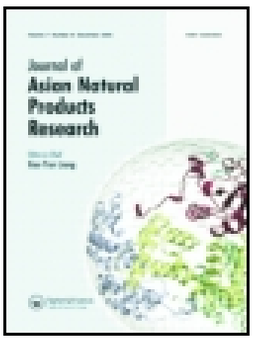

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A cycloartenol synthase from the steroidal saponin biosynthesis pathway of *Paris polyphylla*

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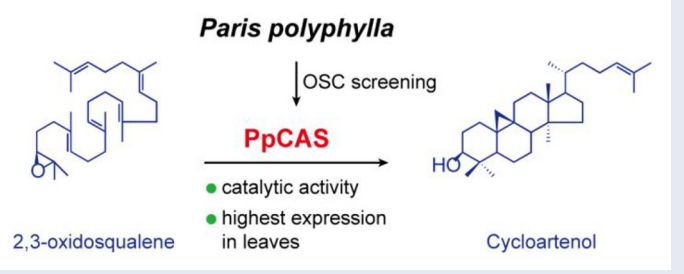
ABSTRACT

Steroidal saponins named polyphyllin are the major active components of *Paris polyphylla*. Cycloartenol synthase (CAS) is a key enzyme that catalyzes the formation of the sterol scaffold. In this study, we cloned a putative *CAS* gene from *Paris polyphylla*. Heterologous expression in yeast indicated that PpCAS can convert 2,3-oxidosqualene into cycloartenol. qRT-PCR analysis showed that the expression of *PpCAS* was highest in leaves and lowest in roots. To our best knowledge, this is the first report of the functional characterization of cycloartenol synthase from *Paris polyphylla*, which lays the foundation for further analysis of the biosynthesis pathway of polyphyllins.



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Cycloartenol synthase; oxidosqualene cyclase; polyphyllins; steroidal saponin biosynthesis; *Paris polyphylla*

1. Introduction

Paris polyphylla Smith var. *yunnanensis* (Franch.) Hand. -Mazz. is the main source plant of the traditional Chinese medicinal herb Paridis rhizoma, which contains a variety of chemical components with a wide range of pharmacological activities. Many Chinese medicines containing the rhizome of this plant as the main raw material, including "Yunnan BaiYao," "ReDuQing" and "GongXueNing," have significant

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clinical efficacy in the treatment of tumors, functional uterine bleeding and various inflammatory conditions [1]. The major active components of this plant are steroidal saponins named polyphyllins, which have anticancer activity, antimicrobial activity, anti-inflammatory activity, anthelmintic activity and a hemostatic effect [2, 3].

The cyclization of 2,3-oxidosqualene, catalyzed by oxidosqualene cyclases (OSC), is the first committed step in the biosynthesis of sterols and triterpenes. Many types of OSCs have been cloned and identified, such as β-amyrin synthase [4], cycloartenol synthase [5], multifunctional triterpene synthase [6] and so on. Different from triterpenoids with a chair-chair-chair conformation, cycloartenol synthase (CAS) can convert oxidosqualene into cycloartenol, with chair-boat-chair conformation as a leading precursor in the biosynthesis of steroids [7]. This step is an important branch of the steroid and terpenoid biosynthesis pathways in higher plants [8]. In fact, as a common precursor, cycloartenol plays an important role in the biosynthesis of phytosterols and steroidal saponins (Figure 1). Cycloartenol can be further converted into phytosterols via the catalysis of S-adenosyl-L-methionine-sterol C24 methyltransferase 1 (SMT1) and cytochrome P450 (CYP450) [9]. It can also undergo a series of modifications to form cholesterol, including oxidation and reduction reactions [10]. After that, the C-16, C-22, and C-26 positions of cholesterol are hydroxylated and cyclized to form a hemiketal, which further yields steroidal sapogenins with a spiroketal structure, such as diosgenin [11].

Because of their potential ability to modify the chemical structures of their substrates, OSCs have attracted the attention of many investigators [12]. However, to our knowledge there are no reports of the functional characterization of OSCs in *Paris polyphylla*. In this study, the full-length cDNA of a putative CAS gene was cloned from *Paris polyphylla* and characterized by heterologous expression in yeast cells. Furthermore, the expression of the *PpCAS* gene in different tissues was investigated by quantitative real-time PCR.

2. Results and discussion

2.1. Sequence analysis of PpCAS

The open reading frame of the candidate gene *PpCAS* (GenBank: MN368727) was 2286 bp long and was predicted to encode a protein comprising 761 amino acids with a molecular weight of 86973.36 Da and a theoretical isoelectric point of 6.54. The protein was predicted to contain a transmembrane domain encompassing residues 606 to 628. Protein domain analysis showed that PpCAS has a terpenoid cyclase/protein prenyltransferase alpha-alpha toroid domain and the conserved site of terpene synthases. The amino acid sequence of PpCAS showed high similarity (76.79%−84.75%) to homologous genes from several other species. Multiple sequence alignments showed that PpCAS contains one DCTAE motif and four QW (QXXXGXW and QXXXGXXXW) motifs (Figure 2), which may be associated with substrate binding and stabilization of the carbocation intermediates, respectively [13]. In addition, PpCAS also has the MWCHCR motif, which is commonly found in cycloartenol synthases [14]. In fact, it has been demonstrated that Ile and Tyr are conserved sites in CAS. The *AtCAS1* Ile481Val, Tyr410Thr, Tyr410Cys and Tyr532His mutants can convert 2,3-oxidosqualene to produce lanosterol [15], while the His477Asn/Ile481Val double mutant is an accurate lanosterol synthase [16].

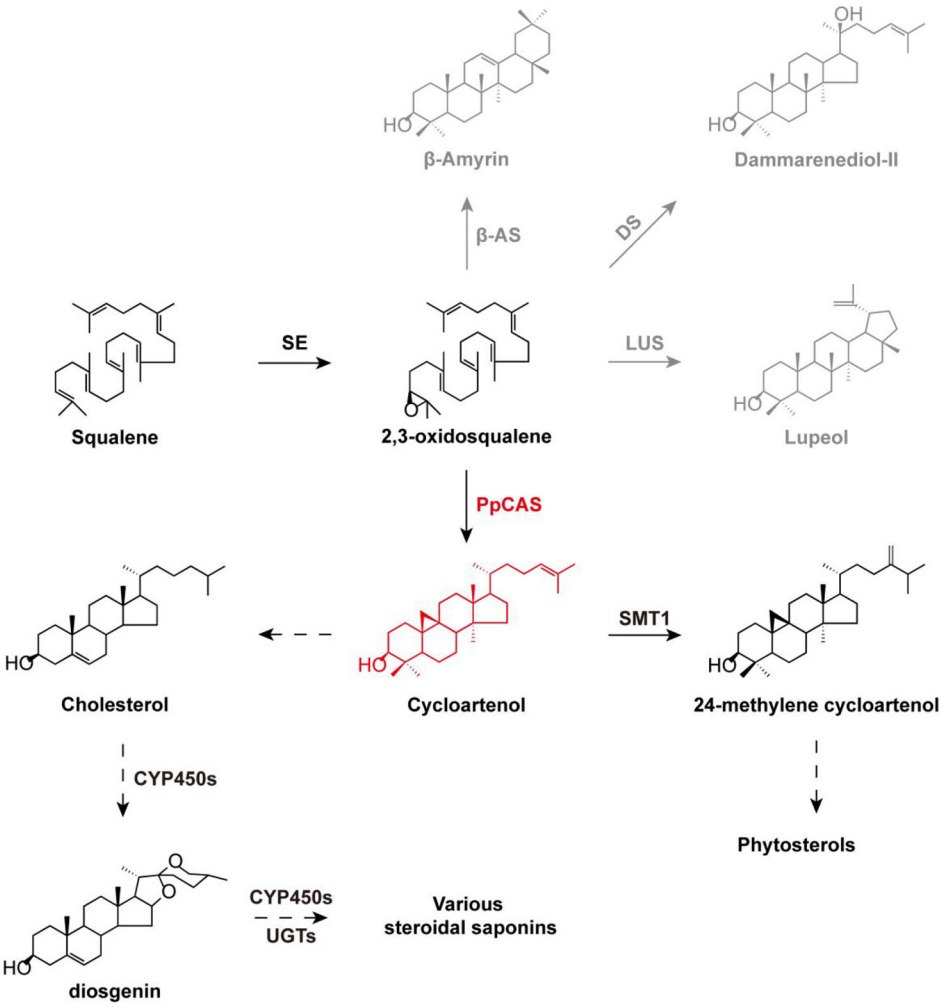

Figure 1. Putative biosynthetic pathway of steroidal saponins and phytosterols in *Paris polyphylla*. The solid- and dashed lines indicate single- and multiple catalytic steps, respectively. The enzyme identified in this study is shown in red. Other OSCs are shown in gray. SE, squalene epoxidase; β-AS, β-amyrin synthase; DS, dammarenediol synthase; LUS, lupeol synthase; SMT1, sterol C24-methyltransferase 1; CYP450s, cytochrome P450s; UGTs, UDP-glycosyltransferases.

2.2. Phylogenetic analysis

The analysis involved 28 sequences of OSCs from different plants, all of which were downloaded from NCBI (www.ncbi.nlm.nih.gov/). The phylogenetic tree showed that these OSCs are mainly grouped into 3 categories: *β*-amyrin/multifunctional triterpene synthase, lupeol synthase and cycloartenol synthase (Figure 3). As predicted, PpCAS clustered with cycloartenol synthase, forming a branch with FtCAS [17] and CSOSC1 [18], both of which are monofunctional cycloartenol synthases. This result prompted us to further characterize the function of PpCAS in yeast.

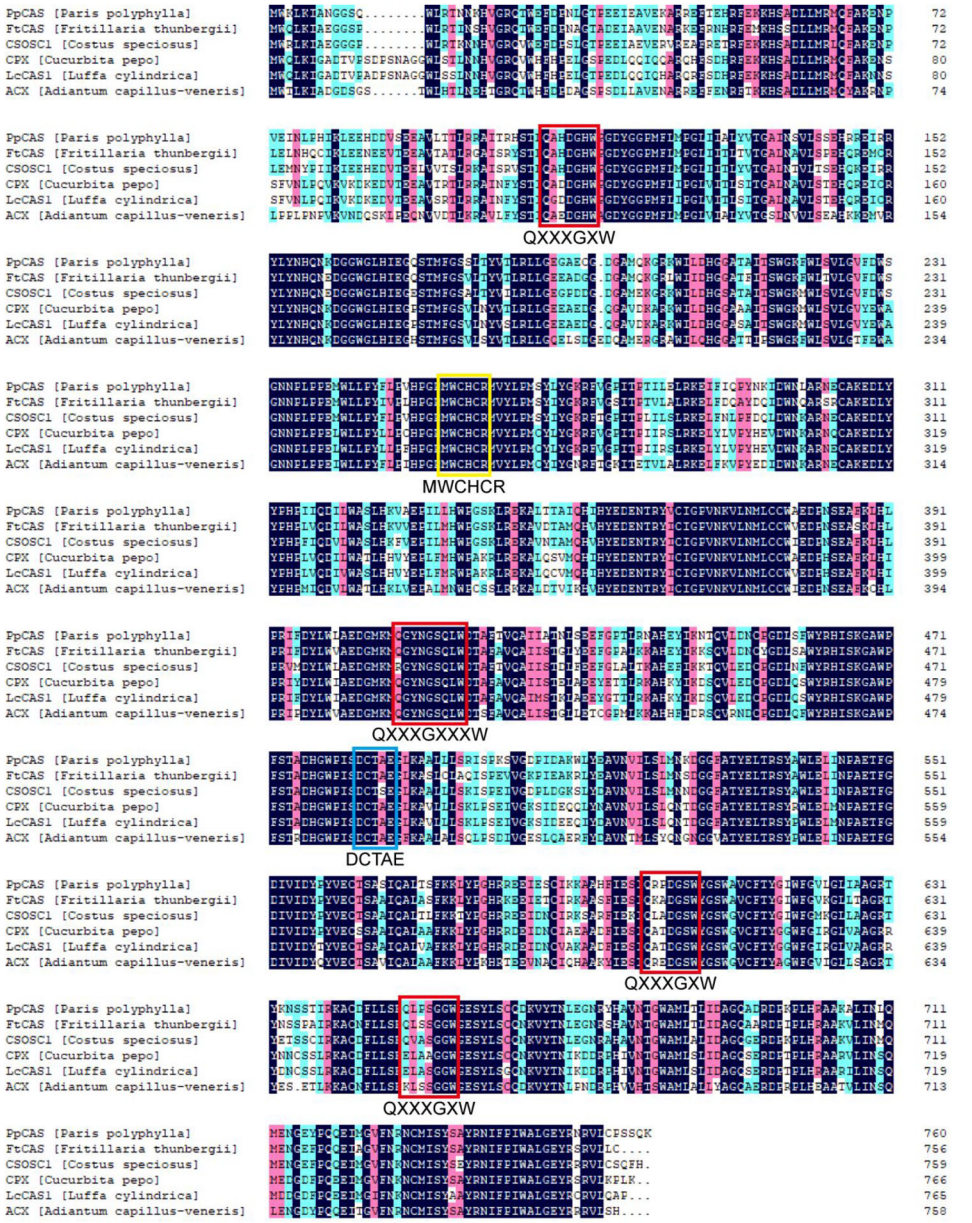

Figure 2. Amino acid sequence alignment of PpCAS with CASs from other species. The QW motifs, DCTAE motifs and conserved MWCHCR motifs are shown in red, blue and yellow boxes, respectively. The proteins and GenBank accession numbers are: FtCAS (AEO27878.1), CSOSC1 (AB058507.1), CPX (AB116237.1), LcCAS1 (AB033334.1), ACX (AB368375.1).

2.3. Functional characterization of PpCAS in yeast

To biochemically confirm the predicted function of *PpCAS*, we transferred the recombinant plasmid pYES2-PpCAS into a lanosterol synthase-deficient yeast strain. After culturing and induction, the extract of the recombinant yeast strain was analyzed

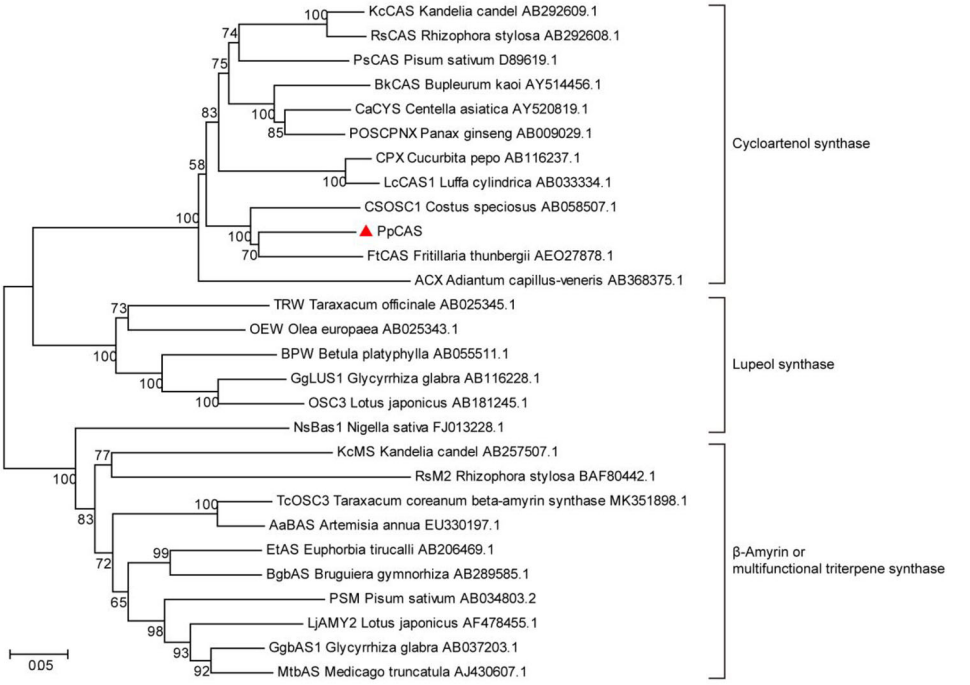

Figure 3. Phylogenetic tree of PpCAS and OSCs from other species. The tree was constructed in MEGA 6.0 software using the bootstrap method with 1000 iterations.

using GC-MS. A new peak appeared in the recombinant yeast at 9.9 minutes that was not present in the vector control (Figure 4), and it had the same retention time and mass spectral characteristics as the authentic standard of cycloartenol. The GC-MS results therefore indicate that PpCAS can produce cycloartenol from 2,3-oxidosqualene. To our best knowledge, this is the first report of the functional characterization of cycloartenol synthase from *Paris polyphylla*.

While some plants, such as *Betula platyphylla* [19] and *Polygala tenuifolia* [14] contain two *CAS* genes, only one *CAS* gene was found in the *Paris polyphylla* transcriptome, similar to many other species [20, 21]. The presence of multiple *CAS* genes may protect the phytosterol biosynthetic pathways against fatal mutations, or enable specific expression in response to different environmental conditions.

2.4. Relative expression of PpCAS in different tissues of Paris polyphylla

To investigate whether *PpCAS* has tissue-specific expression, the relative expression levels of the gene in roots, stems, leaves and flowers of *Paris polyphylla* were determined by qRT-PCR. As shown in Figure 5, the transcript levels of *PpCAS* were highest in the leaves, followed by stems and flowers, and lowest in the roots. The melting curves were shown in supplementary material Figure S1. The results of qRT-PCR analysis suggested that the initial step of polyphyllin biosynthesis may occur mainly in leaves, not in roots. Our findings are consistent with other studies [22], which

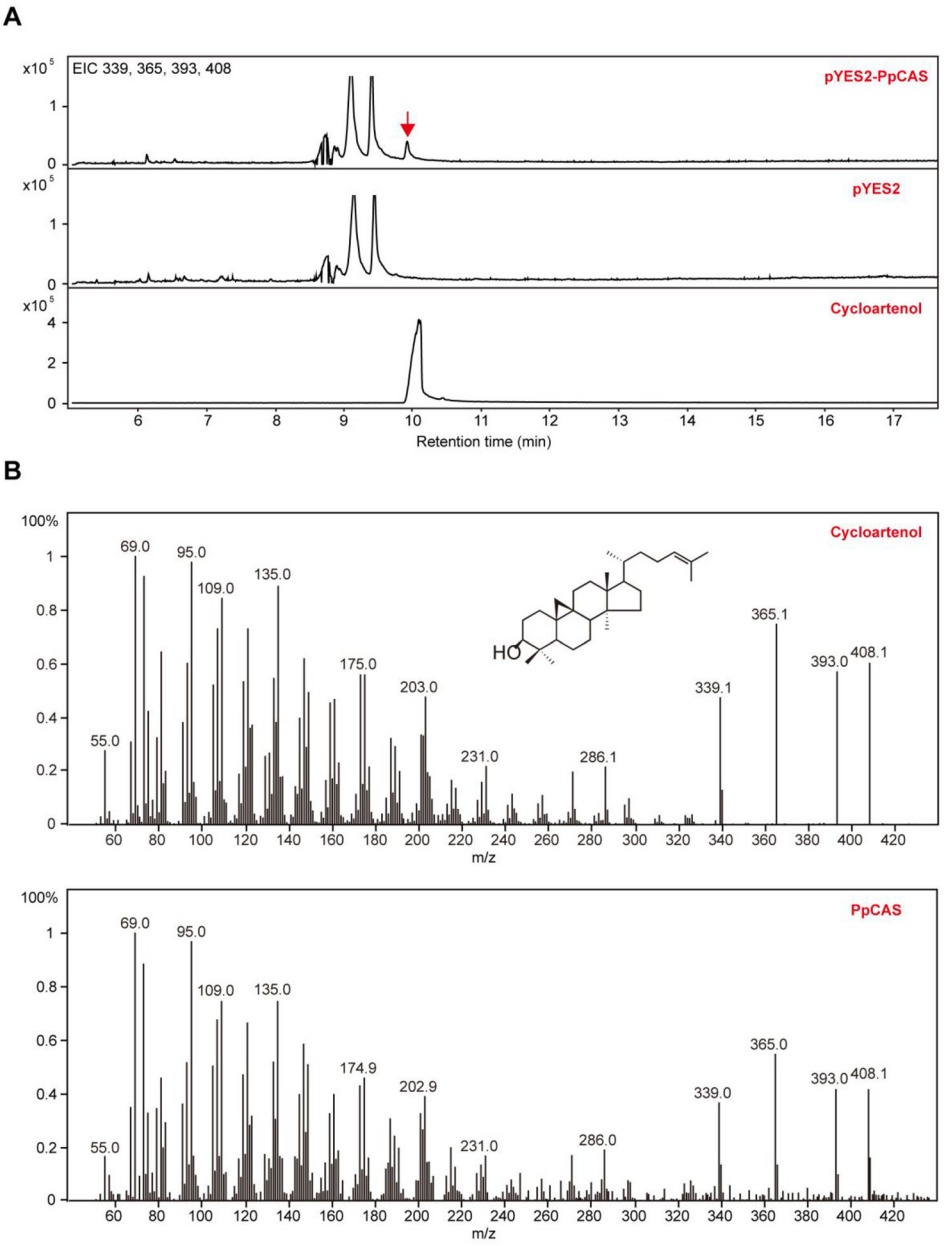

Figure 4. Gas chromatography-mass spectrometry (GC-MS) analysis of steroids extracted from yeast overexpressing the *PpCAS* gene. (A) GC chromatograms of yeast transformed with pYES2-PpCAS, control yeast transformed with the empty pYES2 vector and cycloartenol authentic standard; (B) MS of cycloartenol authentic standard and cycloartenol produced by the pYES2-PpCAS yeast.

reported that polyphyllins are mainly synthesized in chloroplasts, transported down through stems, and finally accumulated in roots. This proves that leaves are important organs for the biosynthesis of steroidal saponins in *Paris polyphylla*.

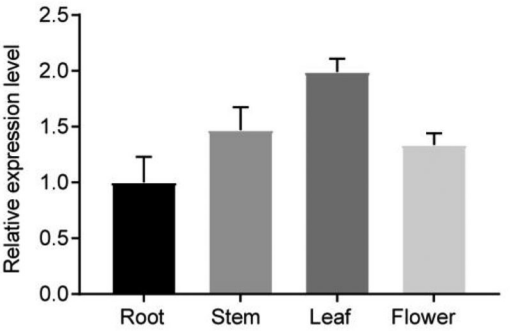

Figure 5. qRT-PCR analysis of *PpCAS* gene expression in the roots, stems, leaves and flowers of *Paris polyphylla*. The results are presented as the means ± standard error (n = 3).

3. Experimental

3.1. RNA extraction and reverse transcription

Total RNA was isolated from *Paris polyphylla* using the Eastep® Super Total RNA Extraction Kit (Promega Biotech, Beijing, China) and was converted to cDNA using the GoScript™ Reverse Transcription Mix, Oligo(dT) (Promega Biotech, Beijing, China) according to the manufacturer's instructions.

3.2. Cloning of the candidate gene

The full-length cDNA fragment of the candidate gene was amplified by PCR (Polymerase Chain Reaction) using 2× Phusion HF Master Mix (New England Biolabs, Beijing, China) in conjunction with the specific primers shown in Table 1. The PCR conditions were as follows: 98 °C for 30 s; 35 cycles at 98 °C for 10 s, 60 °C for 20 s and 72 °C for 1 min; 72 °C for 7 min. The PCR products were cloned into the pEASY- Blunt Zero cloning vector (TransGen Biotech, Beijing, China) and transformed into *E. coli* Trans1-T1 competent cells (TransGen Biotech, Beijing, China). The positive colonies were selected on LB (Luria-Bertani) medium with ampicillin and confirmed by sequencing.

3.3. Bioinformatic analysis of the protein sequence

The amino acid sequence was searched against the NCBI database (http://www.ncbi.NLM.NIH.gov) using BLAST (Basic Local Alignment Search Tool). The molecular mass and theoretical isoelectric point were predicted using the ProtParam tool (http://web.expasy.org/protparam). The transmembrane helices were predicted using TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Functional domains were identified using Interpro (www.ebi.ac.uk/Tools/InterProScan). Multiple sequence alignments were constructed using DNAMAN software. Phylogenetic analysis was performed using MEGA 6.0 software [23].

Table 1. PCR primers used in this study.

Purpose	Primer	Sequence (5′ to 3′)
Cloning	PpCAS-F	CTATCCCTCTTGGCTGGTAATGTGG
	PpCAS-R	GCTGATGAGCTGTCATTGTTTCTGG
pYES2-PpCAS	EcoRI-PpCAS-F	CCGCCAGTGTGCTGGAATTCATGTGGAAGCTGAAGATCGC
	NoTI-PpCAS-R	GCATGCTCGAGCGGCCGCTCACTGTTTCTGGGAAG
qRT-PCR	PpCAS-RT-F	TTCGCCGATACCTCTACAACC
	PpCAS-RT-R	CCACTTCCGCCCTTTCTGC
	GAPDH-F	TTGTAGGTCGGTTAATCGTGG
	GAPDH-R	TTTGATTTGGCACAGGGTCT

3.4. Expression of PpCAS in yeast

PpCAS was ligated into the pYES2 expression vector (Invitrogen, California, USA) using the pEASY®-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China) in conjunction with the specific primers shown in Table 1. In order to heterologously express and investigate the function of *PpCAS*, pYES2 (vector control) and the recombinant plasmid pYES2-PpCAS were respectively introduced into the yeast mutant GIL77 (-erg7, -ura; ATCC, Virginia, USA) using the Frozen-EZ Yeast Transformation II^{TM} kit (Zymo Research, California, USA). The positive colonies were grown in 50 ml of liquid synthetic complete medium without uracil (SC-URA) with 20 g/L glucose at 30 °C and 220 rpm for 2 days. To induce expression, the cells were collected by centrifugation at 2000 × g for 2 min, resuspended in 50 ml SC-URA with 20 g/L galactose, and cultivated under the same conditions for another 2 days.

3.5. GC-MS analysis

Transgenic yeast cells were harvested by centrifugation at 10000 × g for 1 min and refluxed with 15 ml 20%KOH/50%EtOH for 5 min. After the reflux mixture was completely cooled, it was extracted 3 times with 15 ml hexane. The organic phases were combined, evaporated to dryness, and derivatized with 100 μl of pyridine (Macklin, Shanghai, China) and 100 μl of BSTFA (Bis(trimethylsilyl) trifluoroacetamide) (containing 1% TMCS (Trimethylchlorosilane); Solarbio Science & Technology, Beijing, China) at 70 °C for 1 h. The derivatized extract was evaporated under N_2, reconstituted in 200 μl of hexane, centrifuged at 15000 × g for 10 min, and the supernatant was taken as the test sample.

The analysis was performed on a 7890B gas chromatography system (Agilent, California, USA) equipped with a DB-5ms column (15 m × 250mm × 0.1 mm), and interfaced with a 7000C triple-quadrupole mass spectrometer (Agilent, California, USA). A sample (1 μl) was injected at a He flow rate of 1 ml/min at 250 °C in splitless mode. The oven temperature program was as follows: 180 °C for 1 min, followed by a gradient from 180 °C to 280 °C at 20 °C/min, rising to 300 °C at 2 °C/min, with a final hold for 2 min at 300 °C. The ion trap temperature was 230 °C. The electron energy was 70 eV. Spectra were recorded in the range of 50-550 m/z.

3.6. Quantitative RT-PCR analysis

Total RNA from roots, stems, leaves and flowers was extracted and reverse transcribed using the Fast King RT Kit (TIANGEN Biotech, Beijing, China). The KAPA

SYBR FAST Universal qPCR Kit (Sigma-Aldrich, Shanghai, China) was used for quantification on a QuantStudio® 5 Real-Time PCR System (Thermo Fisher, Shanghai, China). The GAPDH gene was used as the internal standard [24], and the specific primers are shown in Table 1. The PCR conditions were as follows: 95 °C for 3 min, 40 cycles at 95 °C for 3 s and 60 °C for 30 s, 95 °C for 1 s, 60 °C for 20 s and 95 °C for 1 s. The results were analyzed using the $2^{-\Delta\Delta\ CT}$ method [25] and presented as the means ± SEs of three independent samples, each of which was measured in technical triplicate.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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