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Characterization of spermidine synthase and spermine synthase – The polyamine-synthetic enzymes that induce early flowering in *Gentiana triflora*

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ABSTRACT

Polyamines are essential for several living processes in plants. However, regulatory mechanisms of polyamines in herbaceous perennial are almost unknown. Here, we identified homologs of two *Arabidopsis* polyamine-synthetic enzymes, spermidine synthase (SPDS) and spermine synthase (SPMS) denoted as *GtSPDS* and *GtSPMS*, from the gentian plant, *Gentiana triflora*. Our results showed that recombinant proteins of GtSPDS and GtSPMS possessed SPDS and SPMS activities, respectively. The expression levels of *GtSPDS* and *GtSPMS* increased transiently during vegetative to reproductive growth phase and overexpression of the genes hastened flowering, suggesting that these genes are involved in flowering induction in gentian plants.

1. Introduction

Polyamines are low molecular weight polycations that are ubiquitous to all living organisms, except for some Archaea. As common diamines and polyamines, putrescine (Put), spermidine (Spd), and spermine (Spm) are distributed in all eukaryotic cells [1]. In plants, these polyamines are reported to be involved in gene expression, protein and DNA synthesis, cell division, growth and developmental processes, and stress responses [2,3]. Polyamines often conjugate with molecules such as amides, hydroxycinnamic acids, lignins and proteins [4,5]. Conjugation may be a way of regulating the concentration of free polyamines [6,7]. Free polyamine concentrations are also regulated by transport from cytoplasm to vacuoles, mitochondria, and chloroplasts [2]. Because polyamines involve a number of cellular processes, their concentrations must be strictly regulated in several ways.

Put and other polyamines are synthesized by two alternative pathways — through ornithine decarboxylase (ODC; EC 4.1.1.17) or arginine decarboxylase (ADC; EC 4.1.1.19). Put is converted to Spd by Spd synthase (SPDS; EC 2.5.1.16) and Spd is then converted to Spm by Spm synthase (SPMS; EC 2.5.1.22). SPDS and SPMS are categorized as aminopropyltransferases that require decarboxylated *S*-adenosylmethionine (dcAdoMet) as an aminopropyl donor, which is produced by *S*-adenosylmethionine (AdoMet) decarboxylase (SAMDC; EC 4.1.4.50). The genes encoding these polyamine synthetic enzymes have been cloned from several plant species [8]. For example, in *Arabidopsis*, the genes *ADC1* and *ADC2* encode ADC, *SPDS1* and *SPDS2* encode SPDS, and *SPMS* encodes SPMS [9–12]. The *Arabidopsis ACAULIS5* (*ACL5*) gene shows high homology to *SPDS* and *SPMS*, and ACL5 protein has both Spm and thermospermine synthetic activities [9,13]. Additionally, four genes, *SAMDC1*, *SAMDC2*, *SAMDC3*, and *SAMDC4*, have also been identified as encoding SAMDC [14,15]. ODC and ADC are key enzymes of polyamine synthesis, but *Arabidopsis* does not possess a gene coding for ODC [16], suggesting that polyamine-synthetic pathway is different among plant species. Among these polyamine-related genes, a few plant *SPMS* genes have been isolated and studied [8,17], but, to our knowledge, no polyamine-synthetic enzyme genes have been isolated or characterized from any perennial herbaceous species so far, and information about this enzyme is scarce.

Gentians (*Gentiana* spp.) are herbaceous perennials native to the alpine regions of the world and are popular ornamental flowers in Japan. Prior to winter, gentians produce overwintering buds

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(OWBs) and enter dormancy. When spring comes, OWBs sprout and start vegetative growth [18]. Since gentians are cultivated for four to five years, growth control and disease protection are important problems in gentian-producing area. Yellow dwarf-like symptom (renamed as gentian Kobu-sho disease) caused by gentian Kobu-sho-associated virus, which induces polyamine accumulation in leaves and stems [19], is one of the most serious diseases that threaten gentian production [20]. Furthermore, the polyamine levels showed fluctuations in plants exposed to mineral deficiency as well as in OWBs during dormancy to budbreak [18,21]. Although the modulation of polyamine levels have been observed in several organs during different seasons, no attempts have been made to characterize the polyamine-synthetic enzymes.

In this study, we isolated the gentian homologs of *Arabidopsis SPDS* and *SPMS* (denoted *GtSPDS* and *GtSPMS*) and investigated their molecular characteristics. Recombinant proteins of GtSPDS and GtSPMS produced in *Escherichia coli* possessed SPDS and SPMS activity, respectively, indicating that these genes are functional in gentian. Our results also showed that the expression levels of *GtSPDS* and *GtSPMS* changed during dormancy, vegetative growth and reproductive growth stages. We generated transgenic *Arabidopsis* plants overexpressing *GtSPDS* and *GtSPMS* to investigate the effect of these genes. In this report, we discuss the possible functions of GtSPDS and GtSPMS in gentians.

2. Materials and methods

2.1. Plant materials

Gentians (*Gentiana triflora* cv. SpB) grown in an agricultural field at the Iwate Biotechnology Research Center were used. Overwintering buds (OWBs) were harvested during September 2012 to March 2013. Leaves and shoot apical meristems (SAMs) were harvested during April to August 2013. Samples were frozen in liquid nitrogen, freeze-dried, and stored at −20 °C until use. *Arabidopsis* plants were grown at 22 °C under short day (SD, 8/16 h light/dark) conditions.

2.2. Molecular cloning of polyamine-synthetic enzyme genes

Total RNA was extracted from freeze-dried tissues of *G. triflora* and *Arabidopsis* as described previously [18]. First-strand cDNA was synthesized from 1 μg of total RNA using a RNA PCR kit (Takara) with oligo(dT) primer according to the manufacturer's instructions. Partial cDNA sequences of *GtSPDS* and *GtSPMS* were obtained from our *G. triflora* EST-library [22]. Rapid amplification of cDNA ends (RACE) was performed to determine the complete nucleotide sequences using the GeneRacer kit (Invitrogen). Amplified fragments containing full-length ORF of *GtSPDS*, *GtSPMS* and *Arabidopsis SAMDC1* (*AtSAMDC1*; At3g02470) were cloned into pENTR TOPO vector (Invitrogen). The resulting plasmids, pENTR-*GtSPDS*, pENTR-*GtSPMS*, and pENTR-*AtSAMDC1*, were sequenced with the universal M13 forward and reverse primers using BigDye terminator chemistry and an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The primers used are listed in Supplementary Table 1.

2.3. Bacterial expression and purification of recombinant proteins

The cDNA of *GtSPDS*, *GtSPMS*, and *AtSAMDC1* cloned into a pENTR vector was transferred into pET-DEST42 vector (Invitrogen) using the Gateway recombination system. The resulting plasmids were transformed into *E. coli* BL21 (DE3). Cells were cultured in Luria–Bertani broth supplemented with 50 μg mL^{-1} ampicillin with shaking at 37 °C until an A_{600} of 0.5 was reached. The genes were expressed with induction at 25 °C for 18 h with 500 μM isopropylthiogalactoside and the bacterial pellets were disrupted with sonication in sonication buffer (20 mM Tris–HCl, pH 7.5, 250 mM NaCl). After centrifugation at 6800 *g* for 20 min, the His-tagged recombinant protein was purified by passing through a His GraviTrap column (GE Healthcare) and using a TALON cobalt affinity resin (Clonetech), according to the manufacturer's instructions. Purified recombinant proteins were quantified with a protein assay kit (Bio-Rad Laboratories). The *E. coli* cell crude extracts containing purified recombinant proteins were diluted with a loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 5% glycerol). After boiling for 5 min, the proteins were analyzed by SDS-PAGE (using 10% gels) and visualized with Coomassie Brilliant Blue R250 (Bio-Rad Laboratories).

2.4. Enzyme assays

For the estimation of SPDS and SPMS activities, dcAdoMet was synthesized in 200 μl of reaction mixture containing 50 mM Tris buffer (pH8.0), 0.5 mM pyridoxial phosphate (PLP), 10 mM AdoMet, and 400 μg of recombinant protein of AtSAMDC1 incubated at 37 °C for 30 min. After filtration through a Millipore 3-kD cutoff filter (Amicon), produced dcAdoMet was identified by capillary electrophoresis mass spectrometry (CE-MS; Agilent Technologies), and the solution was used as dcAdoMet solution. SPDS and SPMS activities were determined in 50 μl of reaction mixture containing 50 mM hydroxymethyl aminomethane (Tris) buffer (pH 9.0), 0.5 mM PLP, 1 μl of dcAdoMet solution, 100 ng of recombinant proteins of SPDS or SPMS, and 1 mM Put or Spd incubated at 37 °C for 10 min. Produced Spd and Spm were quantified using a CE-MS with selected ion mode (SIM) monitoring according to the method described previously [21].

2.5. Alignment and phylogenic tree of deduced amino acid sequences

Deduced amino acid sequences of *GtSPDS* (LC027438) and *GtSPMS* (LC027439) were aligned with SPDS and SPMS isolated from other plant species using the ClustalW algorithm [23]. The phylogenetic tree was constructed using the neighbor joining algorithm by the Mega5 software [24].

2.6. Quantitative PCR (qPCR) analysis

First-strand cDNA was synthesized from 500 ng of total RNA using a High Capacity cDNA Reverse transcription kit (Applied Biosystems) with random primers according to the manufacturer's instructions. qPCR was carried out using StepOnePlus (Applied Biosystems) with the KAPA SYBR FAST ABI Prism qPCR Kit (Kapa Biosystems, Woburn, MA). Amplification conditions were 95 °C for 10 min, followed by 40 cycles each consisting of 15 s at 95 °C and 1 min at 60 °C, and plate reading after each cycle. *G. triflora Ubiquitin* (*GtUBQ*) and *Arabidopsis elongation factor 1α* (*EF1α*) genes were used as the normalization control. The data was analyzed using the StepOne software (Applied Biosystems). Primer pairs used for qPCR are listed in Supplementary Table 1.

2.7. Generation of transgenic plants

To generate transgenic plants of *Arabidopsis thaliana* overexpressing *GtSPDS* and *GtSPMS* under the transcriptional control of the cauliflower mosaic virus 35S promoter, pENTR-GtSPDS and pENTR-GtSPMS were recombined with the pH2GW7 destination vector [25] using the Gateway recombination system. The binary plasmids, pH2GW7-GtSPDS and pH2GW7-GtSPDS, were introduced into wild-type *Arabidopsis* (Col-0) by *Agrobacterium*