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ORIGINAL RESEARCH

Cloning and sequence characterization of a partial Piper colubrinum phytoene desaturase (PcPDS) gene homologue for virus-induced gene silencing studies

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ABSTRACT

In the present study, we report the partial cloning and characterization of Phytoene desaturase (PDS) gene from the plant Piper colubrinum, a distant relative of Piper nigrum (Black pepper). P. colubrinum is the only species reported to be totally immune to Phytophthora capsici infection. PDS gene is widely used as a visual reporter gene for functional genomics studies in various plant species. We have amplified a 1 kb PDS fragment from P. colubrinum cDNA using degenerate primers and cloned it into pCR®8/GW/TOPO® vector. Sequencing and further phylogenetic analysis confirmed the sequence as Phytoene desaturase. The PDS gene fragment was re-amplified using gene specific primers and sequence was confirmed for its further use in virus-induced gene silencing studies.

KEY WORDS: Reporter gene, Piper colubrinum, Phytoene desaturase

Introduction

Piper colubrinum also known as wild pepper is a distant relative of cultivating black pepper (Piper nigrum). The foot rot disease caused by Phytophthora capsici is the major devastating disease of black pepper (Anandaraj and Sarma 1995). The wild pepper plant *P. colubrinum* shows high level of resistance towards P. capsici infection (Kelkar et al., 1996; Yusuf et al., 2001) and our group has been successful in identifying a set of defense gene candidates from this plant (Dicto and Manjula 2005; Mani and Manjula 2010; Mani et al. 2012). Functional genomic approaches are yet to be attempted to understand the defense mechanism of this nonmodel plant. So far, the plant is reported to be recalcitrant to Agrobacterium-mediated stable genetic transformation which makes the functional genomics studies based on Agrobacterium-mediated stable genetic transformation

difficult (Mani and Manjula 2011). Virus-induced gene silencing (VIGS) is a reverse genetics tools for analysis of gene function and it is a transient technique that does not rely on the generation of transgenic plants (Lu et al., 2003). VIGS will be a promising alternative for functional genomics in P. colubrinum plant. For successful application, VIGS need to be initially optimized using a reporter gene which produces an observable phenotype in the host plant, when silenced (Senthil-Kumar et al., 2007).

Phytoene desaturase (PDS) has been widely and successfully used as a reporter gene in VIGS studies (Liu et al., 2002; Ratcliff et al., 2001). Carotenoids play an essential role in photosynthesis by protecting chlorophyll against photooxidative destruction by singlet oxygen (Sandmann et al., 1991a). Phytoene desaturase is the major rate limiting enzyme in the carotenoid pathway, which converts the

colorless phytoene to colored carotenoids(Lopez *et al.*, 2008). Disruption of *PDS* expression in plants leads to the depletion of chlorophyll accumulation in newly emerging leaves (Sandmann *et al.*, 1991b) resulting in a bleached phenotype in the *PDS* silenced / down regulated areas in the leaf. In this paper we report the partial cloning and sequence characterization *P. colubrinum PDS* gene in order to construct a reporter VIGS construct for our future VIGS studies in the plant.

Materials and Methods

Plant material

P. colubrinum in vitro cultures were established as described earlier (Mani and Manjula 2011) and the cultures were incubated at $25 \pm 2^{\circ}$ C under 16/8-h photoperiod per day. Direct shoots derived from the leaf explants were rooted in basal MS solid medium and hardened in soil contained in the sterile pots. Healthy plants of *Piper colubrinum* Link, maintained in the growth chamber (Conviron CMP6010) 16-h photoperiod, irradiance of 200 - 400 µmol m⁻² s⁻¹, day/night temperature of 24 °C and relative humidity 70 % served as source material for the experiments.

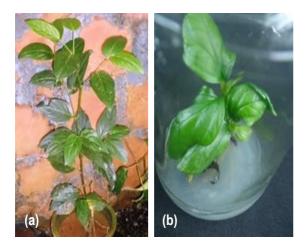


Figure 1: *Piper colubrinum* plants. a. Healthy plant maintained in the net house. b. *In-vitro* maintained plant

RNA Isolation and cDNA synthesis

Total RNA was isolated from young *P. colubrinum* leaves using Trizol (Invitrogen, CA, USA) method. The isolated RNA sample was checked for integrity on 2 % EtBr agarose gel and quantified by a NanoDrop ND-1000 Spectrophotometer (Thermo scientific, USA) at 260 nm. First

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strand cDNA was generated using 1 µg of total RNA, oligo d(T) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA).

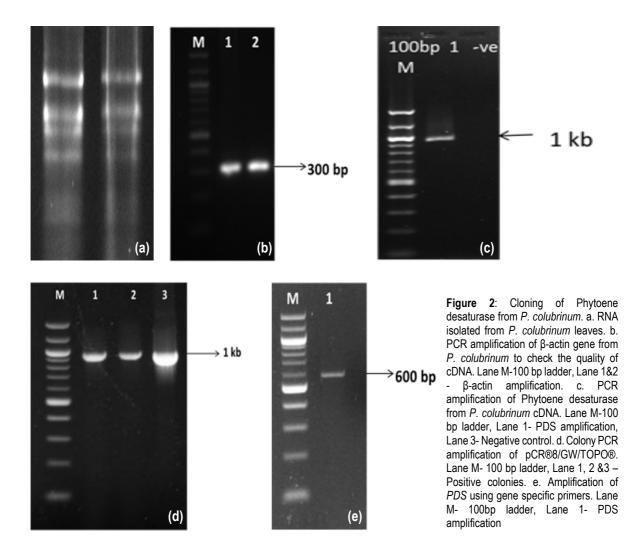
RT-PCR and cloning of PDS gene

Degenerate primers were designed by aligning the conserved regions of PDS from different plant species using primer 3 plus software (Untergasser et al., 2007). P. colubrinum PDS gene fragments were amplified by a gradient PCR using the degenerate primers DPDS F and DPDS R (Table 1). The PCR cycling parameters were - initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 50°C - 60°C (gradient) for 30s and 72°C for 1 min and a final extension of 72°C for 5 min. The *P. colubrinum* β actin gene (ACTIN F and ACTIN R Table 1) served as an internal control. The PCR cycling parameters were initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30s and 72°C for 1 min and a final extension of 72°C for 5 min. PCR products were separated on a 1% TBE agarose gel and visualized by EtBr. The amplified PDS gene fragments were purified and cloned into pCR8/GW/TOPO cloning vector (Invitrogen, CA, USA) and transformed into DH5a E. coli cells. GW1 and GW2 primers (Table 1) were used for colony PCR. PDS F and PDS R (Table 1) gene specific primers were used to re-amplify PDS gene fragment.

Table 1: Primers used in the study		
DPDS F	5'-TGGAARGARCAYTCIATGATWTTTGCWATG-3	
DPDS R	5'- ACRACATGRTACTTIAVDATYTTWGCTTT-3'	
PDS F	5'- GAGGCTCAAGACGGTTTGAC-3'	
PDS R	5'- GGAACGGTTTGGATCATAATATTC-3'	
GW1	5' GCTACTGCCCAGACTTGCATT 3'	
GW2	5' CATGGATCACTCAACATTTC 3'	
ACTIN F	5'- CTGCTGGTATTCACGAGAC-3'	
ACTIN R	5'- GCACTTCCTGTGGACTATTG-3'	

Sequencing PCR and post reaction clean up

The Big Dye v3.1 sequencing kit (Applied Biosystems, USA) was used to amplify the plasmid DNA, which was amplified using GW1 and GW2 primers, in preparation for sequencing in ABI instruments (ABI PRISM^R 3730 DNA Analyzer, Applied Biosystems, USA). The PCR cycling parameters were 30 cycles of 95°C for 30 s, 45°C for 30s and 60°C for 2 min. Post reaction clean up were carried out using 125mM EDTA, 3M sodium acetate and 100% alcohol.



Bioinformatic analysis

BLAST program of NCBI was used for identity searches for *PDS* sequence at the National Center for Biotechnology Information (<u>www.ncbi.nlm.nih.gov</u>). Nucleotide sequence alignment to determine the identity of *PDS* and its similarity with the nucleotide sequences of known *PDS* from other taxa and phylogenetic tree were constructed using the Phylogeny.fr software (Dereeper *et al.*, 2008).

Results and Discussion

Good quality RNA was isolated (Fig 2 a) from the young leaves of *in vitro* maintained *P. colubrinum* plants (Fig 1). An OD 260/280 ratio greater than 1.8 (~2) is considered as an acceptable indicator of good RNA quality. For pure RNA the

expected 260/230 values are commonly in the range of 2.0-2.2 (Sambrook *et al.*, 1989). The 260/280 ratio of 1.9 and 260/230 ratio of 2.1 confirmed the purity of the isolated RNA. The synthesized cDNA was checked by PCR amplification of housekeeping gene β -actin which gave the expected amplification of 300 bp (Fig 2 b).

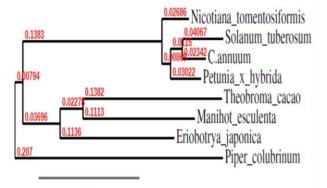
Gradient PCR using DPDS F and DPDS R degenerate primers amplified a *PDS* fragment of 1 kb size at 55° C Tm (Fig 2 c). Purified *PDS* gene fragment were cloned into pCR®8/GW/TOPO® cloning vector and transformed into to DH5 α *E. coli*. Colonies grown on spectinomycin antibiotic selection was screened for the presence of insert by colony PCR amplification using GW1 and GW2 primers which span the multiple cloning sites. All selected colonies were positive and showed an expected amplification of 1 kb (Fig 2 d).

2/13/2015 phy	ylogeny.lirmm.fr/phylo_cgi/get_result.cgi?task_id=ee39ecf642d51f23cacc4e0bf1b07834&results_in_list=4&raw=1&file=alignment.html
Piper_colu	GTCAAAGGCACTgAAtTTtATtAAtCCTGATGAgCTgTCtATGCAGTGCATCcTcATTGC
Solanum_tu	GTCAAAGGCACTTAACTTTATAAACCCTGACGAACTgTCAATGCAGTGCATCTTGATTGC
Petunia_x	GTCAAAGGCACTTAACTTCATAAACCCTGAcGAgCTTTCAATGCAGTGCTTCTTGATcGC
Nicotiana	GTCAAAGGCACTTAACTTCATAAACCCTGAcGAgCTTTCgATGCAGTGCATtTTGATTGC
C.annuum	GTCAAAGGCACTTAACTTCATAAAtCCTGATGAgCTTTCgATGCAGTGCATCTTGATCGC
Theobroma	GTCAAAGGCACTgAACTT <mark>C</mark> ATtAACCCaGA <mark>T</mark> GA <mark>ACTT</mark> TC <mark>A</mark> ATGCAGTGtATaTTGAT <mark>T</mark> GC
Eriobotrya	GTCAAAGGCcCTTAACTTtAATtAACCCTGATGAACTTTCAATGCAGTGCATATTGATTGC
Manihot_es	GTCAAAGGCACT <mark>T</mark> AACTTtAAttCCaGA <mark>T</mark> GA <mark>A</mark> CTTTCgATGCAGTGtATtTTGATaGC
Piper_colu	TTTGAAtcGtTTTCTTCAGGAGAAACATGGcTCAAAgATGGCCTTCcTcGATGGcAATCC
Solanum tu	aTTGAACAGgTTTCTTCAGGAGAAACATGGTTCAAAAATGGCCTTTTTAGATGGTAACCC
Petunia_x	TTTGAACAGATTTCTTCAGGAGAAACATGGTTCAAAAATGGCCTTTTTAGATGGTAATCC
Nicotiana	TTTGAACAGATTTCTTCAGGAGAAACATGGTTCAAAAATGGCCTTTTTAGATGGTAACCC
C.annuum	gTTGAAC <mark>AGATTTCTT</mark> CAGGAGAAACATGG <mark>T</mark> TCAAA <mark>A</mark> ATGGCCTTtTT <mark>A</mark> GATGG <mark>T</mark> AA <mark>T</mark> CC
Theobroma	TTTGAAtcGATTTCTgCAGGAGAAAaATGGaTCAAAgATGGCaTTCTTgGATGGCAAcCC
Eriobotrya	TTTGAAtcGATTcCTcCAGGAGAAACAcGGcTCcAAgATGGCtTTCTTgGATGGTAgTCC
Manihot_es	aTTGAACcGATTTCTTCAGGAGAAACATGGTTCAAAgATGGCtTTCTTAGATGGaAATCC
Piper_colu	TCCaGAGAGACTaTGCATGCCaATTGTaaAtCAcATTacGTCAttAGGTGGagAAGTCAG
Solanum_tu	TCCTGAGAGACTTTGCATGCCGATTGTTGAACACATcGAGTCAAAAGGTGGCCAAGTCAG
Petunia_x	TCCTGAGAGACTTTGCATGCCGATTGTTGAACATATTGAGTCAAAAGGTGGCCAAGTCAG
Nicotiana	TCCTGAGAGACTTTGCATGCCGATTGTTGAACATATTGAGTCAAAAGGTGGCCAAGTCAG
C.annuum	TCCTGAGAGACTTTGCATGCCGATTGTTGAACATATCGAGTCAAAAGGTGGaCAAGTCAG
Theobroma	cCC <mark>T</mark> GAGAGgCT <mark>T</mark> TGCATGCCtATTGTTaAtCA T AT <mark>TG</mark> AGTCActgGGTGGtgAgGTCtG
Eriobotrya	cCCcGAGAG <mark>A</mark> CTcTGtgctCCaATTGTTGAtCA <mark>T</mark> ATccAGTCAttgGGTGGtgAAGTCcG
Manihot_es	cCCaGAGAGgCT <mark>T</mark> TGCATGCCaATTGTTGAtCA <mark>T</mark> AT <mark>T</mark> cAGTCAttgGGTGGtgAAGTCcG
Piper_colu	ACTTAACTCgCGATTACAgAAAATcGAatTGAATcAtGATGGAACaGTCAAacaTTTTgT
Solanum_tu	ATTgAACTCACGAATAAAAAAAgATTGAGTTGAATGAGGATGGgAgTGTCAAGTGTTTTAT
Petunia_x	ACTAAACTCACGAATAAgAAAgATcGAGCTGAAcGAGGATGGAAgTGTCAAGTGTTTTAT
Nicotiana	ACTAAACTCACGAATAAAAAAAAAATTGAGCTCAATGAGGATGGAAgTGTCAAaTGTTTTAT
C.annuum	ACTgAACTCACGAATAAAAAAAgATTGAGCTGAATGAGGATGGAAgTGTCAAGTGTTTTAT
Theobroma	gCTtAACTC <mark>A</mark> CGAATA <mark>A</mark> AgAAAATaGA <mark>G</mark> CTcAAT <mark>G</mark> AtGATGGAA <mark>CT</mark> GTgAA <mark>G</mark> aGTTTTcT
Eriobotrya	AactAAtTCcCGAATAcAgAAAATTGAtCTaAATaAtGATGGAA <mark>C</mark> aGTCAAGaGTTTTgT
Manihot_es	gCTAAAtTCACGAATAAAgAAAATTGA <mark>G</mark> tTaAATaAtGATGGAACaGTgAAaaGaTTTtT
Piper_colu	tCTtAcaAATGGaAGTACAATTGAgGGAGATGCgTATGTtgTTGCCACTCCAGTtGAcAT
Solanum_tu	ACTGAATgAcGGtAGTACAATTGAgGGcGATGCTTtTGTGTTTGCCACTCCAGTGGATAT
Petunia_x	ACTGAATAATGGCACTtCAATTGAgGGAGATGCaTtcGTGTTTGCCgCTCCAGTGGATAT
Nicotiana	ACTGAATAATGGCAGTACAATTAAAGGAGATGCTTTTGTGTTTGCCACTCCAGTGGATAT
C.annuum	ACTGAAcgATGGtAGTACAATTGAgGGAGATGCTTtTGTGTTTGCgACTCCAGTGGATAT
Theobroma	tCTaAc <mark>TAATGGC</mark> AaT <mark>ACA</mark> ATTGAAGGAGATGCTTATGTaaTgGCagCTCCAGTtGA <mark>T</mark> AT
Eriobotrya	ACTaAATAATGGgAGTgtgATTGAAGcAGATGCgTATGTGTTcGCCACTCCAGTtGATAT
Manihot_es	ACTaAATAgctGggagACgATTGAAGGAGATGtTTATGTGTTTGCtACTCCAGTtGAcAT
Piper_colu	CCTaAAGCgTCTTTTGCCTGAAGACTGGAAAGAacTctCtTATTTCCAgAAATTGGAGAt
Solanum_tu	ttTCAAGCTgCTTTTGCCTGAAGACTGGAAAGAGATTCCATATTTCCAAAAgTTGGAGAA
Petunia_x	ttTCAAGCTTCTTTTGCCTGAAGACTGGAAAGAGATTCCATATTTCCAAAAgTTGGAGAA
Nicotiana	CtTCAAGCTTCTTTTGCCTGAAGACTGGAAAGAGATCCCATATTTCCAAAAgTTGGAGAA
C.annuum	ttTCAAGCTTCTTTTGCCTGAAGACTGGAAAGAGATTCCATATTTCCAAAAgTTGGAGAA
Theobroma	tCTgAAGCTaCTTTTGCCTGAAGACTGGAgAGAGATTtCATACTTCaAgAAATTaGAGAA
Eriobotrya	CCTaAAGCTTCTaTTGCCTGAAaACTGGAAAGAGATgCCATATTTCaAgAAATTGGAGAA
Manihot_es	CCTgAAGCTTCTTTTGCCTGACGACTGGAAAGAGATTCC TTACTTCaAgAAATTGGAGAA
Piper_colu	AcTgGTgGGAGTACCaGTTATtAAcGTgCATATATGGTTTGACAGAAAgCTGAAGAACAC
Solanum_tu	gTTÄGTCGGAGTACCTGTTATAAATGTÄCATATATGGTTCGACAGAAAACTGAAGAACAC
Petunia_x	gTTAGTcGGAGTACCTGTTATAAATGTgCATATATGGTTTGACAGAAAACTGAAGAACAC
Nicotiana	gcTAGTgGGAGTtCCTGTgATAAATGTcCATATATGGTTTGACAGAAAACTGAAGAACAC
C.annuum	gTTAGTTGGAGTACCTGTgATAAATGTcCATATATGGTTTGACAGAAAACTGAAGAACAC
Theobroma	ATTAGTTGGAGTCCCaGTaATCAACGTtCACATATGGTTTGAtAGgAAAtTGAAGAACAC

http://phylogeny.lirmm.fr/phylo_cgi/get_result.cgi?task_id=ee39ecf642d51f23cacc4e0bf1b07834&results_in_list=4&raw=1&file=alignment.html

1/2

Figure 3: Multiple sequence alignment. Similar residues are colored as the most conserved one (according to BLOSUM62). Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5 . *P. colubrinum* PDS was showing high level of similarity with *PDS* nucleotide sequences from other plant species



0.1

Figure 4: Phylogenetic tree constructed using *PDS* sequences from different taxa. *Piper colubrinum* stands distinct in the phylogenetic tree.

Further confirmation of the presence of PDS fragment was performed by restriction digestion of plasmids isolated from positive colonies using EcoRI enzyme. Sequencing the plasmid with TOPO vector specific GW1 & GW2 primers, confirmed the sequence as *Phytoene desaturase*. Sequence and phylogenetic analysis showed similarity to phytoene desaturase from other plants. *P. colubrinum PDS* (KM582050) showed 80% similarity to *Theobroma cacao* (XM007034086.1), *Nicotiana tomentosiformis*

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(XM009615723.1), *Eriobotrya japonica* (JN004211.1) ,*Capsicum annuum* (X68058.1), *Solanum tuberosum* (XM006342818.1), *Petunia xhybrida* (AY593974.1), and *Manihot esculenta* (GU120072.1) phytoene desaturase. A 600 bp PDS fragment was re-amplfied using gene specific primers (Fig 2 e).

P. colubrinum is recalcitrant to stable genetic transformation, which makes it difficult for gene functional studies based on stable genetic transformation. In the present study we have amplified and characterized the partial sequence of *PcPDS* which can be used as a reporter gene in gene functional validation by VIGS. In many plant species, VIGS has been standardized using down regulation of *PDS* gene expression. The sequence obtained in this study can be used to construct *PcPDS* VIGS vector which can be further used to optimize virus induced gene silencing in *P. colubrinum*.

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