Cloning and Expression Analysis of the Cycloartenol Synthase Gene from *Paeonia delavayi* Postprint

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Date: 2019-08-26T00:00:00+00:00

Abstract

To investigate the mechanism of action of the Cycloartenol Synthase gene in the biosynthesis of Paeonia delavayi, this study employed RT-PCR technique to clone, for the first time from Paeonia delavayi, a cycloartenol synthase gene (PdCAS) containing a complete open reading frame. The gene has a full length of 2,274 bp and encodes 757 amino acids. The PdCAS protein sequence exhibits over 86% similarity with protein sequences from peach (Prunus persica), Prunus yedoensis var. nudiflora, and grape (Vitis vinifera). Sequence alignment analysis demonstrated that PdCAS possesses the typical DCTAE domain of the oxidosqualene cyclase superfamily and the signature sequence DGSWYGSWGVCFTYG characteristic of triterpene synthases. The PdCAS protein from Paeonia delavayi clusters with CAS proteins from Rosaceae species including apple (Malus domestica XP 008391430.1), pear (Pyrus bretschneideri XP 009370034.1), rose (Rosa chinensis XP 024193310.1), Prunus yedoensis var. nudiflora (PQQ11009.1), and peach (Prunus persica XP 007225240.1). Transcriptional pattern analysis revealed that the PdCAS gene is expressed in all tissues of Paeonia delavayi, with relatively higher expression levels in petals. The PdCAS gene cloned in this study is closely associated with the synthesis of sterol compounds in Paeonia delavayi.

Full Text

Preamble

DOI: 10.11931/guihaia.gxzw201904021

Title: Cloning and Expression Analysis of Cycloartenol Synthase Gene from *Paeonia delavayi*

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Abstract: To investigate the functional mechanism of cycloartenol synthase (CAS) in sterol biosynthesis in *Paeonia delavayi*, we cloned a full-length CAS gene with a complete open reading frame from *P. delavayi* using RT-PCR technology for the first time. The full-length cDNA of the *PdCAS* gene was 2,274 bp, encoding 757 amino acids. The PdCAS protein sequence exhibited over 86% similarity with sequences from peach (*Prunus persica*), Japanese flowering cherry (*Prunus yedoensis* var. *nudiflora*), and grape (*Vitis vinifera*). Sequence alignment analysis revealed that PdCAS possesses the typical DCTAE motif of the oxidosqualene cyclase superfamily and the signature sequence DGSWYGSWGVCFTYG of triterpenoid synthases. Phylogenetic analysis showed that the *P. delavayi* PdCAS protein clustered with CAS proteins from Rosaceae species including apple (*Malus domestica* XP_008391430.1), Chinese white pear (*Pyrus bretschneideri* XP_009370034.1), Chinese rose (*Rosa chinensis* XP_024193310.1), Japanese flowering cherry (*Prunus yedoensis* var. *nudiflora* PQQ11009.1), and peach (*Prunus persica* XP_007225240.1). Transcriptional pattern analysis indicated that *PdCAS* was expressed in all tissues of *P. delavayi*, with relatively high expression levels in petals. The *PdCAS* gene cloned in this study is closely related to the synthesis of sterol compounds in *P. delavayi*.

Keywords: *Paeonia delavayi*, cycloartenol synthase (CAS), cDNA cloning, gene function analysis

Funding: Supported by the National Natural Science Foundation of China (31860177) and Yunnan Applied Basic Research Youth Program (2016FD100).

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Introduction

Cycloartenol is a plant sterol compound widely distributed throughout the plant kingdom. Recent studies have revealed that cycloartenol exhibits significant pharmacological activities, including anti-inflammatory (Akihisa et al., 2000), antioxidant (Islam et al., 2009), antitumor (Prakash et al., 2004), and cholesterol-regulating effects (Fukuoka et al., 2014). It also serves as an important pharmaceutical intermediate and lead compound, representing a key precursor for the biosynthesis and semi-chemical synthesis of phytosterols such

as campesterol and steroidal saponins (Zhang et al., 2017). The content of cycloartenol determines the efficiency of phytosterol biosynthesis, and it significantly influences plant growth and development (Gas-Pascual et al., 2014).

Cycloartenol synthase (CAS) belongs to the oxidosqualene cyclase (OSC) family and represents a key regulatory gene in the synthesis of cycloartenol and plant sterols (Zhang et al., 2017; Liu et al., 2019). To date, CAS genes have been isolated and cloned from over 20 plant species, including *Eleutherococcus senticosus* (Xing et al., 2012), *Salvia miltiorrhiza* (Li et al., 2013), and *Astragalus membranaceus* (Chen et al., 2015), with functional validation through heterologous expression in some cases. However, no studies on the cycloartenol synthase gene from *Paeonia delavayi* have been reported to date.

Paeonia delavayi belongs to the genus *Paeonia* in the family Paeoniaceae. The root bark of *Paeonia* species is a traditional Chinese medicinal material commonly known as "mudanpi" or "danpi," containing active components such as phenols (e.g., paeonol), terpenoids, glycosides (e.g., paeoniflorin), and tannins, with functions including anticoagulation, blood pressure reduction, anti-inflammation, and central nervous system inhibition. As a peony group within the *Paeonia* genus, *P. delavayi* is also widely used as an ethnic medicinal material, with its root bark exhibiting antibacterial activity and applications in analgesia, sedation, anti-inflammation, and treatment of female disorders (Wu et al., 2002).

Based on transcriptome sequencing of *P. delavayi*, this study successfully cloned the cycloartenol synthase (CAS) gene from *P. delavayi*, conducted bioinformatic analysis, functional validation, and transcriptional pattern analysis, laying a foundation for further investigation of CAS gene function in this species.

1.1 Experimental Materials

Paeonia delavayi plants were cultivated at the nursery base of Yunnan Academy of Forestry. Roots, stems, leaves, seeds, flower buds (flowers completely enclosed by calyx), buds with exposed petals, fully bloomed petals, and petals at the withering stage (petals beginning to wilt) were collected and immediately frozen in liquid nitrogen for preservation.

1.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted from roots, stems, leaves, seeds, and flowers at various developmental stages of *P. delavayi* using a plant total RNA extraction kit. RNA quality was assessed using 1% agarose gel electrophoresis, and RNA concentration was measured using a NanoDrop™ 2000 spectrophotometer. The RNA was then reverse-transcribed into cDNA and stored at -20°C for subsequent use.

1.3 Cloning of the *PdCAS* Gene

Using the CAS gene from *Cyclamen persicum* as a template, BLAST searches were performed against the transcriptome data of *P. delavayi* petals to obtain the cDNA sequence of the *P. delavayi* cycloartenol synthase gene (*PdCAS*). Based on this sequence, specific primers containing start and stop codons were designed: *PdCAS1F*: 5' -ATGTGGAAGCTGAAGATCGC-3' and *PdCAS1R*: 5' -TCAGGAGACTTGCAATACCC-3' .

Using *P. delavayi* petal cDNA as template and *PdCAS1F*/*PdCAS1R* as primers, the full-length *PdCAS* gene fragment was amplified using EasyPfu DNA polymerase. After electrophoresis verification, the target fragment was ligated into the pGMT vector, confirmed by PCR detection, and sent to Shanghai Sangon Biotech for sequencing.

1.4 Protein Sequence Analysis of the *PdCAS* Gene

After obtaining the *PdCAS* gene cDNA sequence, the ORF Finder tool on NCBI (http://www.ncbi.nlm.nih.gov/) was used to analyze and obtain its protein amino acid sequence, and ProParam was used to predict its physicochemical properties. The protein amino acid sequence of *PdCAS* was used for BLAST searches on NCBI to select plant cycloartenol synthase protein sequences with high homology to PdCAS, and MEGA 6.0 was used to construct a phylogenetic tree.

1.5 Expression Analysis of *PdCAS* Gene in Different Organs

Total RNA was extracted from various organs, flowers at different developmental stages, and petals of different colors of *P. delavayi* using a plant total RNA extraction kit. After assessing RNA quality and concentration via agarose gel electrophoresis and NanoDrop™ 2000, respectively, the RNA was reverse-transcribed to synthesize the first-strand cDNA. The expression of *PdCAS* in different organs including roots, stems, leaves, and petals was then detected using specific primers *TPdCAS F* (5' -TTGAGAGAGAAGGCTCTTCG-3') and *TPdCAS R* (5' -TCGATGAACATAGCAGCTCT-3'). After agarose gel electrophoresis detection, the integrated absorbance of the *PdCAS* gene was analyzed using GENE-SNAPS image analysis software for relative quantitative analysis.

Results

2.1 RNA Extraction and *PdCAS* Gene Cloning

Total RNA was extracted from *P. delavayi* petals and other organs following the plant total RNA extraction kit protocol, and reverse-transcribed into first-strand cDNA for storage. Using primers *PdCAS1F* (containing the start codon)

and *PdCAS1R* (containing the stop codon), with *P. delavayi* petal cDNA as template, the cDNA fragment was amplified using EasyPfu DNA polymerase. After electrophoresis verification, the target fragment was ligated into a cloning vector and cultured overnight at 28°C, then transformed into *E. coli* DH5 competent cells. Following colony PCR detection, the samples were sent to BGI for sequencing. The full-length coding region cDNA of the *P. delavayi* CAS gene was obtained, measuring 2,274 bp and encoding 757 amino acids, and was designated as *PdCAS*.

[Figure 1: see original paper] Gene cloning of PdCAS from *Paeonia delavayi*

2.2 Protein Sequence Analysis of *P. delavayi PdCAS* Gene

The ORF Finder online program was used to predict the open reading frame of *PdCAS*, and ProParam was used to predict its physicochemical properties. The results showed that the protein consists of 757 amino acids with a molecular weight of 86,117.2 Da and an isoelectric point of 6.12. The deduced 757-amino-acid sequence was compared with known CAS protein sequences from other plants. The results revealed that the *P. delavayi* PdCAS protein sequence exhibited 86.66% similarity with peach (*Prunus persica*) CAS, 86.66% similarity with Japanese flowering cherry (*Prunus yedoensis* var. *nudiflora*) CAS, and 86.49% similarity with grape (*Vitis vinifera*) CAS. Analysis of the *P. delavayi* PdCAS protein sequence identified one typical DCTAE motif of the oxidosqualene cyclase superfamily (Poraiia et al., 1994) and one signature sequence DGSWYGSWGVCFTYG of triterpenoid synthases (Xing et al., 2012) ([Figure 2: see original paper]).

[Figure 2: see original paper] Alignment of CAS protein sequences. Note: Pd, *Paeonia delavayi*; Vv, *Vitis vinifera* (accession No. XP_002264289.1); Pp, *Prunus persica* (accession No. XP_007225240.1); Py, *Prunus yedoensis* var. *nudiflora* (accession No. PQQ11009.1); Black box, conserved site.

To investigate the molecular evolutionary status and phylogenetic relationships of *P. delavayi* with other plant species, the deduced PdCAS protein sequence was used for BLAST searches on NCBI to obtain CAS protein sequences from other plants. Multiple protein sequence alignment was performed using Clustal W integrated in MEGA 6.0, and a phylogenetic tree of PdCAS and CAS proteins from other plants was constructed using the Neighbor-joining algorithm with 1,000 bootstrap replicates ([Figure 3: see original paper]). The results showed that *P. delavayi* PdCAS protein clustered with CAS proteins from Rosaceae species including apple (*Malus domestica* XP_008391430.1), Chinese white pear (*Pyrus bretschneideri* XP_009370034.1), Chinese rose (*Rosa chinensis* XP_024193310.1), Japanese flowering cherry (*Prunus yedoensis* var. *nudiflora* PQQ11009.1), and peach (*Prunus persica* XP_007225240.1) ([Figure 3: see original paper]).

[Figure 3: see original paper] Phylogenetic tree of PdCAS and CAS in other plants

2.3 Transcriptional Pattern Analysis of *PdCAS* Gene in *P. delavayi*

Roots, stems, leaves, seeds, and petals at various stages of *P. delavayi* were collected. Total RNA was extracted from petals at different developmental stages and various organs following the plant total RNA extraction kit protocol and reverse-transcribed into cDNA. Specific primers *PdCAS1F* and *PdCAS1R* were used to detect *PdCAS* gene expression in different tissues including petals and stamens at various stages, as well as roots, stems, leaves, and seeds ([Figure 4: see original paper]). The results demonstrated that *PdCAS* was expressed in roots, stems, leaves, seeds, stamens, and petals at all stages, with relatively high expression in petals and stamens, particularly in petals at the withering stage.

[Figure 4: see original paper] The expression of *PdCAS* gene in different tissues of *P. delavayi*

Discussion

CAS is a key gene that directs the isoprenoid metabolic pathway toward sterol compound synthesis (Kim et al., 2005). Analyzing its expression patterns across different organs can provide insights into sterol synthesis regulation. RT-PCR results indicated that *PdCAS* was expressed in flowers at different stages, seeds, leaves, stems, and roots, similar to the expression characteristics of CAS genes in *Dioscorea zingiberensis* (Tu et al., 2010) and *Acanthopanax senticosus* (Xing et al., 2012), demonstrating constitutive expression. The ubiquitous expression of *PdCAS* in roots, stems, leaves, flowers, and seeds of *P. delavayi* also confirms the gene' s universal expression pattern. However, expression levels varied significantly among organs, with higher expression in petals and lower expression in roots and stems. The maximum expression was observed in petals one day after pollination at the withering stage, while the minimum expression occurred in stems.

In this study, *PdCAS* expression was highest in reproductive organs. Although expression was low in roots and stems, its presence indicates that cycloartenol derivatives exist in these tissues. Previous studies have suggested that *P. delavayi* is primarily used medicinally for its root bark (Wu et al., 2002). If cycloartenol and its derivatives are the main active components of "danpi," then flowers, leaves, and stems may also possess medicinal efficacy. While expression levels are high in flowers and leaves, the accumulation of compounds may not necessarily exceed that in roots because flowers wither and leaves fall. As a storage organ, the root may accumulate active substances over years, and although expression levels are low, the content of active compounds may not be less than that in petals.

The *PdCAS* gene protein sequence cloned in this study showed 80% similarity with CAS protein sequences from Rosaceae plants such as peach and apple, indi-

cating high structural and sequence conservation. Previous studies have isolated five classes of squalene cyclases in different plants: CAS, -amyrin synthase (-AS), dammarenediol synthase, lanosterol synthase, and lupeol synthase (Xing et al., 2012). Both triterpenoids and phytosterols in organisms originate from the same precursor—2,3-oxidosqualene—synthesized via the isoprenoid pathway. 2,3-oxidosqualene is cyclized by these five cyclases to generate phytosterol and triterpenoid skeletons. CAS primarily functions to catalyze the cyclization synthesis of various sterols and steroid-like compounds (Ming et al., 2010), where 2,3-oxidosqualene is converted to cycloartenol under the action of CAS, which then undergoes a series of catalytic reactions to generate phytosterols (a pathway known as the "cycloartenol pathway") (Zhang et al., 2017). The presence of the characteristic DCTAE sequence of the oxidosqualene cyclase superfamily in the PdCAS protein sequence in this study also demonstrates that *PdCAS* belongs to the squalene cyclase gene family. DCTAE can be created through site-directed mutagenesis of DDTAV in squalene synthase, changing the substrate from squalene to 2,3-oxidosqualene (Dang & Prestwich, 2000). Therefore, the *PdCAS* gene is closely related to sterol compound synthesis in *P. delavayi*. Future studies will attempt heterologous expression of *PdCAS* and potentially related P450s to obtain new cycloartenol derivatives.

The "cycloartenol pathway"is the main route for sterol compound synthesis (Gas-Pascual et al., 2014). This pathway and cycloartenol significantly affect plant growth and development, but the specific mechanisms remain inadequately studied. Cycloartenol also exhibits various pharmacological activities, though the underlying mechanisms are not yet clear. Therefore, future research should focus on its pharmacological activities, physiological functions, and related secondary metabolites to establish a foundation for better development and application.

References

Akihisa T, Yasukawa K, Yamaura M, et al., 2000. Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects[J]. *J Agr Food Chem*, 48(6): 2313-2319.

Chen J, Wu XT, Xu YQ, et al., 2015. Global transcriptome analysis profiles metabolic pathways in traditional herb *Astragalus membranaceus* Bge. var. Mongolicus (Bge.) Hsiao[J]. *BMC Genomics*, 16(S7): 1-20.

Chaturvedula VSP, Zhou BN, Gao Z, et al., 2004. New lupane triterpenoids from *Solidago canadensis* that inhibit the lyase activity of DNA polymerase beta[J]. *Bioorg Med Chem*, 12(23): 6271-6275.

Dang T, Prestwich GD, 2000. Site-directed mutagenesis of squalene-hopene cyclase: altered substrate specificity and product distribution[J]. *Chem Biol*, 7(8): 643-649.

Fukuoka D, Okahara F, Hashizume K, et al., 2014. Triterpene alcohols and

sterols from rice bran lower postprandial glucose-dependent insulinotropic polypeptide release and prevent diet-induced obesity in mice[J]. *J Appl Physiol*, 117(11): 1337-1348.

Gas-Pascual E, Berna A, Bach TJ, et al., 2014. Plant oxidosqualene metabolism: cycloartenol synthase-dependent sterol biosynthesis in *Nicotiana benthamiana*[J]. *PLoS One*, 29(10): e109156.

Islam MS, Yoshida H, Matsuki N, et al., 2009. Antioxidant, free radical-scavenging, and NF-kappaB-inhibitory activities of phytosteryl ferulates: structure-activity studies[J]. *J Pharmacol Sci*, 111(4): 328-337.

Kim OT, Kim MY, Hwang SJ, et al., 2005. Cloning and molecular analysis of cDNA encoding cycloartenol synthase from *Centella asiatica* (L.) Urban[J]. *Biotechnol Bioproc Eng*, 10(1): 16-22.

Liang Y, Zhao S, Zhang X, 2009. Antisense suppression of cycloartenol synthase results in elevated ginsenoside levels in *Panax ginseng* hairy roots[J]. *Plant Mol Biol Rep*, 27(3): 298-304.

Li Z, Wang DH, Yao W, et al., 2013. Cloning and expression analysis of cycloartenol synthase gene from *Salvia miltiorrhiza* Bunge[J]. *Acta Bot Boreali-Occident Sin*, 33(7): 1285-1291.

Liu MD, Li CF, Zhang YS, 2019. Molecular cloning of cycloartenol synthase from *Trigonella foenum-graecum* L. and its effect on diosgenin biosynthesis[J]. *Plant Science Journal*, 37(1): 87-92.

Luo ZL, Zhang KL, Ma XJ, et al., 2016. Research progress in synthetic biology of triterpen saponins[J]. *Chin Tradit Herbal Drugs*, 47(10): 1806-1814.

Ming QL, Han T, Huang F, et al., 2010. Advances in studies on ginsenoside biosynthesis and its related enzymes[J]. *Chin Tradit Herbal Drugs*, 41(11): 1913-1917.

Poraiia K, Heweit A, Prestwich GD, et al., 1994. A specific amino acid repeat in squalene and oxidosqualene cyclases[J]. *Trends Biochem Sci*, 19(4): 157-158.

Prakash Chaturvedula VS, Zhou BN, Gao ZJ, et al., 2004. New lupane triterpenoids from *Solidago canadensis* that inhibit the lyase activity of DNA polymerase beta[J]. *Bioorg Med Chem*, 12(23): 6271-6275.

Tu BM, Chen YQ, Yang ZF, 2010. Molecular cloning and analysis of the full-length cycloartenol synthase gene from *Dioscorea zingiberensis* Wright[J]. *Acta Bot Boreali-Occident Sin*, 30(1): 8-13.

Wu SH, Luo XD, Ma YB, et al., 2002. Monoterpenoid derivatives from *Paeonia delavayi*[J]. *J Asian Nat Prod Res*, 4(2): 135-140.

Xing ZB, Long YH, Wu P, et al., 2012. Cloning of cycloartenol synthase gene from *Eleutherococcus senticosus* and its expression analysis[J]. *Chin Tradit Herbal Drugs*, 43(7): 1387-1392.

Zhang ZL, Luo ZL, Shi HW, et al., 2017. Research advance of functional plant pharmaceutical cycloartenol about pharmacological and physiological activity[J]. *Chin J Chin Mater Med*, 42(3): 433-437.

Note: Figure translations are in progress. See original paper for figures.