pmol μl^{-1} each of forward and reverse primers and 7 µl of sterile water. The PCR profile starts with 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 1 min, extension at 72°C for 2 min. A final extension at 72°C for 5 min was included. The PCR products were electrophoresed in a 3% agarose gel with ethidium bromide at 50 V for 2–3 h. The gel was then observed on a UV transilluminator. DNA samples were sent for sequencing to AN Xplorigen Technologies Pvt. Ltd. (New Delhi, India). The sequence obtained was submitted to GenBank. The GenBank accession number after acceptance of sequence is GU930288.

Results

Phytotoxicity tests of culture filtrates and toxin on tomato plants

Treatment with toxin and FCF showed a similar pattern of infection. Cultivar NDT-96 showed tolerance to *A. solani,* but GP-5 showed clear susceptibility to *A. solani.* Significant differences in resistance were identified between the two cultivars (Table 2).

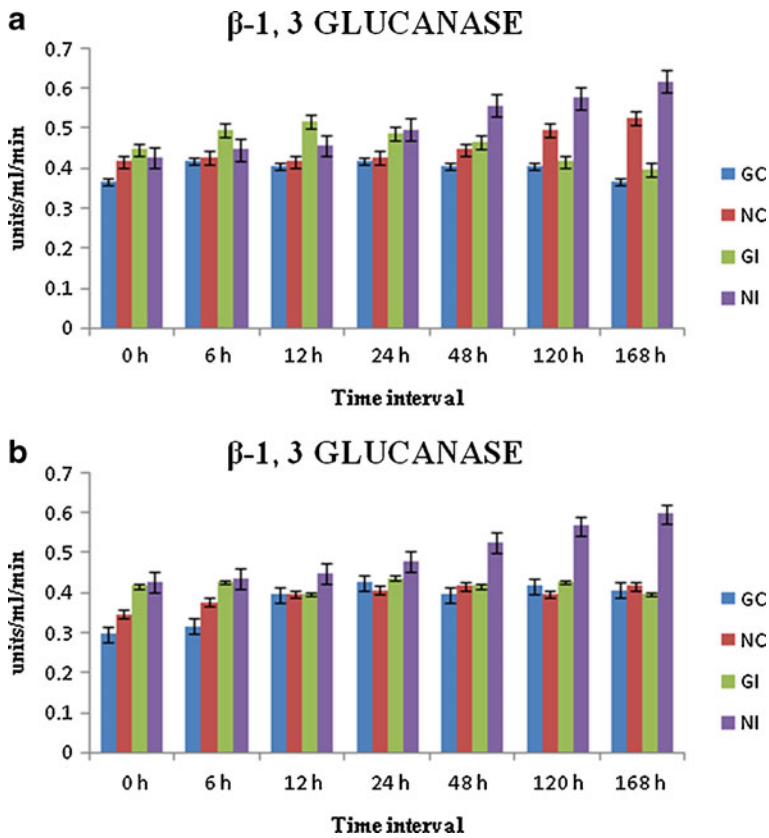

Fig. 4 The enzymatic activity of (**a**) PAL (FCF) and (**b**) PAL (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = P-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

Induction of biochemical defense mechanisms

CAT assay By comparing the treatment of tomato leaves with FCF and with toxin, a similar pattern of induction of defense-related enzymes was observed. Studies of induction of defense mechanism revealed that a high accumulation of CAT was observed in FCF- and toxin-treated GP-5. The levels of CAT increased by 85.97 (t=42.01, P<0.0001) at 168 h (Fig. 1a) with FCF treatment and 84.97 (t=19.31, P<0.0001) (Fig. 1b) with toxin treatment in susceptible cultivar GP-5 at 168 h in response to the said treatments.

POX assay A significant increase in POX activity was observed in FCF- and toxin-treated NDT-96. The levels of POX increased by 7.867 (t=4.667, P< 0.0001) (Fig. 2a) with FCF treatment and by 6.867 (t=1.909, P<0.0001) (Fig. 2b) with toxin treatment in NDT-96 at 168 h.

PAL assay A significant increase in PAL activity was recorded in FCF- and toxin-treated NDT-96 plants. The levels of PAL increased by 93.58 (t=39.48, P< 0.0001) (Fig. 3a) with FCF treatment and by 93.97 (t=37.98, P<0.0001) (Fig. 3b) with toxin treatment in NDT-96 at 168 h.

β-1,3 glucanase assay Treatments of tomato plants with FCF and toxin elicited an increase in β-1,3-glucanase activity. A significant increase in β-1,3-glucanase activity was found: 0.6167 (t=46.67, P< 0.0001) in NDT-96 at 168 h with FCF treatment (Fig. 4a) and 0.5967 (t=42.43, P<0.0001) at 168 h with toxin treatment (Fig. 4b).

PPO assay Upon pathogen infection the activities of PPO increased 3.967 (t=1.909, P<0.0001) at 168 h in NDT-96 with FCF treatment (Fig. 5a), and increased 4.167 (t=1.697, P<0.0001) at 168 h (Fig. 5b) in NDT-96 with toxin treatment.

Chitinase assay Chitinase activity increased rapidly in tomato upon treatment with FCF and with toxin. It increased significantly 7.867 (t=10.18, P<0.0001) at

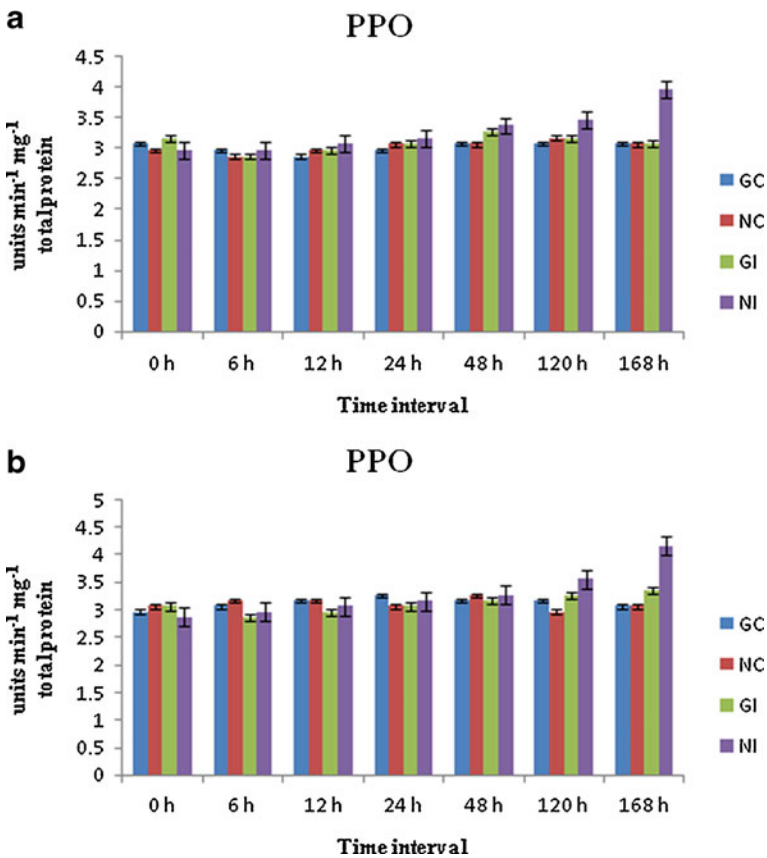

Fig. 5 The enzymatic activity of (**a**) Chitinase (FCF) and (**b**) Chitinase (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

168 h in NDT-96 with FCF treatment (Fig. 6a) and 7.967 (t=6.152, P<0.0001) with toxin treatment (Fig. 6b) in NDT-96. A significantly higher enzyme activity was detected at all sampling days.

Estimation of total phenolic content Phenolic content increased significantly in FCF-and toxin-treated NDT-96. The levels of total phenol increased 94.97 (t= 88.47, P<0.0001) at 168 h in NDT-96 with FCF treatment (Fig. 7a) and 92.87 (t=72.13, P<0.0001) with toxin treatment (Fig. 7b) in NDT-96.

Analysis of total soluble proteins (Figs. 8 and 9) SDS gel (12%) of total soluble protein showed differential expression of PR-proteins in leaves of the tolerant (NDT-96) and susceptible (GP-5) tomato cultivars inoculated with alternaric acid toxin at different periods of inoculation. A specific band of 49.48 kDa was observed to be absent in GP-5 at all time intervals.

Molecular marker analysis (Fig. 10) A 3% PCR agarose gel showed the presence of 108 bp band present in NDT-96.This particular band was absent in GP-5.

Discussion

Reliable and repeatable techniques for large-scale screening are necessary to identify host plant resistance. Techniques have been developed for early blight resistance screening under field, glasshouse, and laboratory conditions. In our studies, in the laboratory as well as under field conditions, FCF and fungal toxin have been used in screening for resistance. Locke (1948) used detached leaflet assays for evaluation of early resistance as a means to circumvent the influence of growth habit, which may affect the reaction of plants in the field or glasshouse. In field tests, large populations can be assessed under normal growing conditions during the whole life cycle of the plants. By both *in vitro* and *in vivo* assays it can be proved that NDT-96 is a tolerant variety and GP-5 is a susceptible variety.

The qualitative estimations of catalase, peroxidase, β-1,3 glucanase, phenylalanine-ammonia-lyase, chitinase and polyphenol-oxidase enzymes indicated the role of these oxidative and hydrolytic enzymes in plant responses towards the pathogen infection.

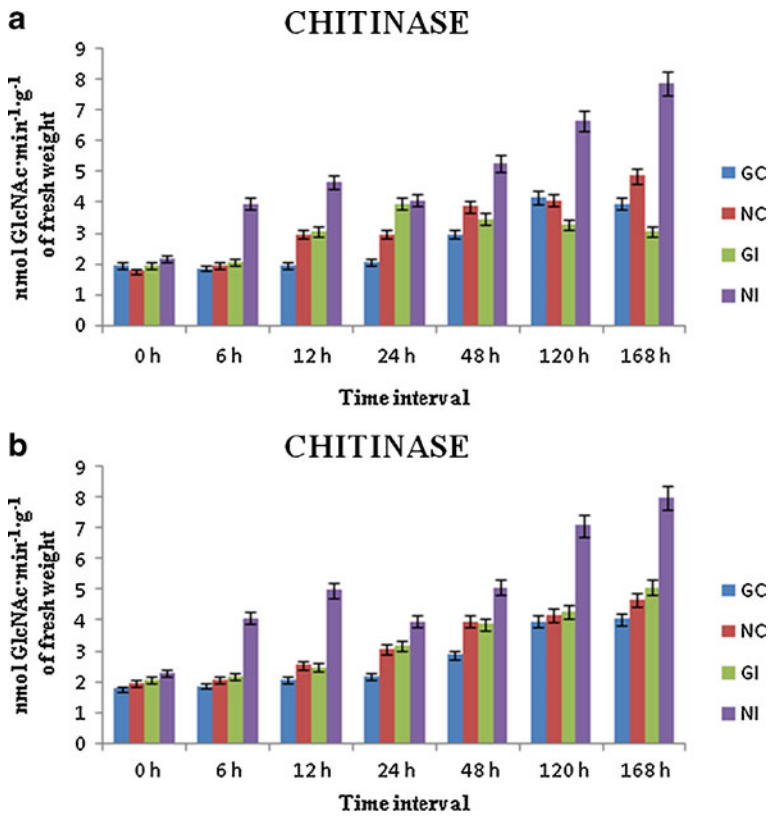

Fig. 6 The enzymatic activity of (**a**) PPO (FCF) and (**b**) PPO (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

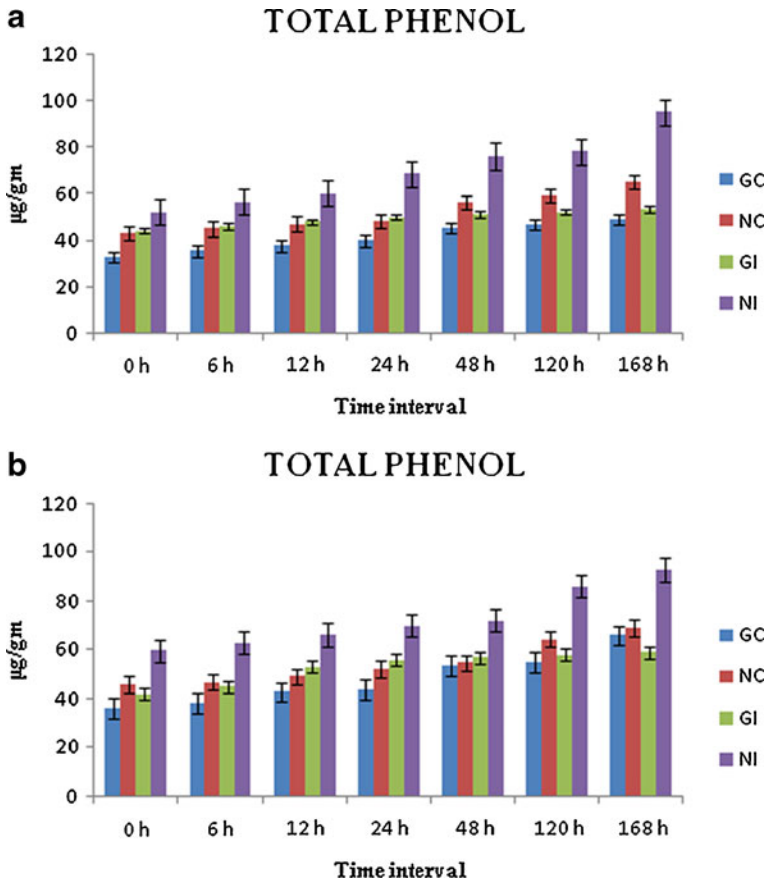

Fig. 7 The enzymatic activity of (**a**) Total phenol (FCF) and (**b**) Total phenol (toxin) was investigated in susceptible (GP-5) and tolerant (NDT-96) tomato genotypes following infection with alternaric acid toxin and fungal culture filtrate (FCF) of *Alternaria solani*. GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. PAL = phenylalanine ammonia-lyase; PPO = polyphenol oxidase. The error bar shows the standard error for the means of three replicates

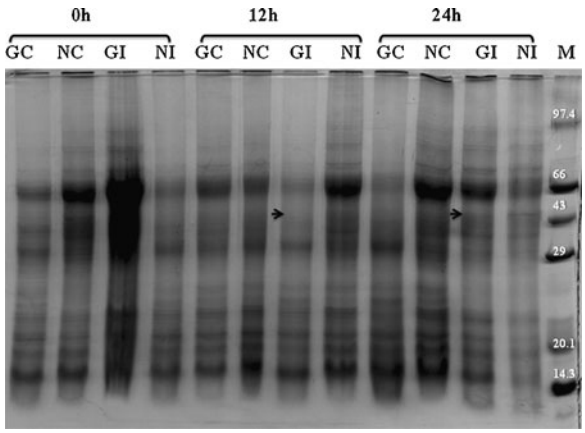

Fig. 8 Polyacrylamide gel electrophoresis (12%) of total soluble protein showing differential expression of PR-proteins in leaves of the tolerant (NDT-96) and susceptible (GP-5) tomato varieties inoculated with alternaric acid toxin at different times. Loaded samples were adjusted to a constant amount of protein (15 µg). M = marker (kDa), GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. Figures 8 and 9 show the time differential expression of total soluble proteins from 0 h to 168 h

Certain quantitative differences in oxidative enzymes in pathogen-infected as well as uninfected plants had occurred. These changes were also prominent between the two varieties of the tomato plants. CAT is an oxygen-scavenging enzyme that removes toxic substrates during development, which are otherwise lethal. Large increases in foliar catalase activity were observed in Algerian-Susceptible but not in Algerian-Resistant barley (*Hordeum vulgare* L.) leaves inoculated with *Blumeria graminis* (Vanacker *et al*. 1998). However, catalases were earlier reported to act as antagonist to the enzyme peroxidase so this could be the reason for the decreased activity of the enzyme in NDT-96. They were also known to be inhibited by the salicylic acid release in the plants due to SAR (Chen *et al*. 1993).

Peroxidase is involved in the production of reactive oxygen species, which are directly toxic to the pathogen or indirectly reduce the spread of the pathogen by increasing the cross-linkage and lignifi-

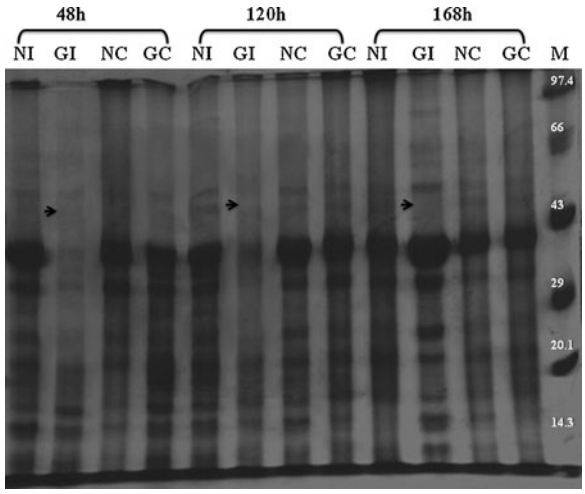

Fig. 9 Polyacrylamide gel electrophoresis (12%) of total soluble protein showing differential expression of PR-proteins in leaves of the tolerant (NDT-96) and susceptible (GP-5) tomato varieties inoculated with alternaric acid toxin at different times. Loaded samples were adjusted to a constant amount of protein (15 μg). M = marker (kDa), GC = GP-5 Control, GI = GP-5 Infected, NC = NDT-96 Control and NI = NDT-96 Infected. Figures 8 and 9 show the time differential expression of total soluble proteins from 0 h to 168 h

cation of the plant cell walls (Hammond-Kosack and Jones 1996). POX is required for the final polymerization of phenolic derivatives into lignin and also involved in suberization or wound healing (Ward *et al*. 1991). Increased POX activity was observed in a number of resistant interactions involving plant-pathogenic fungal and bacterial interactions (Deborah *et al.* 2001; Flott *et al*. 1989; Reimers *et al*. 1992). The results of the present study indicated that POX activity significantly increased in NDT-96 after treatment when compared to GP-5, with both the treatments.

Phenylalanine ammonia-lyase is the key enzyme in the synthesis of the secondary, endogenous signaling molecule salicylic acid, which in turn activates the expression of a variety of PR genes (Mauch-Mani and Slusarenko 1996). Therefore, these results suggest that increased accumulation of PAL with a pathogen could re-establish the notion that in response to invasion by a pathogen, PAL is the first enzyme of phenylpropanoid metabolism and plays a significant role in the regulation of biosynthesis of phenols in plants. Induction of PAL enzyme activity is correlated with increased resistance to pathogenic infection (Bell *et al*. 1984). It was demonstrated that treatment with PAL inhibitors suppressed resistance and increased susceptibility in several host-pathogen interactions. Further, it was demonstrated that reduction of phenylpropanoid metabolism through inhibition of PAL activity in transgenic tobacco also rendered tissues more susceptible to *Cercospora nicotianae* (Maher *et al*. 1994). The results of the present study indicated that PAL activity increased significantly from 0 h after treatment and reached the maximum 168 h after treatment in NDT-96 as compared with GP-5 plants.

β-1,3-glucanases (PR-2 family) and chitinases (PR-3 family) degrade the fungal cell wall and cause lysis of fungal cells. Chitin and glucan oligomers released during degradation of the fungal cell wall act as an elicitor that elicits various defense mechanisms in plants (Frindlender *et al*. 1993). Induction of defense proteins makes the plant resistant to pathogen invasion (Van Loon 1997), and has been correlated with defense against pathogen invasion in tomato (Bashan *et al*. 1985). Chitinases and glucanases probably slow fungal ingress in the plant, as indicated by their inhibition of the *in vitro* growth of *A. solani* (Lawrence *et al*. 1996). Enzyme preparations from resistant lines also induced the *in vitro* release of elicitors of the hypersensitive response from *A. solani*, whereas enzymes from susceptible lines did not (Lawrence *et al*. 2000). The results of the present study indicated that β-1,3-glucanase activity increases in NDT-96 plants. A significant increase in chitinase activity was observed at 168 h in NDT-96 plants.

Polyphenol oxidase is systemically up-regulated in response to *A. solani* infection and is detected in

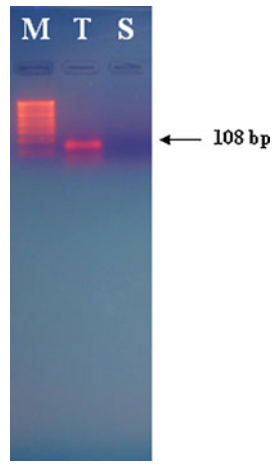

Fig. 10 Polymerase chain reaction gel showing the presence of marker linked to resistance in the tolerant tomato variety. M=100 bp DNA ladder, T = Tolerant variety (NDT-96) and S = Susceptible variety (GP-5)

leaves of upper nodes but not of lower nodes (Thipyapong and Steffens 1997). This induction pattern coincides with the observation of temporary resistance of young leaves to *A. solani* infection (Johanson and Thurston 1990). PPOs catalyze the oxygen-dependent oxidation of *o*-dihydroxyphenols to *o*-quinones, which are more toxic to pathogens than the former. Direct toxicity of quinones against pathogens has also been proposed (Mayer *et al*. 1965). In addition, many studies have shown that PPO is induced in response to mechanical wounding, fungal and bacterial infection, and by treatment with signaling molecules such as jasmonic acid/methyl jasmonate (MeJA), systemin and salicylic acid (Constabel *et al*. 2000; Stewart *et al*. 2001). Systemic induction of PPO expression in response to wounding and pathogens might provide an additional line of defense to protect plants against further attack by pathogen and insects (Stout *et al*. 1999; Thipyapong *et al*. 1995). In the present study, PPO activity increased at 168 h in NDT-96.

The phenolic compounds may contribute to enhancing the mechanical strength of host cell walls and may also inhibit the fungal growth, as phenolics are fungitoxic in nature. Altering the level of phenolic compounds in plants has been demonstrated to change disease susceptibility (Yao *et al.* 1995). Increase in phenolic content in plants has been correlated with increased resistance to pathogens (Velazhahan and Vidhyasekaran 1994). Carrasco *et al*. (1978) showed that treatment of susceptible tomato plants with precursors of phenolic compounds such as quinic acid and phenylalanine increased their phenolic content and their resistance to *Fusarium oxysporum*. However, in the present study, the accumulation of phenolics was higher in the tolerant cultivar than in the susceptible cultivar.

Expressed defense-related genes such as those encoding pathogenesis-related proteins are used as markers for the establishment of SAR (Du and Klessig 1997). PR-proteins are host-coded proteins induced by different types of pathogens and abiotic agents. Synthesis and accumulation of PR proteins have been reported to play an important role in plant disease resistance (Van Loon 1997). In our study, 49.48 kDa protein has been induced by *A. solani* in leaves of a tolerant cultivar, but not of a susceptible cultivar. SSRs provide highly informative markers because they are co-dominant (unlike RAPDs and AFLPs) and generally highly polymorphic (Daugrois *et al*. 1991; Devi and Reddy 2002). Furthermore, the ease and speed of genetic analysis based on SSRs enhance the ability to make a greater number of SSRs available to the scientific community, at least for most of the species of economic value, such as the tomato.

Thus, it can be suggested that there is a correlation between constitutive induced levels of these enzymes and plant resistance. On application of each of these treatments there was an elicitation of array of signals, which helps in turn to increase the levels of the biochemicals that play a role in plant immunity. The induction level of all these enzymes except catalase and total phenols was observed to increase in variety NDT-96 more than in their respective controls. However, a considerable increase of catalase enzyme had been found in variety GP-5. Both FCF and toxin treatments showed a similar pattern of induction of these enzymes as well as total phenols. These findings strongly suggest that alternaric acid induces SAR and is associated with resistance. The result of differential expression of total soluble proteins indicates the possibility of involvement of this particular protein in the defense of tomato against early blight. The presence of the 108 bp band in the tolerant variety may be used as a marker for genomic mapping, variety identification and marker-assisted selection in tomato.

Acknowledgments We are very thankful to Dr. Subhash Patel, Anand Agricultural University, India, for providing the *Alternaria solani* isolate.

References

Barber, J. M. (1980). Catalase and peroxidase in primary leaves during development and senescence. *Journal of Plant Breeding, 97*, 135–144.

Barksdale, T. H. (1971). Field evaluation for tomato early blight resistance. *Plant Disease Reporter, 55*, 807–809.

Bashan, Y., Okon, Y., & Henis, Y. (1985). Peroxidase, polyphenol oxidase and phenols in relation to resistance against *Pseudomonas syringae* pv *tomato* in tomato. *Canadian Journal of Botany, 65*, 366–372.

Bell, J. N., Dixon, R. A., Bailey, J. A., Rowell, P. M., & Lamb, C. J. (1984). Differential induction of chalcone synthase mRNA activity at the onset of phytoalexin accumulation in compatible and incompatible plant–pathogen interactions. *Proceedings of the National Academy of Sciences of the United States of America, 81*, 3384–3388.

Bhatia, I. S., Uppal, D. S., & Bajaj, K. L. (1972). Study of phenolic contents of resistant and susceptible varieties of tomato (*Lycopersicum esculentum*) in relation to early blight disease. *Indian Phytopathology, 25*, 231–235.

Brian, P. W., Elson, G. W., Hemming, H. G., & Wright, J. M. (1952). The phytotoxic properties of alternaric acid in relation to the etiology of plant diseases caused by *Alternaria solani* (Ell. & Mart.) Jones & Grout. *Applied Biology, 39*, 308–321.

Carrasco, A., Boudet, A. M., & Marigo, G. (1978). Enhanced resistance of tomato plants to *Fusarium* by controlled stimulation of their natural phenolic production. *Physiology and Plant Pathology, 12*, 225–232.

Chen, Z., Silva, H., & Klessig, D. F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science, 262*, 1883–1886.

Constabel, C. P., Yip, L., Patton, J. J., & Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar, cloning and expression in response to wounding and herbivory. *Plant Physiology, 124*, 285–295.

Daugrois, J. H., Lafitte, C., Barthe, J. P., & Touze, A. (1991). Induction of β-1,3-glucanase and chitinase activity in compatible and incompatible interactions between *Colletotrichum lindemuthianum* and bean cultivars. *Journal of Phytopathology, 130*, 225–234.

Deborah, S. D., Palaniswami, A., Vidhyasekaran, P., & Velazhahan, R. (2001). Time course study of the induction of defense enzymes, phenolics and lignin in rice in response to infection by pathogen and non-pathogen. *Journal of Plant Diseases and Protection, 108*, 204–216.

Devi, M. C., & Reddy, M. N. (2002). Phenolic acid metabolism of groundnut (*Arachis hypogaea* L.) plants inoculated with VAM fungus and Rhizobium. *Plant Growth Regulators, 37*, 151–156.

Dickerson, D. P., Pascholati, S. F., Hagerman, A. E., Butler, L. G., & Nicholson, R. L. (1984). Phenylalanine ammonia-lyase and hydroxycinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiological Plant Pathology, 25*, 111–123.

Dixon, R. A., Harrison, M. J., & Lamb, C. J. (1994). Early events in the activation of plant defense responses. *Annual Review of Phytopathology, 32*, 479–501.

Doyle, J. J., & Doyle, J. L. (1990). A rapid total DNA preparation procedure for fresh plant tissue. *Focus, 12*, 13–15.

Du, H., & Klessig, D. L. (1997). Systemic acquired resistance in catalase deficient tobacco plant. *Plant Physiology, 10*, 922–925.

Fernandez, A., Solorzano, E., Peteira, B., & Fernandez, E. (1996). Peroxidase induction in tomato leaves with different degrees of susceptibility to *Alternaria solani*. *Revista de Protección Vegetal, 11*, 79–83.

Flott, B. E., Moersehbacher, B. M., & Reisener, H. (1989). Peroxidase isozyme patterns of resistant and susceptible wheat leaves following stem rust infection. *The New Phytologist, 111*, 413–421.

Frindlender, M., Inbar, J., & Chet, I. (1993). Biological control of soil borne plant pathogens by a β-1,3-glucanase producing *Pseudomonas cepacia*. *Soil Biology and Biochemistry, 25*, 1211–1221.

Gaube, C., Dubourg, C., Pawelec, A., Chamont, S., Blancard, D., & Briard, M. (2004). Brûlures foliaires parasitaires de la carotte. *Alternaria dauci* sous surveillance. *PHM Revue Horticulture, 454*, 15–18.

Hammond-Kosack, K. E., & Jones, J. D. G. (1996). Resistance gene-dependent plant defense responses. *The Plant Cell, 8*, 1773–1791.

He, C., Poysa, V., & Yu, K. (2003). Development and characterization of simple sequence repeat (SSR) markers and their use in determining relationships among *Lycopersicon esculentum* cultivars. *Theoretical and Applied Genetics, 106*, 363–373.

Herriot, A. B., Haynes, F. L., Jr., & Shoemaker, P. B. (1986). The heritability of resistance to early blight in diploid potatoes (*Solanum tuberosum* subsp. *phureja* and *stenotonum*). *American Potato Journal, 63*, 229–232.

Jaskani, M. J., Kwon, S. W., Kim, D. H., & Abbas, H. (2006). Seed treatments and orientation affects germination and seedling emergence in tetraploid watermelon. *Pakistan Journal of Botany, 38*, 89–98.

Johanson, A., & Thurston, H. D. (1990). The effect of cultivar maturity on the resistance of potato to early blight caused by *Alternaria solani*. *American Potato Journal, 67*, 615–623.

Jung, W. J., Jin, Y. L., Kim, K. Y., Park, R. D., & Kim, T. H. (2005). Changes in pathogenesis-related proteins in pepper plants with regard to biological control of *Phytophthora* blight with *Paenibacillus illinoisensis*. *Biocontrol, 50*, 165–178.

Keinath, A., DuBose, V. B., & Rathwell, P. J. (1996). Efficacy and economics of three fungicide application schedules for early blight control and yield of fresh-market tomato. *Plant Disease, 80*, 1277–1282.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lawrence, C. B., Joosten, M. H. A. J., & Tuzun, S. (1996). Differential induction of pathogenesis related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. *Physiological and Molecular Plant Pathology, 43*, 361–377.

Lawrence, C. B., Singh, N. P., Qiu, J., Gardner, R. G., & Tuzun, S. (2000). Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solani* and may function as an elicitor release mechanism. *Physiological and Molecular Plant Pathology, 57*, 211–220.

Linthorst, H. J. M. (1991). Pathogenesis-related proteins of plants. *Critical Reviews in Plant Sciences, 10*, 123–150.

Locke, S. B. (1948). A method for measuring resistance to defoliation diseases in tomato and other *Lycopersicon* species. *Phytopathology, 38*, 937–942.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with folin phenol reagent. *The Journal of Biological Chemistry, 193*, 265–275.

Madden, L., Pennypacker, S. P., & MacNab, A. A. (1978). FAST, a forecast system for *Alternaria solani* on tomato. *Phytopathology, 68*, 1354–1358.

Maher, E. A., Bate, N. J., Ni, W., Elkind, Y., Dixon, R. A., & Lamb, C. J. (1994). Increased disease susceptibility of transgenic tobacco plants with suppressed levels of

preformed phenylpropanoid products. *Proceedings of the National Academy of Sciences of the United States of America, 91*, 7802–7806.

Mauch-Mani, B., & Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia lyase in the resistance of Arabidopsis to *Perenospora parasitica*. *The Plant Cell, 8*, 203–212.

Mayer, A. M., Harel, E., & Shaul, R. B. (1965). Assay of catechol oxidase—a critical comparison of methods. *Phytochemistry, 5*, 783–789.

Nash, A. F., & Gardner, R. G. (1988). Tomato early blight resistance in a breeding line derived from *Lycopersicon hirsutum* PI 126445. *Plant Disease, 72*, 206–209.

Neuhoff, V., Arold, N., Taube, D., & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis, 9*, 255–262.

Pan, S. Q., Ye, X. S., & Kuç, J. (1991). Association of β-1,3 glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. *Physiological and Molecular Plant Pathology, 39*, 25–39.

Pound, G. S., & Stahmann, M. A. (1951). The production of toxic material by *Alternaria solani* and its relation to the early blight disease of tomato. *Phytopathology, 41*, 1104–1114.

Powell, W., Machray, G. C., & Provan, J. (1996). Polymorphism revealed by simple sequence repeats. *Trends in Plant Science, 1*, 215–222.

Powell, W., Orozco-Castillo, C., Chalmers, K. J., Provan, J., & Waugh, R. (1995). Polymerase chain reaction-based assays for the characterisation of plant genetic resources. *Electrophoresis, 16*, 1726–1730.

Radhajeyalakshmi, R., Velazhahan, R., Samiyappan, R., & Doraiswamy, S. (2009). Systemic induction of pathogenesis related proteins (PRs) in *Alternaria solani* elicitor sensitized tomato cells as resistance response. *Scientific Research and Essay, 4*, 685–689.

Rafalski, J. A., Vogel, J. M., Morgante, M., Powell, W., Andre, C., & Tingey, S. V. (1996). Generating new DNA markers in plants. In B. Birren & E. Lai (Eds.), *Non-mammalian genomic analysis: A practical guide* (pp. 75–134). New York, NY: Academic.

Rajput, S. G., Wable, K. J., Sharma, K. M., Kubde, P. D., & Mulay, S. A. (2006). Reproducibility testing of RAPD and SSR markers in tomato. *African Journal of Biotechnology, 5*, 108–112.

Rani, P., & Yasur, J. (2009). Physiological changes in groundnut plants induced by pathogenic infection of *Cercosporidium personatum* Deighton. *Allelopathy Journal, 23*, 369–378.

Reimers, P. J., Guo, A., & Leach, J. E. (1992). Increased activity of cationic peroxidase associated with an incompatible interaction between *Xanthomonas oryzae* pv *oryzae* and rice (*Oryza sativa*). *Plant Physiology, 99*, 1044–1050.

Reissig, J. L., Strominger, J. L., & Leloir, L. F. (1959). A modified colorimetric method for the estimation of N-acetyl sugars. *The Journal of Biological Chemistry, 217*, 959–962.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., & Hunt, M. D. (1996). Systemic acquired resistance. *The Plant Cell, 8*, 1809–1819.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Hori, G. T., et al. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science, 239*, 487–491.

Sherf, A. F., & MacNab, A. A. (1986). *Vegetable diseases and their control*. New York, NY: Wiley.

Shtienberg, D., Blachinsky, D., Kremer, Y., Ben-Hador, G., & Dinoor, A. (1995). Integration of genotype and age-related resistance to reduce fungicide use in management of *Alternaria* diseases of cotton and potato. *Phytopathology, 85*, 995–1002.

Siriphanich, J., & Kader, A. A. (1985). Effects of CO_2 on cinnamic acid 4-hydroxylase in relation to phenolic metabolism in lettuce tissue. *Journal of the American Society for Horticultural Science, 110*, 333–335.

Solorzano, E., Fernandez, A., Peteira, B., & Fernandez, E. (1996). Polyphenol oxidases and phenylalanine ammonium lyases induction in tomato leaves infected with *Alternaria solani*. *Revista de Protección Vegetal, 11*, 153–157.

Stewart, R. J., Sawyer, B. J. B., Bucheli, C. S., & Robinson, S. P. (2001). Polyphenol oxidase is induced by chilling and wounding in pineapple. *Australian Journal of Plant Physiology, 28*, 181–191.

Stout, M. J., Fidantsef, A. L., Duffey, S. S., & Bostock, R. M. (1999). Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology, 54*, 115–130.

Sumner, J. B., & Gjessing, E. C. (1943). A method for the determination of peroxidase activity. *Archives of Biochemistry and Biophysics, 2*, 291–293.

Thipyapong, P., Hunt, M. D., & Steffens, J. C. (1995). Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry, 40*, 673–676.

Thipyapong, P., & Steffens, J. C. (1997). Tomato polyphenol oxidase: differential response of the polyphenol oxidase F promoter to injuries and wound signals. *Plant Physiology, 115*, 409–418.

Vanacker, H., Carver, T. L. W., & Foyer, C. H. (1998). Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiology, 117*, 1103–1114.

Van Loon, L. C. (1997). Induced resistance in plants and the role of pathogenesis related proteins. *European Journal of Plant Pathology, 103*, 753–765.

Velazhahan, R., & Vidhyasekaran, P. (1994). Role of phenolic compounds, peroxidase and polyphenol oxidase in resistance of groundnut to rust. *Acta Phytopathologica et Entomologica Hungarica, 29*, 23–29.

Wang, Y., Tang, X., Cheng, Z., Mueller, L., Giovannoni, J., & Tanksley, S. D. (2006). Euchromatin and pericentromeric heterochromatin: comparative composition in the tomato genome. *Genetics, 172*, 2529–2540.

Ward, E., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., et al. (1991).

Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell, 3*, 1085–1094.

Yao, K., De Luca, V., & Brisson, N. (1995). Creation of a metabolic sink for tryptophan alters the phenyl propanoid pathway and the susceptibility of potato to *Phytophthora infestans*. *The Plant Cell, 7*, 1787–1799.

Zieslin, N., & Ben-Zaken, R. (1993). Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. *Plant Physiology and Biochemistry, 31*, 333–339.