A Polyamine Oxidase from *Selaginella lepidophylla* (SelPAO5) can Replace AtPAO5 in *Arabidopsis* through Converting Thermospermine to Norspermidine instead to Spermidine

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**Abstract:** Of the five polyamine oxidases in *Arabidopsis thaliana*, AtPAO5 has a substrate preference for the tetraamine thermospermine (T-Spm) which is converted to triamine spermidine (Spd) in a back-conversion reaction in vitro. A homologue of AtPAO5 from the lycophyte *Selaginella lepidophylla* (SelPAO5) back-converts T-Spm to the uncommon polyamine norspermidine (NorSpd) instead of Spd. An Atpao5 loss-of-function mutant shows a strong reduced growth phenotype when growing on a T-Spm containing medium. When SelPAO5 was expressed in the Atpao5 mutant, T-Spm level decreased to almost normal values of wild type plants, and NorSpd was produced. Furthermore the reduced growth phenotype was cured by the expression of SelPAO5. Thus, a NorSpd synthesis pathway by PAO reaction and T-Spm as substrate was demonstrated in planta and the assumption that a balanced T-Spm homeostasis is needed for normal growth was strengthened.

**Keywords:** polyamine oxidase; norspermidine; thermospermine; *Selaginella lepidophylla*; *Arabidopsis thaliana* mutant

1. Introduction

Polyamines (PAs) are aliphatic compounds derived from amino acids with low molecular masses that are ubiquitously present in all living organisms [1,2]. Plants mainly contain the diamine putrescine (Put), the triamine spermidine (Spd), and the two tetraamines spermine (Spm) and thermospermine (T-Spm) [3–7], an isomer of Spm that was first discovered in thermophilic bacteria [8]. They are implicated in regulating various developmental processes such as embryogenesis, cell division, organogenesis, flowering, and senescence, as well as responses to abiotic and biotic stresses [9–14]. The biosynthesis of the polyamines Put, Spd, Spm, and T-Spm in plants is well elucidated [15]. “Lower” or non-vascular plants, such as bryophytes, mosses, and some eukaryotic algae, contain norspermidine (NorSpd) and norspermine (NorSpm) [16–18]. The biosynthesis of those uncommon PAs starts with 1,3-diaminopropane (DAP), which is produced by the metabolism of Spd and Spm through the action of terminal catabolism-type polyamine oxidase (PAO) [19]. 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, a second APT action converts NorSpd to NorSpm [19]. However, the occurrence of NorSpd and NorSpm
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has also been reported in alfalfa [20] and maize [21]. Catabolism of PAs in plants is executed by two kind of oxidases, copper-dependent amine oxidase (CuAO) and flavin-containing polyamine oxidase (PAO). PAO are reported to act in two different pathways, a terminal catabolic pathway and a back-conversion pathway [22]. The first characterized plant PAOs of maize and barley catalyze the terminal catabolic reactions [23–27]. They oxidize the carbon at the endo-side of the N4-nitrogen of Spm and Spd, producing N-(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, and concomitantly 1,3-diaminopropane and H₂O₂ in both reactions [28,29]. A back-conversion reaction was first shown for Arabidopsis thaliana [30] that produces Spd from Spm and NorSpd from NorSpm in vitro [30]. The Arabidopsis thaliana gene family of PAO comprises five members named AtPAO1–AtPAO5 with well characterized gene products that all function in the back-conversion of tetraamines to triamines and/or triamines to diamines, albeit with different substrate specificities [22]. AtPAO1 localizes in the cytoplasm and oxidizes Spm, T-Spm, and NorSpm, but not Spd [30], while AtPAO2, AtPAO3, and AtPAO4 localize in peroxisomes [31,32]. AtPAO2 and AtPAO3 convert Spm to Put via Spd, whereas AtPAO4 produces less Put from Spm, which is explained by the very low affinity for Spd [33]. AtPAO5 localizes in the cytoplasm and shows a preference to convert T-Spm (or Spm) to Spd [34]. Arabidopsis pao5 mutants contain 2-fold higher T-Spm levels exhibit aerial tissue growth retardation and growth inhibition of stems and leaves at an early stage of development after external T-Spm application [4]. These findings are in accord with observations made in Arabidopsis plants with mutated aculis5 (ACL5) gene encoding T-Spm synthase. In this mutant (acl5), T-Spm content is reduced producing a dwarf phenotype with over-proliferated xylem vessels, suggesting a role of T-Spm in xylem differentiation [4,5]. Taken together, a fine-tuned T-Spm homeostasis secured by regulation of T-Spm synthase (Aculis5) and T-Spm oxidase (AtPAO5) activities is necessary for proper xylem development and growth. A PAO from the lycophyte Selaginella lepidophylla (SelPAO5) with the highest sequence identity to AtPAO5 was shown to prefer T-Spm and Spm as substrates like the Arabidopsis homologue, but instead back-converts T-Spm to NorSpd not to Spd [35]. Here, for further characterization, we used the SelPAO5 encoding cDNA to complement the Arabidopsis Atpao5 mutant.

2. Results

2.1. Phylogenetic Classification of SelPAO5 and Cellular Localization

Recombinant proteins of Arabidopsis AtPAO5 and rice OsPAO1 both prefer Spm and T-Spm as substrates and back-convert it to Spd in vitro [34,36]. These two PAOs are considered to convert T-Spm to Spd in plants. Phylogenetic relationship of PAOs identified in the genome of Selaginella moellendorffii [37] and SelPAO5 of S. lepidophylla to PAOs of Arabidopsis and rice is shown in Figure 1. PAO6 and PAO7 of S. moellendorffii (SmPAO6 and SmPAO7) are members of the clade III plant PAOs that comprise AtPAO5 and OsPAO1. SelPAO5 of S. lepidophylla belongs to this clade and is the homologue to SmPAO6 and SmPAO7.
Figure 1. Phylogenetic relationship between SelPAO5, other Selaginella PAOs, and selected angiosperm PAOs. The tree was made by alignment of the amino acid sequences using Molecular Evolutionary Genetics Analysis (MEGA 6.0) software [38]. Bootstrap values obtained with 1000 replicates are indicated at the nodes. 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_002985959.1), SmPAO8 (XP_002986593.1), OsPAO1 (NM_001050573), OsPAO2 (NM_001055782), OsPAO3 (NM_001060458), OsPAO4 (NM_001060753), OsPAO5 (NM_001060874), OsPAO6 (NM_001060754), OsPAO7 (NM_001069545), AtPAO1 (NM_121373), AtPAO2 (AF364952), AtPAO3 (AY143905), AtPAO4 (AF364953), AtPAO5 (AK118203).

2.2. SelPAO5 Complementation of Atpao5-2 Mutant Rescues T-Spm-Induced Growth Inhibition

For complementing the mutated AtPAO5 gene in Atpao5-2 with SelPAO5, the cDNA of SelPAO5 was introduced into Atpao5-2. Atpao5-2 plants and Atpao5-2 plants transformed with empty vector displayed reduced growth on T-Spm containing medium compared to wild type plants (Figure 2A), while Atpao5-2 plants transformed with the vector containing SelPAO5 cDNA did not. Instead, they looked similar to wild type plants (Figure 2A). For quantification of plant growth, the average fresh weight of ten seedlings each was compared (Figure 2B). While wild type and Atpao5-2 plants expressing SelPAO5 had an average weight of about 75 mg; the growth reduced Atpao5-2 plants and Atpao5-2 plants transformed with the empty vector had an average weight of 50 mg (Figure 2B). Expression of SelPAO5 was confirmed by RT-PCR with RNA samples of wild type Arabidopsis plants, Atpao5-2 plants, and Atpao5-2 plants transformed with the empty vector or the vector containing SelPAO5 cDNA (lines S5#5, S5#11 and S5#13), respectively (Figure S1A). AtPAO5 expression could only be detected in wild type plants but not in the Atpao5-2 mutants. SelPAO5 expression was confirmed in three independent Atpao5-2 lines that were transformed with the SelPAO5 cDNA-containing vector but not in plants that have been transformed with the empty vector only. While T-Spm had a negative effect on growth of Atpao5-2
plants, and Atpao5-2 plants transformed with the empty vector (Figure S1B), other polyamines, Put, Spd, and Spm, respectively, did not have such an effect (Figure S1C). In all the three lines of Atpao5-2 expressing SelPAO5, no growth inhibition could be seen on T-Spm containing medium.

![Figure 2. Recovery of thermospermine (T-Spm)-induced growth arrest in Atpao5-2 by complementation with SelPAO5. Wild type plants (WT, Col-0), Atpao5-2 mutant (AtPAO5-2), Atpao5-2 transgenic carrying the control empty binary vector pPZP2Ha3(+) [39] (AtPAO5-2/EV), and Atpao5-2 transgenic line S5#11 carrying the CaMV3SS-driven SelPAO5 (AtPAO5-2/SelPAO) were grown vertically for 24 days on half-strength Murashige and Skoog agar medium containing 5 µM T-Spm. Seedlings were carefully picked from the plates and photographed (A). The fresh weight of ten seedlings each was determined and the calculated mean including standard deviations displayed in a bar chart (B). Asterisks indicate significant differences to fresh weight of WT plants using Student’s t-test: ** p < 0.01.

2.3. SelPAO Produces NorSpd in Arabidopsis Plants

Polyamine patterns in the Atpao5-2 mutant expressing SelPAO5 were compared to that of wild type Arabidopsis (Col-0) by HPLC analysis (Figure 3). In Col-0 plants, the major plant PAs Put, Spd, T-Spm, and Spm were detected but not NorSpd. The Atpao5-2 mutant expressing SelPAO5 (pao5-2/SelPAO OX) contained NorSpd in addition to the four other PAs. Quantification of PAs revealed that three lines of Atpao5-2 mutant expressing SelPAO5 (S5#5, S5#11, and S5#13) contained more Put (10–12 nmol/gFW) than wild type Arabidopsis plants, Atpao5-2 plants, and Atpao5-2 plants transformed with the empty vector (7–8 nmol/gFW, Figure 4A). Spd levels were similar (~50 nmol/gFW) among these plants (Figure 4B). T-Spm content in Atpao5-2 plants and Atpao5-2 plants transformed with the empty vector were higher (~8 nmol/gFW) than in wild type and Atpao5-2 plants expressing SelPAO5 (~5 nmol/gFW). The Spm content did not vary much (10–15 nmol/gFW) within the plants tested (Figure 4D). NorSpd was only detected in Atpao5-2 plants expressing SelPAO5 (5–9 nmol/gFW, Figure 4E). The data show that
SelPAO5 produces NorSpd when expressed in the Atpao5-2 background. Furthermore, since the level of T-Spm drops in the SelPAO5 expressing Atpao5-2 mutant compared to untransformed Atpao5-2 and Atpao5-2 transformed with the empty vector, and the Spm contents stay almost same, it can be assumed that SelPAO5 converts T-Spm to NorSpd in planta.

**Figure 3.** Chromatograms of HPLC analysis of polyamine patterns from Arabidopsis wild type plants (Col-0) and Atpao5-2 mutant expressing SelPAO5, respectively. Std = chromatogram of polyamine standards.
3. Discussion

A recent phylogenetic analysis using a plant PAO protein sequence database identified four subfamilies: three subfamilies comprising PAOs with back conversion activity named PAO back conversion 1–3 (PAObc1, PAObc2, PAObc3), and one subfamily formed by terminal catabolism PAOs (subfamily PAOtc) [40]. PAObc1 was present on every lineage in the survey, pointing out important roles of back conversion-type PAOs in plants. PAObc2 was exclusively present in vascular plants, supporting the idea that T-Spm oxidase activity plays an important role in the development of the vascular system [34,40]. Arabidopsis AtPAO5 and rice OsPAO1 belong to this subfamily. Based on phylogenetic relationship, polyamine oxidase SelPAO5 of Selaginella lepidophylla is an orthologue of...
Arabidopsis AtPAO5 and rice OsPAO1 which both convert Spm and T-Spm to Spd in a back-conversion reaction. Therefore, it was expected that SelPAO5 produces Spd when using T-Spm as a substrate. However, in a previous work we could show that the recombinant SelPAO5 protein produces NorSpd in vitro [35]. To further characterize SelPAO5, we wanted to answer the questions i) does SelPAO5 convert Spm and/or T-Spm to NorSpd in vivo, and ii) can SelPAO5 replace AtPAO5 function and cure the reduced growth phenotype of Atpta5-2 mutant? In the Atpta5-2 mutant, T-Spm levels were increased, and plants showed a reduced growth phenotype [34]. A reduced growth phenotype was also observed in the Arabidopsis Acaulis5 mutant (acl5) lacking T-Spm synthase activity and thus had decreased T-Spm levels [4,5,41]. Therefore, it is assumed that deviation from normal T-Spm levels, both an increase and decrease, cause reduced growth of plants [34]. A balanced homeostasis of T-Spm is necessary for normal growth. When SelPAO5 was expressed in the Arabidopsis Atpta5-2 mutant, the T-Spm content decreased to almost normal levels of wild type plants while Spm levels did not decrease. NorSpd was only detected in the Atpta5-2 mutant that expressed SelPAO5. In total, these results suggest that SelPAO5 uses T-Spm as substrate and converts it into NorSpd in a back-conversion reaction when expressed in Arabidopsis. Reduction of T-Spm content to almost wild type levels by SelPAO5 action also cured the growth retardation effect that is caused by increased T-Spm levels and enables normal development. Whether Spd or NorSpd is produced by the T-Spm specific PAO activity does not make a difference concerning the effect of T-Spm homeostasis. The presence of NorSpd does not seem to disturb development of Arabidopsis, although it is usually not detectable in this plant. NorSpd is an unusual triamine in eukaryotes, which is present in lower, single-celled eukaryotes including Euglena, cryptophytes, diatoms, and also in Chlamydomonas and Volvox [18,42,43], but also in Bryophytes [16], in the leguminous plant Medicago sativa (alfalfa) [20], and in maize [21]. A NorSpd synthesis pathway like in the Gram-negative bacterium Vibrio cholerea is not found in eukaryotes [7], and NorSpd synthesis in alfalfa from the precursor DAP (1,3-diaminopropane), which is a co-product of Spd oxidation by PAO, could not be demonstrated [44]. The production of NorSpd by SelPAO5 using T-Spm as a substrate is a demonstration of a NorSpd synthesis pathway in plants. The idea that T-Spm back-conversion by a PAO results in NorSpd in plants is backed by the finding that presence of homologues of the Arabidopsis ACL5-encoded T-Spm synthase in genomes correlates with the presence of NorSpd in the organism [7]. In the unicellular green alga Chlamydomonas reinhardii, NorSpd stimulated cell division [45]. The role that NorSpd could play in higher plants is yet unknown. Further work should be done to follow how NorSpd is further metabolized in Arabidopsis and what kind of effect it has by making use of the Atpta5-2 mutant expressing SelPAO5.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Arabidopsis italiana wild-type (WT) plants [accession Columbia-0 (Col-0)] and the T-DNA inserted Atpta5-2 line (SALK_053110) [35] were used in this work. All seeds were surface sterilized by wetting with 70% ethanol for 1 min and subsequent treatment in a solution of 1% sodium hypochloride and 0.1% Tween-20 for 15 min. After extensive washing with sterile distilled water, sterilized seeds were placed onto vermiculite or on 1/2 Murashige and Skoog medium-1.5% agar plates (pH 5.6) containing 1% sucrose. Agar plates were kept upright under the angle of 75° to ground to allow plant growth on the agar surface by gravity. Growth conditions were 22 °C with a 14 h light/10 h dark photocycle.

4.2. Determination of Plant Fresh Weight

Seedlings grown for 24 days on agar surface containing 5 µM T-Spm where carefully picked with forceps and immediately weighed on a precision scale. Statistical analysis was done using MS-Excel software.
4.3. Chemicals

Put, Spd, and Spm were purchased from Nacalai-Tesque Ltd. (Kyoto, Japan). T-Spm and Nor-Spd was chemically synthesized [46]. 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.

4.4. Generation of Arabidopsis pao5 T-DNA Insertion Mutant Transgenic Lines Expressing SelPAO5 ORF

The fragment encompassing the coding region of the SelPAO5 cDNA was amplified by PCR with the primer pair listed in Table S1. It was digested with XbaI and SacI and subcloned into the corresponding sites of the pPZP2Ha3(+) vector [39], yielding pPZP2Ha3(+)‐SelPAO5. This plasmid was introduced into Agrobacterium tumefaciens strain GV3101, and the Agrobacterium transformant then introduced into pao5-2 plants using the floral dip method [47]. The resulting seeds were selected on MS agar medium containing 25 mg/mL hygromycin (hyg) and 50 mg/mL carbenicillin. T2 seeds, obtained from self-fertilization of primary transformants, were surface-sterilized and grown on hyg-containing plates. Seedlings showing a 3:1 (resistant:sensitive) segregation ratio were selected to produce homozygous (hygR/hygR) T3 lines that were used for further study.

4.5. RT-PCR Analysis

Total RNA was extracted from whole aerial parts of two-week-old Arabidopsis seedlings using Sepasol-RNA I Super (Nacalai-Tesque, Kyoto, Japan). First-strand cDNA was synthesized with ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) and oligo-dT primers. Quantitative real-time RT-PCR was performed in triplicate using Fast-Start Universal SYBR Green Master (ROX; Roche Molecular Systems, Indianapolis, IN, USA) on a StepOne real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using the above cDNA and the primers listed in Table S1. Constitutively expressed AtActin (accession number, NC_008396.2) was used as an internal control for the analysis to which the amount of target mRNA was normalized.

4.6. PA Analysis by High-Performance Liquid Chromatography (HPLC)

PA analysis was performed as described previously [5]. The benzoylated PAs were analyzed with a programmable Hewlett Packard series 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 consisted of a total of 60 min at a flow rate of 1 mL/min at 30°C; i.e., 42% acetonitrile for 25 min for PA separation, increased up to 100% acetonitrile during 3 min, then 100% acetonitrile for 20 min for washing, decreased down to 42% acetonitrile during 3 min, and finally 42% acetonitrile for 9 min. Statistical analysis was done using MS-Excel software.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/8/4/99/s1, Table S1. Oligonucleotide primers used in this study, Figure S1. Recovery of T-Spm-induced growth reduction in Atpao5-2 by complementation with SelPAO5.

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References

1. Tabor, C.W.; Tabor, H. Polyamines. *Annu. Rev. Biochem.* 1984, 53, 749–790. [CrossRef] [PubMed]
2. Cohen, S.S. *A Guide to the Polyamines*; Oxford University Press: Oxford, UK, 1998.
3. Knott, J.M.; Römer, P.; Sumper, M. Putative spermine synthases from *Thalassiosira pseudonana* and *Arabidopsis thaliana* synthesize thermospermine rather than spermine. *FEBS Lett.* 2007, 581, 3081–3086. [CrossRef] [PubMed]
4. Kakehi, J.; Kuwashiro, Y.; Niitsu, M.; Takahashi, T. Thermospermine is required for stem elongation in *Arabidopsis thaliana*. *Plant Cell Physiol.* 2008, 49, 1342–1349. [CrossRef] [PubMed]
5. Naka, Y.; Watanabe, K.; Sagor, G.H.M.; Niitsu, M.; Pillai, M.A.; Kusano, T.; Takahashi, Y. Quantitative analysis of plant polyamines including thermospermine during growth and salinity stress. *Plant Physiol. Biochem.* 2010, 48, 527–533. [CrossRef]
6. Takano, A.; Kakehi, J.I.; Takahashi, T. Thermospermine is not a minor polyamine in the plant kingdom. *Plant Cell Physiol.* 2012, 53, 606–616. [CrossRef]
7. Michael, A.J. Polyamines in eukaryotes, bacteria, and archea. *J. Biol. Chem.* 2016, 291, 14896–14903. [CrossRef] [PubMed]
8. Ohshima, T. Unique polyamines produced by an extreme thermophile, *Thermus thermophilus*. *Amino Acids* 2007, 33, 367–372. [CrossRef]
9. Groppa, M.D.; Benavides, M.P. Polyamines and abiotic stress: Recent advances. *Amino Acids* 2008, 34, 35–45. [CrossRef]
10. Kusano, T.; Berberich, T.; Tateda, C.; Takahashi, Y. Polyamines: Essential factors for growth and survival. *Planta* 2008, 228, 367–381. [CrossRef]
11. Alcázar, R.; Altabella, T.; Marco, F.; Bortolotti, C.; Reymond, M.; Koncz, C.; Carrasco, P.; Tiburcio, A.F. Polyamines: Molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 2010, 231, 1237–1249. [CrossRef]
12. Mattoo, A.K.; Minocha, S.C.; Minocha, R.; Handa, A.K. Polyamines and cellular metabolism in plants: Transgenic approaches reveal different responses to diamine putrescine versus higher polyamines spermidine and spermine. *Amino Acids* 2010, 38, 405–413. [CrossRef]
13. Minocha, R.; Majumdar, R.; Minocha, S.C. Polyamines and abiotic stress in plants: A complex relationship. *Front. Plant Sci.* 2014, 5, 175. [CrossRef]
14. Berberich, T.; Sagor, G.H.M.; Kusano, T. Polyamines in Plant Stress Response. In *Polyamines, A Universal Molecular Nexus for Growth, Survival, and Specialized Metabolism*; Kusano, T., Suzuki, H., Eds.; Springer: Tokyo, Japan, 2015; ISBN 978-4-431-55211-6.
15. Takahashi, T.; Tong, W. Regulation and diversity of polyamine biosynthesis in plants. In *Polyamines, A Universal Molecular Nexus for Growth, Survival, and Specialized Metabolism*; Kusano, T., Suzuki, H., Eds.; Springer: Tokyo, Japan, 2015; ISBN 978-4-431-55211-6.
16. Hamana, K.; Matsuzaki, S. Distinct difference in the polyamine compositions of Bryophyta and Pteridophyta. *J. Biochem.* 1985, 97, 1595–1601. [CrossRef]
17. Kuehn, G.D.; Rodriguez-Garay, B.; Bagga, S.; Phillips, G.C. Novel occurrence of uncommon polyamines in higher plants. *Plant Physiol.* 1990, 94, 855–857. [CrossRef]
18. Hamana, K.; Aizaki, T.; Arai, E.; Uchikata, K.; Ohnishi, H. Distribution of norspermidine as a cellular polyamine within micro green algae including non-photosynthetic aclorophyllous Polytoma, Polytomella, Prototheca and Helicosporidium. *J. Gen. Appl. Microbiol.* 2004, 50, 289–295. [CrossRef]
19. Fuell, C.; Elliot, K.A.; Hanfrey, C.C.; Franceschetti, M.; Michael, A.J. Polyamine biosynthetic diversity in plants and algae. *Plant Physiol. Biochem.* 2010, 48, 513–520. [CrossRef]
20. Rodriguez-Garay, B.; Phillips, G.C.; Kuehn, G.D. Detection of norspermidine and norspermine in *Medicago sativa*, L. (alfalfa). *Plant Physiol.* 1989, 89, 525–529. [CrossRef]
21. Koc, E.C.; Bagga, S.; Songstad, D.D.; Betz, S.R.; Kuehn, G.D.; Phillips, G.C. Occurrence of uncommon polyamines in cultured tissues of maize. *In Vitro Cell Dev. Biol. Plant* 1998, 34, 623–631. [CrossRef]
22. Kusano, T.; Kim, D.W.; Liu, T.; Berberich, T. Polyamine catabolism in plants. In *Polyamines, A Universal Molecular Nexus for Growth, Survival, and Specialized Metabolism*; Kusano, T., Suzuki, H., Eds.; Springer: Tokyo, Japan, 2015; ISBN 978-4-431-55211-6.
23. Federico, R.; Angelini, R.; Cona, A.; Niglio, A. Polyamine oxidase bound to cell walls from *Zea mays* seedlings. *Phytochemistry* 1992, 31, 2955–2957. [CrossRef]
24. Tavladoraki, P.; Schinina, M.E.; Cecconi, F.; Di Agostino, S.; Manera, F.; Rea, G.; Mariotti, P.; Federico, R.; Angelini, R. Maize polyamine oxidase: Primary structure from protein and cDNA sequencing. FEBS Lett. 1998, 426, 62–66. [CrossRef]

25. Radová, A.; Sebela, M.; Galusuzka, P.; Frébort, I.; Jacobsen, S.; Faulhammer, H.G.; Pec, P. Barley polyamine oxidase: Characterisation and analysis of the cofactor and the N-terminal amino acid sequence. Phytochem. Anal. 2001, 12, 166–173. [CrossRef]

26. Cervelli, M.; Cona, A.; Angelini, R.; Polticelli, F.; Federico, R.; Mariotti, P. A barley polyamine oxidase isoform with distinct structural features and subcellular localization. Eur. J. Biochem. 2001, 268, 3816–3830. [CrossRef]

27. Hanzawa, Y.; Takahashi, T.; Komeda, Y. ACL5: An Arabidopsis gene required for internodal elongation after flowering. Plant J. 2003, 34, 410–418. [CrossRef]

28. Liu, T.; Kim, D.W.; Niitsu, M.; Berberich, T.; Kusano, T. The polyamine oxidase responsible for a full back-conversion pathway in Arabidopsis. Plant Physiol. 2008, 147, 1845–1857. [CrossRef]

29. Kamada-Nobusada, T.; Hayashi, M.; Fukazawa, M.; Sakakibara, H.; Nishimura, M. A putative peroxisomal polyamine oxidase AtPAO4 is involved in polyamine catabolism in Arabidopsis thaliana. Plant Cell Physiol. 2008, 49, 1272–1282. [CrossRef] [PubMed]

30. Fincato, P.; Moschou, P.N.; Spedaletti, V.; Tavazza, R.; Angelini, R.; Federico, R.; Roubelakis-Angelakis, K.A.; Tavladoraki, P. Functional diversity inside the Arabidopsis polyamine oxidase gene family. J. Exp. Bot. 2011, 62, 1155–1168. [CrossRef]

31. Liu, T.; Kim, D.W.; Niitsu, M.; Berberich, T.; Kusano, T. Oraya sativa polyamine oxidase 1 back-converts tetraamines, spermine and thermospermine to spermidine. FEBS Lett. 2003, 589, 3071–3078. [CrossRef] [PubMed]

32. Banks, J.A.; Nishiyama, T.; Hasebe, M.; Bowman, J.L.; Glibskov, M.; de Pampfils, C.; Albert, V.A.; Aono, N.; Aoyama, T.; Ambrose, B.A.; et al. The Selaginella genome identifies genetic changes associated with evolution of vascular plants. Science 2012, 332, 960–963. [CrossRef]

33. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 2013, 30, 2725–2729. [CrossRef]

34. Fuse, T.; Sasaki, T.; Yano, M. Ti-plasmid vectors useful for functional analysis of rice genes. Plant Biotech. 2001, 18, 219–222. [CrossRef] [PubMed]

35. Bordenave, C.D.; Granados Mendoza, C.; Jiménez Bremont, J.F.; Gárriz, A.; Rodriguez, A.A. Defining novel plant polyamine oxidase subfamilies through molecular modeling and sequence analysis. BMC Evol. Biol. 2019, 19, 28. [CrossRef] [PubMed]

36. Hanzawa, Y.; Takahashi, T.; Komeda, Y. ACL5: An Arabidopsis gene required for intermodal elongation after flowering. Plant J. 1997, 12, 863–874. [CrossRef] [PubMed]

37. Harashima, K.; Niitsu, M.; Samejima, K. Unusual polyamines in aquatic plants: The occurrence of homospermidine, norspermidine, thermospermine, norspermine, aminopropylhomospermidine, bis(aminopropyl)ethanediamine, and methylspermidine. Can. J. Bot. 1998, 76, 130–133. [CrossRef]

38. Hamana, K.; Matsuzaki, S. Widespread occurrence of norspermidine and norspermine in eukaryotic algae. J. Biochem. 1982, 91, 1321–1328. [CrossRef] [PubMed]
44. Bagga, S.; Rochford, J.; Klaene, Z.; Kuehn, G.D.; Phillips, G.C. Putrescine aminopropyltransferase is responsible for biosynthesis of spermidine, spermine, and multiple uncommon polyamines in osmotic stress-tolerant Alfalfa. *Plant Physiol.* **1997**, *114*, 445–454. [CrossRef]

45. Tassoni, A.; Awad, N.; Griffiths, G. Effect of ornithine decarboxylase and norspermidine in modulating cell division in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol. Biochem.* **2018**, *123*, 125–131. [CrossRef] [PubMed]

46. Niitsu, M.; Samejima, K. Synthesis of a series of linear pentaamines with three and four methylene chain intervals. *Chem. Pharm. Bull.* **1986**, *34*, 1032–1038. [CrossRef]

47. Clough, S.J.; Bent, A.F. Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **1998**, *16*, 735–743. [CrossRef] [PubMed]