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ORIGINAL PAPER

Virus-induced gene silencing (VIGS) for elucidation of pathogen defense role of serine/threonine protein kinase in the non-model plant *Piper colubrinum* Link.

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Abstract *Piper colubrinum* Link., an exotic species, is a wild relative of cultivated black pepper (*Piper nigrum* L.), and shows high degree of resistance to the oomycete pathogen *Phytophthora capsici*, which causes the devastating 'quick wilt' disease in *P. nigrum*. Serine/threonine protein kinase (*PcSTPK*) is a prospective candidate gene identified from *P. colubrinum* for future genetic improvement of *P. nigrum*. RACE PCR amplified a full-length *PcSTPK* of 1.7 kbp which encodes 575 amino acid residues with a calculated molecular weight of 64.45 kDa. *PcSTPK* forms a part of a distinct clade in phylogeny analysis, sharing higher similarity to receptor-like protein kinase class of Ser/Thr protein kinases. Real-time qRT-PCR showed the enhanced expression of *PcSTPK* gene upon *P. capsici* inoculation and the maximum accumulation of *STPK* transcripts was observed at 12 h post inoculation. A Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) was established using Phytoene desaturase endogenous gene as a reporter. TRV:*PcSTPK* VIGS vector was infiltrated into young leaves of *P. colubrinum.* A time course study revealed that *STPK* transcript levels were significantly downregulated. Knock down of *PcSTPK* by VIGS increased the susceptibility to *P. capsici* infection, as evidenced by the appearance of foliar necrotic lesions and increased proliferation and sporulation of *P. capsici* on the leaf surface. We report the successful implementation of VIGS as a systemic strategy for defense gene functional validation in *P. colubrinum* and our data provide direct evidence for the possible role of *PcSTPK* in modulating antifungal defense response in the plant.

Keywords Tobacco rattle virus · Agroinfiltration · Phytoene desaturase · *Phytophthora capsici*

Introduction

Plant receptor-like kinases (RLKs) act as an important class of sentinels acting in both broad-spectrum, elicitor-initiated defense responses and as dominant resistance (R) genes in race-specific pathogen defense. Most defense-related RLKs are of the leucine-rich repeat (LRR) subclass although new data are highlighting other classes of RLKs as important players in defense responses (Goff and Ramonell 2007). Plant genomes contain a large number of genes encoding proteins having the basic receptor serine/threonine kinase structure, which have been termed RLKs because in most cases receptor function (i.e., specific ligand binding) has not been demonstrated. RLKs likely play a major role in signal perception and transduction, controlling a wide range of physiological responses in plants. Experimental evidences suggest that phosphorylation of specific Ser and Thr residues controls kinase activation and signal transduction to downstream components of brassinosteroid (BR) signaling pathway (Eckardt 2005). Serine/threonine protein kinases (STPKs) are potential targets for genetic manipulation for transgenic solution for crop

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improvement as they regulate cascades of molecular events in plant stress reactions (Coello et al. 2010).

Crop wild relatives are the possible progenitors of crops and species that can serve as gene donors to crops. They are in fact an important natural resource and deserve urgent attention for systematic conservation and substantially expanded use (Ford-Lloyd et al. 2011). *Piper colubrinum* Link., is a woody shrub which is a wild relative of cultivated black pepper (*Piper nigrum* L.). It is native to the northern part of South America. This species is important because of its resistance to the oomycete *Phytophthora capsici*, which causes the devastating disease 'quick wilt' in *P. nigrum* (Purseglove et al. 1981; Ravindran and Remashree 1998). *P. capsici* presents an oomycete worst-case scenario to growers as it has a broad host range, often produces long-lived dormant sexual spores, has extensive genotypic diversity and has an explosive asexual disease cycle. It is becoming increasingly apparent that novel control strategies are needed to safeguard food production from *P. capsici* and other oomycetes (Lamour et al. 2012). Ten resistance gene analogues were recently identified from *P. colubrinum* and clustering analysis revealed that NBS-containing R genes comprise a large gene family in *Piper* species (Lau et al. 2012).

In our previous work, a salicylic acid induced subtracted library was generated in *P. colubrinum* for discovery of candidate genes and regulatory elements as an essential step towards genetic improvement of cultivated pepper (Dicto and Manjula 2005; Mani and Manjula 2011; Mani et al. 2012). The serine/threonine protein kinase gene (*PcSTPK*) was identified in the subtracted library as a differentially over expressed partial clone (EB104037). The specific pathogen defense role of this gene needs to be explored to enable its efficient utilization for crop improvement programmes.

The analysis of gene functions in a non-model plant species like *P. colubrinum* is often hampered by the fact that stable genetic transformation to over express/down-regulate gene expression is laborious and time-consuming, or, sometimes, even not achievable. The past several years have seen the complete sequencing of many plant genomes and the generation of large databases of sequence information as a result of which alternative approaches to traditional forward genetics can be implemented to identify the genes involved in a process of interest. Reverse genetics investigates the function of a gene or DNA sequence directly by altering the expression of the sequence of interest and then identifying the mutant phenotype that is produced. Most reverse genetics approaches described in plants to date rely on posttranscriptional gene silencing (PTGS; Watson et al. 2005). The mechanism of PTGS involves the sequence-specific degradation of RNA and several different techniques have been developed to harness PTGS phenomena for gene functional studies. One of these is virus-induced gene silencing (VIGS; Baulcombe 1999; Dinesh-Kumar et al. 2003). The observation that plants could overcome infection by viruses and then be rendered resistant to subsequent infection by closely related viruses was the first suggestion that PTGS was an innate antiviral defense in plants (Lindbo et al. 1993; Ratcliff et al. 1999; Soosaar et al. 2005). VIGS takes advantage of this defense system to silence endogenous RNA sequences that are homologous to a sequence engineered into the viral genome, which generates the double-stranded RNA that mediates silencing (Burch-Smith et al. 2006). VIGS can serve as an alternative to mutant collections or stable transgenic plants to allow the characterization of gene functions in a wide range of angiosperm species, albeit in a transient way. This strategy is nowadays widely used in plant genetics for gene knockdown due to its ease of use and the short time required to generating phenotypes (Lange et al. 2013; Demircan and Akkaya 2010; Martin et al. 2013). It is a virus vector technology that exploits RNA-mediated defense and the most widely used VIGS vectors are derived from the *TRV*. TRV provides the most robust results in terms of efficiency, ease of application, and absence of disease symptoms (Padmanabhan and Dinesh-Kumar 2009). Engineered TRV vectors carrying host-derived segments are transformed into *Agrobacterium tumefaciens*, which is then introduced into the plant (Liu et al. 2002; Tian et al. 2014).

Phytoene desaturase (*PDS*) is a rate-limiting enzyme in carotenoid synthesis that converts phytoene to colored ξ-carotene in a two-step desaturation reaction (Bartley and Scolnik 1995). Therefore, *PDS* gene is very important in carotenoid metabolism and photosynthesis. It was reported earlier that in VIGS experiments, leaf with *PDS* silencing was albinotic and this gene was used as a silencing marker (Kumagai et al. 1995; Wang et al. 2009).

We report in this paper, full-length cloning of Ser/Thr Protein Kinase gene from the resistant wild *Piper* species, *P. colubrinum*, and corroboration of its pathogen defense role by TRV-induced VIGS. This is the first report of application of VIGS technique for elucidation of gene functions in *Piper* species and will open up newer possibilities for molecular studies in this important but underutilized non-model plant species.

Materials and methods

Plant material and growth conditions

In vitro cultures of *P. colubrinum* were established from leaf explants as described earlier (Mani and Manjula 2011) and the cultures were incubated at 25 ± 2 °C under 16/8-h

photoperiod per day, with light provided by white fluorescent lighting at an intensity of 54 l mol m^{-2} s^{-1}. Direct shoots derived from the leaves were rooted in basal MS solid medium and hardened in soilrite mixture (peat moss: vermiculite: perlite, 1:1:1, v/v/v—Keltech Energies Ltd, Bangalore, India) contained in sterile cups. The plants were maintained in a growth chamber (Conviron CMP6010) with the temperature adjusted to 24 °C, humidity of 70 % and under a 16/8-h light/dark regime. Young leaves (2nd or 3rd) of 2 month old plants were collected for RNA isolation and whole plants were used for VIGS experiments.

P. capsici inoculation

The virulent *P. capsici* strain obtained from Kerala Agricultural University (Thiruvananthapuram, India) was used to inoculate *P. colubrinum* plants. To prepare the fungal inocula, *P. capsici* was grown on potato dextrose agar medium at 28 °C overnight. *P. capsici* infection was carried out by the mycelium agar disc method on detached leaves (Godoy et al. 1990). Mycelial agar plugs of 5.0 mm diameter punched from growing margins of a 24 h old *P. capsici* culture was applied on the pin pricked abaxial surface of the leaves. Leaves were kept under 16 h photoperiod and 100 % humidity. The disease symptoms were observed every 24 h, over a period of 1 week. Plain agar plugs (without mycelia) applied at pin pricks on the abaxial side of leaves, served as control.

P. capsici infection scoring

P. capsici infections were scored based on the increase in the diameter of lesions. Leaf lesions >2 mm diameter which progressed with time were taken as lesions caused by *P. capsici* infection. Smaller dark spots (<2 mm diameter), which did not show an increase in size with time were taken as spots representing hypersensitive cell death response.

RNA isolation and rapid amplification of cDNA Ends (RACE) PCR

Total RNA was isolated from leaf of *P. capsici* challenged *P. colubrinum* plant by Trizol (Invitrogen, CA, USA) method. Approximately 1 µg of DNase (Sigma, USA) treated RNA was used for cDNA synthesis. The isolated RNA sample was checked for integrity on 2 % EtBr agarose gel and quantified by NanoDrop1000 spectrophotometer (Thermo Fischer Scientific, USA). *PcSTPK* was cloned full-length following 5′ and 3′ RACE protocol. RACE ready cDNA synthesis was carried out using SMARTScribe™ reverse transcriptase following set procedures (SMARTer™ RACE cDNA amplification kit, Clontech, CA, USA). The standard RACE PCR protocol was carried out using the *P. colubrinum* serine/threonine protein kinase (EB104037) gene specific primers (STPK1 for 3′ RACE and STPK 2 for 5′ RACE and universal primer UPM; Table 1). The PCR conditions were as follows: 5 cycles of 94 °C for 30 s, 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min and 20 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min. The products were checked on 1.2 % EtBr-agarose gel. Primers were designed based on the sequences of the RACE products to amplify the complete ORF (forward primer PcSTPK F, reverse primer PcSTPK R; Table 1). The PCR was carried out using the Advantage 2 polymerase mix (Clontech, CA, USA). The PCR cycling parameters were: Initial denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min and a final extension of 72 °C for 5 min. The PCR product was cloned into the pGEM-T cloning vector (Promega, Madison, WI, USA) and subjected to sequencing (ABI PRISM® 3730 DNA Analyzer, Applied Biosystems, Foster City, CA, USA).).

BLAST program of NCBI was used for identity searches for *PcSTPK* sequence at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). ALIGN program of the European Bioinformatic Institute (EBI) server (www.ebi.ac.uk) was used to determine the identity of *PcSTPK* and its similarity with the amino acid sequences of known receptor-kinases from other taxa. Multiple alignment of the protein sequences were carried out using BioEdit sequence alignment editor version 7.0.5.3. Motif searches were performed by the PROSITE program using ExPASy proteomics server (www.us.expasy.org). A phylogenetic tree was constructed using the MEGA software version 4.0 by the neighbour joining (NJ) method.

Cloning of *P. colubrinum PDS* gene

One kbp *PDS* gene fragment was amplified from *P. colubrinum* by PCR using degenerate primers PcPDS F and PcPDS R (Table 1) from conserved regions of the *PDS* gene sequences of other plants. The amplified product was purified and cloned into pCR8/GW/TOPO cloning vector (Invitrogen, CA, USA) and transformed into DH5α *E. coli* cells. Recombinants were confirmed by colony PCR using degenerate *PDS* primers. Plasmids from putative recombinants were isolated and digested using *EcoRI* (New England Biolabs). Sequencing with TOPO vector specific GW1 & GW2 primers (Table 1), confirmed the PCR product as *Phytoene desaturase*.

Construction of TRV: VIGS vectors

First, *PcSTPK* sequence was analyzed on a web-based computational tool (http://bioinfo2.noble.org/RNAiScan.htm) developed by Xu et al. (2006), to identify potential

Table 1 List if primers used in the present study

Primer name	Sequence (5′–3′)	Binding site	Accession no
STPK1	CTCAGCAAAATCAGGTCTTCCAT	1859–1882	KM582051.1
STPK2	GTAGGTGTTGTTCAAAAGGGTC	1763–1784	KM582051.1
PcSTPK F	ATGGGGAGGGAGGACAACGA	266–285	KM582051.1
PcSTPK R	ATGGTTTCGTCTGAGAACTGA	1970–1990	KM582051.1
RTSTPK1	TTCCAACGTTGCTAAAAGTGG	1434–1454	KM582051.1
RTSTPK2	AGTCTTCAAGTCCCGGTGAAC	1502–1522	KM582051.1
TRVSTPK F	TTCACCGGGACTTGAAGACT	1503–1522	KM582051.1
TRVSTPK R	CTTGTTGCCAGCATCTCTCA	1833–1853	KM582051.1
RT PDS F	TGGCAACAACATACGCATCTC	353–373	KM582050.1
RTPDS R	GAATCATGATGGAACAGTCAAACAT	297–321	KM582050.1
PBIN-F	GTTTGTGGACGGTAGGAG	5408–5425	D00155.1
PBIN-R	ATGAACCCAGGCGTATCT	5600–5618	D00155.1
PTV-F	CCTGCTGACTTGATGGAC	603–620	AF406991.1
PTV-R	CAGTGTTCGCCTTGGTAG	943–960	AF406991.1
Tvect F	GGGTTTTCCCAGTCACGACGT	2955–2975	Commercial vector
Tvect R	CGCCAAGCTATTTAGGTGACAC	147–168	
TRV F	GCTGCTAGTTCATCTGCAC	[a]	
TRV R	GCACGGATCTACTTAAAGAAC		
UPM	TAATACGACTCACTATAGGGCAAGC-AGTGGTATCAACGAGAGT	[b]	
GW1	GCTACTGCCCAGACTTGCATT	607–631	[b]
GW2	CATGGATCACTCAACATTTC	733–757	[b]
M13F	TGTAAAACGACGGCCAGT	537–552	[b]
M13R	CAGGAAACAGCTATGAC	836–852	[b]
Pc PDS F	TGGAARGARCAYTCIATGATWTT-TGCWATG	Degenerate primers	
Pc PDS R	ACRACATGRTACTTIAVDATYTT-WGCTTT		
ACT F	CTGCTGGTATTCACGAGAC	NA	
ACT R	GCACTTCCTGTGGACTATTG		
RT ACT F	ACATCCGCTGGAAGGTGC		
RT ACT R	TCTGTATGGTAACATTGTGCTC		

NA Sequences not submitted in NCBI

[a] http://www.plantsci.cam.ac.uk/research/davidbaulcombe/methods/ptv

[b] Commercially available vector primers (universal primers)

off-target silencing from the VIGS vector containing the insert. Target dataset were set to "plant" which includes a large library of transcript sequences from model and non-model plants. The query sequence fragment analyzed did not have hits in target dataset which suggests that the sequence selected might not produce an off-target gene silencing. TRV-RNA1 (pBIN) and TRV-RNA2 (pTV00) VIGS vectors were kindly offered by Dr. David Baulcombe (University of Cambridge, UK). To construct TRV:*PcPDS* vector, a *PcPDS* fragment of 600 bp from 1 kbp *PcPDS* PCR product was cloned into pTV00 by restriction digestion using *HindIII* (New England Biolabs) and *SpeI* (New England Biolabs) and ligated using *T4 DNA ligase* (Promega, Madison, WI, USA). To generate TRV:*PcSTPK,* 354 bp *STPK* fragment was amplified by PCR from *P. colubrinum* cDNA using gene specific primers TRVSTPK F and TRVSTPK R (Table 1). The amplified product was purified and cloned into pGEM-T easy vector and transformed into DH5α *E. coli* cells. Recombinants were confirmed by colony PCR using gene specific primers. Plasmid was isolated and sequence was confirmed by sequencing using pGEM-T easy vector specific primers Tvect F and Tvect R (Table 1). pGEM-T vector carrying *STPK* gene and pTV00 vector were digested using *HindIII* and *SpeI* and ligated using *T4 DNA ligase*.

The resulting construct TRV:*PcPDS*/TRV:*PcSTPK* was transformed into DH5α competent *E. coli cells* and plated on selective LB media containing 50 μg/ml kanamycin.

Colonies were PCR screened for the presence of the modified constructs using primers TRV F and TRV R which span the multiple cloning site in TRV-RNA2. Plasmids were isolated from selected positive colony and the identity of the final constructs was verified by sequencing with the TRV F and TRV R primers.

Agroinfiltration

VIGS vectors carrying Pc*PDS*, Pc*STPK* gene fragments as well as TRV-RNA1 and TRV-RNA2 were transformed into *A. tumefaciens* strains *GV3103* by freeze–thaw method (Weigel and Glazebrook 2002). *Agrobacterium tumifaciens GV3103* cultures containing TRV-RNA1, TRV-RNA2, TRV*:PcPDS* and TRV*:PcSTPK* were separately grown overnight at 28 °C in LB medium containing antibiotics (50 μg/ml kanamycin, 25 μg/ml rifampicin). Subsequently, bacterial cells (OD600 of 1.5) were harvested and resuspended in infiltration media (10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; SIGMA), pH 5.7 and 200 μM acetosyringone (SIGMA), and incubated at room temperature for 3 h without shaking. *Agrobacterium* cultures containing the *Agrobacterium* mixtures of TRV-RNA1 and TRV-RNA2 or TRV*:PcPDS* or TRV*:PcSTPK* (1:1ratio) were infiltrated with a needle-less 1 ml syringe into lower leaves of four to five leaf stage plants following the protocol by Liu et al. (2002). Experimental and control plants were transferred to growth chamber and maintained under set conditions as mentioned before. Each experiment was repeated three times with three replicates each. Accumulation of virus in the upper uninoculated leaves was analyzed by RT PCR every 2 for 8 weeks. Leaves inoculated with empty vector (TRV-RNA1 + TRV-RNA2) served as control.

RT PCR for determining viral movement

Total RNA was extracted from fresh leaf tissue of silenced (TRV-RNA1 and TRV-RNA2:*PcPDS/PcSTPK)*, non-silenced control (infiltrated with empty vector TRV-RNA1 and TRV-RNA2) and untreated plants (without agroinoculation) using TRIzol reagent following the manufacturer's instructions and treated with RNasefree DNase. The first strand cDNA was generated using 1 μg of total RNA, oligo d(T) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). To detect the presence of TRV virus, fragments of RNA1 and RNA2 of TRV were amplified by two pairs of specific primers (PBIN F and PBIN R, PTV F and PTV R, Table 1). The *P. coubrinum* β-actin gene (Table 1) served as an internal control. PCR products were separated on a 1 % TBE agarose gel and visualized by EtBr staining.

Real-time PCR analysis

New leaves were collected after 15, 21, 30 and 45 days of inoculation from control, TRV*:PcPDS* and TRV*:PcSTPK* infiltrated plants to determine the efficiency of silencing. All the qRT-PCR primers are listed in Table 1. First-strand cDNA synthesis was conducted using the M-MLV reverse transcriptase on purified RNA for each sample and all amplifications were performed on the ABI 7900 realtime PCR machine (Applied Biosystems, CA, USA) using the SYBR green PCR reagent (Applied Biosystems, CA, USA). cDNA equivalent to 1 μg of total RNA was used. The reaction was set up in a final volume of 20 μL containing 10 μL SYBR green PCR reagent, 2 μL of diluted cDNA and 300nM each of the designed primers and the conditions were: 50 °C for 2 min initially followed by 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a real-time PCR machine. The housekeeping gene β-actin cloned from *P. colubrinum* was used for normalisation and the relative expression level of the genes was determined by comparing the Ct values using $2^{-}_{\Delta\Delta CT}$ method. Each experiment included three independent samples (biological replicates), each performed in triplicate (technical replicates). Statistical significance were analysed by calculating one way ANOVA followed by Bonferroni method using GraphPad prism (www.graphpad.com).

Light microscopy

Lactophenol cotton blue staining was carried out using the protocol described by Chung et al. (2010). Pathogen inoculated leaf samples were cut into 1 × 1 cm^2 segments, incubated first in an acetic acid: ethanol (1:3, v/v) solution overnight, followed by treating in an acetic acid: ethanol: glycerol (1:5:1, v/v/v) solution for at least 3 h. The samples were subsequently incubated overnight in a staining solution of 0.01 % (w/v) trypan blue (Loba Chemie, India) in lactophenol, rinsed 3–4 times with water and then stored in 60 % glycerol until examination. Specimens were transferred onto microscopic slides and examined under a 40× magnification in a compound microscope (Leica Microsystems DM750).

Quantification of H_2O_2

H_2O_2 was measured by ferrous oxidation using the Xylenol Orange assay (Gay et al. 1999). The Xylenol orange reagent was freshly prepared and remained stable for 6–8 h. One milliliter of solution [25 mm $FeSO_4$ and 25 mm $(NH_4)_2SO_4$, dissolved in 2.5 m H_2SO_4] was added to 100 ml of 125 μm xylenol orange and 100 mM sorbitol. Leaf discs (0.5 cm^2) were floated on 1 ml distilled water for 10 min, followed by centrifugation at 5,000 g in a

microcentrifuge (Eppendorf, Germany) for 1 min. The supernatant (100 µL) was incubated for 30 min in 1 ml of xylenol orange reagent. After incubation for 30 min at room temperature, the absorbance (A_{560}) was measured in spectrophotometer (Shimadzu, Japan) against the blank containing 100 µL distilled water. H_2O_2 was determined at 560 nm using a standard H_2O_2 curve.

DAB staining

For in situ detection of H_2O_2, DAB staining was carried out using an adaptation of the method of Thordal-Christensen et al. (1997). Silenced and control leaves were infiltrated under gentle vacuum with 1 mg ml^{-1} DAB containing 0.05 % v/v Tween 20 and 10 mm sodium phosphate buffer pH 7.0. The reaction was terminated at 6–7 h post-inoculation, when a brown precipitate started to be visible in the leaves. Leaves were fixed and then boiled for 15 min in ethanol:acetic acid:glycerol (3:1:1) solution. Bleaching solution was replaced and leaves were incubated until the chlorophyll was completely bleached. Leaves were observed by light microscopy under bright field at 4× magnification (Leica Microsystems DM750).

Estimation of chlorophyll content

Chlorophyll extracted from 100 mg of leaf tissue in an acetone:DMSO mix was centrifuged and the supernatant was made up to a known volume. The absorbance was recorded at 663 and 645 nm using a UV–visible spectrophotometer (UV 2450, Shimadzu). Total chlorophyll was estimated (Hiscox and Israelstam 1979; Nageswara Rao et al. 2001) and the percentage reduction over control was calculated.

Statistical analysis

Results are presented as mean ± SD. The statistical significance of differences was analyzed by one-way ANOVA followed by post hoc (Bonferroni's multiple comparison test) using GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com). Data with asterisks are significantly different ($P < 0.05$) according to the post hoc ANOVA statistical analysis.

Results

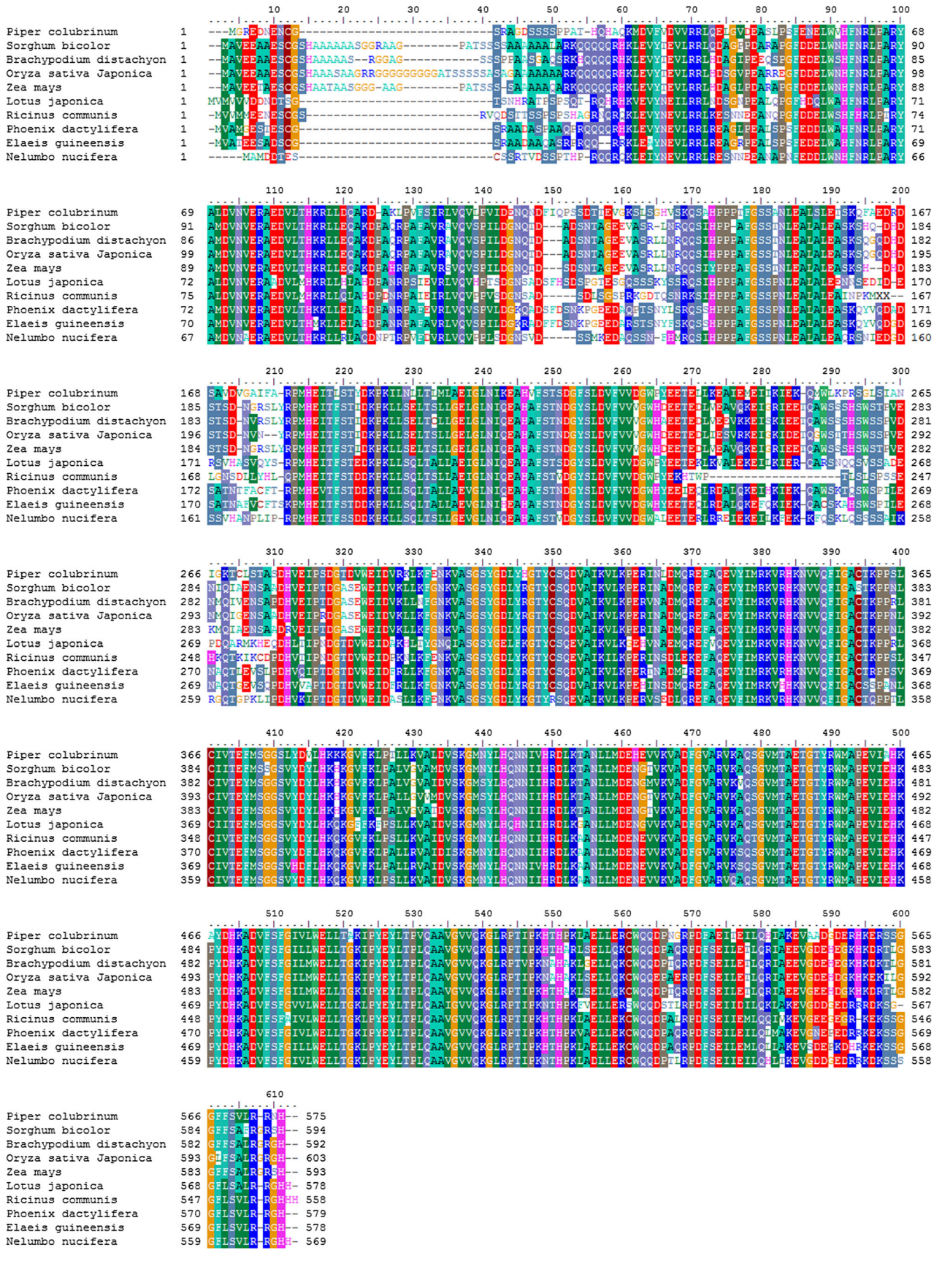
Full-length amplification and sequence analysis of *PcSTPK*

5′ RACE and 3′ RACE primers designed based on the sequence of an SSH fragment were used for amplification of ends of *PcSTPK*. Amplicon sizes of 2 kbp and 500 bp were obtained from 5′ and 3′ RACE reactions respectively (Supplementary Fig. 1a, b). Each of the products was cloned separately and sequenced. Full-length of the gene was deduced by aligning and assembling the sequences of 5′ RACE, 3′ RACE and the SSH fragment (EB104037) obtained earlier in our lab. Primers designed based on the sequences of 5′ RACE and 3′ RACE successfully amplified the *PcSTPK* as a single amplicon (Supplementary Fig. 1)

Analysis of the resultant nucleotide sequence showed that the full-length *PcSTPK* cDNA was 2170 bp in length and contained a 1725 bp ORF, with a 5′ UTR of 265 bp upstream of the start codon and a 3′ UTR of 177 bp downstream from the stop codon. The deduced *PcSTPK* protein consisted of 575 amino acid residues with a calculated molecular weight of 64.45 kDa and an isoelectric point (pI) of 5.95. The identities of the PCR products were confirmed by homology search at NCBI database. Homology search of amino acid sequence using the BLAST programme (NCBI) showed that *PcSTPK* shared significant identity with protein kinase sequences of other plant species like *Lotus japonicus* (71 % identity to accession number AB353300.1), *Ricinus communis* (78 % identity to accession number XM002521189.1), *Nelumbo nucifera* (69 % identity to accession numbers XP 010266065.1 and *Oryza sativa* (72 % identity to accession number NM001052241.1). The *PcSTPK* nucleotide sequence is deposited in the NCBI Gen Bank under Accession number KM582051.

Multiple alignment of *PcSTPK* with amino acid sequences of RLKs from different taxa is shown in Fig. 1, which suggests that *PcSTPK* shares significant sequence homology with other receptor-like protein kinase class. PROSITE analysis of deduced protein sequence of *PcSTPK* identified residues 295–549 in the sequence as the protein kinase domain, of which 412–424 (IVHRDLKTANLLM) were identified as serine/threonine protein kinase active-site signature. Signal P software predicted no signal peptides and target P software predicted the protein as non-secretory protein. *PcSTPK* forms a part of a distinct clade in phylogeny analysis, sharing higher similarity to receptor-like protein kinase class than other classes like SNF-1 and CDPK of ser/thr protein kinases (Fig. 2). Sequence and phylogenetic analyses of *PcSTPK* reveals its cytoplasmic

Fig. 1 Multiple alignment of *PcSTPK*. Multiple alignment of *PcSTPK* amino acid sequence with sequences from different taxa viz. *Sorghum bicolour* (XP 002460599.1), *Brachypodium distachyoni* (XP 003578561.1), *Oryza sativa* Japonica Group (NP 001063830.1), Zea mays (DAA62681.1), *Lotus japonicus* (BAI63585.1), *Ricinus communis* (XP002521235.1), *Arabidopsis thaliana* (BAD93724.1), *Aegilops tauschii* (EMT07451.1), *Ricinus communis* (XP 002533015.1), *Piper colubrinum* (KM582051). The conserved region representing identical/similar residues are coloured. The multiple alignment was carried out using the Bioedit Sequence Alignment Editor version 7.0.5.3 (Hall 1999)


Piper colubrinum
Sorghum bicolor
Brachypodium distachyon
Oryza sativa Japonica
Zea mays
Lotus japonica
Ricinus communis
Phoenix dactylifera
Elaeis guineensis
Nelumbo nucifera

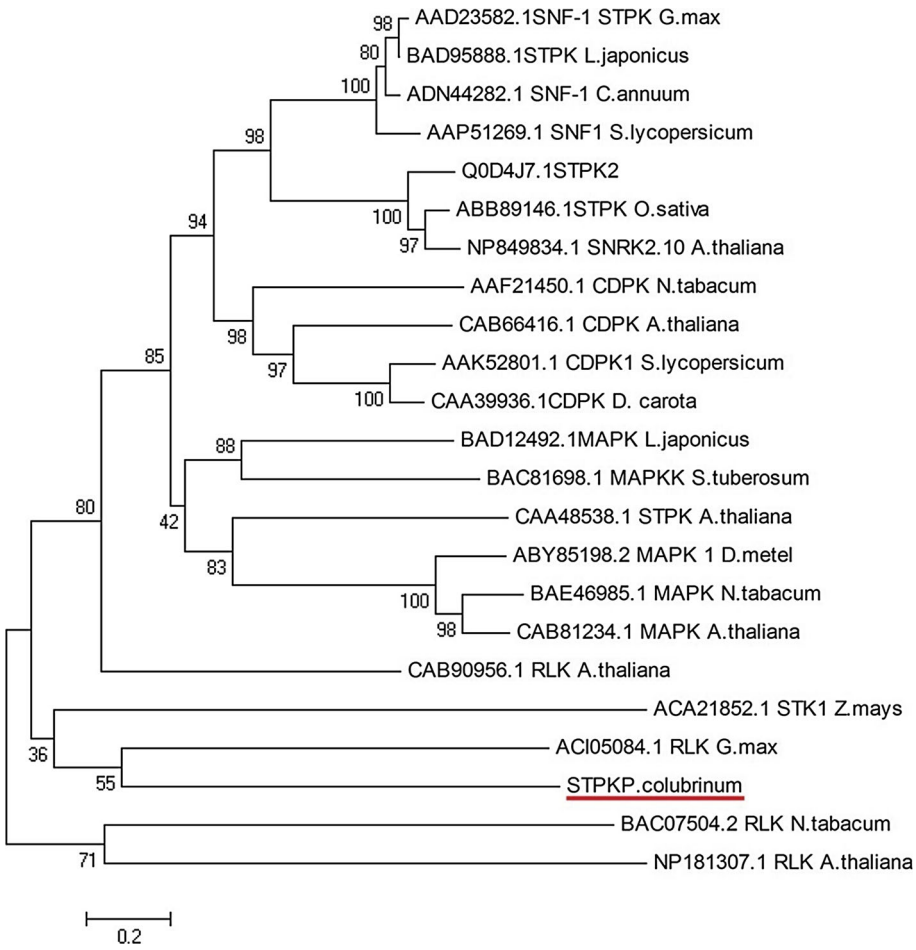


Fig. 2 Phylogenetic analysis of *PcSTPK*. The tree was constructed from amino acid sequences obtained from NCBI for protein kinases with the Neighbor-joining method using the MEGA program version 4.0 (Tamura et al. 2007) Accession numbers for each sequence is shown. *PcSTPK* is *underlined* in *red* (*STPK P. colubrinum* KM582051). (Color figure online)

localization, with sequence similarity to receptor-like protein class.

Regulation of *PcSTPK* transcripts in response to pathogen attack

qRT-PCR analysis was conducted to study the expression profile of *PcSTPK* transcripts in leaf tissues in a period of 0–72 h inoculation with *P. capsici*. The expression of *PcSTPK* gene was upregulated upon *P. capsici* inoculation and the maximum accumulation of *STPK* transcripts were observed in inoculated leaves after 12 h, which was not significantly ($P > 0.05$) altered up to 24 h. But beyond this time point, the transcript levels were significantly reduced ($P < 0.05$; Fig. 3).

VIGS in *P. colubrinum*

Recombinant TRV infects P. colubrinum

The ability of TRV vector to cause systemic infection in *P. colubrinum* was assessed in order to employ it in VIGS experiments. Twenty-one days post-agroinfiltration, total RNA was prepared from the upper un-infiltrated leaves, followed by RT-PCR analysis with primers derived from the RNA1 and RNA2 sequences of TRV. Amplicons corresponding to RNA1 and RNA2, of size 210 and 345 bp respectively, were detected only in those leaves infiltrated with *Agrobacterium*-containing TRV clones (Fig. 4b, Lanes 3 and 4) and were absent in the control agroinfiltrated plants. TRV infection however did not result in any visible symptoms. The results clearly show that recombinant TRV can efficiently replicate and spread systemically in *P. colubrinum* plants.

Silencing of *PcPDS* by VIGS

A mixture of *Agrobacterium* cultures containing TRV-RNA2 carrying *P. colubrinum PDS* (TRV2*: PcPDS)*, and TRV-RNA1 was infiltrated onto the lower leaves of plants. Plants infected with TRV2*:PcPDS* developed a yellow coloration in the upper newly formed leaves 21 days post-agroinfiltration (Fig. 5a), which persisted for at least 4 months (Fig. 5c). The presence of viral particles in the *PDS* silenced leaves was confirmed by RT-PCR (Fig. 5d) after 21 days of infiltration in each experiment. Real-time

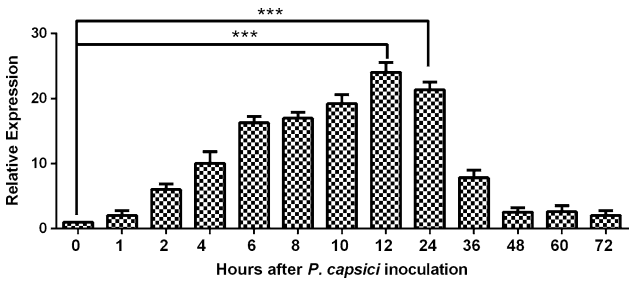


Fig. 3 qRT-PCR analysis of *STPK* expression in *P. colubrinum* leaves in a period of 0-72 h after *P. capsici* inoculation. *STPK* gene was significantly upregulated 12–24 hpi. Wounded (pin pricked) leaves were used as control and set as the calibrator. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Data are the mean ± SD from three independent experiments. Data with asterisks are significantly different ($P < 0.05$) according to the post hoc ANOVA statistical analysis

PCR was performed to quantify *PDS* silencing. In TRV*:PcPDS* infected plants, the *PDS* transcripts were reduced by more than 50 % compared with the TRV infected controls (Fig. 5e), and the efficiency of *PcPDS* VIGS was about 83 % (Supplementary Table 1). *P. colubrinum* actin gene served as an internal control for RNA quality and RT-PCR amplification. Total chlorophyll was extracted from control and *PDS* downregulated leaves. The concentration of total chlorophyll was reduced by 58 % in *PDS* silenced leaves compared with empty vector control leaves (Fig. 5f). The fact that TRV effectively caused the VIGS of *PDS* in *P. colubrinum* suggests that other nuclear genes could be targeted for silencing using TRV:VIGS vector.

PcSTPK silencing by VIGS

To validate the role of *PcSTPK* by VIGS, TRV*:PcSTPK* VIGS vector containing 354 bp *PcSTPK* insert was infiltrated into young leaves of *P. colubrinum* maintained in a growth chamber. 15, 30 and 45 days after infiltration, the upper newly formed leaves were collected and checked for viral presence. RT-PCR analysis using TRV-RNA1 indicated systemic movement and multiplication of viral particles in the infiltrated plants (Fig. 6a). Real-time PCR analysis was performed to quantify *STPK* mRNA levels. A time course study revealed that *STPK* transcript levels were significantly ($P < 0.05$) downregulated (40–64 %) at 15–45 dpi, with a maximum down regulation of 64 % observed in silenced leaves at 30 days of post infiltration (Fig. 6b), and an efficiency of 93 % (Supplementary Table 2).

Increased susceptibility of TRV: *PcSTPK* infiltrated leaves of *P. colubrinum* to *P. capsici*

In a susceptible reaction (*P. nigrum* × *P. capsici*), primary necrotic spots develop into water-soaked lesions within 24–48 h (Fig. 7a; image on the right). In an incompatible reaction as in *P. colubrinum* × *P. capsici* interaction, fungal development is restricted to 1 or 2 few small, dark brown spots [non-spreading lesions, Fig. 7a image on the left; 7b (control)]. At 30 dpi, newly emerged leaves from both empty vector infiltrated control plants and *STPK* silenced plants were challenged with *P. capsici.* Disease symptoms on leaves were monitored 12–72 h after inoculation with virulent pathogen *P. capsici*. Control leaves showed very small dark spots representing points of HR (Fig. 7b), with no visible symptoms of disease initiation and progression. *STPK* down regulated leaves on the contrary, were marked by the development of larger lesions which progressed in size with time. Disease symptoms of *P. capsici* infection were observed in 93 % of the silenced leaves (Supplementary Table 2). The lesions observed were water soaked and necrotic, typical of *P. capsici* foliar symptoms (Fig. 7b). Staining with lactophenol blue displayed very sparse germination of fungal mycelia on the leaf epidermis in control leaves (Fig. 7c). Microscopic examinations revealed profuse hyphae producing copious chlamydospores in *STPK* silenced leaves. There was also a significant increase in the number of spots on the leaf showing hyphal development (Fig. 7c).

To investigate the role of the *PcSTPK* gene in the regulation of oxidative burst, H_2O_2 generated was quantified by Xylenol Orange assay and visualized by DAB

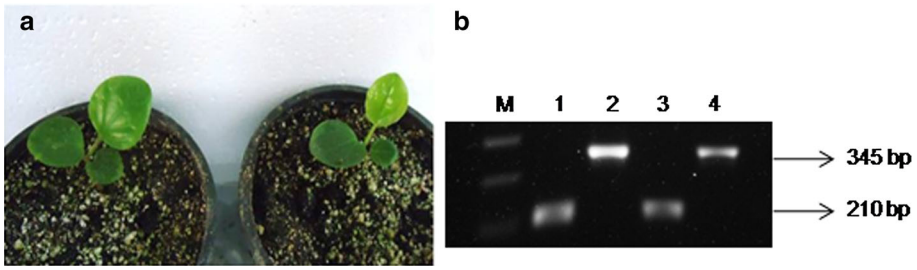


Fig. 4 Agroinfiltration with TRV empty vector. **a** Control plants of *P. colubrinum* maintained in the growth chamber for VIGS experiments. **b** Amplification of viral transcripts (TRV-RNA1 and TRV-RNA2) from upper uninfiltrated leaves of empty vector control-infiltrated plants by RT-PCR. *Lane M*—Marker (NEB 100 bp ladder), *Lane 1*—TRV-RNA1 Positive control, *Lane 2*—TRV-RNA2 Positive control, *Lane 3*—TRV-RNA1 amplification, *Lane 4*—TRV-RNA2 amplification

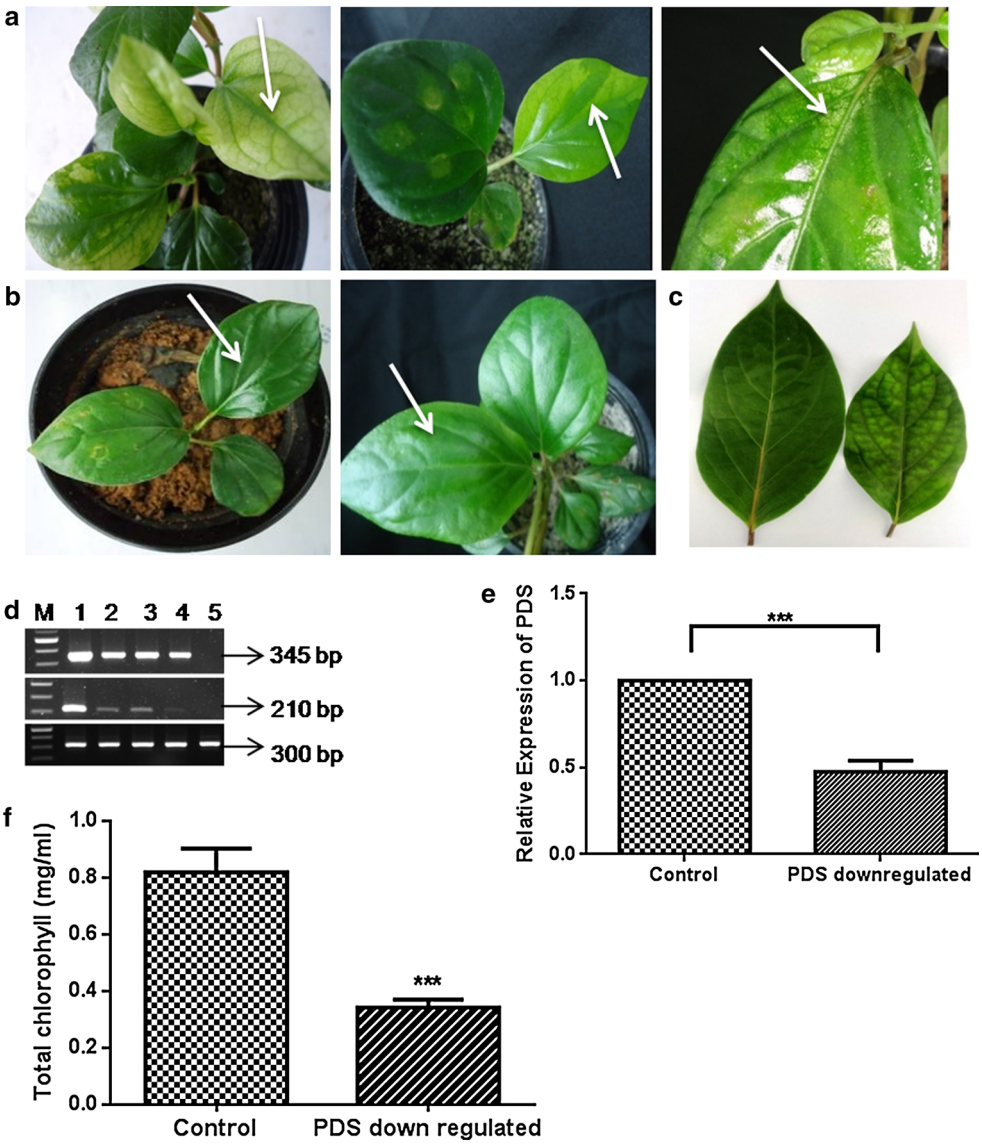


Fig. 5 Tobacco rattle virus (TRV)-induced *PDS* silencing in *P. colubrinum.* **a** Newly formed leaves of *P. colubrinum* plant infiltrated with TRV:*PcPDS* showing *yellowing* after 21 dpi. **b** Empty vector control infiltrated plant showing normal phenotype. **c** Persistence of *PDS* downregualtion 4 months post infiltration. **d** RT PCR amplification of TRV viral particles from new leaves of empty vector control and *PDS* downregulated plants. Lane M-Marker (NEB 100 bp ladder), *Lane 1*- Positive control, *Lanes 2* and *3*- leaves from *PDS* down regulated plant, *Lane 4*-Empty vector infiltrated plant, *Lane 5*-Uninfiltrated plant. **e** Real-time PCR of *PDS* gene expression in empty vector infiltrated and *PDS* downregulated plant. *PDS* expression was significantly (52.4 %) downregulated by silencing of *P. colubrinum PDS* gene. **f** Comparison of total chlorophyll content in control and *PDS* downregulated leaves. Total chlorophyll was reduced 58 % in silenced leaves. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Data are the mean ± SD from three independent experiments. Data with asterisks are significantly different ($P < 0.05$) according to the post hoc ANOVA statistical analysis. (Color figure online)

staining in leaves of control and *STPK* silenced plants at 48 hpi with *P. capsici*. DAB staining at 48 hpi showed abundant dark brown spots as well as diffused staining concentrated around the leaf veins and around infection sites in control leaves (Fig. 7d). H_2O_2 quantification in leaves of *STPK*-silenced plants displayed significantly lower (50 % of control; $P < 0.05$) accumulation of H_2O_2, which was also evident microscopically (Fig. 7d, e). These data indicate that the *PcSTPK* gene is involved in the early events of cell death and defense response of pepper plants.

Discussion

Over the past few years, protein kinases have been identified after non race- and race-specific elicitation for each step in the induction of plant defence responses. They participate in the direct perception of elicitors and *Avr* products, mediate signaling required for the induction of defence mechanisms, including the activation of transcription factors and systemic responses, and function as negative regulators of defence responses (Romeis 2001; Zhang et al. 2010). Protein phosphorylation/dephosphorylation are major signaling events induced by biotic and abiotic stress as well as developmental pathways in higher plants (Afzal et al. 2008; Cao et al. 2011a, b). Serine/threonine protein kinases (EC 2.7.11.1) are host immune receptors which are known to play a role in Effector-triggered immunity (ETI) of plants, through phosphorylation of the OH group of serine or threonine residues leading to a functional change of the target protein (Hardie 1999; Romeis 2001). Elucidation of physiological roles of specific protein kinases in response to abiotic and biotic stress in plants is very crucial for better understanding and

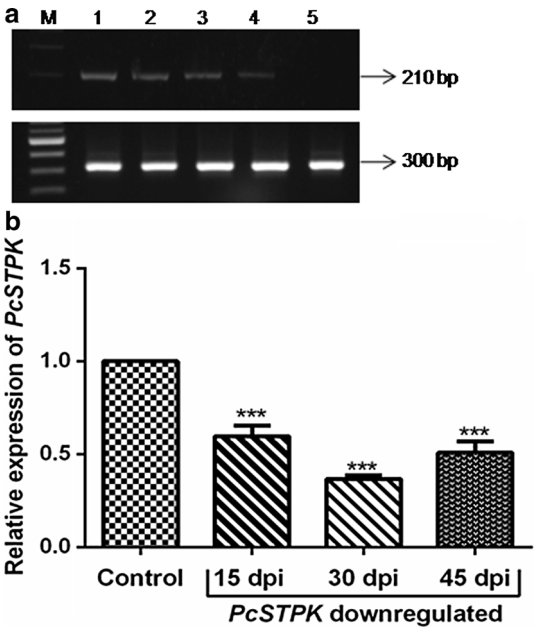


Fig. 6 *PcSTPK* silencing by VIGS**. a** RT-PCR confirmation of viral movement in control and TRV:*PcSTPK* infiltrated plants using TRV-RNA1 primers. *Lanes 1* and *2* - Empty vector control, *Lanes 3* and *4*- Leaves from TRV:*PcSTPK* infiltrated plant, *Lane 5*- uninfiltrated leaf, Lane M- Marker (NEB 100 bp ladder). Expected amplicon of 210 bp was observed in empty vector control and TRV:*PcSTPK* infiltrated plants. **b** Real-time PCR of STPK gene expression in systemic leaves of empty vector infiltrated and STPK downregulated plant. Leaves samples were taken 15, 30 and 45 days of post infiltration (dpi). Maximum downregulation of 64 % observed at 30 dpi. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Data are the mean ± SD from three independent experiments. Data with asterisks are significantly different ($P < 0.05$) according to the post hoc ANOVA statistical analysis

identification of key players in the defense pathway, and protein kinases are perceived as suitable targets for genetic manipulation for crop improvement (Ramonell and Somerville 2002; Sheen 2010).

The present study includes the successful cloning of a pathogen inducible serine/threonine protein kinase gene from *P. colubrinum*. Sequence analysis of *PcSTPK* revealed its cytoplasmic location and it was predicted as a member of the receptor-like protein class as indicated by phylogenetic analysis. Recent studies have highlighted the importance of receptor-like cytoplasmic kinase (RLCK) in mediating pathogen triggered immunity as well as effector triggered immunity (Kim and Hwang 2011; Liu et al. 2011). This is the first report of identification of a RLCK from *Piper* species, which attains more relevance as it is identified from a highly resistant, wild species like *P. colubrinum*, and is highly perceptive to the fungal pathogen *P. capsici*, a highly devastating pathogen infecting *P. nigrum*. Wild species have been widely used as genetic resources for introgression of useful traits into cultivated species by wide hybridization (Li et al. 2008), and *P. colubrinum* is one such species which is yet to be fully explored for its potential as a gene donor for *P. nigrum* genetic improvement programmes. An interspecific hybrid between *P. colubrinum* and *P. nigrum* was developed by Vanaja et al. (2008) having partial resistance to the dreaded disease *Phytophthora* foot rot.

Lack of reports on genetic manipulation in *P. colubrinum*, and our own experience so far prompt us to believe that the plant is recalcitrant to genetic transformation. New techniques need to be developed to promote genetic studies in *P. colubrinum* and our group had earlier optimized gene silencing by hairpin-mediated RNAi strategy (Mani and Manjula 2011). However, the scope for application of this technology for downstream applications including gene functional evaluation is limited because of the absence of a good transformation system and ensuing lack of reproducibility. The ability to generate gene knockdown phenotypes without having to genetically manipulate the plant genome is one of the advantages of VIGS compared to other gene suppression methods like mutagenesis and RNA interference (RNAi; Senthil-Kumar and Mysore 2011).

In this paper, we describe the successful optimization of VIGS as a transient silencing strategy and demonstrate the utility of this method as a tool for gene functional evaluation in *P. colubrinum*. Most marker genes used in VIGS experiments are related to chlorophyll synthesis because of the visible phenotypic effects that result from silencing. The *PDS* gene is a commonly used marker gene for VIGS which is known to cause loss of chlorophyll and carotenoids when silenced (Rotenberg et al. 2006). Photo bleaching occurs due to failure of chlorophyll synthesis, which if strong and persistent, can eventually lead to death of the leaves due to shortage of nutrients (Estevez et al. 2000; Gao et al. 2011). *PDS* down regulation in *P. colubrinum* led to mild photobleaching, though *PDS* transcripts were significantly reduced. The silencing phenotype observed in *P. colubrinum* is less pronounced than that observed in many other plants including *N. benthamiana* (Ruiz et al. 1998), Petunia (Reid et al. 2009), Tomato (Liu et al. 2002), etc. The location and amount of silencing within a plant can be highly variable in VIGS (Chen et al. 2004). The difference in phenotype observed may be due to genetic differences between plants with respect to viral resistance or antiviral silencing machinery, as host RNA-dependent RNA polymerase (RDRP) and Dicer-like enzymes are implicated in viral accumulation and antiviral silencing (Donaire et al. 2009). Useful viruses for VIGS must replicate quickly while suppressing the plant's suppressing machinery, and there may be a balance between viral proliferation and gene silencing (Broderick and Jones 2014). This might explain the decreased symptoms of PTGS in *P. colubrinum* that may be the consequence of

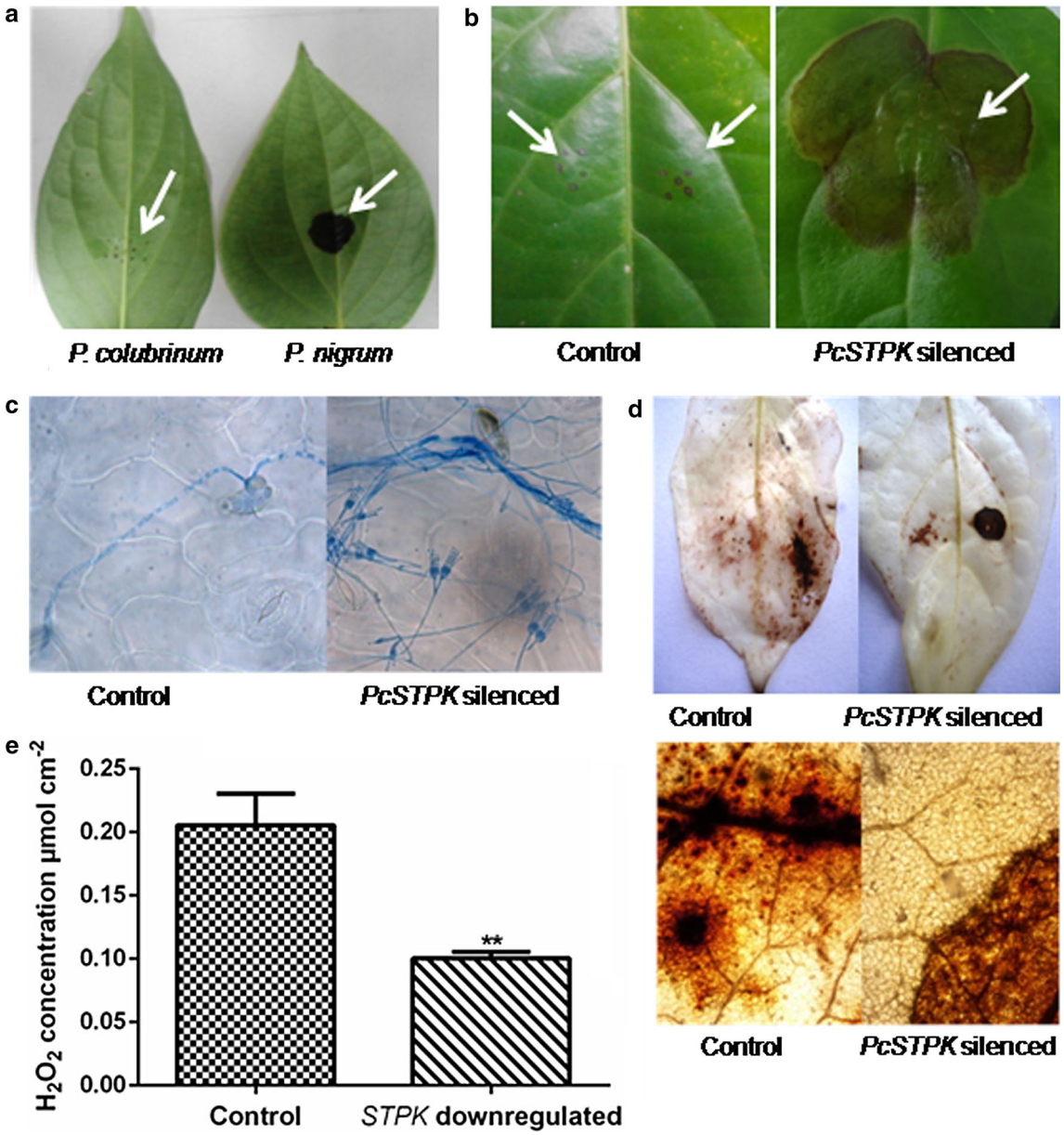


Fig. 7 Enhanced susceptibility of *PcSTPK* downregulated *P. colubrinum* leaves to infection by *P. capsici.* All observations were made in a period of 24–72 hpi with *P. capsici.* **a** Foliar symptoms of *P. capsici* incompatible (*P. colubrinum* × *P. capsici)* and compatible (*P. nigrum* × *P. capsici*) interactions. 24 h post *P. capsici* infected *P. colubrinum* leaves showed hypersensitive cell death response where as *P. nigrum* leaves were suceptible for infection. **b** Photographs of *P. capsici* infected leaves of empty vector control plant and *STPK* downregulated plant. Control leaves showed very small dark spots representing points of HR, with no visible symptoms of disease initiation and progression. *STPK* down regulated leaves on the contrary, were marked by the development of larger lesions which progressed in size with time. **c** Micrographs of lactophenol blue staining. Control leaves showed very sparse germination of fungal mycelia on the leaf epidermis where as in STPK silenced showed profuse hyphae producing copious chlamydospores. **d** DAB staining for the detection of H_2O_2 production. After infection with *P.capsici*, *dark-brown* sites stained with DAB were observed in the empty vector control leaves, but distinctly reduced in the *STPK* silenced leaves. **e** Quantification of H_2O_2 accumulation in empty vector control and *STPK* silenced leaves. Data are the mean ± SD from three independent experiments. Data with *asterisks* are significantly different ($P < 0.05$) according to the post hoc ANOVA statistical analysis. (Color figure online)

high disease resistance and resulting strong antiviral defense manifested by the plant. *PDS* gene therefore can be considered an efficient marker for VIGS in the plant, as it does not produce a detrimental effect on the leaf survival.

Persistence of VIGS for longer duration is influenced by age of the plant, viral titer, and environmental conditions that favour virus multiplication (Fu et al. 2006). Our results provide evidence that VIGS can be maintained for

5 months in *P. colubrinum*, during which the plants show persistence of *PDS* silencing phenotype. Ongoing experiments will furnish further evidence on the exact duration of silencing effect by VIGS. The observation of longer duration of VIGS is highly relevant in *P. colubrinum* which is predominantly vegetatively propagated and hence transmittance of VIGS through stem cuttings is a potential application for adopting VIGS as a tool to study gene function in plant responses to stress and development.

PcSTPK transcripts were significantly downregulated by VIGS and the down regulation was systemic. A notable observation was the increased susceptibility of *STPK* downregulated leaves to *P. capsici* infection. This is concurrent to our knowledge that serine/threonine protein kinase is one of the important proteins responsible for defense signal transduction. The serine/threonine kinase domain is the major constituent of the tomato *Pto* gene which not only interacts with the avirulence proteins from *Pseudomonas syringae*, but also functions as a signal transduction mediator (Martin et al. 1993). The STK domain is also contained in the rice *Xa21* gene which confers resistance to *Xanthomonas oryzae* pv oryzae (Song et al. 1995). Overexpression of an SNF1-type serine/threonine protein kinase of wheat (*Triticum aestivum* L.) *TaSnRK2.4*, was reported to confer enhanced multistress tolerance in *Arabidopsis* (Mao et al. 2010). The cloning of genes from wild relatives and the use of these genes in transgenic studies is an efficient way for modern genetic improvement. In a recent report, Cao et al. (2011a, b) demonstrated that a putative serine/threonine protein kinase *Stpk-V* cloned from a wild relative species of wheat confers strong durable resistance in cultivated wheat to powdery mildew.

Most pathogen infections are accompanied by production of activated oxygen species including superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). These are recognized and can either intensify the damage or trigger multiple host defence responses (Vranova et al. 2002; Huckelhoven and Kogel 2003). Controlled modulation of active oxygen species (AOS) production/dissociation determines the host response to specific pathogen. AOS perform variety of phytochemical roles: anti-microbial action, inactivation of enzymes, signaling leading to activation of genes involved in biosynthesis (PR-proteins, phenolics and phytoalexins), promotion of lignin precursors and cell wall proteins cross-linking, oxidative burst during HR (Heath 1998; Vranova et al. 2002). In our experiments, deep brown polymerization product occurred at sites of DAB reaction with H_2O_2. In control plants, accumulation and extensive spread of H_2O_2 suddenly follows the contact with pathogen (6–12 h). Moreover, more diffuse reaction products were visualized, denoting possible spread of H_2O_2 signal from infected sites to surrounding cells. This supports H_2O_2 involvement and HR in signaling and mediation of plant defense responses. In leaves of TRV:*PcSTPK* infiltrated plants, however, site of H_2O_2 accumulation was restricted to the area surrounding the lesions, with no sign of diffusion to neighboring cells when observed 72 h after infection. Our study suggests the tight connection between oxidative burst and exhibition of hypersensitive reaction in *P. colubrinum* × *P. capsici* incompatible reaction, an observation coinciding with previous similar reports in other plants (Sedlarova et al. 2004). Contrarily, *STPK* down regulation resulted in increased foliar susceptibility to *P. capsici*, which was correlated to localized production of H_2O_2 around the infection sites, representing areas of necrosis. *Phytophthora* spp. are hemi-biotrophic pathogens, having a lifestyle that features a biotrophic phase, followed by a switch to necrotrophy (Judelson and Blanco 2005; Jupe et al. 2013). It is known that some necrotrophic pathogens may produce ROS to induce cell death that facilitates subsequent spread of the pathogen (Gonner and Schlosser 1993; Tiedemann 1997). Thus, similar to previous observations, H_2O_2 accumulation may be regarded as a non-specific stress response of the host tissue resulting from cell disorganization caused by fungal colonization and deprivation of available nutrients (Shetty et al. 2003; Wang et al. 2007).

In summary, the paper describes the functional role played by *PcSTPK* in pathogen defense, as evidenced by the observation of increased susceptibility as a result of its transient down regulation, and the implication of H_2O_2 in limiting the spread of the oomycete, *P. capsici* in control non-silenced plants. This study demonstrates that VIGS can be used for reverse genetics or functional genomics studies in *P. colubrinum*. This approach also ensures that the gene validation can proceed without laboring with stable transformation in this recalcitrant host. The future challenge of this study will be the biochemical and genetic characterization of regulators, phosphorylation targets and other interacting partners involved in the defense signaling event.

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