RESEARCH

Overexpression of *Camellia sinensis* Thaumatin-Like Protein, *CsTLP* in Potato Confers Enhanced Resistance to *Macrophomina phaseolina* and *Phytophthora infestans* Infection

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Abstract Thaumatin-like proteins (TLPs), a class of pathogenesis related proteins are induced in response to pathogens and exhibit antifungal property when overexpressed in transgenic plants. In the present study, we have raised transgenic potato plants overexpressing TLP gene of *Camellia sinensis* (*CsTLP*). Fungal resistance assays of transgenic potato elucidated the potential role of *CsTLP* in imparting tolerance to fungal pathogens, *Macrophomina phaseolina* (necrotrophic) and *Phytophthora infestans* (hemi-biotrophic). Transgenic tubers with higher resistance to *M. phaseolina*, showed a concomitant and significant increase in transcripts of *StPAL*, *StLOX*, and *StTLP* genes involved in phenylpropanoid, lipoxygenase, and general defense response pathway, respectively after infection. Importantly, leaves of CsTLP transgenic lines inoculated with *P. infestans* spores under in vitro conditions also showed a resistant phenotype. The resistant phenotype recorded for the two important fungal pathogens by *CsTLP* transgenic potato plants is remarkable, since no effective control methods and no resistant cv. against *M. phaseolina* has been identified so far in potato.

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Abbreviations

PR	Pathogenesis related
TLP	Thaumatin-like protein
PAL	Phenyl-alanine ammonia lyase
LOX	Lipoxygenase
CsTLP	*Camellia sinensis* thaumatin-like protein

Introduction

Potato (*Solanum tuberosum* L.) is an important cash crop of the world and is exposed to various bacterial and fungal diseases throughout its life cycle, causing huge losses all over the world. In some regions, the reduction in crop yield and quality due to fungal infections is between 30 and 40 % of the potential yield. Some of the important fungal diseases, such as late blight and charcoal rot, caused by *Phytophthora infestans* and *Macrophomina phaseolina*, respectively, are of primary concern. Recent changes in the population structure of the late blight fungus have led to the advent of new races that are more aggressive and resistant to previously effective fungicides [1]. *M. phaseolina* causes severe potato yield losses in hot and dry years which are predicted to become more frequent due to global warming. Since, conventional breeding for disease resistance is a problem due to the tetraploid nature of the potato crop, presently chemical control is an alternative to the growers. However, considering the adverse impact of pesticides on environment or/and resistance gained by the pathogens against these pesticides, plant genetic engineering opens

possibilities for alternative strategies to control plant diseases [2].

Upon pathogen infection, plants protect themselves by generating various defense responses, such as accumulation of phenylpropanoid compounds [3–5], oxylipin biosynthesis [6, 7], and induction of antimicrobial proteins [8]. Induction of majority of these components is mediated through activation of phenylpropanoid, lipoxygenase (LOX) pathway, and deployment of pathogenesis-related (PR) proteins. Phenyl-alanine ammonia lyase (PAL) is the key enzyme of the phenylpropanoid pathway and is involved in the synthesis of phenylpropanoid compounds viz. flavonoids, iso-flavonoids, and stilbenes that serve as phytoalexins [9–11]. While the LOX pathway begins with α-linoleic acid and LOX is the key enzyme for this pathway and is involved in the synthesis of oxylipins [12, 13]. It has been demonstrated that PAL and LOX genes are induced in response to fungal attack or elicitors and their expression is positively and significantly correlated with enzyme levels and also the end products of their respective pathways [14–16]. Fungitoxic action of these phenylpropanoid compounds [17, 18] and oxylipins [19] impart resistance toward ingress of the pathogen. Constitutive expression of thaumatin-like proteins (TLPs) is typically absent in healthy plants but is induced exclusively in response to wounding or pathogenic attack [20]. Altogether, several lines of evidences revealed that either elicitor or fungal infection stimulates, individually or collectively, the transcription of *TLP*, *LOX*, and *PAL* in plants [21–26].

The susceptibility or resistance toward pathogens, is often determined by the speed and magnitude with which defense related genes are activated and expressed and also by their effectiveness against individual pathogen with different modes of attack. Over the past decades, large number of reports exists on the development of genetically engineered plants for disease resistance by overexpressing PR-proteins. These proteins are of particular interest because they are involved in plant's defense system [27–29]. Several PR-proteins belonging to the group 5 (PR5) share homology with thaumatin, sweet protein, originally identified from the fruits of the West African shrub, *Thaumatococcus daniellii* (Benett) Benth [30], and hence are called thaumatin-like proteins (TLPs). TLPs have been purified from Kewlin chestnut seeds [31], edible fruits [32], *Cynanchum komarovii* [33] and known to be inhibitory to fungal pathogens. The protection provided by the constitutive expression of TLP gene from different plant sources is remarkably strong. For example, Liu et al. [34] and Zhu et al. [35] reported that the expression of a tobacco TLP gene in transgenic potato plants caused a substantial increase in resistance to *P. infestans*. Constitutive expression of rice TLP gene in transgenic tobacco [36], rice [37], and wheat [38, 39] have shown the enhanced resistance against *Alternaria alternata*, *Rhizoctonia solani*, and *Fusarium graminearum*, respectively, indicating its antifungal activity against a variety of fungal pathogens. The mechanism of action associated with TLP is through inhibition of hyphal growth and reduction of spore germination, probably by a membrane permeabilization mechanism and/or by interaction with pathogen receptors [40]. Recent studies illustrated that besides antifungal activity, this class of PR-proteins is also involved in improving seed germination [41], senescence [42], increased H^+-ATPase activity [43], and abiotic stress tolerance [44].

In the present study, we overexpressed *Camellia sinensis* TLP gene (*CsTLP*) in potato and studied the potential of *CsTLP* in conferring resistance against two fungal pathogens which differ in life style from each other viz. *M. phaseolina* (necrotrophic) and *P. infestans* (hemi-biotrophic). In addition, using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) approach, we studied the defense response in transgenic and un-transformed wild type (WT) potato plant tubers at the molecular level after infection with *M. phaseolina* and analyzed whether the nature of defense response launched by *CsTLP* transgenic plants was different from their counterpart WT plant tubers. The results obtained suggested that *CsTLP* transgenic tubers, in addition to having an antimicrobial shield provided by the CsTLP are at a heightened level of defense as manifested by increase in the relative expression ratio of *StPAL* and *StLOX* after infection with *M. phaseolina*.

Materials and Methods

Plasmid Construction and Plant Transformation

Full length thaumatin-like protein cDNA was cloned from *C. sinensis* plant (here onwards termed as *CsTLP*) from Western Himalaya. Coding nucleotide sequence was amplified using forward primer (5′-cca tgg ATG GCA AAG GGC GTT GCT G-3′), and reverse primer (5′-tct aga TCC TTG AAG GCC AAT AAT ACC-3′) containing *Nco*I and *Spe*I restriction sites, respectively. PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and 72 °C for 7 min. PCR product was cloned into a cloning vector pGEM-T easy (Promega). The cloned cDNA sequence was confirmed by nucleotide sequence analysis. The cloned cDNA was isolated by digesting with the restriction enzymes *Nco*I and *Spe*I, and purified with gel extraction method (Qiagen). The cDNA was sub cloned into binary vector pCAMBIA1302 under the cauliflower mosaic virus 35S promoter. The resulting

plasmid construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 by tri-parental mating technique [45] and used for plant transformation.

Potato (*S. tuberosum* L. cv. Kufri Giriraj) shoot cultures were grown in vitro on MS basal medium [46] containing 2.0 % sucrose and 0.8 % agar at 24 ± 2 °C and 16-h photoperiod. Internodal explants were incubated for 30 min in a saturated culture of *A. tumefaciens* harboring *CsTLP* construct, blotted dry on sterile Whatman paper, and co-cultivated in solid MS medium for 48 h under dark at 25 °C. Thereafter, explants were subcultured onto selection and regeneration medium (PSR) containing NAA (0.01 mg/l), zeatin (0.25 mg/l), cefotaxime (500 mg/l), and hygromycin (20 mg/l) and incubated for three weeks under similar conditions. To make the selection procedure more stringent, hygromycin resistant shoots were excised and further shifted to MS medium supplemented with hygromycin (20 mg/l). As a control, un-transformed WT potato plants were transferred to the same medium. For each transformation, four independent transgenic plants were regenerated.

Molecular Characterization of Transgenic Plants

Genomic DNA Isolation and PCR Analysis

Genomic DNA was isolated from the leaves of 3–4 week old putative transgenic and WT control plants [47]. In order to confirm the presence of *CsTLP* in transgenic plants, PCR was performed using genomic DNA as template and the *CsTLP* -specific (F) 5′-GCA CTG GAG ACA ACT TTA AT-3′ and (R), 5′-GTG ACT ATC TTG GTT GTA ATC C-3′ primers. A 25 μl PCR mixture contained 1× reaction buffer, 0.2 mM nucleotide mix, 1 μM *CsTLP*(F) and *CsTLP*(R) primers, 1 U *Taq* DNA polymerase, and 1 μg of template DNA. Amplification was performed in a thermal cycler programed for one cycle of 3 min at 94 °C followed by 35 cycles (1 min at 94 °C, 45 s at 55 °C, 2 min at 72 °C) and finally an extension cycle of 10 min at 72 °C. PCR products were visualised by electrophoresis on 1.0 % agarose gel containing 1 μg/ml ethidium bromide.

Southern Analysis

The genomic DNA (30 μg) samples of WT and *CsTLP* transgenic plants digested with *Hin*dIII (Fermentas Inc.) were electrophoresed on 0.8 % agarose gel followed by DNA denaturation and transfer to nylon membrane (Hybond N⁺, Amersham, UK) by capillary blotting. Probe of *CsTLP* was labeled using Biotin DecaLabel™ DNA labeling kit (Fermentas Inc.). Hybridization was carried out overnight at 65 °C. Detection procedure was followed as provided in Biotin Chromogenic detection kit (Fermentas Inc.).

Expression Analysis of Transgenic Plants Using Semi-Quantitative RT-PCR

Total RNA was isolated from 100 mg of frozen potato leaves (WT and *CsTLP* transgenic plants) using *iRIS* solution following the method of Ghawana et al. [48]. The 2 μg RNA was treated with 2 U amplification grade DNase I (Invitrogen, Carlsbad, CA) and the cDNA was synthesized in the presence of 400 U reverse transcriptase Superscript II (Invitrogen) and 1.0 μg oligo (dT)12–18 [49]. The resultant cDNA was used as a template for PCR using *CsTLP* -specific (forward) 5′-ATG AGC TTC CCC AAA AGC CT-3′ and (reverse) 5′-TCA AGG GCA GAA GGT AAT AGC-3′ primers. The cycling conditions were optimized to obtain amplification in the exponential phase as, 1 cycle of 94 °C for 3 min; 28 cycles (94 °C for 30 s, 54 °C for 40 s, and 72 °C for 30 s), and 1 cycle of 72 °C for 7 min. The expression of *26S rRNA* gene was used as an internal control for expression studies [49]. PCR amplified products were visualised by electrophoresis on 1.0 % agarose gel containing 1 μg/ml ethidium bromide.

Field Transfer of Transgenic and WT Seedlings and Identification of Fungal Pathogen Infecting Tubers

Selected transgenic lines (TL1 and TL2) along with the WT were hardened and planted in plastic pots (30 cm × 30 cm) containing sandy clay loam soil in contained polyhouses (photoperiod 14 h, mean air temperature 24 °C, RH 65 %, PAR 500 μmol/m²/s and atmospheric pressure 87.6 kPa) at Palampur (1,300 m a.s.l., 32°6′N, 76°33′E). At the time of tuber harvesting, it was observed that a greater percentage of WT tubers was infected with fungus. To identify the fungal pathogen, the tubers were surface sterilized (0.01 % HgCl₂ and 70 % Ethanol) and excised into slices using sterile surgical blade. Pith of infected and healthy tubers was placed onto potato dextrose agar (PDA) in triplicates and incubated at 28 °C. Fungal strain was identified on the basis of ITS region sequencing. For this, mycelium from 5-day-old colony was scarped from the petriplate, frozen in liquid nitrogen, and ground to fine powder. Genomic DNA was isolated using Qiagen Plant DNeasy Kit (Qiagen Gmb H, Hiden). The amplification of ITS 1, 5.8S ribosomal RNA gene and ITS 2 was achieved using forward primer ITS 1: 5′-TCC GTA GGT GAA CCT GCG G-3′ and reverse primer ITS 4: 5′-GCT GCG TTC ATC GAT GC-3′ [50] and cloned into pGEM-T easy vector (Promega, Madison) followed by transformation in *E. coli* DH5α cells. The nucleotide sequence of the cloned DNA fragment was confirmed using M13F/M13R

primers through ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA). The sequence was analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm and aligned to the nearest neighbors [51]. The phylogenetic tree was constructed using the MEGA4 software package after aligning the sequences with ClustalW [52] and generating evolutionary distance matrix, inferred by the neighbor-joining method using Kimura's two-parameter model [53].

Validation of Charcoal Rot Tolerance of *CsTLP* Transgenic Plants

Tuber Bioassay

Randomly selected healthy and uniform tubers of WT and *CsTLP* transgenic plants (TL1 and TL2) were washed thoroughly under tap water without any bruise. Under sterile conditions, the tubers were swabbed gently with 70 % ethanol followed by rinsing in autoclaved distilled water and finally air dried. Mycelial mat of *M. phaseolina* culture grown in PDA medium at exponential phase was sliced into pieces of equal size (3 × 5 mm). Tubers of WT, TL1, and TL2 plants were punctured (6 mm long and 5 mm deep) using sharp surgical blade and inoculum was inserted into the periderm. Punctured surface of tubers was sealed by pressing the excised portion transversely followed by coating of molten wax (40 °C). Tubers inoculated with sterile PDA medium were used as reference for experimental procedure (C1). To provide a reference for the healthy tuber skin, tubers of WT, TL1, and TL2 were left intact throughout out the experiment and referred as non-inoculated (C2). All the tubers were incubated at 25 °C for 3 weeks. After 3 weeks, inoculated tubers (WT, TL1, and TL2) along with their respective controls (C1 and C2) were sliced into two sections through the middle and photographed to demonstrate inner surface damage caused by *M. phaseolina*. Damage to the sprouts/lateral eyes and discoloration of tubers was also recorded. The experiment was repeated three times over two season crops, consisting of five WT and *CsTLP* transgenic tubers for each replicate.

Effect of Leaf Extract from Transgenic Plants on *M. phaseolina* Mycelial Growth

Leaf tissues (1 g each) from WT and *CsTLP* transgenic (TL1 and TL2) plants growing under contained polyhouse were harvested, crushed in sodium phosphate buffer (pH 7.0) and incubated at room temperature for 30 min [44]. The leaf extract was centrifuged at 1,700×g and the supernatant was filter sterilized (FS). Individually, 1 ml of FS leaf extract of WT, TL1 and TL2 was added to 100 ml PDA after cooling the medium to about 45 °C followed by

pouring equal volume (20 ml) into five petriplates (90 mm) and referred as WTLE, TL1LE and TL2LE, respectively. As a control 100 ml PDA medium was poured into five petriplates. For bioassay controls, same volume (1 ml) of phosphate buffer, boiled crude protein extract of leaves of WT and *CsTLP* transgenic plants were added to the PDA medium as mentioned above and referred as CB, WTC, TL1C and TL2C, respectively. The mycelial disks of *M. phaseolina* were inoculated in the center of all the controls and leaf extract-amended PDA medium. Petriplates were incubated at 28 °C for 72 h, growth zone diameter of the fungal colony in centimeters (cm) was measured and photographed. The experiment was repeated three times with five replicates for each control and treatment.

Qualitative Analysis of *P. infestans* Infection in *CsTLP* Transgenic Potato Leaves

For this experiment, leaf tissues were collected from *CsTLP* and WT potato plants grown for 6 weeks in growth chambers set for 16 h photoperiod, 25/20 °C day/night temperature cycles, and light intensity (500 μmol/m^2/s). *P. infestans* race 1.2.3.4.5.7.8.9.10.11 was kindly provided by Dr. S.K. Chakorborty (Division of Crop Protection, CPRI, Shimla) and grown on Rye agar-A medium and to maintain the pathogenicity *P. infestans* was cultured on tuber slices of late blight susceptible potato variety Kufri Chandramukhi (KCM) at 18 °C (RH > 90 %). A zoospore suspension of the *P. infestans* was prepared from the cultures grown on rye agar plates for 14 days in the dark at 18 °C. Sporangia were harvested from the petridishes by rinsing the mycelia in cold (4 °C) sterile, distilled water and scrapping the mycelial mat using sterile glass rod. The suspension was incubated at 4 °C for 1 h to release zoospores. The concentration was adjusted to about 1 × 10^5 zoospores/ml based on the haemocytometer readings. Inoculum (25 μl) was placed in the center of abaxial surface of detached leaflets of TL1, TL2, and WT plants. The inoculated leaves were kept in controlled-environment chambers (temperature 18 °C and RH > 90 %) and the degree of infection was analysed visually by the appearance of *P. infestans* induced necrotic lesion at 2, 5, and 10 days post inoculation (dpi).

Defense-Related Gene Expression in the *CsTLP* Transgenic Potato Tubers upon *M. phaseolina* Infection

Activation of defense response in terms of transcript accumulation of defense response phenylpropanoid pathway gene (*StPAL*), LOX pathway gene (*StLOX*), and TLP gene (*StTLP*) in *M. phaseolina* inoculated WT, TL1, and TL2 tubers was monitored by qRT-PCR assay in a time course experiment. Surface sterilized healthy tubers of WT, TL1, and TL2 plants were sliced into 1 cm thick sections

Table 1 Primer sequences used in performing the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Gene	Accession number	Primer sequence (5′–3′)
StUb (ubiquitin)	EU294350	F: CGC CAC TGA TTT CTC TCC TC
		R: AAA TCA AAC GCT GCT GGT C
StLOX (LOX)	AF039651	F: CTG AAT TAC ATG CCC TGT TTG
		R: AAG GTC CTT GAG GAA GTT TTG A
StPAL (phenylalanine ammonia-lyase)	X63103	F: ATT GTG AAA CTT TGG GGT GA
		R: CAC CAG CTC TTG CAC TTT C
StTLP (thaumatin-like protein)	AY737315	F: TGC TCT AAA TCA GCC CAA TAA
		R: GGT GCG TTG CAT AAG AGA TTA
CsTLP (*C. sinensis* thaumatin-like protein)	DQ444296	F: CAA GTT CTT CAA GGA TAG GTG
		R: CAA GGG CAG AAG GTA ATA GC

using sterile blade and uniform size *M. phaseolina* culture bits were placed in the center of all the tuber slices. As a control, tubers of WT, TL1, and TL2 were mock inoculated with sterile PDA medium. Petriplates were sealed with parafilm and incubated at 25 °C and samples (50 mg tuber tissue) were harvested and frozen in liquid nitrogen and stored at −80 °C until used. Total RNA was isolated and cDNA was synthesized from 2 μg of DNA–free RNA (as described above). The final concentration and quality of cDNA was determined spectrophotometrically (Nano Drop). Gene-specific primers were designed based on the potato nucleotidic sequences published in NCBI data base, using web-based primer picking service Primer3 ver. 0.4.0 [54] (Table 1). The PCR reactions were performed with an MX 3000P Real-Time PCR system (Stratagene). The reactions contained 5 μl of 2× Brilliant SYBR Green QPCR master mix (Stratagene), 2.5 μl cDNA (diluted 1:10), 0.5 μl gene-specific forward and reverse primers (5 pM), in a final volume of 10 μl. The following thermal profile was used: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and final extension at 72 °C for 5 min. A melting curve was performed for each reaction to determine the specificity of the reaction. Reactions were set up in triplicate, including a control with no template (NTC). Ct values were determined as the numbers of amplification cycles needed to reach a fixed threshold in the exponential growth region of the amplification curve. To normalize the variance in cDNA input, *S. tuberosum* ubiquitin gene (*StUb*) was used as an internal control in each case [55]. The relative expression ratio at 2, 4, and 8 dpi in WT and *CsTLP* transgenics (TL1 and TL2) was calculated with respect to mock inoculated control using REST 2009 software [56]. To study the role of *CsTLP* overexpression on the transcriptional changes in intrinsic potato defense response genes, relative expression ratio was determined in TL1 and TL2 tubers before and after inoculation (8 dpi) with *M. phaseolina* relative to WT tubers before and after inoculation, respectively. Mean

values and standard error were obtained from three technical and two biological replicates.

Statistical Analysis

For experiment to study effect of leaf extract on *M. phaseolina* mycelia growth (shown in Fig. 4), statistical significance between the mean values were assessed through one-way analysis of variance (ANOVA) applying Duncan's multiple range test (DMRT) using STATISTICA software program version 7. A probability of $P < 0.05$ was considered significant.

Results

Molecular Characterization of Transgenic Potato Plants

The potato cv. Kufri Giriraj was used for generating transgenics. Figure 1a shows the schematic of construct used for the transformation experiment. A total of four independently derived, putative transgenic potato lines were selected based on their ability to grow and root on hygromycin supplemented MS medium. While, un-transformed WT plants (control) failed to grow on the same medium (Fig. 1b). Transgenic lines were referred as ″TL″ for the *CsTLP* gene, and each number represented an independent transgenic line. PCR analysis revealed amplification of 681 bp corresponding to the complete CDS of the *CsTLP* in all the four *CsTLP* transgenic lines (Fig. 1c). Southern blot analysis revealed stable integration of the transgene in all four transgenic lines (Fig. 1d). However, single copy insertion was observed only in line TL1 and TL2. RT-PCR analysis showed amplification of expected fragment (681 bp) from cDNA of transgenic lines, while no amplification was observed from cDNA of WT plant (Fig. 1e, upper panel). An uniform expression of *26S rRNA* was recorded in WT and all the *CsTLP* transgenic lines (Fig. 1e, lower panel). This observation indicates that transgene, *CsTLP* was expressed in transgenic lines. On the
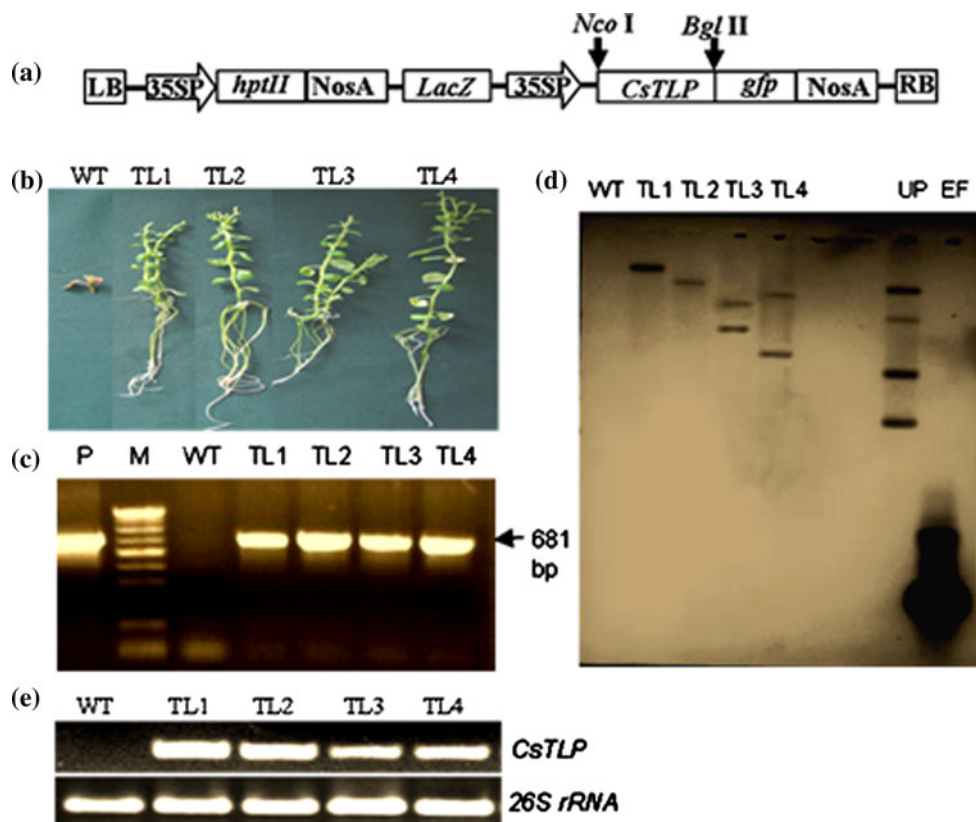
Fig. 1 Overexpression of *CsTLP* in transgenic potato plants. **a** Schematic representation of the T-DNA region harboring *C. sinensis TLP* (*CsTLP*) used for genetic transformation. LB, *left border*; RB, *right border*; 35SP, CaMV 35S promoter; NosA, nopaline synthase 3′ UTR; *gfp*, green fluorescent protein as a reporter gene; *hptII*, hygromycin phosphotransferase gene as a plant selection marker gene. **b** Putative *CsTLP* transgenic potato lines selected on the basis of their ability to grow and root on hygromycin supplemented MS medium. **c** PCR analysis of genomic DNA from putative *CsTLP* transgenic and WT potato plants. P, plasmid DNA; M, 100 bp ladder; WT, DNA from un-transformed potato plant; TL1–TL4, DNA from putative transgenic plants. **d** Southern blot analysis of genomic DNA digested with *Hin*dIII and probed with biotin labeled *CsTLP* eluted PCR product. WT, un-transformed potato plants; TL1–TL4, *CsTLP* transgenic potato lines; UP, un-cut plasmid harboring pCAMBIA *CsTLP*; EF, eluted gene -specific *CsTLP* fragment. **e** Semi-quantitative reverse transcriptase PCR (RT-PCR) analysis. *Upper panel* RT-PCR analysis of RNA isolated from leaf tissue of WT and transgenic potato plants using *CsTLP* gene -specific primers; *lower panel* RT-PCR analysis of same RNA using *26S rRNA* primers. WT, un-transformed potato plants; TL1–TL4, *CsTLP* transgenic potato plants

basis of southern analysis and RT-PCR we continued the subsequent characterization with TL1 and TL2 lines.

Molecular Identification of Fungal Pathogen Causing Tuber Rot

CsTLP transgenic plants (TL1 and TL2) and WT plants grown under contained conditions were in-distinguishable from each other in terms of plant height, leaf shape, and maturity time and tuber size (data not shown). However, at the time of tuber harvesting, it was observed that approximately 30 % tubers of WT plants were infected with unknown fungal pathogen, while TL1 and TL2 tubers remained un-infected. This interesting observation prompted us to characterize this fungal pathogen. Pure culture of the fungus was raised and maintained on PDA medium. A single band of ∼600 bp was obtained on amplification of the Internal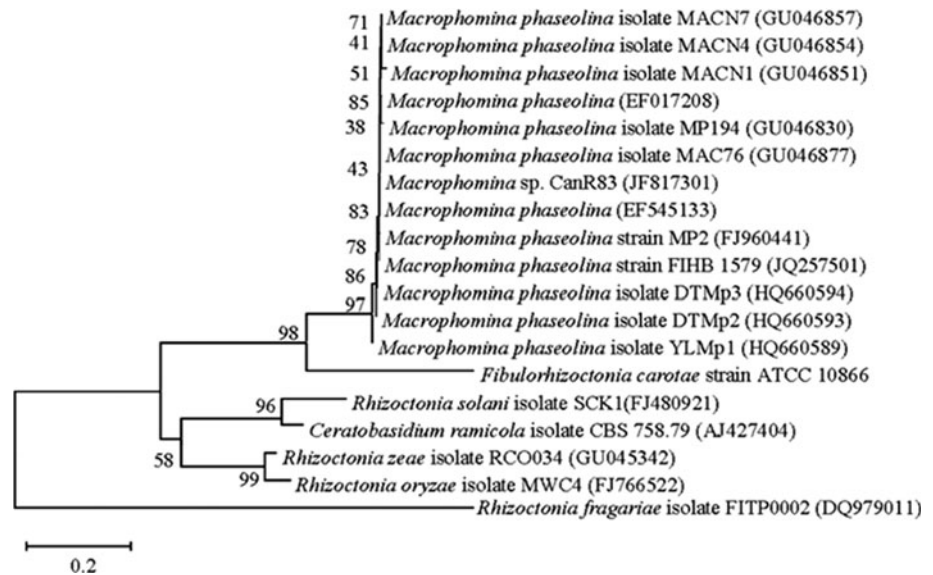 Transcribed Spacer (ITS) region of the fungal isolate. BLASTn analysis of 583 bp ITS region sequence of the fungus showed 100 % identity with *M. phaseolina* that is responsible for causing charcoal rot in potato, soybean, cotton, and maize (data not shown). The phylogenetic tree constructed with the MEGA4 software is shown in Fig. 2. As illustrated in phylogenetic tree, the strain FIHB 1579 was grouped with reference strains of *M. phaseolina* reported for causing charcoal rot in potato. The sequence was deposited in GenBank (Accession No. JQ257501).

Protection of *CsTLP* Transgenic Plants Against *M. phaseolina*

Tuber Bioassay

Tuber bioassay revealed that WT tubers challenged with *M. phaseolina* inoculum were severely infected all along the excision; right from periderm to the pith of the tuber

Fig. 2 Phylogenetic tree showing relationship among *M. phaseolina* strain FIHB 1579 and representatives of some related taxa, based on ITS region sequence. The numbers on the nodes indicate how often (number of times, %) the species to the right are grouped together in 1000 bootstrap samples. *Bar* 0.2 substitution per site


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71  Macrophomina phaseolina isolate MACN7 (GU046857)
41  Macrophomina phaseolina isolate MACN4 (GU046854)
51  Macrophomina phaseolina isolate MACN1 (GU046851)
85  Macrophomina phaseolina (EF017208)
38  Macrophomina phaseolina isolate MP194 (GU046830)
43  Macrophomina phaseolina isolate MAC76 (GU046877)
    Macrophomina sp. CanR83 (JF817301)
83  Macrophomina phaseolina (EF545133)
78  Macrophomina phaseolina strain MP2 (FJ960441)
86  Macrophomina phaseolina strain FIHB 1579 (JQ257501)
97  Macrophomina phaseolina isolate DTMp3 (HQ660594)
    Macrophomina phaseolina isolate DTMp2 (HQ660593)
    Macrophomina phaseolina isolate YLMp1 (HQ660589)
98  Fibulorhizoctonia carotae strain ATCC 10866
96  Rhizoctonia solani isolate SCK1(FJ480921)
    Ceratobasidium ramicola isolate CBS 758.79 (AJ427404)
58  Rhizoctonia zeae isolate RCO034 (GU045342)
99  Rhizoctonia oryzae isolate MWC4 (FJ766522)
    Rhizoctonia fragariae isolate FITP0002 (DQ979011)
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0.2

Fig. 3 Tuber bioassay for resistance against charcoal rot caused by *M. phaseolina*, in WT and *CsTLP* transgenic potato tubers. In the photograph, **a** health of tubers at the beginning of the experiment i.e., on day 0. **b** Physical condition of the excised tubers after 3 weeks of *M. phaseolina* inoculation. C1, tuber skin control; C2, assay control; IN, tuber inoculated with *M. phaseolina*; WT, un-transformed potato; TL1, *CsTLP* transgenic line 1; TL2, *CsTLP* transgenic line 2. *Arrow* indicates the ingress or and damage caused by the *M. phaseolina*. Necrotized lateral eyes (%) and discoloration of tuber pith (%) are shown in Table 2

(Fig. 3, lower panel; WT/IN). *M. phaseolina* infection caused 71.6 % lateral eye necrotization and 80 % tuber discoloration in case of WT plants (Table 2). In contrast, TL1 tubers showed resistance phenotype against *M. phaseolina* with absolutely no lateral eye necrotization or tuber discoloration (Fig. 3, lower panel; TL1/IN; Table 2). However, TL2 tubers were infected with *M. phaseolina* with 45 % lateral eye necrotization and 40 % tuber discoloration, which is relatively lower than infected WT tubers (Fig. 3, lower panel; TL2/IN; Table 2). No infection was observed in any of the non-inoculated and mock inoculated tubers of WT and *CsTLP* transgenics as shown in Fig. 3 lower panel (WT/C1, WT/C2, TL1/C1, TL1/C2, TL2/C1, TL2/C2). The experiment was repeated three times over two season crops and consistent results were found.

Effect of Leaf Extract from *CsTLP* Transgenic Plants on the Growth of *M. phaseolina*

Figure 4a, b show results of the experiment performed to compare the antifungal activity of crude leaf extracts of WT and transgenic lines. The leaf extracts from both, TL1 (TL1LE) and TL2 (TL2LE) inhibited the growth of mycelia with mean zone diameter (MZD) of 2.8 ± 0.1 and 3.2 ± 0.1, respectively. Although leaf extract from WT (WTLE) also inhibited the mycelial growth (MZD 4.5 ± 0.2), but the inhibition was lower than that of TL1

Table 2 Evaluation of *CsTLP* transgenic and un-transformed (WT) tubers after 3 weeks of inoculation with *M. phaseolina*

Plants	Necrotized lateral eyes (%)[a]	Discoloration in tuber pith[b]
WT	71.6 ± 8.3	++++
TL1	0.0	−
TL2	45.0 ± 5.1	++

Data represent mean ± SE; mean obtained by averaging response of five tubers each of WT, TL1 and TL2 plants

[a] Necrotized lateral eyes (%) = (number of necrotized lateral eyes/total number of lateral eyes) × 100

[b] Degree of discoloration was scored in *M. phaseolina* infected tuber of WT, TL1 and TL2 plants on a comparative scale as compared to the response in the mock inoculated tubers: −, no discoloration; +, 20 % discoloration; ++, 40 % discoloration; +++, 60 % discoloration; ++++, 80 % discoloration and +++++, 100 % discoloration

and TL2. This finding indicated enhanced antifungal activity of leaf extracts from *CsTLP* transgenic plants. The mycelial growth of *M. phaseolina* in PDA medium (C) was similar to that of FS extraction buffer (CB), boiled leaf extracts of WT (WTC), TL1 (TL1C), and TL2 (TL2C) supplemented PDA medium indicating that neither the buffer components nor the leaf extract constituents left after boiling have any effect on growth of fungus.

Protection Against *P. infestans* Infection in *CsTLP* Transgenic Leaves

In order to assess whether the fungal resistance displayed by the *CsTLP* overexpression is extended to *P. infestans*, leaflets of Kufri Chandramukhi (KCM; a *P. infestans* susceptible potato cv.), WT (Kufri Giriraj; moderately tolerant to *P. infestans*), and *CsTLP* transgenic plants (TL1 and TL2) were challenged with a virulent strain of *P. infestans* (Fig. 5). As expected, symptoms of late blight in KCM (necrotic lesion around the inoculation site) appeared as early as 2 dpi and infection spread all along the leaf surface at 10 dpi. In case of WT, symptoms of late blight appeared 5 dpi and became more prominent at 10 dpi. Interestingly, inoculated leaflets of TL1 and TL2 both remained free of visible symptoms of *P. infestans* infection till 5 dpi and a mild lesion appeared only at 10 dpi. These results show that *P. infestans* induced disease symptoms are delayed in *CsTLP* transgenics.

Defense-Related Gene Expression in WT and *CsTLP* Transgenic Potato Tubers During *M. phaseolina* Infection

The transcript accumulation of potato self-defense response genes, *StLOX*, *StPAL,* and *StTLP* in WT and *CsTLP* transgenic (TL1 and TL2) tubers of potato in response to

M. phaseolina infection was determined using qRT-PCR assay. Transcript levels of *CsTLP* were also determined in both, WT and *CsTLP* transgenic tubers. The *CsTLP* transcript levels in TL1 and TL2 were 4,482 and 3,432 fold, respectively, relative to WT (Fig. 6a). In case of WT tubers, expression of *StPAL* was 18.2, 19.6, and 12.5 fold higher and that of *StTLP* 1795, 2943, and 103.8 fold upregulated at 2, 4, and 8 dpi, respectively as compared to their respective mock inoculated control tubers. Importantly, *StLOX* remained downregulated over 2, 4, and 8 dpi in comparison to its mock inoculated WT tubers.

In case of TL1 as shown in Fig. 6c, *StPAL* and *StTLP* transcript levels were upregulated by 201.3 and 116.9 fold, respectively with respect to mock inoculated TL1 tubers at 2 dpi. Over time, *StPAL* transcription levels at 4 dpi increased slightly ensuing 220.3 fold relative expression and remained almost constant at a value of 224.9 fold at 8 dpi with respect to TL1 mock inoculated. But for *StTLP*, a gradual decline in relative expression levels was recorded at 4 dpi (65.2 fold) and a sharp decline at 8 dpi in comparison to mock inoculated TL1 tubers. Initially at 2 dpi, the expression of *StLOX* was downregulated by 5 fold (relative expression 0.2) relative to TL1 mock inoculated control tubers. With time, *StLOX* transcript accumulated in TL1 and relative expression level of *StLOX* upregulated by 10.2 fold relative to its mock inoculated control tubers. In case of TL2 at 2 dpi, relative expression of *StLOX* was similar to mock inoculated TL2 tubers. Over time at 4 and 8 dpi, down-regulation in transcript levels of *StLOX* was observed in comparison to mock inoculated TL2 tubers (Fig. 6d). Up-regulation of *StPAL* and *StTLP* was recorded at 2, 4, and 8 dpi in comparison to mock inoculated TL2 tubers. The relative expression of *StPAL* increased from 4.7 fold at 2 dpi to 12.4 fold at 4 dpi and was followed by a 3.1 fold up-regulation at 8 dpi relative to mock inoculated TL2 tubers. A prominent increase in relative expression levels of *StTLP* was observed at 2 and 4 dpi which was 1835 and 4529 fold, respectively.

As shown in Fig. 6b–d, the abundance of *StTLP*, *StPAL,* and *StLOX* transcripts were different for both *CsTLP* transgenics and WT tubers upon infection with *M. phaseolina*. This prompted us to investigate the role of *CsTLP* overexpression on the transcriptional changes occurring in the aforementioned intrinsic potato defense response genes. Thus, we calculated relative expression ratio of these defense response genes in TL1 and TL2 tubers before and after inoculation (8 dpi) with *M. phaseolina* relative to WT tubers before and after inoculation, respectively (Table 3). Before inoculation of *M. phaseolina* in TL1, expression of *StLOX* and *StPAL* was downregulated with relative expression value 0.196 and 0.193, respectively, relative to WT, while it was upregulated for *StTLP* by 6.8 fold. However, a reverse trend was observed in TL2, where
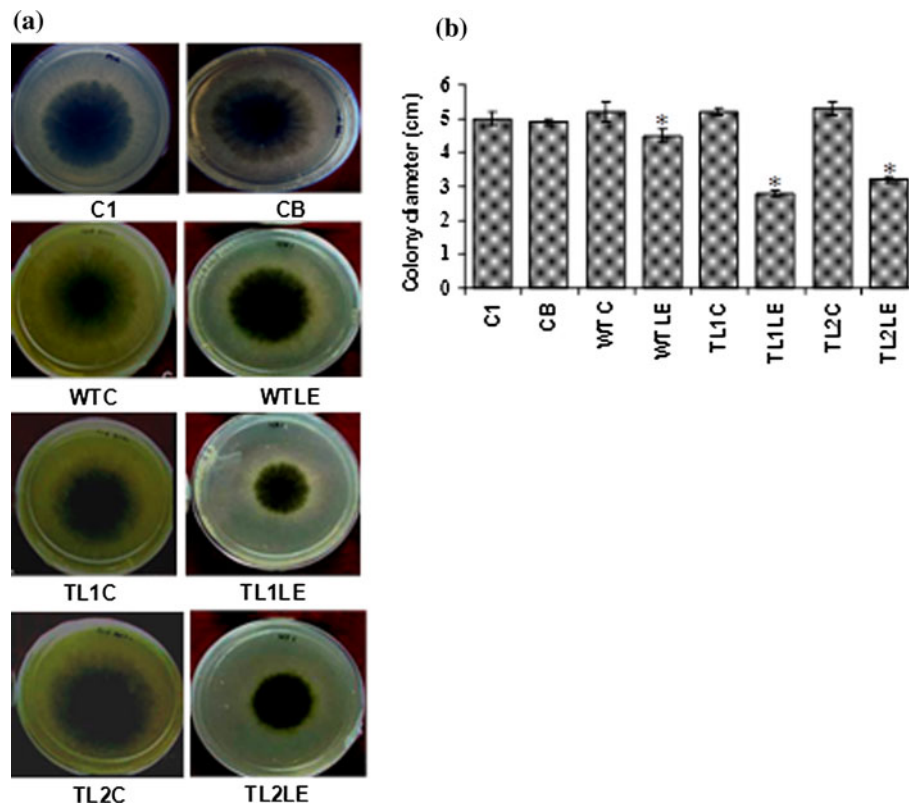
Fig. 4 Effect of crude leaf extract of un-transformed (WT) and *CsTLP* transgenic potato plants (TL1 and TL2) on the mycelial growth of *M. phaseolina*. **a** Mycelial growth of *M. phaseolina* after 72 h of culture. **b** Average colony diameter (in cm) of *M. phaseolina* (data represent mean ± SE; mean obtained by averaging results from three independent experiments each consisting of five petriplates for each of control and treatments). Statistically significant values at $P < 0.05$ are indicated by asterisks. C1, PDA medium; CB, C1 supplemented with 1 ml of FS extraction buffer; WTC, C1 supplemented with 1 ml of boiled (10 min) WT leaf extract; WTLE, C1 supplemented with 1 ml of FS crude leaf extract of WT plants; TL1C, C1 supplemented with boiled crude leaf extract (1 ml) of TL1 plants; TL1LE, C1 supplemented with 1 ml of FS crude leaf extract of TL1 plants; TL2C, C1 supplemented with boiled crude leaf extract (1 ml) of TL2 plants; TL2LE, C1 supplemented with 1 ml of FS crude leaf extract of TL2 plants

StLOX (2.36) and *StPAL* (4.75) exhibited higher relative expression than WT, but *StTLP* (0.36) showed a lower expression than WT before inoculation. In response to *M. phaseolina* infestation in TL1, both *StLOX* (106 fold) and *StPAL* (3.5 fold) were upregulated, while *StTLP* (0.05) was downregulated. In TL2, *StLOX* was upregulated by 4.9 fold relative to WT after inoculation, while there was no change in expression of *StPAL*, which could be attributed to lower resistance of TL2 as compared to TL1. Similar to TL1, *StTLP* was also downregulated in TL2 upon infection with a relative expression value 0.05 (Table 3). The elevated expression of defense-related *StPAL* and *StLOX* genes may provide resistance to *CsTLP* transgenic tubers against *M. phaseolina*.

Discussion

TLPs are a class of PR-proteins that are induced in vascular plants in response to pathogen/elicitors and believed to be effective PR-proteins that inhibit the growth of microorganisms [33, 57]. Overexpression of *TLP* genes in plants have been shown to provide enhanced tolerance to fungal pathogens [36, 37]. In the present study, heterologous expression of *CsTLP* in potato further provides evidence of its role in conferring tolerance against fungal pathogens.

Transgenic and WT potato plants were grown under contained facility and at the time of tuber harvesting it was observed that 30 % WT tubers were infected with a fungal pathogen but none of the *CsTLP* transgenic tuber was infected. This intriguing observation prompted us to identify the fungal pathogen and further analyze the fungal resistance phenotype of the transgenics. Sequencing of complete ITS region of fungal genomic DNA from a pure culture showed that a soil borne pathogen, *M. phaseolina*, was responsible for the rot. *M. phaseolina* is known to infect many economical important hosts, including cereals, legumes, and potato [58].

Tuber bioassay demonstrated that *CsTLP* conferred resistance to *M. phaseolina* in TL1; while TL2 exhibited partial resistance relative to WT infected tubers. The

Fig. 5 Inoculation of detached leaflets with *P. infestans*. Development of disease symptoms (appearance of lesion, its necrotization and chlorosis around the site of inoculation) after inoculation with *P. infestans* in KCM (susceptible potato variety), un-transformed potato plants (WT), *CsTLP* transgenic line 1 (TL1) and *CsTLP* transgenic line 2 (TL2). *Upper row* showing late blight symptoms at 2-day post inoculation (dpi); middle row at 5 dpi and lower row at 10 dpi. *Arrow* in the center of abaxial surface of leaflet shows the necrotization caused by *P. infestans* in the leaves of KCM, WT, TL1, and TL2 plants
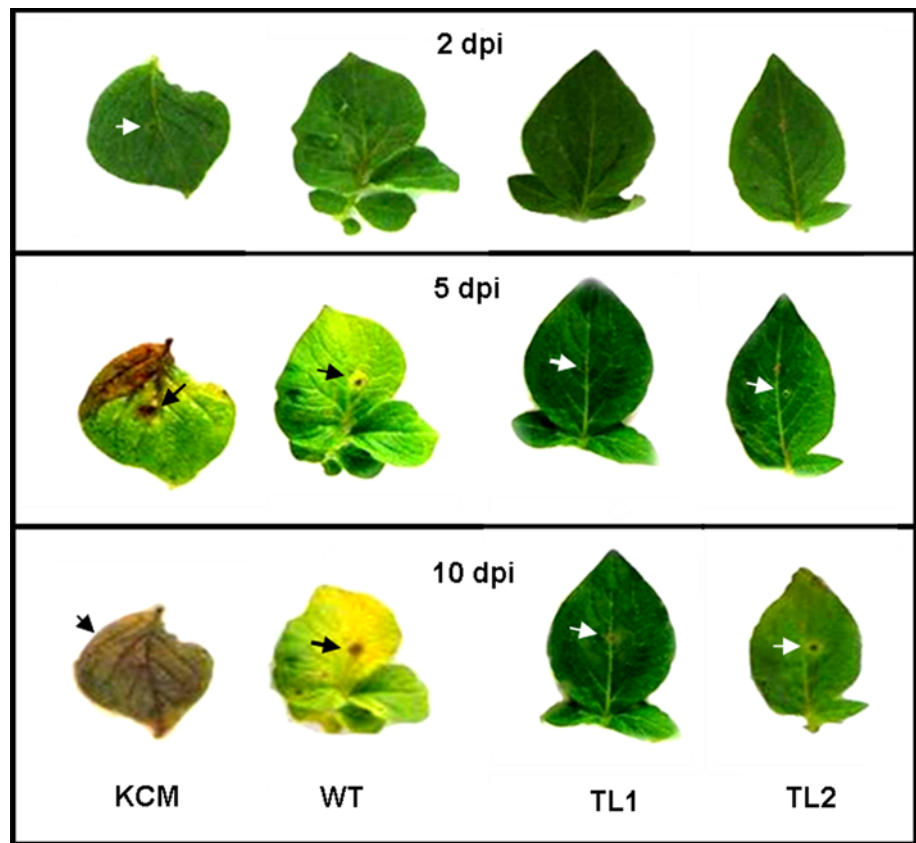
difference in resistance among lines TL1 and TL2 may be attributed to different expression levels of *CsTLP* (Fig. 6a) and other defense-related genes such as, *StPAL*, *StLOX*, and *StTLP* before and after infection with *M. phaseolina* (Table 3). Results from both, tuber bioassay and leaf extract assay established the antifungal role of *CsTLP* toward necrotrophic fungus *M. phaseolina*. In previous reports, TLPs have been shown to have antifungal action toward several types of fungi such as *Alternaria dauci*, *Alternaria petroselini*, *Alternaria radicini*, *Botrytis cine-rea*, *R. solani*, and *Sclerotinia sclerotiorum* [59]. CsTLP protein has 60 % similarity (Fig. S1) with the characterized tobacco osmotin by Singh et al. [60]. This osmotin gene when overexpressed in potato exhibited delayed development of late blight symptoms caused by *P. infestans* [34]. Similarly, *CsTLP* transgenics also display delayed *P. in-festans* induced symptoms, which further extends the role of CsTLP in imparting tolerance to late blight. Hence, CsTLP is potent in imparting tolerance against two different fungal pathogens which differ in life style from each other and cause significant yield loss in potato around the world.

There are few studies that unveil the role of PR/PR5-proteins in the activation of defense response pathway genes in plants. For example, Kumar et al. [61] illustrated that overexpression of chitinase gene led to enhanced expression of LOX, *β*-1,3-glucanase, and peroxidase in transgenic cotton plants. El-kereamy et al. [57] showed that overexpression of *Prunus domestica* PR5 gene in *Arabid-opsis* activates the phenylpropanoid pathway. To verify if, fungal resistance conferred by *CsTLP* overexpression in potato is also due to induction of defense-related genes, we studied the expression of endogenous defense response pathway genes, viz. *StTLP*, *StPAL* and *StLOX* in response to *M. phaseolina* infection using qRT-PCR. In vascular plants PR5, PAL and LOX are shown to be induced after inoculation with pathogen [21, 22, 25]. Similarly, upregulation of *StTLP* and *StPAL* in *M. phaseolina* infected tubers of WT plants was observed. Despite of high and early expression of *StTLP*, WT plants were susceptible to fungal pathogen. Lindqvist-Kreuze et al. [62] also observed higher and early up-regulation of *TLP* in potato upon *P. infestans* infection. It has been reported that some PR-proteins play a defensive role during fungal infection when overexpressed in heterologous system. Interestingly, a high level of PR protein accumulation in transgenic plants of the same species from which the PR gene was obtained does not have anti-pathogen effect [34, 63]. This observation indicates that TLP is the first line of defense against *M. phaseolina* infection. This first line of defense was found to be active not only in WT but also in TL1 and TL2 tubers inoculated with *M. phaseolina*. Apart from inducible TLPs,
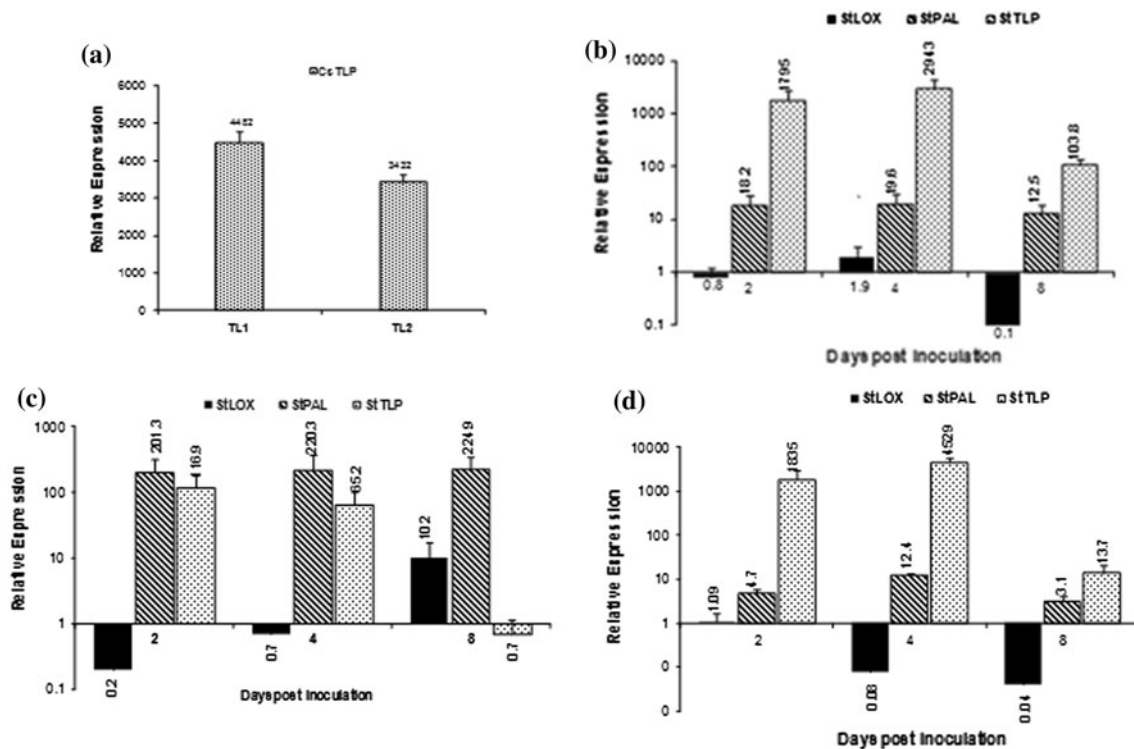
Fig. 6 qRT-PCR analysis. **a** Relative expression of *CsTLP* in transgenic potato tubers (TL1 and TL2) relative to WT tubers. Values are the average fold difference of non-inoculated tubers of TL1 and TL2 relative to non-inoculated WT tubers. Relative expression of *StLOX*, *StPAL*, and *StTLP* in tubers of **b** WT, **c** TL1 and **d** TL2 at 2, 4 and 8 dpi of *M. phaseolina* relative to their respective mock inoculated control tubers. Standard errors of the means are represented by the *error bars*. Values on the top of the bar represents relative expression ratio

Table 3 Relative expression of *StLOX*, *StPAL*, and *StTLP* in transgenic potato tubers overexpressing *CsTLP* before and after inoculation with *M. phaseolina* relative to WT

Relative expression	Before inoculation (0 day)[a]	After inoculation (8 dpi)[b]
Relative expression in TL1		
StLOX	0.196 (0.09)	105.9 (1.9)
StPAL	0.193 (0.11)	3.49 (0.9)
StTLP	6.8 (0.37)	0.05 (0.02)
Relative expression in TL2		
StLOX	2.36 (0.31)	4.9 (0.1)
StPAL	4.75 (0.8)	1.2 (0.6)
StTLP	0.36 (0.21)	0.05 (0.01)

Relative expression (fold difference) was determined by quantitative reverse transcriptase-polymerase chain reaction in *CsTLP* transgenic (TL1 and TL2) tubers before inoculation and 8 day after inoculation with *M. phaseolina*. Mean values and standard error (in parenthesis) were obtained from three technical and two biological replicates

[a] Fold difference calculated with un-transformed (WT) plants before inoculation

[b] Fold difference calculated with inoculated un-transformed (WT) plants at 8 dpi

active plant defenses against pathogens also include the phenylpropanoid pathway enzymes, which are known to be involved in plant disease resistance. PAL is the key enzyme of phenylpropanoid pathway and catalyzes the transformation of phenylalanine into cinnamic acid, which is the core molecule for the synthesis of phenylpropanoid compounds such as flavonoids, phytoalexins, lignins, and benzoic acid derivatives involved in the defense reactions against *P. infestans* [64, 65] and *Erwina carotovera* [66]. Tobacco plants overexpressing *PAL* produces high levels of phenylpropanoid compounds and exhibited a phenotype less susceptible to fungal pathogens [18]. The expression of *LOX* was shown to be upregulated by wounding, pathogen infection or their elicitors [67, 68]. Induction of LOX is believed to be the part of the defense mechanism and tightly related with accumulation of *LOX* mRNA in potato [69] and other plant systems [70, 71]. Overexpression of *LOX* in tobacco decreased the susceptibility to a virulent strain of *P. parasitica nicotinae* [12] and inactivation of LOX (ZnLOX3) increased the susceptibility of maize to *Aspergillus* spp. [72]. In our study, *StLOX* expression remained unaltered up to 4 dpi, but was downregulated at

8 dpi with a relative expression value 0.1 in *M. phaseolina* inoculated WT tubers. However, in the *CsTLP* transgenic tubers (TL1) expression of *StLOX* was downregulated at 2 and 4 dpi but was upregulated at 8 dpi when challenged with the *M. phaseolina* pathogen. The difference observed between WT and *CsTLP* transgenics is the magnitude of expression of these defense response genes at different time intervals of post inoculation.

In conclusion, we provide evidence that constitutive expression of *C. sinensis* TLP gene in potato confers enhanced resistance to *M. phaseolina* and *P. infestans*. Current study also provides insight of the mechanism underlying the resistance to *M. phaseolina* in potato plants expressing *CsTLP* gene. Our results suggested that CsTLP also has a role in activating other defense pathways including LOX and phenylpropanoid pathways.

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