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The polyamine oxidase from lycophyte *Selaginella lepidophylla* (SelPAO5), unlike that of angiosperms, back-converts thermospermine to norspermidine

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ABSTRACT

In the phylogeny of plant polyamine oxidases (PAOs), clade III members from angiosperms, such as *Arabidopsis thaliana* PAO5 and *Oryza sativa* PAO1, prefer spermine and thermospermine as substrates and back-convert both of these substrates to spermidine in vitro. A clade III representative of lycophytes, SelPAO5 from *Selaginella lepidophylla*, also prefers spermine and thermospermine but instead back-converts these substrates to spermidine and norspermidine, respectively. This finding indicates that the clade III PAOs of lycophytes and angiosperms oxidize thermospermine at different carbon positions. We discuss the physiological significance of this difference.

1. Introduction

Polyamines (PAs) are biogenic amines with low molecular masses that are present in all living organisms. PAs function in growth and development, as well as adaptive responses against various stresses [1–4]. Major common PAs in angiosperms include putrescine (Put), spermidine (Spd), spermine (Spm) and thermospermine (T-Spm) [3,5,6]. Norspermidine (NorSpd) and norspermine (NorSpm) occur in Bryophyta, mosses and some eukaryotic algae [7–9]. In 1988, NorSpd and NorSpm were detected in alfalfa [10]. The biosynthesis of those uncommon PAs begins with 1,3-diaminopropane (DAP), which is generated by the action of a terminal catabolism-type of polyamine oxidase (PAO) on Spd and Spm [11]. The aminopropyl residue derived from decarboxylated S-adenosylmethionine is transferred to DAP by a putative aminopropyltransferase (APT) with relaxed substrate specificity, resulting in NorSpd, and subsequently, the second APT action converts NorSpd to NorSpm [12].

Here, we isolated the PAO gene (named *SelPAO5*) from a lycophyte, *Selaginella lepidophylla*, which shows the highest sequence identity to *Arabidopsis thaliana PAO5* (here referred to as *AtPAO5*) [13,14] and *Oryza sativa PAO1* (*OsPAO1*) [15,16]. The recombinant SelPAO5 prefers Spm and T-Spm, which it converts to Spd and NorSpd, respectively. The results of this study reveal a novel route for generating NorSpd, and also show that SelPAO5 oxidizes T-Spm at a different carbon position from that of AtPAO5 and OsPAO1 in angiosperms.

Abbreviations: APT, aminopropyltransferase; DAP, 1,3-diaminopropane; HPLC, high performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; NorSpd, norspermidine; NorSpm, norspermine; ORF, open-reading frame; PA, polyamine; PAO, polyamine oxidase; Put, putrescine; Spd, spermidine; Spm, spermine; T-Spm, thermospermine; WT, wild type

Author contributions: T.K. designed the experiments. G.H.M.S., M.I., D.W.K., S.K., and T.B. performed the experiments and analyzed the data. M.N. synthesized the uncommon PAs. T.K. and T.B. wrote the manuscript.

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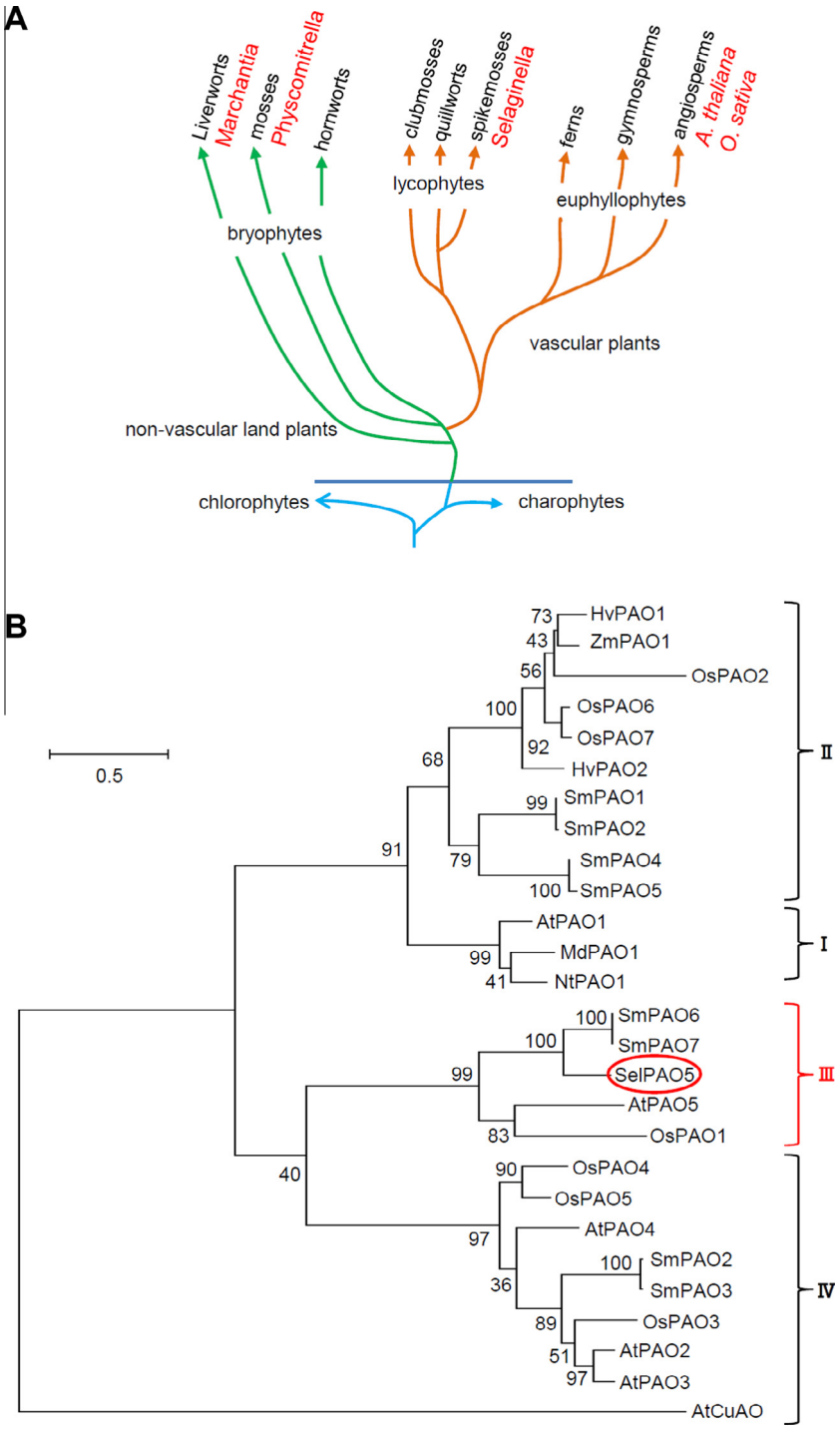

Fig. 1. Isolation of the clade III PAO, SelPAO5, from *S. lepidophylla*. (A) Phylogenic relationship between spikemosses and angiosperms in the plant kingdom. (B) Phylogenetic tree of SelPAO5, other *Selaginella* PAOs and selected angiosperm PAOs. The amino acid sequences of SelPAO5, eight PAOs from *S. moellendorffii* and several PAOs from angiosperms were subjected to phylogenetic analysis. The maximum likelihood tree was built by alignment of the amino acid sequences of PAOs and Arabidopsis copper-dependent amine oxidase 1 (as an outgroup) using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 [25]. Bootstrap values obtained with 1000 replicates are indicated at the nodes. Roman numerals (I–V) indicate clade numbers. The genes and accession numbers used are as follows: SelPAO5 (LC036642), SmPAO1 (XP_002965265.1), SmPAO2 (XP_002965599.1), SmPAO3 (XP_002968082.1), SmPAO4 (XP_002969966.1), SmPAO5 (XP_002981437.1), SmPAO6 (XP_002984796.1), SmPAO7 (XP_002985859.1), SmPAO8 (XP_002986593.1), OsPAO1 (NM_001050573), OsPAO2 (NM_001055782), OsPAO3 (NM_001060458), OsPAO4 (NM_001060753), OsPAO5 (NM_001060754), OsPAO6 (NM_001069545), OsPAO7 (NM_001069546), AtPAO1 (NM_121373), AtPAO2 (AF364952), AtPAO3 (AY143905), AtPAO4 (AF364953), AtPAO5 (AK118203), ZmPAO1 (*Zea mays* PAO1, NM_001111636), NtPAO1 (*Nicotiana tabacum* PAO, AB200262), HvPAO1 (*Hordeum vulgare* PAO1, AJ298131), HvPAO2 (*Hordeum vulgare* PAO2, AJ298132), and MdPAO1 (*Malus domestica* PAO, AB250234).

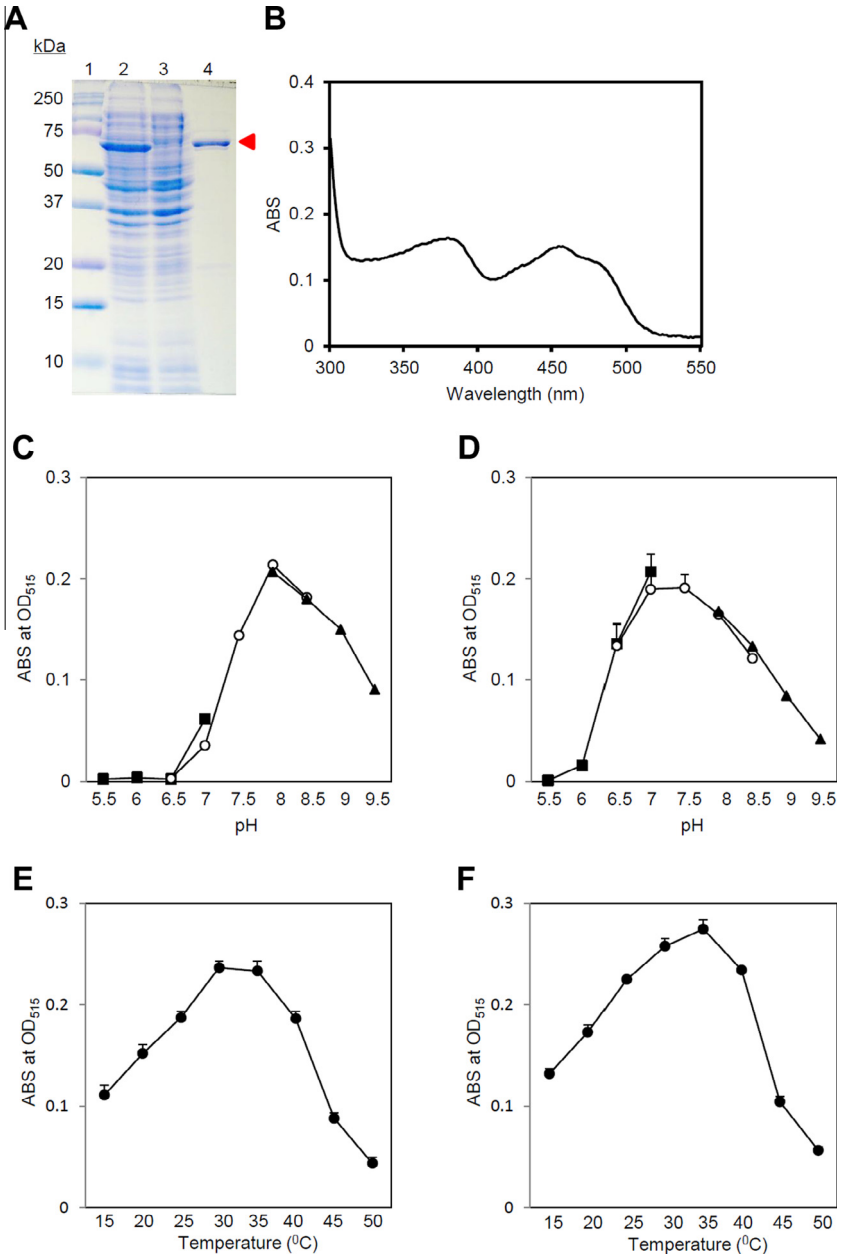

Fig. 2. Characterization of recombinant SelPAO5. (A) Purification of SelPAO5. Lane 1, Molecular size marker; lane 2, IPTG-induced crude extract after sonication; lane 3, non-induced crude extract after sonication; lane 4, SelPAO5 purified by Ni-affinity chromatography (25-fold concentrated sample compared with that of lanes 2 and 3). (B) Absorbance spectrum of purified sample ranging from 300 nm to 550 nm. (C) Optimal pH for SelPAO5 activity using Spm as substrate. The buffers used are as follows: pH 5.5–7.0, 100 mM MES buffer (■), pH 6.5–8.5, 100 mM HEPES buffer (○), pH 8.0–9.5, 100 mM Tricine buffer (▲). (D) Optimal pH for SelPAO5 activity with T-Spm as substrate. (E) Optimal temperature for SelPAO5 activity with Spm as substrate at pH 8.0. (F) Optimal temperature for SelPAO5 activity with T-Spm as substrate at pH 7.0.

2. Results

2.1. Identification of SlPAO5, the clade III PAO from S. lepidophylla

We recently characterized *AtPAO5* and *OsPAO1* [14,15]. Although we proposed that the products of these genes, AtPAO5 and OsPAO1, function as T-Spm oxidases, because the loss-of-function mutant of *AtPAO5* specifically accumulates twice as much T-Spm as wild-type (WT) plants, both of the recombinant enzymes prefer Spm and T-Spm in vitro and back-convert them to Spd [14–16]. Considering the phylogeny of plants [17], we found *AtPAO5* orthologs in the lycophyte *Selaginella moellendorffii* [18] but not in bryophytes such as *Marchantia polymorpha* and *Physcomitrella patens* (Kim et al. unpublished data; Fig. 1A). Based on this information, we tried to isolate *AtPAO5* ortholog(s) from *S. lepidophylla*. We designed a primer pair based on *SmPAO6* and *SmPAO7* cDNA sequences (Accession numbers XP_002984796.1 and XP_002985859.1) from *S. moellendorffii* (Supplemental Table 1). PCR amplification using this primer pair and *S. lepidophylla* genomic DNA resulted in a 813-bp fragment that showed 62% and 61% identity to *SmPAO6* and *SmPAO7* cDNAs, respectively. Next, we performed inverse-PCR (http://labs.mcdb.lsa.umich.edu/labs/maddock/protocols/PCR/inverse_pcr_protocol.html/) of *S. lepidophylla* genomic DNA using two pairs of primers. The genome template was prepared as follows: *S. lepidophylla* genomic DNA was digested with either *Hin*dIII or *Sal*I and then self-ligated according to the protocol. The resulting fragments were sequenced and a 1584-bp open-reading frame (ORF) was identified. Based on

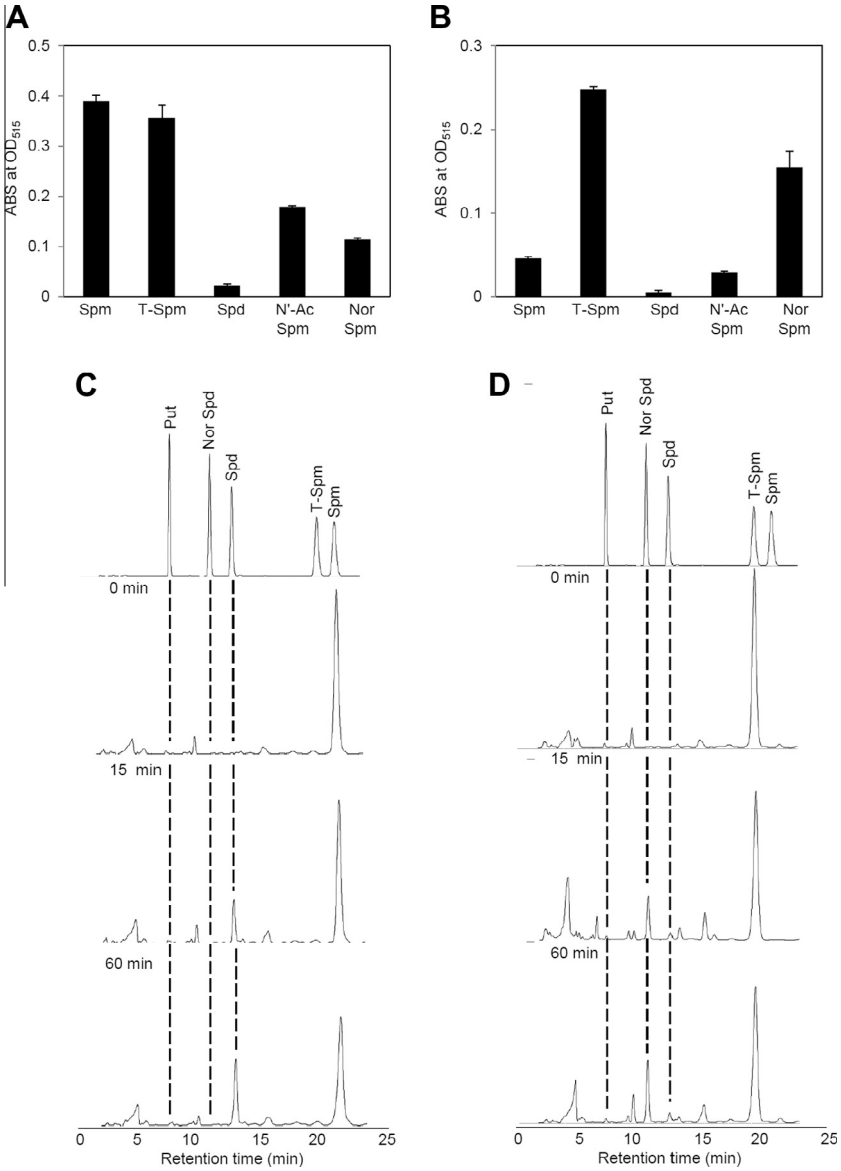

Fig. 3. Substrate specificity of recombinant SelPAO5 and SelPAO5-catalyzed reaction product(s) of T-Spm and Spm. The enzymatic activity of recombinant SelPAO5 was determined in 100 mM HEPES buffer at pH 8.0 (A) and 100 mM MES pH 7.0 (B) at 35 °C. (A and B) Substrate (500 μM each) was incubated at 35 °C for 10 min, and the resulting production of H_2O_2 was measured. Enzyme activity is displayed as relative Abs at 515 nm. The experiments were repeated at least three times, and the mean ± S.D. values are displayed. (C) HPLC analysis after conversion of Spm at pH 8.0. (D) HPLC analysis after conversion of T-Spm at pH 7.0. (C and D) top panels, PA (Put, NorSpd, Spd, T-Spm, and Spm) standard; second, third and bottom panels show product analysis after 0, 15 and 60 min incubation with SelPAO5, respectively.

the results of RT-PCR of total RNA prepared from *S. lepidophylla* using a new primer pair covering the above ORF, we confirmed that this ORF is the correct one, and we designated the corresponding transcript *SelPAO5* (accession number LC036642). *SelPAO5* is intron-less, which is consistent with the structures of *SmPAO6*, *SmPAO7*, *AtPAO5* and *OsPAO1* [14,15]. The translation product of *SelPAO5* is a 527-amino acid protein, which we designated SelPAO5. We generated a phylogenetic tree using the sequences of SelPAO5, seven *O. sativa* PAOs, five *A. thaliana* PAOs, eight *S. moellendorffii* PAOs and several other angiosperm PAOs. Based on this tree, SelPAO5 is classified in clade III (Fig. 1B).

2.2. Enzymatic characteristics of the recombinant SelPAO5

The recombinant SelPAO5 protein was homogenously purified (Fig. 2A). The absorbance spectrum shows that the purified enzyme has two peaks at approximately 380 and 460 nm, which is indicative of the association of flavine adenine dinucleotide with the enzyme (Fig. 2B). The optimal pH and temperature for maximum SelPAO5 activity using Spm as a substrate were pH 8.0 and 30 °C, respectively (Fig. 2C and E). The maximum SelPAO5 activity with T-Spm as a substrate occurred at pH 7.0 at a temperature of 35 °C (Fig. 2D and F). As SelPAO5 activity was highest at pH 8.0 for Spm and pH 7.0 for T-Spm (Fig. 2C and D), we examined recombinant SelPAO5 substrate specificity at pH 8.0 and pH 7.0 using 300 μM substrate. At pH 8.0, the substrate preference of SelPAO5 in decreasing order was as follows: Spm = T-Spm > N^1-acetyl Spm > NorSpm > Spd (Fig. 3A). At pH 7.0, the substrate preference of SelPAO5 in decreasing order was as follows: T-Spm > NorSpm > Spm > N^1-acetyl Spm > Spd (Fig. 3B). At both pH levels, SelPAO5 catalyzed Spd at a very low rate (Fig. 3A and B; Supplemental Fig. 1B).

2.3. SelPAO5 back-converts Spm to Spd and T-Spm to NorSpd

We analyzed the reaction product(s) of Spm and T-Spm catalyzed by SelPAO5. Using Spm as a substrate, Spd was produced

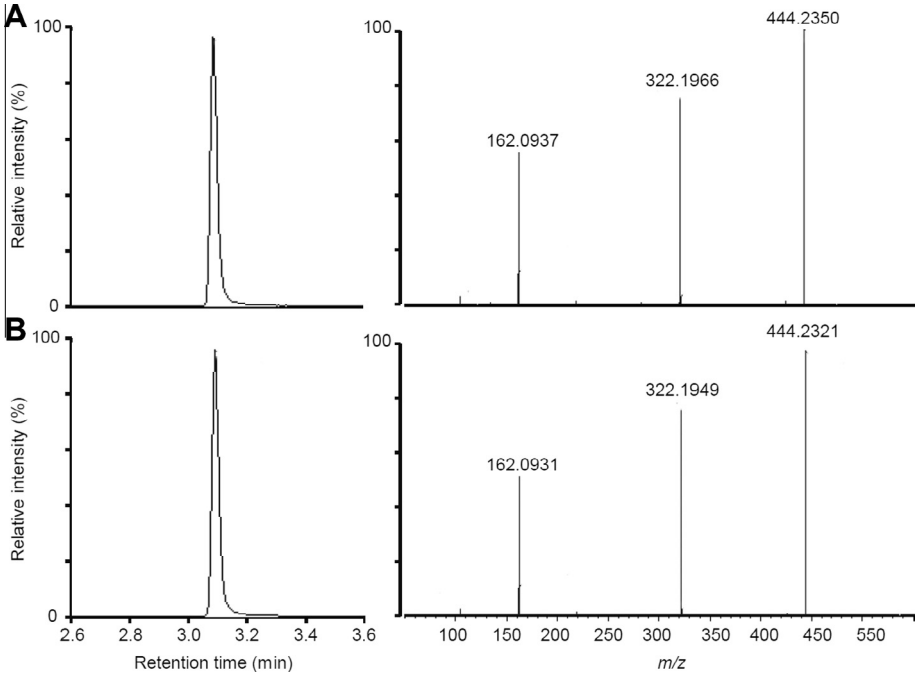

Fig. 4. Identification of NorSpd as the reaction product of T-Spm oxidation by SelPAO5. (A) LC–MS/MS analysis of tri-benzoylated NorSpd (standard). Selected reaction monitoring (Left) and full-scan spectra of fragment ions (Right) of authentic NorSpd. (B) LC–MS/MS analysis of the benzoylated reaction product of T-Spm oxidation by SelPAO5. Selected reaction monitoring (Left) and full-scan spectra of fragment ions (Right).

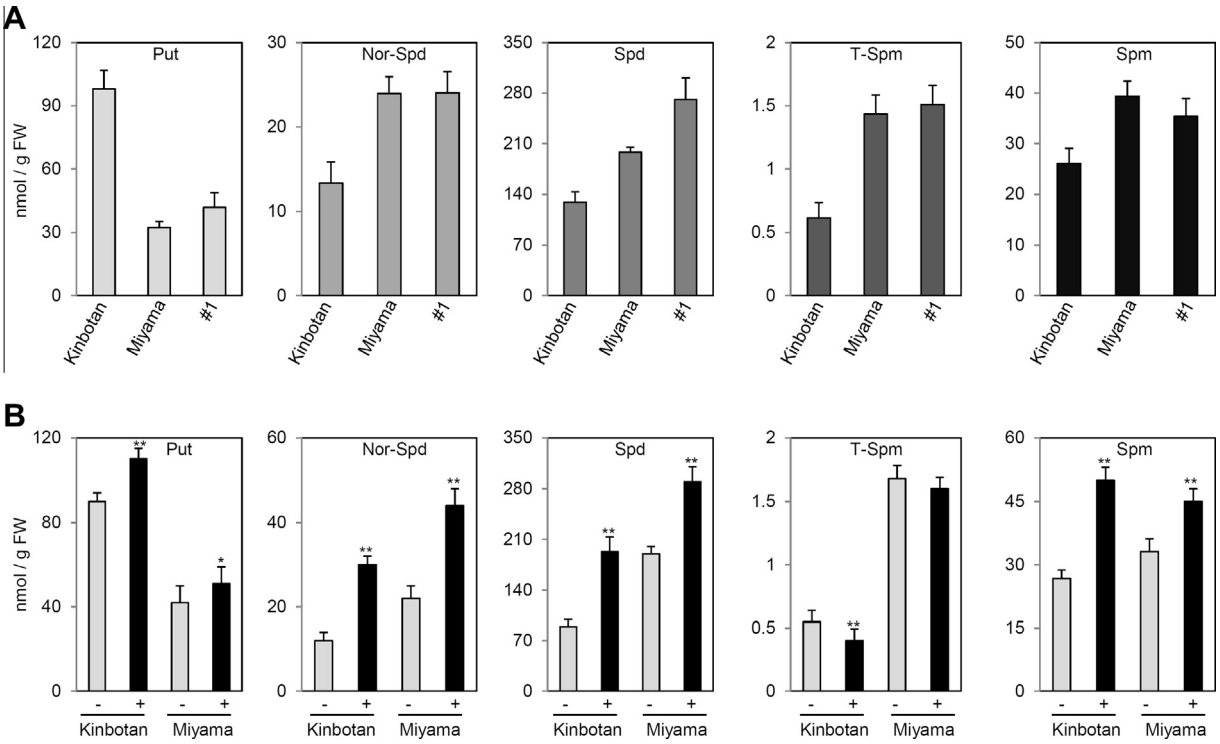

Fig. 5. PA composition in *Selaginella* varieties and the effect of dehydration stress. (A) PA composition in three *Selaginella* varieties. Kinbotan, Miyamanishiki (Miyama) and #1 (no name) were analyzed see Supplemental Fig. 1. (B) Effect of dehydration stress on PA content. Detached leaflets were placed onto water-soaked filter and incubated for 12 h, then one half each of them were transferred onto another wet filter (−) and dry filter (+), respectively, and further incubated for 12 h. Three independent experiments were performed and means ± S.D. were displayed. $^{**}P < 0.01$, $^{*}P < 0.05$.

in a time-dependent manner (Fig. 3C), indicating that SelPAO5 catabolizes Spm in a back-conversion pathway, as do AtPAO5 and OsPAO1 [14,15]. Unexpectedly, when T-Spm was used as a substrate, the peak, which appears to correspond to NorSpd, increased in a time-dependent manner at both pH 7.0 and pH 8.0 (Fig. 3D, Supplemental Fig. 1B). To verify our prediction,

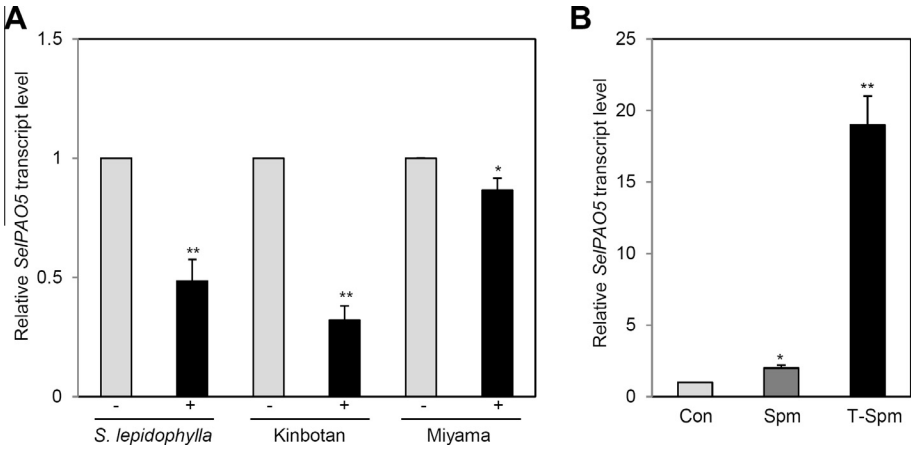

Fig. 6. The response of *SelPAO5* to dehydration stress (A) and exogenous tetraamines, Spm and T-Spm (B). Three independent experiments were performed and means ± S.D. were displayed. $^{**}P < 0.01$, $^{*}P < 0.05$.

the peak fraction was subjected to LC–MS/MS analysis along with tri-benzoylated NorSpd (Supplemental Fig. 2) as a standard. The candidate chemical was clearly identified as NorSpd (Fig. 4).

2.4. Selaginella plants contain NorSpd as well as four common PAs

Next, we obtained three *Selaginella* varieties (see Supplemental Fig. 3) and analyzed their PA composition. These *Selaginella* plants contained Put, Spd and Spm at levels greater than 25 nmol/g fresh weight (FW) (Fig. 5A). Interestingly, the NorSpd content was greater than 10 nmol/g FW, whereas the T-Spm content was less than one-tenth that of NorSpd and Spm (Fig. 5A). As one of the unique features of the *Selaginella* genus is tolerance to desiccation [18–20], we examined the effect of dehydration stress on PA contents in *Selaginella* plants. The levels of Put, NorSpd, Spd and Spm contents increased significantly after dehydration treatment, whereas that of T-Spm did not change or decreased compared to that of well-watered leaflet (Fig. 5B).

2.5. Expression of SelPAO5 is down-regulated by dehydration stress and up-regulated by T-Spm treatment

We then addressed whether *SelPAO5* is responsive to dehydration stress. The levels of *SelPAO5* transcripts decreased distinctly after dehydration stress (Fig. 6A). To find a clue of the SelPAO5 function, we tested the levels of *SelPAO5* transcripts after Spm- and T-Spm-treatment and found that the *SelPAO5* transcripts markedly accumulated by T-Spm treatment but not much by Spm treatment (Fig. 6B).

3. Discussion

3.1. SelPAO5 is involved in NorSpd formation in Selaginella

The *Selaginella* plants that we examined contained NorSpd at levels of over 10 nmol/g FW and T-Spm levels one-tenth those of NorSpd. The *S. moellendorffii* genome contains a putative T-Spm synthase gene [18] (Supplemental Fig. 4). The recombinant SelPAO5 catalyzes the conversion of T-Spm to NorSpd (Figs. 3, 4 and 7). Based on these results, we propose that NorSpd is generated via T-Spm catabolism by SelPAO5. As previously mentioned, NorSpd and NorSpm are synthesized by the sequential transfer of the aminopropyl residue to DAP [10,12]. The latter is formed by the action of a terminal catabolism-type PAO [11]. Therefore, the route from T-Spm to NorSpd catalyzed by PAO is a novel PA metabolic pathway.

3.2. SelPAO5 oxidizes T-Spm at a different carbon position compared to the equivalent enzymes in angiosperms

T-Spm is an asymmetric isomer of Spm, which has a symmetrical structure. Here, we tentatively numbered the carbon atoms of T-Spm C_1 to C_{10} (Fig. 7). AtPAO5 and OsPAO1 prefer Spm and T-Spm in vitro and produce Spd from both of these substrates [13–15], indicating that both enzymes oxidize the C_3-carbon. Unlike these enzymes, SelPAO5 produces NorSpd from T-Spm (Figs. 3 and 4), indicating that SelPAO5 oxidizes the C_7-carbon of T-Spm (Fig. 7).

3.3. Physiological significance of T-Spm conversion to NorSpd in Selaginella

It has been proposed that the T-Spm synthase gene (*ACL5*) was horizontally transferred from thermophilic bacteria or archaea to an ancestral lineage of plants [3,21]. One particular trait of *Selaginella* is desiccation-tolerance [18–20]. A research group proposed that NorSpd may function in dehydration response because drought-tolerant genotypes of alfalfa accumulated significant amounts of NorSpd upon water-deficit stress [9]. However, it may not be the case in *Selaginella* because the expression of *SelPAO5* was negatively regulated by dehydration stress (Fig. 6A). Interestingly *SelPAO5* was extremely up-regulated by exogenous T-Spm (Fig. 6B). We, therefore, speculate that T-Spm, produced by *Selaginella* ACL5 (Supplemental Fig. 4), has high cytotoxicity in *Selaginella* and that to reduce its toxicity, SelPAO5 catabolizes T-Spm to NorSpd, which is less toxic. During subsequent plant evolution, it is likely that the PAO in this clade acquired the ability to oxidize C_3-carbon instead of C_7-carbon to generate Spd by a back-conversion reaction, which occurs in the representative angiosperms AtPAO5 and OsPAO1.

4. Materials and methods

4.1. Plant materials and growth conditions

S. lepidophylla was purchased from Gekihana flower shop (http://shop.plaza.rakuten.co.jp/gekihana/). Other *Selaginella* varieties (Iwahiba in Japanese) were provided by a professional gardener, Mr. Kiyoshi Ishikawa.

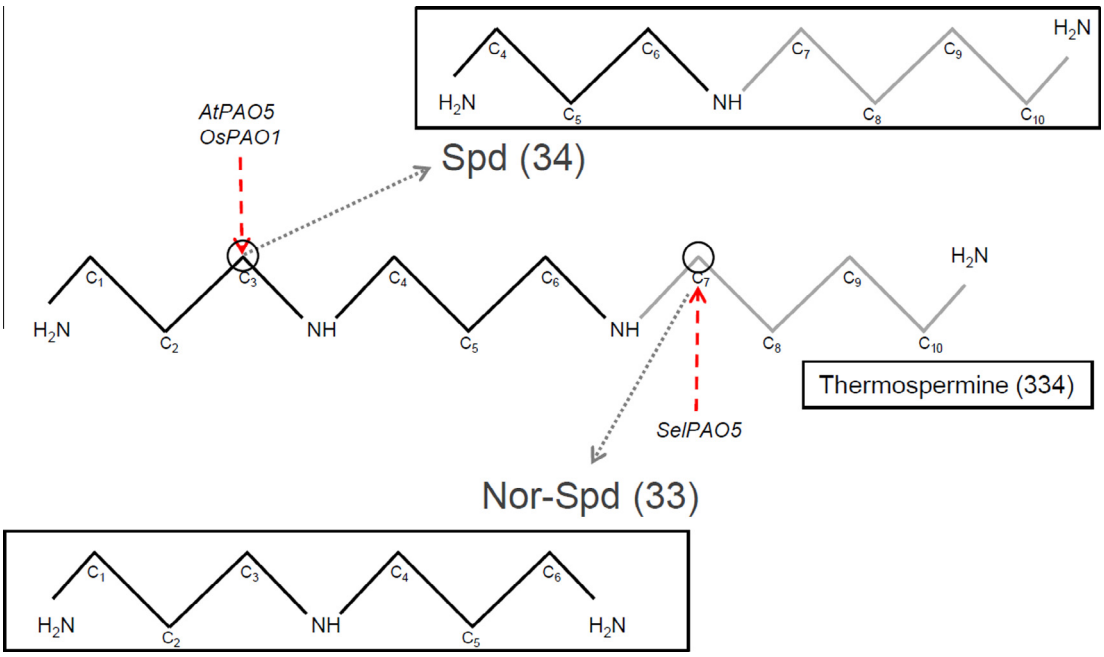

Fig. 7. SelPAO5 oxidizes the C_7-carbon of T-Spm whereas AtPAO5 and OsPAO1 oxidize its C_3-carbon.

4.2. Chemicals

NorSpd and T-Spm were chemically synthesized [22]. All other analytical grade chemicals were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Nacalai-Tesque Ltd. (Kyoto, Japan).

4.3. Production of recombinant SelPAO5 protein in Escherichia coli

The *SelPAO5* coding region was amplified by RT-PCR from total RNA from *S. lepidophylla* using gene-specific primers (see Supplemental Table S1). The amplified PCR products were digested with *Bam*HI and *Sal*I and cloned in-frame with the 6× His tag of the pCold vector (Takara Bio, Shiga, Japan), resulting in pCold-SelPAO5. After confirmation of the cloned fragments by DNA sequence analysis, pCold-SelPAO5 was transformed into *E. coli* Rosetta2 (DE3) cells, and recombinant SelPAO5 protein tagged with 6× His at the N-terminus was produced according to the manufacturer's instructions (Takara Bio, Shiga, Japan) as described [14].

4.4. PAO activity assay

Recombinant SelPAO5 oxidation activities for Spm, T-Spm, Spd, N^1-acetyl Spm and NorSpm were determined spectrophotometrically by following the formation of a pink adduct resulting from oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid catalyzed by horseradish peroxidase as described by Kim et al. [14]. In a typical experiment, 0.5–3.0 μg of protein was added to a buffered solution containing 500 μM of each substrate, 100 μM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxy benzenesulfonic acid and 10 U/ml horseradish peroxidase, and the increase in absorbance at 515 nm was measured using a multiplate reader (Tecan-M200) or spectrophotometer (Hitachi U-2900).

4.5. PA analysis

PA analysis was performed as described in Naka et al. [23]. In brief, plant samples (0.3–0.5 g per sample) were pulverized with a mortar and pestle under liquid nitrogen. Five volumes (2.5 ml per 0.5 g of plant sample) of 5% (v/v) cold perchloric acid were added to the resulting fine powders. The mixtures were transferred to plastic tubes and kept on ice for 1 h. After centrifugation at 15 000×*g* for 30 min at 4 °C, the supernatants were combined and filtered using a filter syringe (pore size, 0.2 μm). One milliliter of 2 N NaOH was added to 1.5 ml of plant extract, the mixture was vortexed, 10 μl of benzoyl chloride was added, the mixture was mixed and incubated at room temperature for 20 min, and then 2 ml of saturated NaCl was added. After the addition of 2 ml of diethyl ester, samples were vigorously mixed and then centrifuged at 3000×*g* for 10 min at 4 °C for phase-separation. An aliquot (1.5 ml) of the organic solvent phase was evaporated and the residue was resuspended in 50 μl of methanol.

Benzoylated PAs were analyzed with a programmable Agilent 1200 liquid chromatograph using a reverse-phase column (4.6 × 250 mm, TSK-GEL ODS-80Ts, TOSOH, Tokyo, Japan) and detected at 254 nm. One cycle of the run took 60 min in total with a flow rate of 1 ml/min at 30 °C i.e., 42% acetonitrile for 25 min for PA separation, increased to 100% acetonitrile over 3 min, 100% acetonitrile for 20 min for washing, decreased to 42% acetonitrile over 3 min, and finally 42% acetonitrile for 9 min.

4.6. LC–MS/MS analysis of the reaction product of T-Spm catalyzed by SelPAO5

The column used for LC was ACQUITY BEH C18 (2.1 × 50 mm, Waters). The sample was separated using 10–60% (linear gradient) acetonitrile containing 0.05% acetic acid. MS/MS analysis was performed at a declustering potential of 40 and a collision energy of 20 eV [24].

4.7. Dehydration treatment and tetraamine-treatment

Dehydration stress: detached *Selaginella* leaflets were placed on wet filter papers for 12 h under normal light condition, then one half of them was transferred onto dry filter papers and the residual half was transferred onto wet filter papers, and further incubated

for another 12 h. Tetraamine treatment: After 12 h incubation described above, the leaflets were separated into three groups: the first group was transferred to wet filter papers as control, and the second and third groups were transferred onto either 0.5 mM Spm or 0.5 mM T-Spm solution contained-filter papers, and further incubated another 12 h.

4.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay

qRT-PCR analysis was performed by StepOne Real-Time PCR System (Applied Biosystems) using SYBR ®Green RT-PCR Kit (FastStart Universal SYBR Green Master, ROX). A standard curve was constructed from different genes and the values were normalized to *Actin* levels. The primers used for qRT-PCR were described in Supplemental Table 1.

4.9. Statistical analysis

Student's *t* tests were used for statistical analysis and were performed using Microsoft Excel statistical tools.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.08.045.

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