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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*

tumefaciens-mediated transformation utilizing the floral dip method [26]. Transgenic plants were selected on a 0.8% (w/v) agar MS medium containing 50 μg mL^{-1} hygromycin. Homozygous T3 plants harboring the *GtSPDS* or *GtSPMS* were used for further analysis.

2.8. Reference regents

Put, Spd, and Spm were purchased from WAKO pure chemicals, and standard solutions of polyamines for quantification were prepared by adjusting these compounds to 50 and 100 μM.

3. Results

3.1. Isolation and phylogenetic analysis of gentian polyamine synthesis genes

We initially isolated gentian cDNAs that contained full-length ORFs homologous to *Arabidopsis SPDS* and *SPMS* to characterize the polyamine biosynthesis of gentian. We obtained partial sequences of gentian *SPDS* and *SPMS* by searching the gentian EST-library data. Using the RACE method, we obtained full-length ORFs of *SPDS* and *SPMS* genes, denoted as *GtSPDS* and *GtSPMS*. The deduced amino acid sequences of GtSPDS and GtSPMS had 317- and 351-amino acid residues, respectively. GtSPDS and GtSPMS had 82% identity to AtSPDS2 and 75% identity to AtSPMS, respectively (Fig. 1). The sequences also had homology with each other; GtSPDS shared 69% identity with GtSPMS. Both GtSPDS and GtSPMS possessed AdoMet-dependent methyltransferase domain [27] and AdoMet or dcAdoMet binding regions (Motif I to VI) [28]. Furthermore, phylogenic analyses revealed that GtSPDS clustered into an SPDS-like group, whereas GtSPMS could be placed into an SPMS-like group (Supplementary Fig. 1). Therefore, GtSPDS and GtSPMS could be related to polyamine synthesis, but may have different activities.

3.2. Polyamine biosynthetic activity of the recombinant GtSPDS and GtSPMS

To determine whether cloned GtSPDS and GtSPMS possess polyamine-synthetic activities, their recombinant proteins were expressed in *E. coli*. The recombinant GtSPDS and GtSPMS proteins appeared as bands with a molecular mass of 35 kDa and 39 kDa, respectively, on SDS-PAGE (Fig. 2A). The sizes were in agreement with the calculated molecular mass based on deduced amino acid sequences of GtSPDS (34864.78 Da) and GtSPMS (38997.84 Da). To confirm that these recombinant proteins possess SPDS or SPMS activities, the proteins were incubated with or without the corresponding substrates. GtSPDS produced Spd in the presence of Put, whereas no accumulation of Spd was observed when boiled GtSPDS was used or in the absence of Put. Likewise, GtSPMS produced Spm only when Spd was presented (Fig. 2B).

3.3. Seasonal expression of GtSPDS and GtSPMS in field-grown gentian plants

G. triflora cv. SpB enter dormancy from October to November and start to grow in April [18]. After vegetative growth, floral initiation occurred in June (data not shown). To elucidate the relationship between the polyamine-synthetic enzymes and gentian life cycle, we investigated seasonal changes of gene expression of *GtSPDS* and *GtSPMS* in OWBs, leaves, and SAMs (Fig. 3B). The expression levels of *GtSPDS* in OWBs were low, but slightly increased from October to February (Fig. 3B), whereas the levels in leaves gradually increased from April and reached a 5-fold increase in June. *GtSPDS* levels in SAM were maintained at a basal

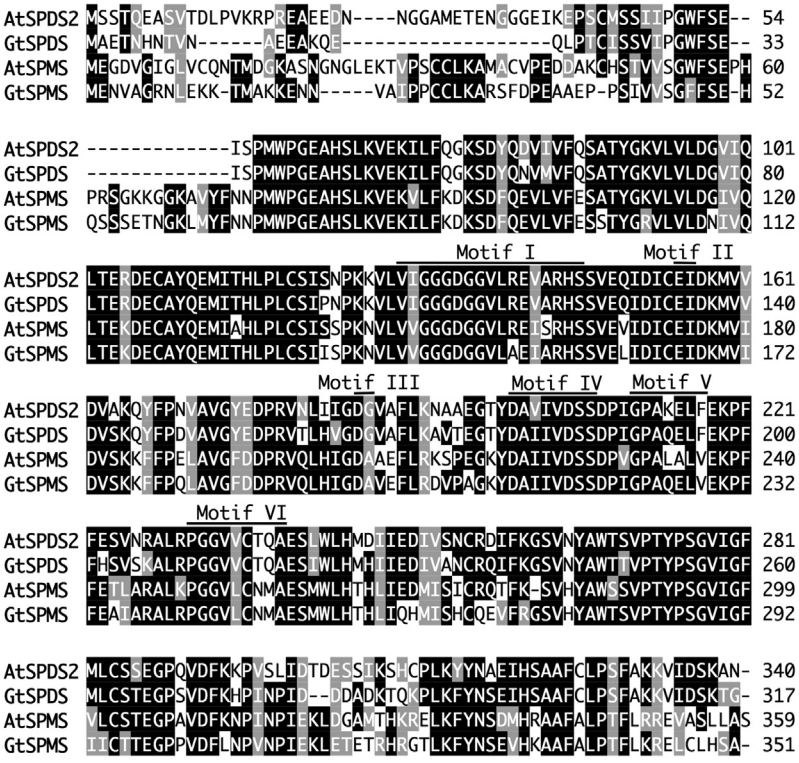

Fig. 1. Comparison of the deduced amino acid sequences of GtSPDS, GtSPMS, and *Arabidopsis* SPDS2 (AtSPDS2) and SPMS (AtSPMS). Overbars indicate the proposed binding site for AdoMet and dcAdoMet (motifs I to VI).

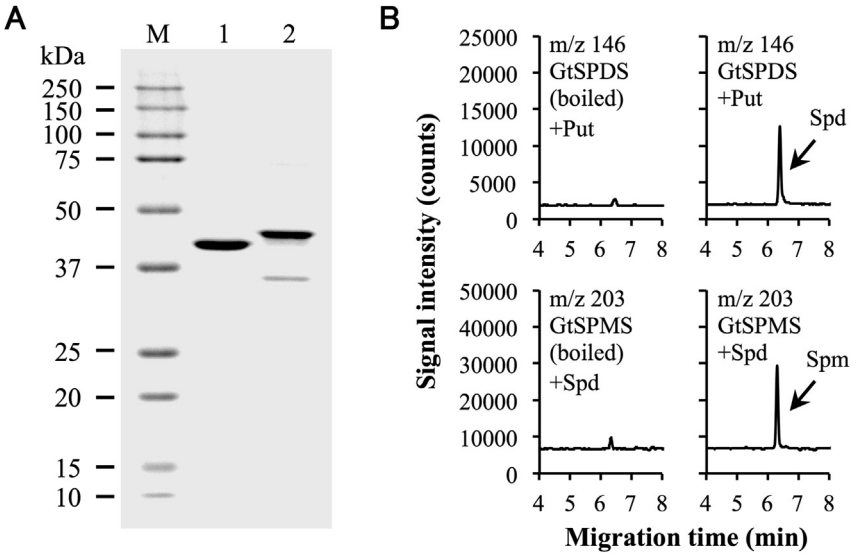

Fig. 2. Characterization of GtSPDS and GtSPMS. (A) Analysis of recombinant GtSPDS and GtSPMS proteins. The proteins were subjected to SDS-PAGE using a 10% polyacrylamide gel and stained by Coomassie Brilliant Blue R-250. Lane M, molecular size markers; lane 1, purified recombinant GtSPDS; lane 2, purified recombinant GtSPMS. (B) CE-MS electropherograms of the enzymatic reaction mixture. The mixture contained 200 ng of proteins, 1 mM Put or Spd as a substrate, and the negative control reaction was performed using boiled enzyme.

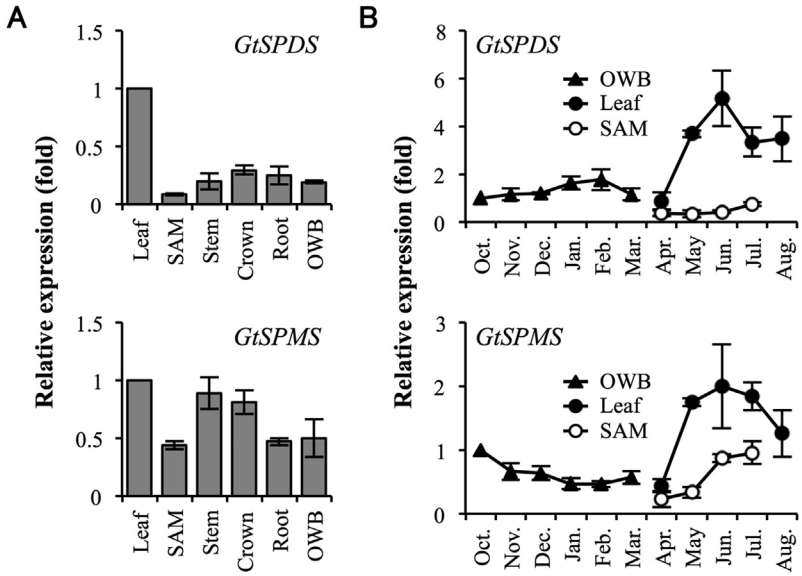

Fig. 3. Gene expression profiles of *GtSPDS* and *GtSPMS* in field-grown gentian. (A) *GtSPDS* and *GtSPMS* expression in different organs. Values were normalized to expression levels obtained in leaves. (B) Seasonal expression of *GtSPDS* and *GtSPMS* in leaves, SAMs, and OWBs of field-grown gentian. Values were normalized to expression levels obtained in October. Each value represents the mean ± SD from three independent measurements.

level throughout, with a marginal increase in July. The expression levels of *GtSPMS* in OWBs slightly decreased from October to February (Fig. 3B). Similar to that of GtSPDS, the levels of *GtSPMS* in leaves also increased and reached a maximum in June and gradually decreased in August. GtSPMS levels in SAMs increased from April to July.

3.4. Overexpression of GtSPDS and GtSPMS in Arabidopsis

To verify the effect of *GtSPDS* and *GtSPMS*, we generated transgenic *Arabidopsis* plants overexpressing *GtSPDS* or *GtSPMS* and selected the significant expression line (GtSPDS-OX1 and OX2; GtSPMS-OX1 and OX2) (Fig. 4A and B). We found that these transgenic plants significantly accelerate flowering under SD condition (Fig 4C). To obtain further details, we examined the expression of flowering-related genes of 7-week-old plants grown under SD condition (Fig 4D). In all transgenic lines, expression level of *FLOWERING LOCUS C* (*FLC*) and Gamma isoform of *FLOWERING CONTROL LOCUS A* (*FCAγ*) decreased and that of *FLOWERING LOCUS T* (*FT*) increased compared to vector control. Interestingly, the expression levels of *TEMPRANILLO 1* (*TEM1*) decreased only in GtSPDS-OX lines. The expression levels of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *gibberellin 20 oxidase 1*(*GA20ox*) tended to increase in GtSPDS-OX, but not in GtSPMS-OX, while those of *TWIN SISTER OF FT* (*TSF*), *FY*, *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*), *APETALA2* (*AP2*), *SCHLAFMUTZE* (*SMZ*), *SHORT VEGETATIVE PHASE* (*SVP*), and *FRUITFULL* (*FUL*) fluctuated in some transgenic lines, without any easily discernible pattern.

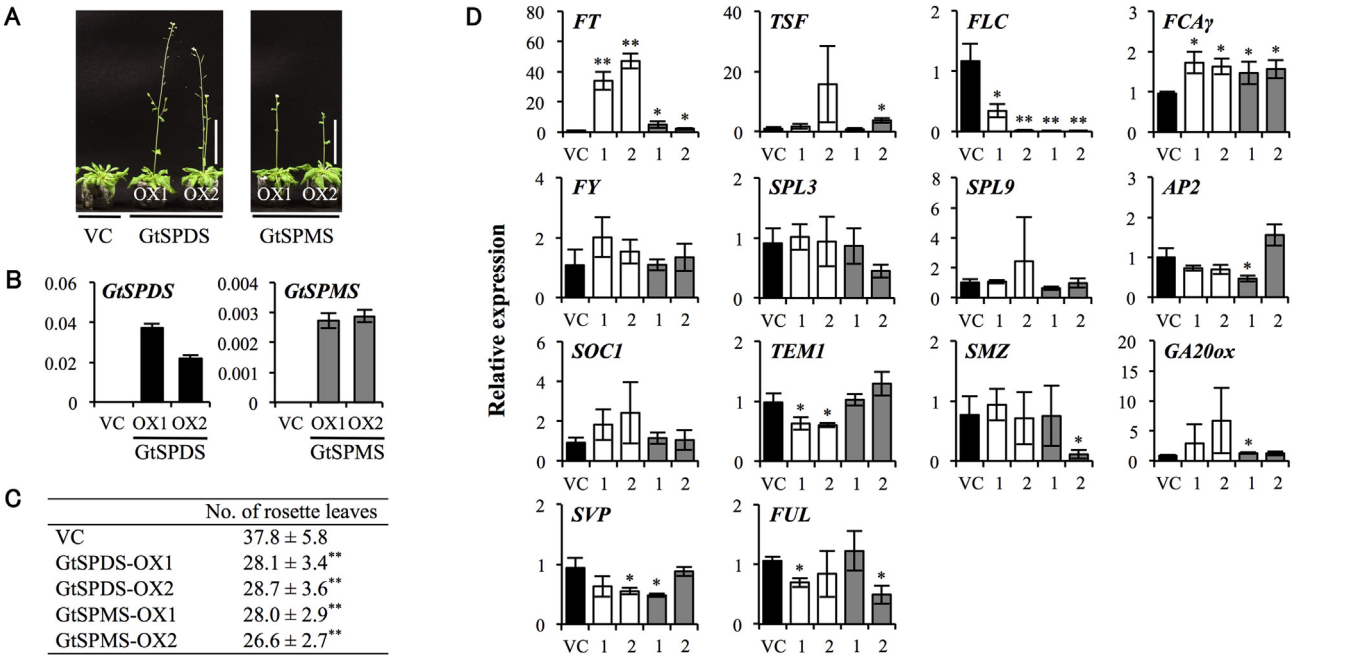

Fig. 4. Overexpression of *GtSPDS* and *GtSPMS* in *Arabidopsis* plants. (A) Transgenic *Arabidopsis* plants overexpressing *GtSPDS* and *GtSPMS*. (B) Expression levels of *GtSPDS* and *GtSPMS* in the shoots of 3-week-old transgenic lines. (C) Flowering time of growth chamber plants measured by total number of rosette leaves at bolting under SD condition. (D) Expression levels of flowering-related genes in the shoots of 7-week-old transgenic lines. Black, white and gray bar indicate the expression of vector control (VC), GtSPDS-OX, and GtSPMS-OX, respectively. Amounts of transcripts are shown as relative values normalized against transcription levels of *EF1α*. Each value represents the mean ± SD from three independent measurements. Significant differences from the control were evaluated by Student's *t*-test (**$p < 0.01$, *$p < 0.05$).

4. Discussion

In this study, we characterized *GtSPDS* and *GtSPMS*, the gentian homologues of *Arabidopsis* polyamine-synthetic enzymes. Since the deduced amino acid sequences of the genes possessed a catalytic domain and AdoMet or dcAdoMet-binding site motifs, and the recombinant proteins showed corresponding activities, *GtSPDS* and *GtSPMS* could be considered functional in gentian. This is the first report about a polyamine-synthetic enzyme gene in gentian, and the function of these enzymes is not known. *SPDS* and *SPMS* genes are widely conserved in animals and plants, and *Arabidopsis* possesses two *SPDS* and one *SPMS* genes. Despite this conservation, the essentiality of these enzymes for survival is still unclear. While the *spds1-1 spds2-1* double-mutant produced abnormal seeds whose development was arrested [29], *spms* mutant with low Spm expression showed no distinct phenotype, and *acl5 spms* double mutant with no Spm expression exhibited a dwarf phenotype [30]. On the other hand, overexpression of *SPDS* and *SPMS* resulted in enhanced tolerance against biotic and abiotic stresses with an up-regulation of defense-related genes [31,32]. The result from alignment analysis shows that *GtSPDS* and *GtSPMS* also may possess similar functions as their counterparts in *Arabidopsis* and other plant species.

To obtain information about *GtSPDS* and *GtSPMS* in gentian plants, we measured seasonal expression of these genes. The pattern of gene expression in OWBs and leaves was similar in that the expression levels of *GtSPDS* and *GtSPMS* were low during dormancy, but exhibited an increase during the growth and developmental stages (Fig. 3). Both Spd and Spm have been reported to be involved in the regulation of cell proliferation and differentiation [33], therefore, the expression of *GtSPDS* and *GtSPMS* may be suppressed during dormancy and increased during the growth stage. OWBs of *G. triflora* begin sprouting and start vegetative growth in April [18]. The expression levels of both *GtSPDS* and *GtSPMS* were low in April and started to increase in May, suggesting that these genes are not involved in budbreak of OWBs. Our results also revealed that the expression levels of these genes were lower in SAMs than in the leaves, suggesting that their expression may not be required during high cell proliferation periods. Since floral initiation of *G. triflora* cv. SpB occurred in June when the expressions of these genes were high (Fig. 3), it is possible that these genes are involved in the transition from vegetative to reproductive phase in gentians. This hypothesis was supported by the results from transgenic *Arabidopsis* plants with overexpressed *GtSPDS* or *GtSPMS* where significant promotion of flowering was observed under SD condition. Previous studies have shown that the fluctuation of polyamine concentrations affected floral initiation and that exogenous Spd promoted floral transition in *Arabidopsis* [34]. These findings indicated that polyamines play a role in flowering as well as other developmental stages. However, the molecular mechanism of polyamine in flowering has not been studied extensively. Our results highlighted that the expression level of *FT* was significantly up-regulated, with a concomitant down-regulation of *FLC* expression in all transgenic lines. Previous studies using microarray analysis of transgenic *Arabidopsis* overexpressing *AtSPMS* have also shown decreased expression of *FLC* [32]. We also found an increased expression level of *FCAγ*, an autonomous pathway gene repressor of *FLC* expression. *FCAγ* encodes only the functional FCA protein among the splice variants of *FCA* and the *fca* mutant showed a late-flowering phenotype owing to the increased expression of *FLC* [35]. These results suggest that *GtSPDS* and *GtSPMS* might facilitate the FLC-mediated flowering pathway. Interestingly, the expression level of *FT* was significantly higher in GtSPDS-OX than in GtSPMS-OX. A possible reason for this could be the involvement of *TEM1*, a direct repressor of *FT* [36]. The expression level of *TEM1* decreased in GtSPDS-OX lines, whereas no effect was observed in GtSPMS-OX lines. This result suggests that *GtSPDS* overexpression also affected *TEM1* in addition to *FLC* and *FCAγ* and the synergistic activation of *FT* might be responsible for the accelerated flowering in the GtSPDS-OX more than the *GtSPMS* overexpression in GtSPMS-OX lines. It is not known how *GtSPDS* and *GtSPMS* induce the expression of flowering genes.

Further analysis involving polyamine feeding and evaluation of these flowering gene mutants would be needed to reveal the relationship between polyamine-biosynthesis genes and transcriptional regulation of these flowering genes.

In conclusion, we have isolated the polyamine biosynthesis genes, *GtSPDS* and *GtSPMS*, from gentian. Recombinant proteins of GtSPDS and GtSPMS possessed the SPDS and SPMS activity, respectively. Transgenic plants overexpressing *GtSPDS* or *GtSPMS* showed an early flowering phenotype through FLC-mediated flowering pathway. This is the first demonstration of the involvement of SPDS and SPMS in gentian flowering and the molecular mechanism of the flowering pattern in gentian. Demonstration of flowering regulation through SPDS and SPMS will provide further insight into this novel mechanism of flowering and thus help advance the understanding of polyamine functions in plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.013.

Transparency document

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