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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 non-model 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 ± 2^0C 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.

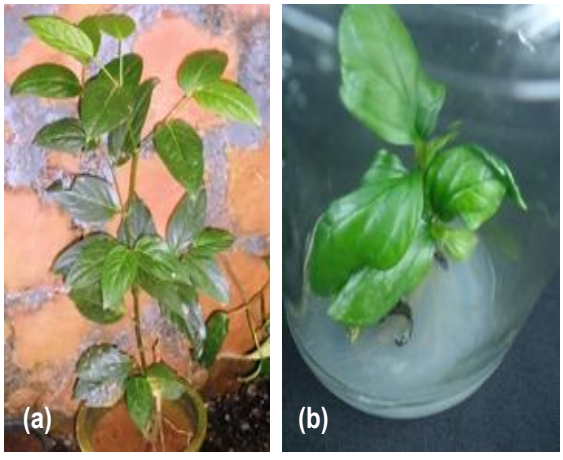

Figure 1: *Piper colubrinum* plants. a. Healthy plant maintained in the net house. b. *In-vitro* maintained plant

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 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 DH5α *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.

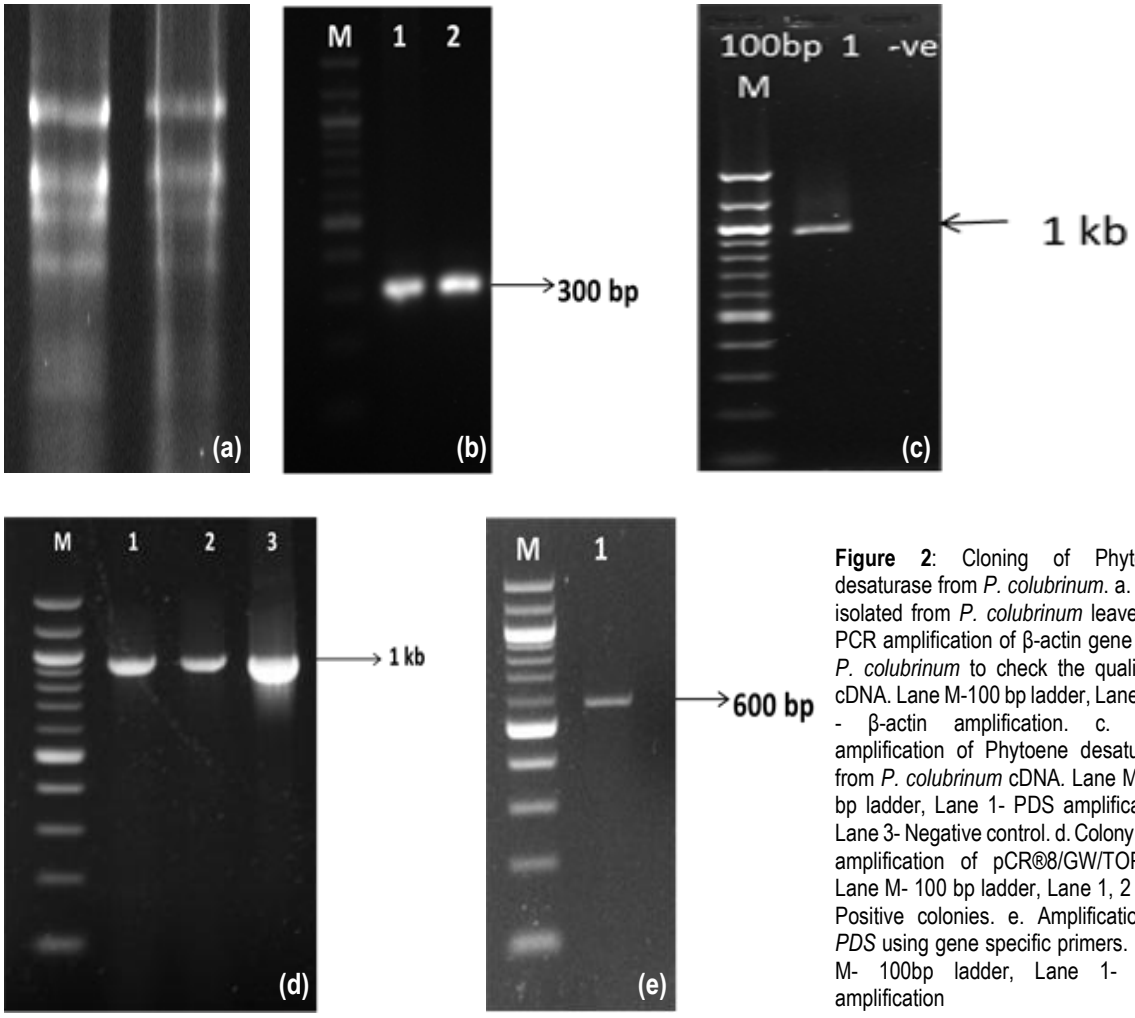

Figure 2: Cloning of Phytoene desaturase from *P. colubrinum*. a. RNA isolated from *P. colubrinum* leaves. b. PCR amplification of β-actin gene from *P. colubrinum* to check the quality of cDNA. Lane M-100 bp ladder, Lane 1&2 - β-actin amplification. c. PCR amplification of Phytoene desaturase from *P. colubrinum* cDNA. Lane M-100 bp ladder, Lane 1- PDS amplification, Lane 3- Negative control. d. Colony PCR amplification of pCR®8/GW/TOPO®. Lane M- 100 bp ladder, Lane 1, 2 &3 – Positive colonies. e. Amplification of *PDS* using gene specific primers. Lane M- 100bp ladder, Lane 1- PDS amplification

Bioinformatic analysis

BLAST program of NCBI was used for identity searches for *PDS* sequence at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). 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 55^0C Tm (Fig 2 c). Purified *PDS* gene fragment were cloned into pCR®8/GW/TOPO® cloning vector and transformed into to DH5α *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).

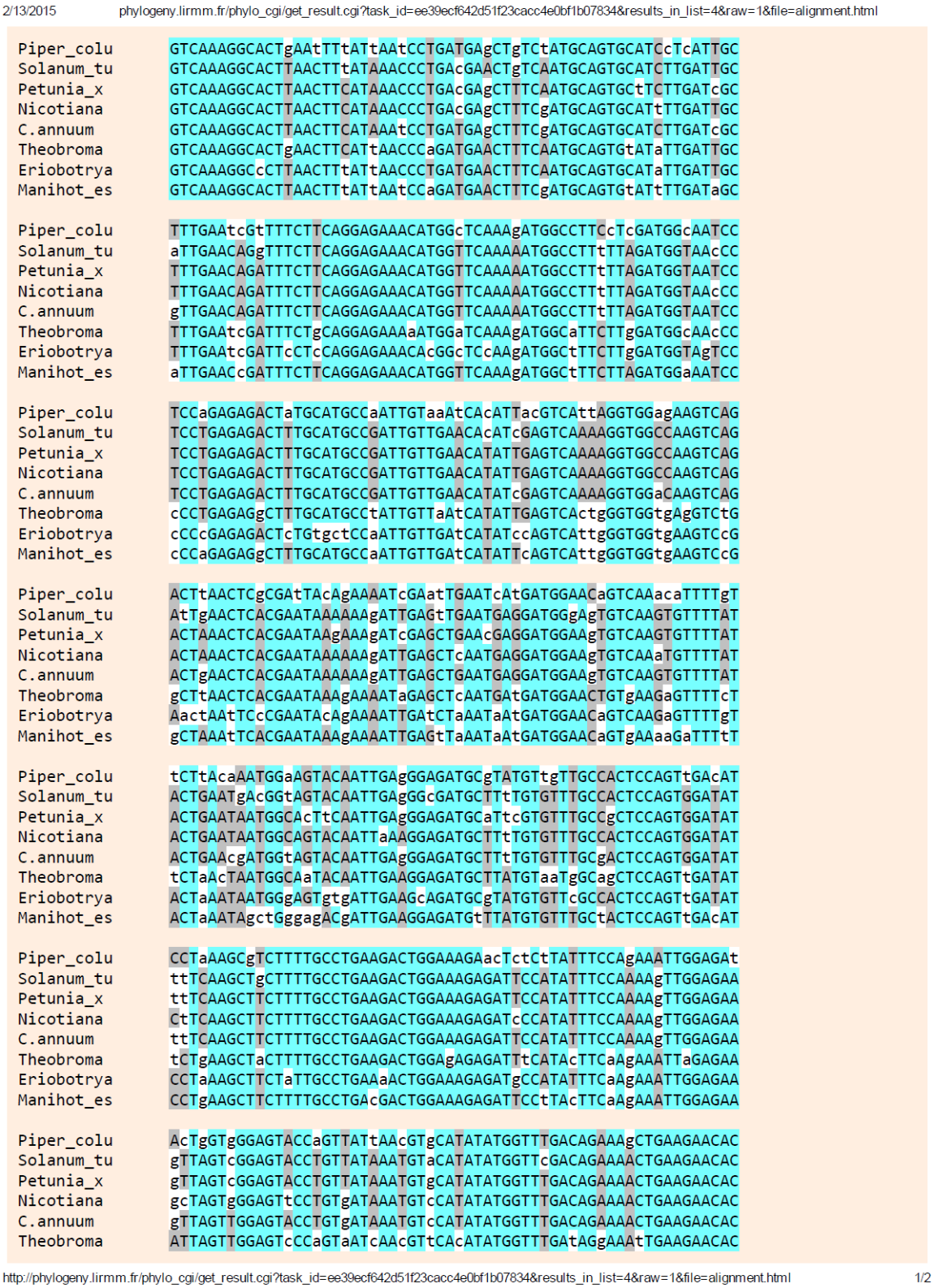

Figure 3: Multiple sequence alignment. Similar residues are colored as the most conserved one (according to BLOSUM62). Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5 . *P. colubrinum* PDS was showing high level of similarity with *PDS* nucleotide sequences from other plant species

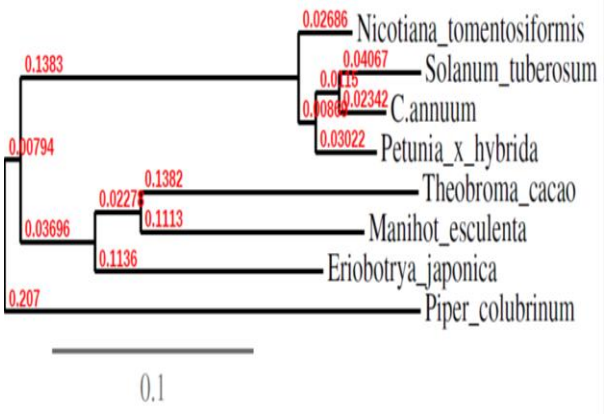

Figure 4: Phylogenetic tree constructed using *PDS* sequences from different taxa. *Piper colubrinum* stands distinct in the phylogenetic tree.

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* (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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