Genome-wide identification and expression analysis of SPL membrane-bound (STM) transcription factors in *Dendrobium officinale* postprint

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Abstract

SPL transcription factors are widely involved in plant growth, development, and stress response processes. Currently, there are no studies on SPL membrane-bound (SPL with Transmembrane Motif) transcription factors, i.e., STM transcription factors, in Dendrobium officinale. To investigate the roles of STM transcription factors in the growth, development, and stress response of Dendrobium officinale, this study identified four STM transcription factors at the whole-genome level, performed bioinformatics analysis of the DoSTM gene family members, and examined the expression patterns of DoSTM in different tissues and under various abiotic stress treatments using reverse transcription PCR. The results showed that: (1) DoSTM1-4 are hydrophilic proteins, all possessing the conserved SBP domain and multiple hormone-responsive elements. (2) All four DoSTM genes were expressed in roots, stems, and leaves, with DoSTM2 showing the lowest relative expression level in leaves; no significant differences were observed in the relative expression levels of DoSTM1/3/4. (3) The relative expression levels of DoSTM1-4 exhibited significant changes under low temperature, high temperature, and drought stress conditions, with the expression of DoSTM1/3/4 decreasing most significantly, suggesting that DoSTM is associated with hormone response, temperature change response, and drought resistance in plants. These conclusions lay the foundation for further research on STM transcription factors in Dendrobium officinale.

Full Text

Preamble

Genome-Wide Identification and Expression Analysis of SPL with Transmembrane Motif (STM) Transcription Factors in *Dendrobium*

officinale

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Abstract

SPL transcription factors are widely involved in plant growth, development, and stress responses. However, no studies have investigated SPL membrane-bound (STM) transcription factors in *Dendrobium officinale*. To explore the potential roles of STM transcription factors in the growth, development, and stress responses of *D. officinale*, we identified four STM transcription factors at the whole-genome level and conducted comprehensive bioinformatics analyses of the *DoSTM* gene family. Additionally, we examined the expression patterns of *DoSTM* genes in different tissues and under various stress treatments using reverse transcription PCR. The results revealed that: (1) DoSTM1-4 are hydrophilic proteins containing conserved SBP domains and several hormone-responsive motifs; (2) All four *DoSTM* genes are expressed in roots, stems, and leaves, with DoSTM2 showing the lowest relative expression in leaves, while DoSTM1/3/4 exhibited no significant differences in expression levels across tissues; and (3) The relative expression levels of DoSTM1-4 changed significantly under low temperature, high temperature, and drought stress, with DoSTM1/3/4 showing the most pronounced decreases. These findings suggest that DoSTM proteins are involved in hormone signaling, temperature sensing, and drought resistance in plants, providing a foundation for further functional characterization of STM transcription factors in *D. officinale*.

Keywords: *Dendrobium officinale*, STM transcription factor, gene identification, functional analysis, gene expression

Introduction

Dendrobium species are predominantly distributed in tropical and subtropical regions of Southeast Asia and Oceania, thriving in warm, humid environments as epiphytes on tree trunks or rocks. *Dendrobium officinale*, a perennial herbaceous plant in the Orchidaceae family, possesses exceptional medicinal value and is used both as food and traditional Chinese medicine. Its primary bioactive components include polysaccharides, alkaloids, flavonoids, and phenolic acids, which exhibit anti-tumor and immune-enhancing properties. Due to overexploitation, wild *D. officinale* resources have declined dramatically in recent years, making molecular-level investigations of its growth and development crucial for conservation efforts.

The SPL (SQUAMOSA promoter binding protein-like) gene family represents a plant-specific class of transcription factors that regulate downstream gene

expression by binding to cis-acting elements in promoter regions. SPL transcription factors play vital roles in plant growth and development, signal transduction, and stress responses. First discovered in snapdragon (*Antirrhinum majus*), SPL transcription factors have since been shown to be essential in various plant species. In *Brassica napus*, numerous *BoSPL* genes are expressed in callus, roots, stems, leaves, buds, flowers, and siliques, potentially contributing to cold tolerance. Recent reports indicate that Arabidopsis SPLs may regulate vegetative phase transition, reproductive morphogenesis, anthocyanin biosynthesis, and stress defense. Specifically, SPL3 plays a crucial role in inflorescence and floral organ development, while SPL1 mediates responses to low-phosphorus stress and regulates phosphorus starvation-induced gene expression.

Membrane-bound transcription factors represent a unique class of regulatory proteins that contain transmembrane domains, enabling direct integration into cellular membranes such as the plasma membrane, endoplasmic reticulum, or nuclear envelope. These factors typically remain dormant until environmental stimuli trigger their release from membranes, activation, and subsequent translocation to the nucleus. While SPL transcription factors include such membrane-bound members, no dedicated studies have focused on SPL membrane-bound transcription factors to date. Therefore, this study employed BLASTP searches against the *D. officinale* genome to identify four SPL membrane-bound transcription factors, designated as DoSTM (SPL with transmembrane motif) proteins, followed by comprehensive bioinformatics and expression analyses.

Materials and Methods

Plant Materials and Stress Treatments

Dendrobium officinale seedlings were subjected to various treatments for 6 hours: 1/2 MS liquid medium (control), 1/2 MS with 100 mmol $\cdot$ L^{-1} NaCl, 1/2 MS with 5 mol $\cdot$ L^{-1} abscisic acid (ABA), 1/2 MS with 10% polyethylene glycol (PEG), 1/2 MS at 40 °C, and 1/2 MS at 4 °C. After treatment, seedlings were blotted dry, 200–300 mg samples were collected in 1.5 mL centrifuge tubes, snap-frozen in liquid nitrogen for 1 minute, and stored at –80 °C. For tissue-specific expression analysis, young roots, stems, and leaves were harvested from potted *D. officinale* seedlings.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using the RNAprep Pure Polysaccharide and Polyphenol Plant Total RNA Extraction Kit (Tsingke, Beijing). First-strand cDNA synthesis was performed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara). PCR amplification was conducted using 2×TSINGKE Master Mix with gene-specific primers.

Bioinformatics Analyses

Identification and Characterization of DoSTM Proteins Arabidopsis STM protein sequences were downloaded from NCBI and used as queries for BLASTP searches against the *D. officinale* genome. Redundant sequences were removed, and transmembrane domains were predicted using TMHMM v2.0. Four proteins (XP_{020685848}, XP_{020681923}, XP_{020672795}, XP_{020688542}) were selected and designated DoSTM1–DoSTM4. Physicochemical properties were analyzed using ProtParam, and subcellular localization was predicted using Plant-mPLoc.

Phylogenetic Analysis STM protein sequences from nine plant species (*Musa balbisiana*, *Dendrobium officinale*, *Arabidopsis thaliana*, *Phalaenopsis equestris*, *Populus tomentosa*, *Erycina pusilla*, *Zea mays*, *Oryza sativa*, *Zingiber officinale*) were retrieved from NCBI. A neighbor-joining phylogenetic tree was constructed using MEGA-X with ClustalW alignment, 500 bootstrap replications, p-distance model, and partial deletion for gaps/missing data. The tree was visualized using iTOL.

Protein Structure Prediction Secondary structure was predicted using SOPMA with default parameters. Conserved motifs were identified using MEME Suite (10 motifs). Multiple sequence alignment was performed using ClustalW in MEGA-X, and visualization was generated using ESPript 3.0. Conserved domains were obtained from NCBI CDD and plotted using TBtools.

Gene Structure and Cis-Acting Element Analysis Genomic information was downloaded from NCBI. Gene structures were plotted using TBtools, and 2,000 bp upstream sequences were extracted for cis-acting element prediction using PlantCARE. Results were visualized with TBtools.

Primer Design Primers were designed using Primer-BLAST based on *DoSTM* cDNA sequences (18–22 bp, product size 400–800 bp). *EF1-α* was used as the internal reference. Primer sequences are listed in Table 1.

Table 1. Primer sequences used for RT-PCR analysis

Gene name	Primer sequence (5' -3')
DoSTM1	F: GTCTATCAGCGTCTCCTGCCR: CGCGCATAGTCTTCAGGAGT
DoSTM2	F: CTTTCTGATGCAATCGGGCGR: ATAAGGCATGTTGACGGCCA
DoSTM3	F: TGAGCCGAGCAGTGAAGAAGR: GGAGCACCTCGGAAGAACAA
DoSTM4	F: AGCAGTGATTGTGGGCATGAR: ATGCAAGTGCAGGAAGCTCA
EF1-α	F: CCACCACCCCCAAATACTCCR: TCCCTAACAGCGAAACGTCC

Expression Analysis by RT-PCR Total RNA was extracted from treated seedlings and root/stem/leaf tissues. cDNA synthesis and PCR amplification

were performed as described above. The PCR program consisted of: 98 °C for 2 min; 30 cycles of 98 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min; and final extension at 72 °C for 10 min. Products were separated on 1% agarose gels.

Statistical Analysis Band intensities were quantified using ImageJ. Data were processed in Excel and analyzed using GraphPad Prism 5 with Student' s t-test. Significance was set at $P < 0.05$.

Results

Physicochemical Properties and Subcellular Localization of DoSTM Proteins

Transmembrane domain prediction revealed that all DoSTM proteins contain a transmembrane motif located at the C-terminal 22 amino acid residues (Fig. 1). Physicochemical analysis showed that DoSTM1–4 contain 1,025, 977, 1,104, and 830 amino acids, with molecular weights of 113.53, 109.09, 121.67, and 93.18 kDa, respectively (Table 2). The isoelectric points (pI) ranged from 6.07 to 8.06. Instability indices ranged from 44.37 to 58.41, indicating relatively unstable proteins. Aliphatic indices were between 78.15 and 83.04. All proteins had negative GRAVY scores (-0.405 to -0.335), confirming their hydrophilic nature. Subcellular localization predicted DoSTM1–3 in the nucleus and DoSTM4 in the chloroplast.

Table 2. Physicochemical properties of the DoSTM protein family

Protein ID	Amino acid size	Molecular weight (kDa)	pI	Instability index	Aliphatic index	GRAVY
DoSTM1	XP_{022585848}	113.53	6.07	58.41	78.15	-0.405
DoSTM2	XP_{020681923}	109.09	8.06	44.37	83.04	-0.335
DoSTM3	XP_{020672795}	121.67	6.61	55.84	78.30	-0.389
DoSTM4	XP_{020688542}	93.18	6.36	54.71	81.12	-0.368

Phylogenetic Analysis of the DoSTM Gene Family

Phylogenetic analysis of 34 STM proteins from nine species revealed four distinct groups (A–D) (Fig. 2). Group A contained 3 STM proteins, group B contained 6, group C contained 9, and group D contained 16. DoSTM1 and DoSTM2 clustered together in group B, suggesting a recent gene duplication event. DoSTM4 was placed in group C, while DoSTM3 belonged to group D.

Fig. 2. Evolutionary relationships of STM proteins from nine plant species. Species abbreviations: *Mb. Musa balbisiana*; *Do. Dendrobium officinale*; *At. Arabidopsis thaliana*; *Pe. Phalaenopsis equestris*; *Pt. Populus tomentosa*; *Eg. Elaeis guineensis*; *Ep. Erycina pusilla*; *Zm. Zea mays*; *Os. Oryza sativa*; *Zo. Zingiber officinale*.

Secondary Structure of DoSTM Proteins

Secondary structure prediction revealed that DoSTM proteins comprise α-helices, extended strands, β-turns, and random coils (Table 3, Fig. 3). The α-helix content ranged from 28.62% to 33.76%, extended strands from 11.68% to 14.70%, β-turns from 4.09% to 4.78%, and random coils from 49.56% to 55.34%.

Table 3. Proportion of secondary structures in DoSTM proteins

Gene name	α-helix (%)	Extended strand (%)	β-turn (%)	Random coil (%)
DoSTM1	33.76	11.68	4.09	50.24
DoSTM2	32.96	12.59	4.40	49.56
DoSTM3	28.62	14.70	4.78	52.17
DoSTM4	30.36	13.86	4.34	51.33

Fig. 3. Secondary structure of DoSTM proteins predicted by SOPMA. Blue represents α-helices, red represents extended strands, green represents β-turns, and purple represents random coils.

Protein Structure and Conserved Motif Analysis

Multiple sequence alignment revealed high homology between DoSTM1 and DoSTM2 (Fig. 4). Conserved motif analysis identified an SBP domain in all four DoSTM proteins. Additionally, DoSTM1 and DoSTM3 contain an Ank_2 superfamily domain, while DoSTM2 possesses an ANKYR domain (Fig. 5). DoSTM1 and DoSTM2 share motifs 1–10, whereas DoSTM3 lacks motifs 7 and 10, and DoSTM4 lacks motifs 3, 4, 7, and 10, indicating that the four DoSTM proteins are not highly conserved in structure.

Fig. 4. Multiple sequence alignment of DoSTM proteins.

Fig. 5. Conserved motif prediction of DoSTM proteins.

Gene Structure and Cis-Acting Element Analysis

Gene structure analysis revealed that all four *DoSTM* genes contain 10 coding regions (CDS) (Fig. 6). Cis-acting element analysis identified light-responsive elements in all genes (Fig. 7). *DoSTM1/2/3* contain methyl jasmonate-responsive elements, while defense-responsive elements were found

only in *DoSTM2/4*. *DoSTM4* additionally contains auxin-responsive elements, and *DoSTM1* responds to abscisic acid. *DoSTM3*, which harbors the most cis-acting elements, also responds to gibberellin, salicylic acid, auxin, and low temperature.

Fig. 6. Gene structure of *DoSTM* genes. CDS represents coding regions; black lines represent introns.

Fig. 7. Analysis of cis-acting elements in *DoSTM* promoters.

Expression Analysis of *DoSTM* Genes

Tissue-Specific Expression RT-PCR analysis revealed that all *DoSTM* genes are expressed in roots, stems, and leaves (Fig. 8). *DoSTM2* showed significantly lower expression in leaves compared to other tissues, while its expression in stems and roots was similar. DoSTM1/3/4 exhibited no significant differences in expression levels across tissues, though DoSTM1 expression was slightly higher in stems and leaves than in roots, and DoSTM3/4 showed higher expression in leaves than in stems, but lower than in roots.

Fig. 8. Relative expression levels of *DoSTM* genes in root, stem, and leaf tissues. *EF1-α* was used as an internal reference. ** indicates $P < 0.01$, $n = 3$.

Stress-Responsive Expression Given that *D. officinale* naturally inhabits cliff environments and experiences multiple abiotic stresses, we examined *DoSTM* expression under various stress conditions (Fig. 9). DoSTM1 expression decreased significantly under low temperature (4 °C), high temperature (40 °C), and 10% PEG treatments, but showed no significant change under ABA treatment and only a slight decrease under NaCl stress. DoSTM2 expression was significantly reduced under low and high temperature stresses but remained unchanged under ABA, NaCl, and PEG treatments. DoSTM3 expression decreased markedly under NaCl, low temperature, and high temperature stresses, with only minor changes under ABA and PEG treatments. Similarly, DoSTM4 showed significant expression changes under low temperature, high temperature, and ABA treatments, but no significant difference under NaCl stress.

Fig. 9. Relative expression levels of *DoSTM* genes under various stress treatments for 6 h. CK, control; NaCl, 100 $mmol \cdot L^{-1}$ NaCl; ABA, 5 $mol \cdot L^{-1}$ ABA; PEG, 10% polyethylene glycol; HT, high temperature (40 °C); LT, low temperature (4 °C). *EF1-α* was used as an internal reference. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, $n = 3$.

Discussion and Conclusion

This study identified four STM proteins from the *D. officinale* genome and conducted comprehensive bioinformatics analyses. All DoSTM proteins are hydrophilic, with DoSTM1/3/4 having isoelectric points below 7, while DoSTM2

has a pI above 7—differing from previous reports that most STM family members have theoretical pI values greater than 7. SPL proteins contain a highly conserved SBP-BOX domain essential for DNA binding, consistent with our conserved domain predictions. The subcellular localization of DoSTM1–3 in the nucleus and DoSTM4 in the chloroplast aligns with the typical nuclear function of transcription factors, though chloroplast localization suggests potential dual roles.

The expression patterns of *DoSTM* genes in roots, stems, and leaves provide insights into their developmental functions. Previous studies on birch *BpSPL6* demonstrated increasing expression in roots during vegetative growth, suggesting a role in root development. Our observation that *DoSTM2/3/4* show relatively higher expression in roots supports a similar function for DoSTM proteins in root development.

Hormone signaling pathways are integral to plant development, and SPL genes have been implicated in hormone responses. The *BpSPL6* promoter contains ten hormone-responsive elements, and our cis-acting element analysis revealed similar regulatory features in *DoSTM* genes. The significant expression changes of *DoSTM3/4* under ABA treatment corroborate these findings. Furthermore, Arabidopsis SPL10 influences jasmonic acid, salicylic acid, and auxin responses, while gibberellin regulates flowering through SPL3/4/5. The presence of multiple hormone-responsive elements in *DoSTM* promoters suggests these genes may participate in diverse hormone signaling pathways.

Numerous studies have demonstrated SPL gene involvement in abiotic stress responses, particularly temperature stress. In Arabidopsis, miR156-regulated SPL2/9/11 expression decreases under high temperature, enhancing thermotolerance. In grapevine, low temperature (5 °C) upregulates *VvSBP3/5* and downregulates *VvSBP4/7*, indicating their involvement in cold stress responses. Our results showing significant expression changes of all *DoSTM* genes under low and high temperature stresses are consistent with these findings. Additionally, studies on birch *BpSPL6* and maize *ZmSPL16* have shown SPL involvement in drought and salt stress responses. The significant downregulation of *DoSTM* genes under PEG-induced drought stress in our study suggests their participation in drought response mechanisms, though the specific patterns varied among family members, warranting further investigation.

In conclusion, we identified four STM transcription factors in *D. officinale* that are hydrophilic, predominantly nuclear-localized, and contain conserved SBP domains and hormone-responsive cis-elements. Expression analyses revealed tissue-specific patterns and significant responses to temperature and drought stresses, with some members also responding to ABA. These results establish a foundation for future functional studies of DoSTM transcription factors in orchid biology and stress adaptation.

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