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Research article

Molecular characterization, expression, and regulation of *Gynostemma pentaphyllum* squalene epoxidase gene 1

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ABSTRACT

Gynostemma pentaphyllum (Thunb.) Makino is a perennial medicinal herb widely distributed in China. This herb contains important medicinal components called gypenosides, which belong to dammarane-type triterpenoid saponins. Squalene epoxidase (SE, EC 1.14.99.7) catalyzes the epoxidation of squalene to form oxidosqualene and is a key regulatory enzyme in triterpenoid saponin biosynthesis. In this study, a *SE* gene designated as *GpSE1* was isolated from *G. pentaphyllum* leaves. The deduced protein sequence of *GpSE1* contained two conserved domains involved in the catalytic function of SE. *GpSE1* was expressed as inclusion bodies in *Escherichia coli* cells, and the HIS-tagged recombinant protein was successfully purified and renatured in vitro. Immunofluorescence indicated that the polygonal reticular fluorescence signal of GpSE1 was significantly stronger in young leaves than in mature leaves and rhizomes. This finding is consistent with the tissue-specific expression pattern of *GpSE1* and suggests that the young leaves of *G. pentaphyllum* mainly serve as the active site of gypenoside synthesis. Methyl jasmonate (MeJA) treatment upregulated *GpSE1* expression in both the young and mature leaves of *G. pentaphyllum*, with greater upregulation in young leaves than in mature leaves. However, the expression of *GpSE1* was not enhanced continually with the increase in MeJA concentration. Moreover, the *GpSE1* expression was maximally regulated in response to 50 μM MeJA but not to 100 μM MeJA. This result indicates that MeJA exerts a concentration-dependent effect on *GpSE1* expression.

1. Introduction

Gynostemma pentaphyllum (Thunb.) Makino, which belongs to the family Cucurbitaceae, is a perennial medicinal herb widely distributed in China. The major medicinal components of *G. pentaphyllum* comprise gypenosides, which are dammarane-type triterpenoid saponins that are reportedly effective in the treatment of many illnesses, such as inflammation, cardiovascular diseases, and cancer (Lu et al., 2008; Bai et al., 2010). These saponins are synthesized via the isoprenoid biosynthesis pathway (Lee et al., 2004). In this pathway, two molecules of farnesyl pyrophosphate are condensed by squalene synthase (SS) to form squalene. Afterwards, squalene undergoes the first oxygenation step performed by squalene epoxidase (SE), which catalyzes the epoxidation of squalene to form 2,3-oxidosqualene (Laden et al., 2000; Kim et al., 2011). 2,3-oxidosqualene is cyclized into dammarane triterpenoid skeletons by dammarenediol-II synthase (DS), followed by various modifications to produce dammarane-type triterpenoid saponins with biological activities (Sun et al., 2013; Niu et al., 2014). SE was suggested as an important regulatory enzyme in the above-mentioned pathway (Han et al., 2010).

In medicinal plants, it is important to uncover the biosynthesis site of bioactive components, in order to promote the production of these components by improving fertilization and cultivation conditions (Yokota et al., 2011). However, the biosynthesis site of gypenosides in *G. pentaphyllum* remains uncertain. The localization of some key enzymes involved in the synthesis of bioactive components may reflect the biosynthesis site of these components. Several reports have documented the subcellular localization of

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enzymes involved in the biosynthesis of isoprenoids, benzylisoquinoline alkaloids, or storage proteins, by immunofluorescence labeling in *Arabidopsis*, opium poppy, and tobacco (Leivar et al., 2005; Samanani et al., 2006; Joseph et al., 2012). Thus, we can feasibly identify the biosynthesis site of gypenosides by determining the immunofluorescence location of key enzymes involved in the biosynthesis of gypenosides.

Methyl jasmonate (MeJA) is a plant-specific signaling molecule that plays important roles in plant defense responses, developmental processes, and secondary metabolism (Wasternack, 2007; Kim et al., 2009). The effect of MeJA on triterpene saponin biosynthesis has been documented in *Panax ginseng*. The transcription levels of *PgSS*, *PgSE*, and *PgDS*, which are genes involved in ginsenoside biosynthesis, are upregulated in ginseng hairy or adventitious root cultures after MeJA treatment, and the levels of ginsenosides increase accordingly (Kim et al., 2009; Wang et al., 2013). MeJA treatment also upregulates *BfSS1* expression in the roots of *Bupleurum falcatum* (Kim et al., 2011). However, insufficient information is available regarding the expression of key enzyme genes involved in gypenoside biosynthesis regulated by MeJA.

Considering that *SE* plays an important role in triterpene saponin biosynthesis (Han et al., 2010; Razdan et al., 2013), this study investigated the expression and location of the *G. pentaphyllum SE* gene and the effect of MeJA on the expression regulation of the *SE* gene in *G. pentaphyllum*. In this study, a *SE* gene designated as *GpSE1* was cloned from *G. pentaphyllum* leaves. The gene was expressed in *Escherichia coli* to assay the biochemical activity of the gene products. To investigate the biosynthesis site of gypenosides, a specific polyclonal antibody against the GpSE1 protein was prepared, and subcellular localization of the GpSE1 protein in various organs of *G. pentaphyllum* was performed by immunofluorescence labeling. *GpSE1* expression regulated by MeJA was analyzed by quantitative real-time polymerase chain reaction (PCR). Our results provide further insights into the molecular mechanisms of gypenoside synthesis in *G. pentaphyllum*, and may serve as a guide in increasing gypenoside production through genetic manipulation or improving cultivation and fertilization.

2. Materials and methods

2.1. Plant materials and growth conditions

Two-year-old *G. pentaphyllum* plants were planted in individual pots (5 L) filled with sandy loam soil and grown in a growth chamber at 22 °C ± 2 °C with 16 h light/8 h dark photoperiod.

2.2. MeJA treatment

G. pentaphyllum leaves were mist sprayed with different concentrations of MeJA (25, 50, and 100 μM) until liquid dripped from the leaves. Each plant had a preservative film covering the surface of the pot under the shoot to prevent the liquid from entering the soil. Young and mature leaves were collected from the plants at 6, 24, 48, 72, and 96 h after MeJA treatment. The leaves were immediately frozen in liquid nitrogen and then stored at −80 °C for *GpSE1* expression analysis. Leaf samples from untreated plants served as controls.

2.3. Cloning of GpSE1 and sequence analysis

Total RNA was extracted from *G. pentaphyllum* leaves by using the Easyspin Plant RNA Kit (Aidlab Biotech., Beijing, China), and first-strand cDNA was synthesized using a reverse transcription (RT) kit (Promega, Madison, WI, USA). The open reading frame (ORF) sequence of *GpSE1* was amplified through PCR by using the primers designed from the 5′ and 3′ ends of *GpSE* ORF (GenBank accession number: FJ906798.1). The PCR reaction was performed using Q5 DNA polymerase (NEB) in accordance with the manufacturer's instructions. The primers used in the cloning of *GpSE1* are listed in Table 1.

The physicochemical properties, hydrophilicity, transmembrane region, domain, and motif of the deduced GpSE1 protein sequence, as well as multiple sequence alignment, were analyzed as previously described (Guo et al., 2013). To elucidate the phylogenetic relationship of GpSE1 among plant SEs, SE family members of selected plant species were defined by Phytozome (www.phytozome.net) and PLAZA (bioinformatics.psb.ugent.be/plaza/). Their protein sequences were retrieved by BLASTP homology search using GpSE1 sequences. A manual curation of the retrieved sequences was performed based on multiple sequence alignment with ClustalW program in BioEdit software. A phylogenetic tree was constructed using the neighbor-joining algorithm and Poisson model included in Mega 5.05. Bootstrap with 1000 replicates was used to establish the confidence limit of the tree branches.

2.4. GpSE1 expression in E. coli and SDS-PAGE analysis

The *GpSE1* coding region was subcloned into the pET32a expression vector (Novagen, Madison, WI, USA). The construct pET32a-*GpSE1* and the empty pET32a vector (control) were transformed into *E. coli* BL21 (DE3) as previously reported (Zhao et al., 2015). Single colonies (transformants) were inoculated into liquid Luria–Bertani (LB) medium containing 50 μg mL^{-1} ampicillin and then cultured overnight at 37 °C in a shaker. The overnight cultures were diluted 50-fold for inoculation into fresh liquid LB medium. The cultures were grown to an OD600 of approximately 0.8 and then induced with 1 mM isopropylthiogalactoside (IPTG). Up to 1 mL of induced culture was harvested at 1, 3, 5, and 7 h after IPTG induction and then centrifuged at 13,000×*g* for 5 min at 4 °C. The pellet was resuspended in a buffer (300 mM NaCl, 50 mM Tris-HCl, pH 8.0) and then loaded on a 10% SDS-PAGE gel after heating the sample with an equal volume of 2 × SDS loading dye. To determine whether the HIS-tagged GpSE1 fusion protein was soluble, 1 mL of the induced culture harvested at 7 h of IPTG induction was

Table 1
DNA sequences of oligonucleotide primers used in this study.

Primer names	Oligonucleotide sequence 5′–3′	
	Forward primers	Reverse primers
GpSE1 ORF cloning		
GpSE1 F1 + R1	ATGGTGGATCAATTCTCCTTAGC	TCATCTGCCTCTCACAAGGGGTG
GpSE1 expression in E.coli		
GpSE1 *BamH* I F2 + *Sal* I R2 (subclone into PET32a)	GGATCCATGGTGGATCAATTCTCCTTAGC	GTCGACGTCTGCCTCTCACAAGGGGTGGAG
Real-time RT-PCR		
GpSE1 F3 + R3 (expression analysis)	GATCGGCTCTTGCTTATACTCT	CGCTGGGCATCAATCTCAT
GAPDH F + R (endogenous reference)	TCACGGACAGTGGAAGCATCAT	ACCCTTCAAATGAGCAGCAGC