Plant Cell Physiol. 49(9): 1272–1282 (2008)
doi:10.1093/pcp/pcn114, available online at www.pcp.oxfordjournals.org

Rapid paper

A Putative Peroxisomal Polyamine Oxidase, AtPAO4, is Involved in Polyamine Catabolism in *Arabidopsis thaliana*

Tomoe Kamada-Nobusada [1,3], Makoto Hayashi [1,2], Mitsue Fukazawa [1], Hitoshi Sakakibara [3] and Mikio Nishimura [1,2,*]

[1] *Division of Cell Mechanisms, Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444-8585 Japan*
[2] *Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies, Okazaki, 444-8585 Japan*
[3] *RIKEN Plant Science Center, Tsurumi, Yokohama, 230-0045 Japan*

We characterized three *Arabidopsis* polyamine oxidase genes, *AtPAO2*, *AtPAO3* and *AtPAO4*. Transient expression of these genes as monomeric red fluorescent protein fusion proteins in *Arabidopsis* root cells revealed that all are peroxisomal proteins. Quantitative analysis of their transcripts in various organs suggested that AtPAO4 is the major isoform in root peroxisomes. Analysis of recombinant AtPAO4 protein indicated that it is a flavoprotein that catalyzed the oxidative conversion of spermine to spermidine. *AtPAO4*-deficient mutants established by using T-DNA insertion and RNA interference techniques had markedly increased spermine and decreased spermidine levels in the roots. These results suggest that AtPAO4 is a root peroxisomal polyamine oxidase that participates in polyamine catabolism. Microarray analysis showed that *AtPAO4* deficiency induced alterations in the expression of genes related to the drought stress response and flavonoid biosynthesis.

Keywords: *Arabidopsis* — Peroxisome — Polyamine — Polyamine oxidase — Spermidine — Spermine.

Abbreviations: CaMV, cauliflower mosaic virus; DRE, dehydration-responsive element; EST, expressed sequence tag; GFP, green fluorescent protein; LEA, late embryonic antigen; mRFP, monomeric red fluorescent protein; PAL, phenylalanine ammonia-lyase; PAO, polyamnine oxidase; PTS, peroxisomal targeting signal; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; WT, wild type.

Introduction

The polyamines spermidine, spermine and their diamine obligate precursor putrescine are all small aliphatic amines that are commonly found in both prokaryotic and eukaryotic cells. Previous studies have suggested that in higher plants, polyamines are involved in a wide variety of cellular processes and stress responses, including the control of cell division, flowering, retardation of senescence, the responses to osmotic shock, drought and salt stresses, and plant–pathogen interactions (Galston and Sawhney 1990, Kumar et al. 1997, Walden et al. 1997, Bouchereau et al. 1999, Cona et al. 2006). Putrescine is converted into spermidine and then into spermine by spermidine synthase and spermine synthase, respectively. The genes in *Arabidopsis* that encode spermidine synthase and spermine synthase have been identified (Hanzawa et al. 2000, Hanzawa et al. 2002, Panicot et al. 2002). It is also known that polyamines are further conjugated to a variety of compounds. In plant cells, the only well-characterized conjugated forms are the hydroxycinnamic acid amides, which are conjugates composed of polyamine and hydroxycinnamic acid. That hydroxycinnamic acid amides also participate in various cellular processes is shown by the association between the accumulation of hydroxycinnamic acid amides and flowering, seed/fruit development, and hypersensitive responsiveness to viral and fungal infections (Flores and Martin-Tamguy 1991).

Polyamines are catabolized by the oxidative de-amination activity of polyamine oxidase (PAO). However, the substrate specificity and reaction products of PAO vary depending on the organisms and isoenzymes involved. It has been reported that plant PAO oxidizes the carbon on the *endo* side of the N^4 nitrogens of spermidine and spermine and thereby produces 4-aminobutanal and 1-(3-aminopropyl)-4-aminobutanal, respectively (Federico and Angelini 1991, Cona et al. 2006). Both reactions also produce 1,3-diaminopropane (DAP) and hydrogen peroxide. These reactions are known as polyamine degradation reactions. The degradation of polyamine appears to occur predominantly in the apoplasmic space since PAO activity is predominantly observed in the cell wall fraction (Slocum 1991). An immunohistochemical study in maize has detected the PAO polypeptide in the cell wall

*Corresponding author: E-mail, mikosome@nibb.ac.jp; Fax, 81-564-55-7505.

(Angelini et al. 1995). Moreover, the maize *PAO* genes that have been isolated to date bear a secretory signal, which supports the notion that PAO acts in the cell wall (Tavladoraki et al. 1998, Cervelli et al. 2000). However, PAO (BPAO2) activity has also been detected in the symplast of barley (Cervelli et al. 2001).

In animals and yeast, PAO oxidizes the carbon on the *exo* side of the N^4 nitrogens on spermine and its N^1-acetylated form (N^1-acetylspermine), thus producing spermidine; it does the same with N^1-acetylspermidine, thereby producing putrescine (Nishikawa et al. 2000, Vujcic et al. 2002, Cervelli et al. 2003, Landry and Sternglanz 2003, Vujcic et al. 2003, Wu et al. 2003). These reactions constitute the so-called polyamine back-conversion pathway. There are two types of polyamine back-conversion pathways in animals and yeast, namely one that involves polyamine N^1-acetylation and one that does not. The *PAO* genes involved in the N^1-acetylation-mediated polyamine back-conversion pathway that have been reported to date are known to bear a peroxisomal targeting signal sequence at their C-terminus (an exception to this is *Saccharomyces cerevisiae* Fms1) (Nishikawa et al. 2000, Vujcic et al. 2003, Wu et al. 2003). Peroxisomal PAO is believed to play an important role in regulating cellular polyamine levels in animals (Igarashi 2006).

The *Arabidopsis thaliana* genome encodes at least five putative *PAO* genes denoted as *AtPAO1–5* (*At5g13700*, *At2g43020*, *At3g59050*, *At1g65840* and *At4g29720*, respectively) (Tavladoraki et al. 2006). A recent study on *Arabidopsis* PAO revealed that in vitro, AtPAO1 converts spermine and norspermine to spermidine and norspermidine, respectively. This suggests that the polyamine back-conversion pathway may also take place in plants (Tavladoraki et al. 2006).

Peroxisomes are single membrane-bound organelles that occur ubiquitously in eukaryotic cells and are involved in degradation of hydrogen peroxide. It is known that in higher plants, peroxisomes in germinating seedlings and in senescent leaves contain the enzymes involved in fatty acid β-oxidation, and those in photosynthetic organs contain the enzymes involved in photorespiration (Beevers 1979). Thus, plant peroxisomes in each organ contain distinct sets of enzymes that lead to different metabolic roles. However, in the other organs, such as the roots, stems and flowers, the functions of the peroxisomes remain poorly understood. To elucidate the functions in those organs, we used a custom-made microarray to determine the gene expression profile of a subset of genes that encode putative peroxisomal proteins (Kamada et al. 2003). The analysis allowed us to identify a putative peroxisomal PAO (AtPAO4) that is highly expressed in the roots.

In this study, we show that three AtPAOs (AtPAO2–4) are peroxisomal proteins, and that *AtPAO4* is a major transcript among peroxisomal *AtPAOs* in roots. Analyses of recombinant AtPAO4 protein and *AtPAO4*-deficient mutants suggested that AtPAO4 is involved in polyamine catabolism. Thus, peroxisomes may participate in polyamine catabolism in *Arabidopsis*, and especially in the roots of these plants.

Results

Peroxisomal localization of AtPAO2, AtPAO3 and AtPAO4

Three of the five *AtPAO* genes, *AtPAO2*, *AtPAO3* and *AtPAO4*, are predicted to encode a protein that bears PTS1 at its C-terminus, which is one of the two known peroxisomal targeting signals (Hayashi et al. 1997, Kamada et al. 2003) (Fig. 1A). To examine the subcellular locations of these three AtPAOs, we prepared plant expression vectors harboring DNA constructs that produced each of the AtPAOs fused to monomeric red fluorescent protein (mRFP) (*mRFP–AtPAO2*, *mRFP–AtPAO3* and

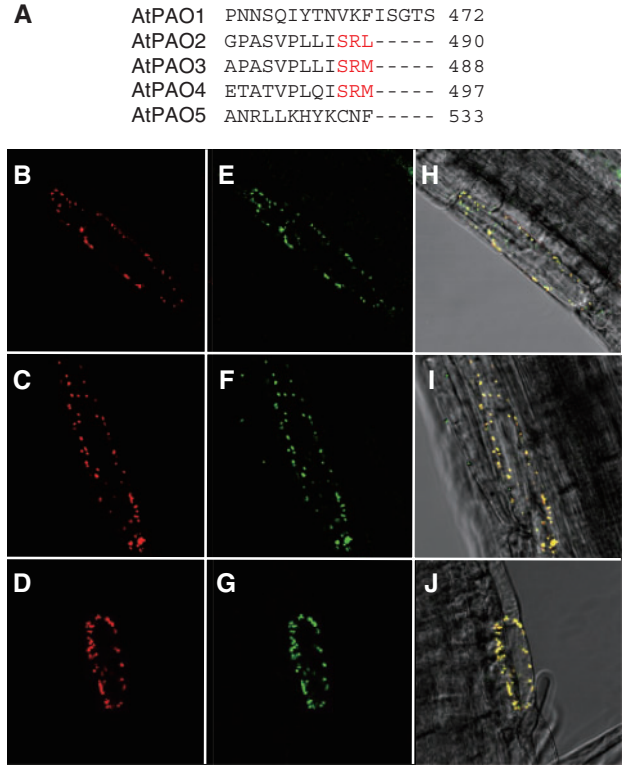

Fig. 1 Subcellular localization of the mRFP–AtPAO fusion proteins in *Arabidopsis* root cells. (A) Alignment of the C-terminal amino acid sequences of AtPAO1–5. The putative PTS1 sequence is highlighted in red. mRFP–AtPAO2 (B), mRFP–AtPAO3 (C) and mRFP–AtPAO4 (D) were introduced into *Arabidopsis* root cells by particle bombardment. As a peroxisomal marker, GFP–PTS1 fusion proteins were concomitantly introduced (E–G). The red and green fluorescence of the root cells was analyzed by laser confocal microscopy. The merged images of mRFP–AtPAO2–4 and GFP–PTS1 together with Nomarski images are shown in (H–J).

mRFP–AtPAO4, respectively). Each of the vectors was introduced into *Arabidopsis* root cells by particle bombardment together with a vector harboring *GFP-PTS1* which produced a peroxisome-targeted green fluorescent protein (Mano et al. 2002). The cells expressing mRFP–AtPAO2, mRFP–AtPAO3 or mRFP–AtPAO4 showed punctate red fluorescence (Fig. 1B–D). The peroxisomes in the cells were labeled with green fluorescence (Fig. 1E–G) due to the co-expression of GFP–PTS1. As shown in Fig. 1H–J, all punctate red fluorescence signals in the cells coincided completely with the green fluorescence of GFP–PTS1. Thus, AtPAO2, AtPAO3 and AtPAO4 appear to be peroxisomal proteins.

Organ-specific expression of peroxisomal AtPAO genes

To evaluate the gene expression of the three AtPAOs in different organs, the RNAs prepared from seedlings and various organs of mature *Arabidopsis* plants were subjected to quantitative reverse transcription–PCR (RT–PCR) (Fig. 2A–C). While *AtPAO2*, *AtPAO3* and *AtPAO4* transcripts were detected in all organs, they differed in their organ-specific abundance. *AtPAO2* transcripts were more abundant in flowers and siliques and less abundant in seedlings and other organs (Fig. 2A). *AtPAO3* transcripts were constitutively expressed at equivalent levels in all tissues (Fig. 2B). In contrast, *AtPAO4* transcripts were abundantly expressed in roots, flowers and greening cotyledons, and more poorly expressed in other tissues (Fig. 2C). It is noteworthy that *AtPAO4* was the most abundantly expressed *AtPAO* among peroxisomal *AtPAO* genes in root cells. We consulted a public gene expression database (ATTEDII; http://www.atted.bio.titech.ac.jp/) containing data about the tissue-specific gene expression of all five AtPAOs. The data suggested that AtPAO4 is the major PAO isoenzyme in roots in terms of its gene expression. This led us to study AtPAO4 further with the hope that this would clarify the contribution of root peroxisomes to polyamine catabolism in the plant.

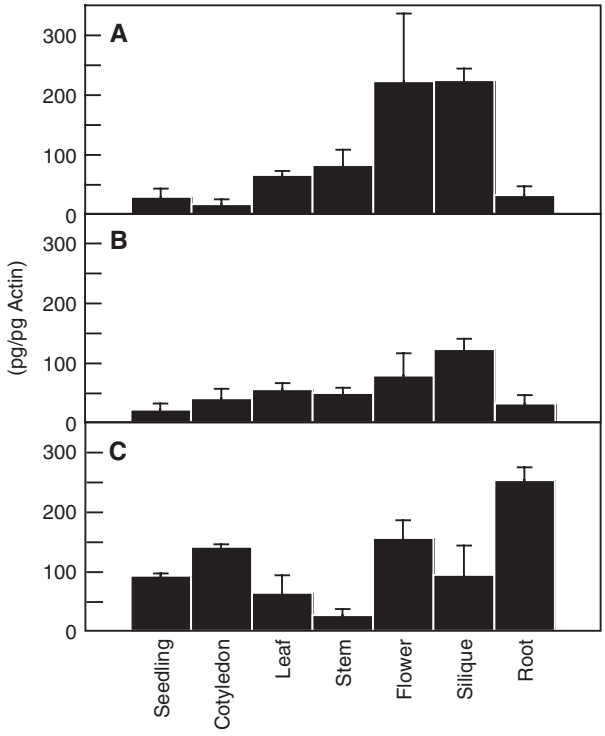

Fig. 2 Quantitative RT–PCR analysis of the *AtPAO2–4* gene expression in various organs. The transcript levels in each organ were measured by using *AtPAO2* (A), *AtPAO3* (B), *AtPAO4* (C) and *actin* gene-specific primers, and are expressed as ng mRNA per 1 ng of actin mRNA.

Establishment of AtPAO4-deficient Arabidopsis mutants

To investigate the physiological function of AtPAO4, we established two *Arabidopsis* mutants that lacked or had reduced *AtPAO4* expression. One was a knock-out mutant that had a T-DNA insertion within the ninth exon of the *AtPAO4* gene (SALK_109229) (Fig. 3A). It was designated

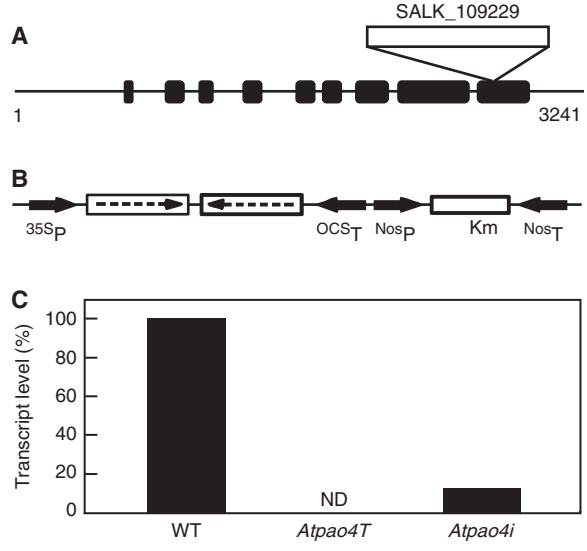

Fig. 3 Establishment of the AtPAO4-deficient mutants *Atpao4T* and *Atpao4i*. (A) Location of the T-DNA insertion in the *AtPAO4* knock-out mutant *Atpao4T* (SALK_109229). The black rectangles indicate the positions and lengths of the exons. (B) The DNA construct that was used to generate the *AtPAO4* knock-down mutant *Atpao4i*. The two white boxes represent two identical 229 bp *AtPAO4* cDNA fragments in reverse orientation (indicated by the dashed arrows). This RNAi construct was expressed under the control of the 35S CaMV promoter and the OCS terminator. (C) Quantitative RT–PCR analysis of *AtPAO4* transcript levels in the roots of 2-week-old WT, *Atpao4T* and *Atpao4i* seedlings. The WT *AtPAO4* transcript levels were set to 100% and the *Atpao4T* and *Atpao4i* transcript levels were expressed as a percentage of the WT levels. ND, not detected. Km, kanamycin; ^{35S}P, 35S CaMV promoter; ^{OCS}T, OCS terminator; ^{NOS}P, NOS promoter; ^{NOS}T, NOS terminator.

as *Atpao4T*. Quantitative RT–PCR analysis failed to detect any *AtPAO4* transcripts in *Atpao4T* (Fig. 3C). The other mutant was a knock-down mutant called *Atpao4i* that expressed *AtPAO4* at reduced levels due to double-stranded RNA interference (RNAi) under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 3B). From 15 independent transgenic plants, we selected the mutant with the lowest *AtPAO4* expression [12.5% of wild-type (WT) plant levels] (Fig. 3C). Neither of the mutants had a visible phenotype in terms of plant size and morphology under normal growth conditions (data not shown).

Polyamine contents in AtPAO4-deficient mutants

We compared the putrescine, spermidine and spermine levels in the roots and shoots of 2-week-old *Atpao4T*, *Atpao4i* and WT *Arabidopsis* seedlings. After extracting the polyamines from these tissues, they were dansylated and analyzed by HPLC. The *Atpao4T* roots had a slightly higher putrescine level (134%) than WT roots, although the levels in *Atpao4i* roots and in each shoot of these two mutants did not differ significantly (Fig. 4A). Both their roots and shoots had markedly lower spermidine levels than those in the WT tissues (*Atpao4T* roots, 51%, and shoots, 74%; *Atpao4i* roots, 60%, and shoots, 68%) (Fig. 4B).

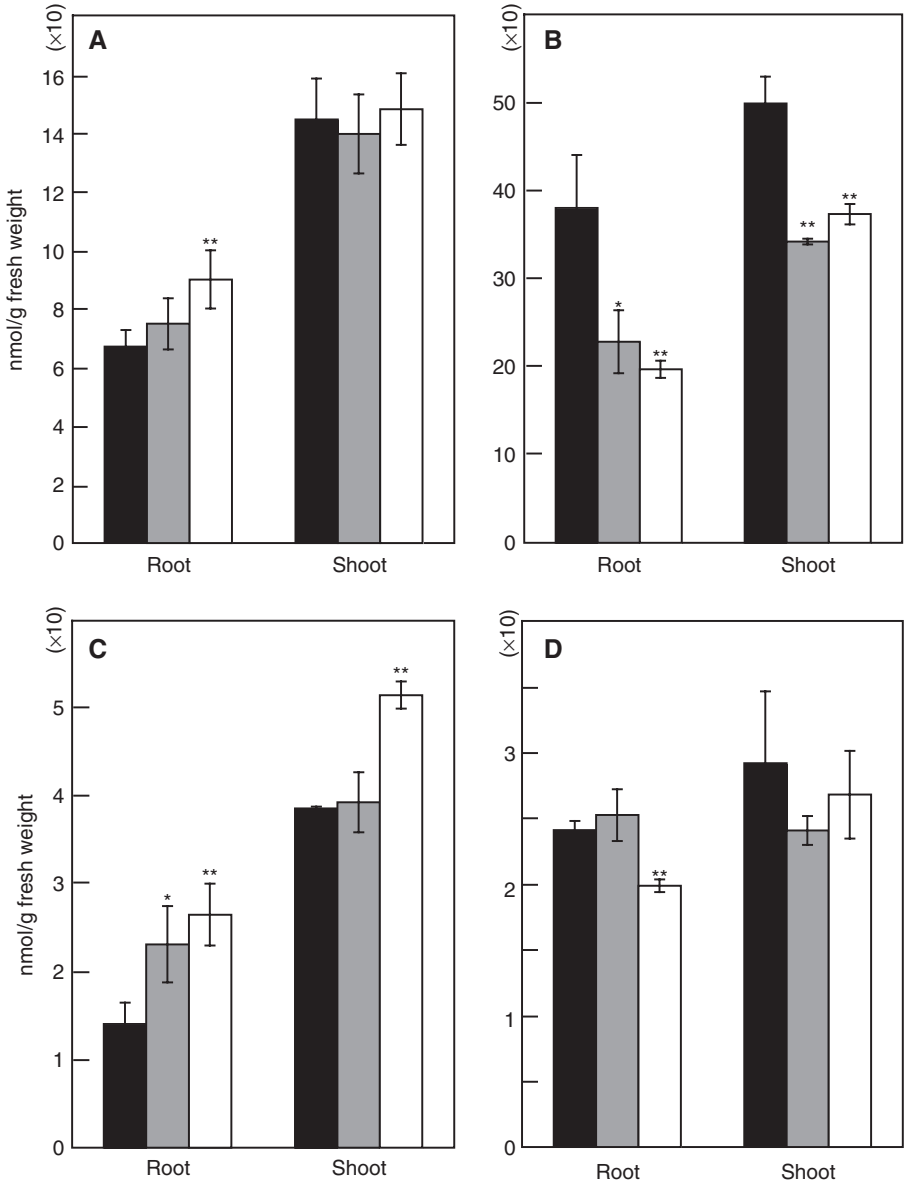

Fig. 4 Polyamine levels in the roots and shoots of AtPAO4-deficient mutants. Putrescine (A), spermidine (B), spermine (C) and N^1-acetylspermine (D) were extracted from the roots and shoots of 2-week-old WT (black bar), *Atpao4i* (gray bar) and *Atpao4T* (white bar) seedlings, after which they were quantified by HPLC. The data shown are the means with SE ($n=3$). $^{**}P<0.05$; $^{*}P<0.055$ by *t*-test.

Their roots also had much higher spermine levels than WT roots (*Atpao4T*, 188%; *Atpao4i*, 164%) (Fig. 4C). In terms of the spermine level in the shoots, it was 133% higher in *Atpao4T* compared with the WT but did not differ significantly in *Atpao4i* (Fig. 4C).

Since the peroxisomal PAOs in mammals and yeast can catalyze the conversion of N^1-acetylspermine to spermidine (Nishikawa et al. 2000, Wu et al. 2003), we also measured the N^1-acetylspermine levels in the *Arabidopsis* mutants. As shown in Fig. 4D, the *Atpao4T* roots had a slightly lower N^1-acetylspermine level (82%) than WT roots, although the levels in the shoots and in *Atpao4i* mutant roots and shoots did not differ significantly from those of the WT. We also checked all other peaks on the chromatograms, but further obvious differences between *Atpao4T*, *Atpao4i* and the WT were not detected. Thus, AtPAO4 deficiency resulted in changes in the spermidine and spermine levels of the mutants, especially in the roots.

Heterologous expression of AtPAO4 in Escherichia coli and characterization of the purified enzyme

To determine the activity of AtPAO4 in vitro, AtPAO4 protein was expressed in *E. coli* as a His-tagged protein, after which it was purified to homogeneity by SDS–PAGE (Fig. 5A). The molecular mass of this protein was 55 kDa, which corresponds well to the molecular mass predicted on the basis of its amino acid sequence. The absorption spectrum of the protein was characterized by three absorbance peaks in the UV-visible part of the spectrum (at 280, 380 and 460 nm), which is characteristic of the oxidized flavoproteins (Fig. 5B).

To determine the substrate specificity of AtPAO4, we incubated purified recombinant AtPAO4 with putrescine, spermidine, spermine or N^1-acetylspermine. The spermine peaks became smaller in size and a new peak appeared only when recombinant AtPAO4 was added to the reaction buffer containing spermine as a substrate (Fig. 6A, B). The retention time of the new peak corresponded to that of spermidine (data not shown). There were no other newly appearing peaks. On the other hand, we did not find any differences between the chromatogram of the reaction with AtPAO4 and the chromatogram of the reaction without AtPAO4 when we used putrescine, spermidine or N^1-acetylspermine as substrates (data not shown). These results suggested that AtPAO4 specifically catalyzed the conversion of spermine to spermidine under these conditions. The observations are consistent with the

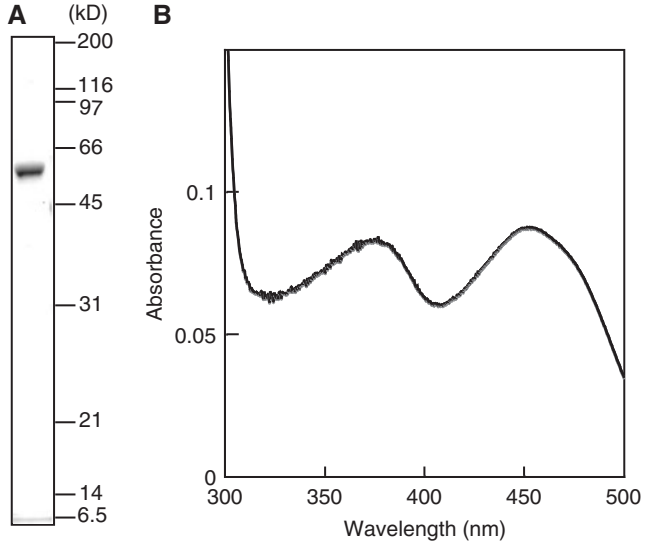

Fig. 5 Purification of recombinant AtPAO4. (A) Recombinant AtPAO4 proteins were purified from *E. coli* by using an Ni-NTA column and then analyzed by SDS–PAGE and Coomassie Brilliant Blue staining. A 1 μg aliquot of the protein preparation was applied to the gel. (B) Absorption spectrum of purified recombinant AtPAO4 dissolved in elution buffer.

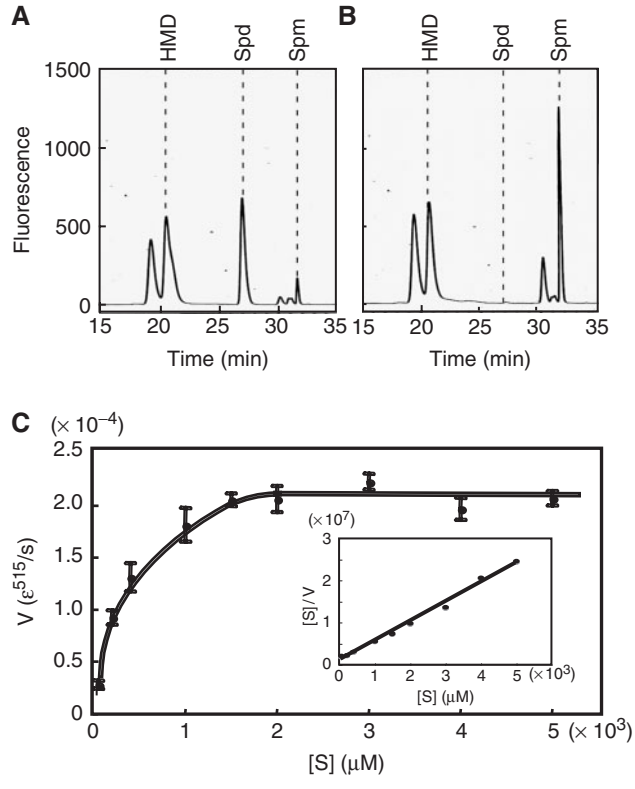

Fig. 6 Catalytic properties of recombinant AtPAO4. Chromatograms show the HPLC analysis of enzymatic reactions after 10 μg of recombinant AtPAO4 (A) or elution buffer only (control, B) were added to the 150 μM spermine (Spm) substrate. The dashed lines indicate the retention times of the internal control polyamine hexamethylenediamine (HMD) and spermidine (Spd) and spermine (Spm). (C) The kinetic parameters of recombinant AtPAO4 for spermine were examined as described in Materials and Methods. The data shown are the means with SE ($n = 3$). The K_m value was calculated from a Hanes–Woolf plot (inset).

polyamine phenotype of the *AtPAO4*-deficient mutants described above.

Furthermore, the analysis of the catalytic properties of the purified AtPAO4 indicated a k_{cat} value of $1.4\,s^{-1}$ for spermine. This is similar to that of recombinant AtPAO1 ($2.7\,s^{-1}$) (Tavladoraki et al. 2006) and recombinant mouse spermine oxidase ($4.5\,s^{-1}$) (Cervelli et al. 2003), but lower than that of recombinant maize PAO ($54\,s^{-1}$) (Polticelli et al. 2005). The K_m value of the protein reaction with spermine was 0.23 mM (Fig. 6C), which is similar to the K_m value of recombinant AtPAO1 (0.11 mM) (Tavladoraki et al. 2006) and recombinant mouse spermine oxidase (0.09 mM) (Cervelli et al. 2003), but much higher than the K_m value of recombinant maize PAO (1.6 μM) (Polticelli et al. 2005). These data suggested that recombinant AtPAO4 is a similar but less efficient enzyme ($k_{cat}/K_m = 6.1\,s^{-1}\,mM^{-1}$) than the recombinant AtPAO1 ($k_{cat}/K_m = 24.1\,s^{-1}\,mM^{-1}$) (Tavladoraki et al. 2006), recombinant mouse spermine oxidase ($k_{cat}/K_m = 50.0\,s^{-1}\,mM^{-1}$) (Cervelli et al. 2003) and recombinant maize PAO ($k_{cat}/K_m = 23.9 \times 10^3\,s^{-1}\,mM^{-1}$) (Polticelli et al. 2005) in catalyzing spermine oxidation.

The optimum pH of the spermine-to-spermidine conversion reaction catalyzed by AtPAO4 was around 7.5–8.5 (data not shown). This is similar to the optimum pH of murine peroxisomal PAO (around pH 8.0; Wu et al. 2003).

Genome-wide gene expression profiling of AtPAO4-deficient mutants

To estimate the physiological function(s) of AtPAO4, we used a DNA microarray covering nearly all *Arabidopsis* genes to compare the genome-wide gene expression profiles of the roots of *Atpao4T*, *Atpao4i* and WT plants. This analysis revealed that 50 genes were up-regulated (Fig. 7A) and 63 genes were down-regulated (Fig. 7B) in both mutants. Analysis of these genes indicated that the up-regulated genes contained two peculiar groups of genes, namely those involved in abiotic stress responses and those involved in flavonoid and/or lignin metabolism (Table 1).

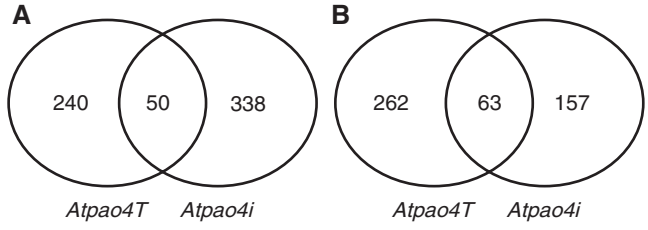

Fig. 7 Venn diagrams indicating the number of genes that are up- (A) and down-regulated (B) in *Atpao4T* and *Atpao4i* relative to the WT, as revealed by microarray analysis of RNAs isolated from the roots of 2-week-old plants.

Table 1 A selection of the genes that are up-regulated in the roots of both *Atpao4i* and *Atpao4T*

Atpao4i[a]	*Atpao4T*[a]	Gene ID	Description
Stress response			
3.98	5.45	At5g66400	Universal stress protein (USP) family protein
3.91	11.88	At5g06760	LEA group 1 domain-containing protein
3.53	8.40	At1g10370	Glutathione S-transferase, putative (ERD9)
3.36	4.05	At3g15670	LEA protein, putative
3.25	5.40	At3g17520	LEA domain-containing protein
3.08	9.05	At5g52310	Desiccation-responsive protein 29A (RD29A)
3.06	8.15	At1g52690	LEA protein, putative
2.54	4.20	At2g15970	Cold acclimation protein, putative (FL3-5A3)
2.32	3.75	At5g05410	DRE-binding protein (DREB2A)
Flavonoid and/or lignin metabolism			
6.77	8.54	At5g08640	Flavonol synthase 1 (FLS1)
5.95	8.77	At2g23910	Cinnamoyl-CoA reductase-related
4.28	5.64	At5g05270	Chalcone-flavanone isomerase family protein
4.05	4.17	At2g22590	Glycosyltransferase family protein
4.03	5.69	At1g65060	4-Coumaroyl-CoA synthase 3 (4CL3)
3.95	5.94	At3g51240	Flavanone 3-hydroxylase (F3H)
2.99	2.80	At5g13930	Chalcone synthase/naringenin-chalcone synthase
2.83	3.09	At5g07990	Flavonoid 3′-hydroxylase (F3′H)
2.02	2.15	At2g37040	Phenylalanine ammonia-lyase 1 (PAL1)

Transcripts annotated as genes involved in stress response and flavonoid and/or lignin metabolism are listed.
[a]Ratio of *Atpao4i* or *Atpao4T* signal intensity relative to WT signal intensity.

Discussion

In this study, we identified three of the five AtPAOs (AtPAO2–4) that are localized to peroxisomes. In previous studies, hydrogen peroxide from the PAO (apoplasmic PAO) reaction was shown to play a specialized role in plant development and pathogen resistance. This is because hydrogen peroxide is derived from polyamine degradation in the plant cell wall, which has been reported to be a major site of PAO activity. The level of hydrogen peroxide produced by the apoplasmic PAOs correlates with cell wall maturation and lignification, as well as with wound healing and cell wall reinforcement during pathogen invasion (Cona et al. 2006). On the other hand, our observations suggest that the hydrogen peroxide produced by AtPAO2–4 is unlikely to play a special physiological role since peroxisomes potently eliminate hydrogen peroxide (Huang et al. 1983). Oxidative catabolism of polyamines itself is likely to be more important as a peroxisomal function.

AtPAO4-deficient mutants had elevated spermine levels and reduced spermidine levels, especially in their roots (Fig. 4). These data suggest that peroxisomes possibly regulate the spermine/spermidine proportion in *Arabidopsis* through AtPAO4 activity. However, the changes in the polyamine contents of the *AtPAO4*-deficient mutants did not cause any visible phenotypes. Polyamine contents and their relative proportions are primarily determined by the inter-relationship between polyamine synthesis and oxidative deamination. Furthermore, AtPAO is encoded by a small multigene family, and AtPAO2 and AtPAO3 are also localized to the peroxisome. Since these PAOs might contribute to polyamine content, there is a possibility that the proportion of changes in the amount of polyamine in the mutants becomes reduced.

Recombinant AtPAO4 could catalyze the oxidative deamination of spermine in vitro. Its catalytic properties with spermine were similar to those of recombinant AtPAO1 (Tavladoraki et al. 2006) and recombinant AtPAO3 reported recently by Moscou et al. (2008). Recombinant AtPAO1 catalyzed the conversion of norspermine, an uncommon polyamine detected in higher plants, more efficiently than it catalyzed the conversion of spermine in vitro. It is also possible that AtPAO4 catalyzes the conversion of such uncommon polyamines. We could not check if these uncommon polyamines were substrates for AtPAO4 in this work, because they are currently not commercially available.

It has been shown that in animals and yeast, peroxisomal PAO is involved in polyamine back-conversion linked with N^1-acetylation (Nishikawa et al. 2000, Wu et al. 2003). In contrast, we found that purified peroxisomal AtPAO4 did not use N^1-acetylspermine as a substrate in vitro. Moreover, the *AtPAO4*-deficient mutants did not differ from the WT in their N^1-acetylspermine levels. It remains unclear at present whether the acetylation-linked polyamine metabolism observed in animals and yeast exists in higher plants. This question will only be resolved when all of the other plant PAOs are biochemically characterized.

The roots of the *AtPAO4*-deficient mutants had markedly lower spermidine levels and much higher spermine levels than WT roots. This was especially the case for the *Atpao4T* mutant (spermidine, 50%; spermine, 188% compared with the WT). In shoots, the spermidine and spermine contents of the *AtPAO4*-deficient mutants were altered, but not to the extent seen in the roots (spermidine, 74%; spermine, 133% in *Atpao4T* compared with the WT). This is in agreement with the gene expression pattern of *AtPAO4*, which is expressed more in the roots than the shoots. At present, the biological function of root peroxisomes remains poorly understood. These results suggest that polyamine catabolism by AtPAO4 is principally performed in root peroxisomes, although further analyses of the function of other peroxisomal AtPAOs will be needed. *AtPAO4* was also expressed at relatively high levels in flowers in our study. The gene expression database ATTEDII has shown that *AtPAO4* is expressed at high levels at restricted stages of stamen and pollen development as well. Thus, AtPAO4 may also function in reproductive organs.

We subjected the *AtPAO4*-deficient mutants to microarray analysis to obtain further clues about the biological function(s) of the peroxisomal PAO. We observed that two groups of genes were up-regulated in the *AtPAO4*-deficient mutants. One group consisted of genes involved in the drought stress response (Table 1). An example is *RD29A*, which has been found to be induced by dehydration, high salt and low temperature in a dehydration-responsive element (DRE)-dependent manner (Yamaguchi-Shinozaki and Shinozaki 1994). Another example is *DREB2A*, which encodes a transcription factor that helps induce drought-responsive gene expression by interacting with the *cis*-acting DRE element (Liu et al. 1998, Sakuma et al. 2006). Moreover, several genes of late embryogenesis abundant (LEA) protein were up-regulated in the *AtPAO4*-deficient mutants. These proteins are known to be dehydration-inducible proteins whose expression is mediated by DRE-binding proteins (Sakuma et al. 2006). Many studies have reported that accumulation of polyamines enhances the drought tolerance of plants (and vice versa) (Capell et al. 2004, Kasukabe et al. 2004, Alcázar et al. 2006a, Alcázar et al. 2006b). An *Arabidopsis* mutant (*acl5*/*sdms*) that cannot produce spermine is hypersensitive to drought. The phenotype of this mutant could only be rescued by exogenous spermine, but not by exogenous spermidine or putrescine (Yamaguchi et al. 2007). Since *AtPAO4*-deficient mutants accumulated more spermine in this study, we expect that the mutants will be more resistant to drought

stress than the WT. However, our pilot study did not detect enhanced drought stress tolerance in the *AtPAO4*-deficient mutants (data not shown). While this question requires more rigorous testing, it is possible that the altered polyamine levels in the mutants may not be sufficient to induce drought resistance.

The other group of genes that showed up-regulation in the *AtPAO4*-deficient mutants consisted of genes involved in flavonoid and/or lignin biosynthesis (Table 1). Interestingly, most of the potential genes that function in the flavonoid biosynthetic pathway were up-regulated in the mutants (for enzymes in the pathway, refer to Besseau et al. 2007). One of these encodes phenylalanine ammonia-lyase 1 (PAL1), which is the first enzyme of the flavonoid and/or lignin biosynthetic pathway. There is a possibility that changes in the contents of certain metabolites in the mutants results in changes in gene expression. Further analyses will be required to understand the relationship between the up-regulation of expression of these genes and *AtPAO4* deficiency. As one possible candidate, we are interested in *p*-coumaric acid, which is an intermediate of the flavonoid and/or lignin biosynthesis pathway and is located downstream of PAL activity. *p*-Coumaric acid is a *cis*-type hydroxycinnamic acid that is known to be conjugated to polyamines. This results in the production of hydroxycinnamic acid amides (Flores and Martin-Tamguy 1991).

In conclusion, we found that peroxisomal polyamine catabolism affects spermidine/spermine levels, which suggests that the peroxisomal AtPAO4 enzyme plays a role in polyamine homeostasis, especially in the root. By analyzing peroxisomal polyamine catabolism further, we may be able to shed more light on the physiological roles of peroxisomal polyamine catabolism, about which very little is currently understood.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia served as the WT plant. All seeds were germinated on growth medium containing 2.3 mg ml^{-1} MS salt (Wako, Osaka, Japan), 2% (w/v) sucrose, 100 μg ml^{-1} myo-inositol, 1 μg ml^{-1} thiamine-HCl, 0.5 μg ml^{-1} pyridoxine, 0.5 μg ml^{-1} nicotinic acid, 0.5 mg ml^{-1} MES-KOH (pH 5.7) and 0.8% (w/v) agar. Seedlings were harvested after being grown for 1 d in the dark. Cotyledons were harvested after being grown for 4 d in the dark and then for 1 d under illumination. Seedlings were also grown for 2 weeks to generate roots. Leaves, stems, flowers and siliques were harvested from 5-week-old adult plants grown on a 1 : 1 (v/v) mixture of perlite and vermiculite. All plants were grown under a 16 h light (100 μE m^{-2} s^{-1})/8 h dark cycle at 22°C.

Particle bombardment

To generate the *mRFP–PAO2*, *mRFP–PAO3* and *mRFP–PAO4* constructs, the full-length cDNAs of the *AtPAOs* were amplified from expressed sequence tag (EST) clones (RAFL05-17-I08, RAFL07-16-P17 and RAFL11-02-B10, respectively) obtained from the RIKEN BioResource Center (Seki et al. 1998, Seki et al. 2002). The amplified cDNA fragment was introduced into the Gateway entry vector pDONR221 (Invitrogen, Carlsbad, CA, USA) by a BP recombination reaction (Gateway technology, Invitrogen). By using an LR recombination reaction (Gateway technology, Invitrogen), the insert was transferred to the transient expression vector, pUGW54 which contains the *mRFP1* gene (kindly provided by Dr. Nakagawa, Shimane University). The *GFP–PTS1* construct has already been described previously (Mano et al. 1999, Mano et al. 2002). Gold particles of 1 μm diameter were coated with plasmid mixtures composed of equal amounts of *GFP–PTS1* and each of the *mRFP–PAO* constructs and then introduced into *Arabidopsis* root cells by the particle bombardment method described in the manufacturer's instructions (Helios Gene Gun, Bio-Rad, Tokyo, Japan). After overnight incubation, fluorescence in the root cells was observed by using an LSM510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Quantitative RT–PCR analysis

Total RNA was extracted from each organ by using the Concert^{TM} Plant RNA Reagent (Invitrogen) and then further purified by precipitation with LiCl. It was reverse-transcribed by using the SUPERSCRIPT^{TM} First-Strand Synthesis system for RT–PCR (Invitrogen) according to the protocols provided by the manufacturer. Quantitative RT–PCR was performed by using the SmartCycler Instrument (Cepheid, Foster City, CA, USA), and SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and TaKaRa Ex Taq^{TM} R-PCR Version for PCR (TAKARA SHUZO CO. LTD., Shiga, Japan) according to the manufacturer's protocols. Specific primer sets were designed for *AtPAO2* (forward primer, 5′-GGAATGCCGGAAGATCTTCC GTGATTGTGATCGG-3′, reverse primer, 5′-CGATTCCAACA CCGAGATTTGCATACTCCATGCAGC-3′) and *AtPAO3* (forward primer, 5′-GCTGCATGGAGTATGCAAATCTCGGTGTT GGAATCG-3′, reverse primer, 5′-AGAAAGCTGGGTTTTGCA GCTGCTTCATCG-3′). The primer set for *AtPAO4* has been described previously (Kamada et al. 2003). The quantitative RT–PCRs involved 40 cycles of two-step amplifications (95°C for 3 s and 68°C for 20 s) after pre-heating (95°C for 30 s). The amount of target mRNA in each organ was normalized by using actin mRNA.

Establishment of AtPAO4-deficient mutants

The *AtPAO4* knock-out mutant (*Atpao4T*, SALK_109229) was found in a collection of T-DNA-inserted transformants that was established by the Salk Institute Genome Analysis Laboratory (http://signal.salk.edu). The T-DNA insertion of *Atpao4T* was confirmed by PCR using genomic DNA and *AtPAO4* gene-specific primers (forward primer, 5′-AAAAAGCAGGCTCTATGGATA AGAAGAAGAATTCGT-3′ and reverse primer, 5′-AGAAAGCT GGGTTTCACATCCTGGAGATTTGGAG-3′) and T-DNA specific primers (LB-1, 5′-TGGTTCACGTAGTGGGCCATCG-3′ and RB-1, 5′-GCTGATAGTGACCTTAGGCG-3′).

To generate the *Atpao4i* RNAi mutant, we used the Ti vector pHellsgate8 (Wesley et al. 2001). A 229 bp cDNA fragment derived from the *AtPAO4* gene was amplified from the *AtPAO4* cDNA plasmid by PCR using a forward primer (5′-AAAAAGCAG GCTCT-TTCACAAGGCAACAGGC-3′) and a reverse primer (5′-CCGATCACAATCACGGAAGATCTTCCGGCATTCC-3′). These primers were designed so that the 229 bp cDNA fragment

does not perfectly match any other open reading frame in the *Arabidopsis* genome by >20 bp. The 229 bp fragment was subcloned into pDONR221 and then transferred into pHellsgate8 by using Gateway technology according to a protocol provided by the manufacturer (Invitrogen). The recombinant pHellsgate8 plasmid was introduced into *Arabidopsis* (Columbia) by vacuum infiltration using *Agrobacterium tumefaciens* (strain C58C1Rif^R) (Bechtold et al. 1993). Transformants were selected on growth medium containing 50 μg ml^{-1} kanamycin. T_2 progeny showing kanamycin resistance were used for further analyses.

Polyamine contents in plants

To extract polyamines, plants (100 mg) were homogenized in 1 ml of 5% (w/v) perchloric acid containing 1 nmol of hexamethylenediamine (NACALAI TESQUE, INC., Kyoto, Japan) as an internal standard. Subsequent extraction of polyamines and dansylation were performed as described by Imai et al. (2004). We analyzed a mixture of free and acid-soluble conjugated polyamine forms. A portion of the dansylated polyamines was injected onto an HPLC column (Symmetry C18 reverse phase column, particle size 3.5 μl; 2.1 × 100 mm; Waters, Milford, MA, USA) and analyzed by using a flow rate of 0.1 ml min^{-1} and a water to acetone solvent gradient that changed from 20% (v/v) acetone to 100% (v/v) acetone over 85 min. Polyamines were measured by a fluorescence detector set at excitation and emission wavelengths of 365 and 510 nm, respectively. The results were standardized by using equimolar mixtures of dansylated putrescine, spermidine, spermine, N^1-acetylspermine and hexamethylenediamine. We carried out a statistical analysis of the data, which was the Student's *t*-test or Welch's *t*-test depending on the homogeneity of the two data sets compared.

Expression of AtPAO4 in E. coli

Full-length *AtPAO4* cDNA was amplified from the EST clone obtained from the RIKEN BioResource Center (see above, Seki et al. 1998, Seki et al. 2002) by using the gene-specific primers AtPAO4F (5′-ACAAAACATATGGATAAGAAGAAG AATT-3′) and AtPAO4R (5′-AAGCAGGTCGACTCACATC CTGGAGATTTGG-3′). The fragment has *Nde*I and *Sal*I sites. The PCR product was subcloned into pCRII-Blunt-TOPO (Invitrogen), which was then digested and ligated into the *Nde*I and *Sal*I sites of the pCold vector (TAKARA SHUZO CO. LTD.) to yield a plasmid denoted as pCold-AtPAO4. *Escherichia coli* DH5α was transformed with this plasmid and then selected on Luria–Bertani (LB) agar plates containing 100 μg ml^{-1} ampicillin. The transformed *E. coli* was used to amplify the plasmid. To produce recombinant AtPAO4, *E. coli* BL21 (DE3) (Invitrogen) was transformed with pCold-AtPAO4.

Growth of transformed bacteria and purification of recombinant AtPAO4

The expression of recombinant AtPAO4 was induced in the pCold-AtPAO4 transformants according to the pCold vector manufacturer's instructions (TAKARA SHUZO CO. LTD.). After centrifuging the cells, the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0) containing protease inhibitor cocktail and 1 mg ml^{-1} lysozyme chloride, and then disrupted by sonication. After centrifugation at 30,000×*g* for 25 min at 4°C, the cleared supernatant was applied onto an Ni-NTA column equilibrated with lysis buffer at 4°C. After binding the proteins, the column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0). The recombinant AtPAO4 proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0). The purity of the protein preparation was assessed by SDS–PAGE using 12.5% Super-Sep^{TM} (Wako) according to the manufacturer's instructions.

Characterization of recombinant AtPAO4 protein

To determine the substrate specificity of AtPAO4 and its reaction product, purified recombinant AtPAO4 (10 μg) or the same volume of elution buffer as a control was incubated with 150 μM of each substrate, namely putrescine, spermidine, N^1-acetylspermine and spermine, in 100 mM Tris–HCl buffer (pH 8.0) at 30°C for 2 h. After adding an equal volume of 5% (w/v) perchloric acid containing 0.12 mM hexamethylenediamine as an internal standard, the aliquots were dansylated and analyzed by HPLC (see 'Polyamine contents in plants' above) with a water to acetone solvent gradient that changed from 60% (v/v) acetone to 100% (v/v) acetone over 35 min at a flow rate of 0.1 ml min^{-1}. A dansylated reference solution containing hexamethylenediamine, putrescine, spermidine, N^1-acetylspermine and spermine was also analyzed by HPLC to establish the retention times and signal intensities of each compound. To study the pH dependence of recombinant AtPAO4 activity, 0.3 μg of recombinant AtPAO4 and 300 μM spermine in 500 μl of reaction buffer (100 mM Tris–HCl buffer with pHs ranging from 7.0 to 9.0, or 100 mM sodium phosphate buffer with pHs ranging from 6.5 to 7.0 at 25°C) was used. The catalytic parameters (K_m and k_{cat}) for the oxidation of spermine by recombinant AtPAO4 were determined by following spectrophotometrically the formation of a pink adduct ($\varepsilon_{515} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$) arising from the horseradish peroxidase-catalyzed oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzesulfonic acid (Tavladoraki et al. 2006). For the K_m value, the measurements were performed at 25°C by adding 3 μg of recombinant AtPAO4 and 50–5,000 μM spermine as the substrate to 1 ml of 100 mM Tris–HCl buffer (pH 8.0) whose O_2 concentration was constant at the air-saturated level. The K_m value was calculated from a Hanes–Woolf plot. The k_{cat} value was calculated at a saturating concentration of the substrate (5 mM).

Microarray analysis

Microarray analysis was performed by using an Agilent Arabidopsis 3 Oligo Microarray (Agilent Technologies, Palo Alto, CA, USA) as recommended by the manufacturer's instructions. The total RNAs in the roots of plants grown for 2 weeks were extracted (see 'Quantitative RT–PCR analysis' above). Feature extraction software (Agilent Technologies) was used to locate and delineate every spot in the array and to integrate the intensity, filtering and normalization of each spot. Further data analysis was performed by using GeneSpring 7 (Agilent Technologies). The *Atpao4T* and *Atpao4i* strains were compared with the WT in the microarray experiments. Two microarray analyses were performed for each mutant. We only selected the genes that showed meaningful signal intensity in all four microarray data sets. The selected genes were then narrowed down to the genes that had a *P*-value of <0.01 in all four microarray data sets. Finally, we selected the genes whose ratio of induction in both of the *AtPAO4* mutants was >2.0 (up-regulated genes) or <0.5 (down-regulated genes).

Funding

Ministry of Education, Culture, Sports, Science and Technology of Japan Scientific Research on Priority Areas

(16085209 to M.N.); the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (20370024 to M.N.).

Acknowledgments

We thank Dr. Peter Waterhouse (CSIRO Plant Industry of Australia) for providing pHELLSGATE8, and Dr. Tsuyoshi Nakagawa (Shimane University) for providing pUGW54. We are also grateful to Mikiko Kojima and Nanae Ueda at the RIKEN Plant Science Center for their technical assistance and advice.

References

Alcázar, R., Cuevas, J.C., Patron, M., Altabella, T. and Tiburcio, A.F. (2006a) Abscisic acid modulates polyamine metabolism under water stress in *Arabidopsis thaliana*. *Physiol. Plant*. 128: 448–455.

Alcázar, R., Marco, F., Cuevas, J.C., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A.F. and Altabella, T. (2006b) Involvement of polyamines in plant response to abiotic stress. *Biotechnol. Lett*. 28: 1867–1876.

Angelini, R., Federico, R. and Bonfante, P. (1995) Maize polyamine oxidase: antibody production and ultra-structural localization. *Plant Physiol*. 145: 686–692.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) In planta *agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C.R. Acad. Sci*. 316: 1194–1199.

Beevers, H. (1979) Microbodies in higher plants. *Annu. Rev. Plant Physiol*. 30: 159–193.

Besseau, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B. and Legrand, M. (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell* 19: 148–162.

Bouchereau, A., Aziz, A., Larher, F. and Martin-Tanguy, J. (1999) Polyamines and environmental challenges: recent development. *Plant Sci*. 140: 103–125.

Capell, T., Bassie, L. and Christou, P. (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proc. Natl Acad. Sci. USA* 101: 9909–9914.

Cervelli, M., Cona, A., Angelini, R., Polticelli, F., Federico, R. and Mariottini, P. (2001) A barley polyamine oxidase isoform with distinct structural features and subcellular localization. *Eur. J. Biochem*. 268: 3816–3830.

Cervelli, M., Polticelli, F., Federico, R. and Mariottini, P. (2003) Heterologous expression and characterization of mouse spermine oxidase. *J. Biol. Chem*. 278: 5271–5276.

Cervelli, M., Tavladoraki, P., Agostino, S.D., Angelini, R., Federico, R. and Mariottini, P. (2000) Isolation and characterization of three polyamine oxidase genes from *Zea mays*. *Plant Physiol. Biochem*. 38: 667–677.

Cona, A., Rea, G., Angelini, R., Federico, R. and Tavladoraki, P. (2006) Function of amine oxidases in plant development and defence. *Trends Plant Sci*. 11: 80–88.

Federico, R. and Angelini, R. (1991) Polyamine catabolism in plants. *In* Biochemistry and Physiology of Polyamines in Plants. Edited by Slocum, R.D. and Flores, H.E. pp. 41–56. CRC Press, Boca Raton, Florida.

Flores, H.E. and Martin-Tamguy, J. (1991) Polyamines and plant secondary metabolites. *In* Biochemistry and Physiology of Polyamines in Plants. Edited by Slocum, R.D. and Flores, H.E. pp. 57–76. CRC Press, Boca Raton, Florida.

Galston, A.W. and Sawhney, R.K. (1990) Polyamines in plant physiology. *Plant Physiol*. 94: 406–410.

Hanzawa, Y., Imai, A., Michael, A.J., Komeda, Y. and Takahashi, T. (2002) Characterization of the spermidine synthase-related gene family in *Arabidopsis thaliana*. *FEBS Lett*. 527: 176–180.

Hanzawa, Y., Takahashi, T., Michael, A.J., Burtin, D., Long, D., Pineiro, M., Coupland, G. and Komeda, Y. (2000) *ACAULIS5*, an *Arabidopsis* gene required for stem elongation, encodes a spermine synthase. *EMBO J*. 19: 4248–4256.

Hayashi, M., Aoki, M., Kondo, M. and Nishimura, M. (1997) Changes in targeting efficiencies of proteins to plant microbodies caused by amino acid substitutions in the carboxy-terminal tripeptide. *Plant Cell Physiol*. 38: 759–768.

Huang, A.H.C., Trelease, R.N. and Moore, J.T.S. (1983) Plant Peroxisomes Academic Press, New York.

Igarashi, K. (2006) Physiological functions of polyamines and regulation of polyamine content in cells. *YAKUGAKU ZASSHI* 126: 455–471.

Imai, A., Matsuyama, T., Hanzawa, Y., Akiyama, T., Tamaoki, M. et al. (2004) Spermidine synthase genes are essential for survival of *Arabidopsis*. *Plant Physiol*. 135: 1565–1573.

Kamada, T., Nito, K., Hayashi, H., Mano, S., Hayashi, M. and Nishimura, M. (2003) Functional differentiation of peroxisomes revealed by expression profiles of peroxisomal genes in *Arabidopsis thaliana*. *Plant Cell Physiol*. 44: 1275–1289.

Kasukabe, Y., He, L., Nada, K., Misawa, S., Ihara, I. and Tachibana, S. (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol*. 45: 712–722.

Kumar, A., Taylor, M., Altabella, T. and Tiburcio, A.F. (1997) Recent advances in polyamine research. *Trends Plant Sci*. 2: 124–130.

Landry, J. and Sternglanz, R. (2003) Yeast *Fms1* is a FAD-utilizing polyamine oxidase. *Biochem. Biophys. Res. Commun*. 303: 771–776.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10: 1391–1406.

Mano, S., Hayashi, M. and Nishimura, M. (1999) Light regulates alternative splicing of hydroxypyruvate reductase in pumpkin. *Plant J*. 17: 309–320.

Mano, S., Nakamori, C., Hayashi, M., Kato, A., Kondo, M. and Nishimura, M. (2002) Distribution and characterization of peroxisomes in *Arabidopsis* by visualization with GFP: dynamic morphology and actin-dependent movement. *Plant Cell Physiol*. 43: 331–341.

Moschou, P.N., Sanmartin, M., Andriopoulou, A.H., Rojo, E., Sanchez-Serrano, J.J. and Roubelakis-Angelakis, K.A. (2008) Bridging the gap between plant and mammalian polyamine catabolism: a novel peroxisomal polyamine oxidase responsible for a full back-conversion pathway in *Arabidopsis thaliana*. *Plant Physiol*. DOI:10.1104/pp.108.123802. *In press*.

Nishikawa, M., Hagishita, T., Yurimoto, H., Kato, N., Sakai, Y. and Hatanaka, T. (2000) Primary structure and expression of peroxisomal acetylspermidine oxidase in the methylotrophic yeast *Candida boidinii*. *FEBS Lett*. 476: 150–154.

Panicot, M., Minguet, E.G., Ferrando, A., Alcázar, R., Blázquez, M.A., Carbonell, J., Altabella, T., Koncz, C. and Tiburcio, A.F. (2002) A polyamine metabolon involving aminopropyl transferase complexes in *Arabidopsis*. *Plant Cell* 14: 2539–2551.

Polticelli, F., Basran, J., Faso, C., Cona, A., Minervini, G., Angelini, R., Federico, R., Scrutton, N.S. and Tavladoraki, P. (2005) Lys300 plays a major role in the catalytic mechanism of maize polyamine oxidase. *Biochemistry* 44: 16108–16120.

Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell* 18: 1292–1309.

Seki, M., Carninci, P., Nishiyama, Y., Hayashizaki, Y. and Shinozaki, K. (1998) High-efficiency cloning of *Arabidopsis* full-length cDNA by biotinylated CAP trapper. *Plant J*. 15: 707–720.

Seki, M., Narusaka, M., Kamiya, A., Ishida, J., Satou, M. et al. (2002) Functional annotation of a full-length *Arabidopsis* cDNA collection. *Science* 296: 141–145.

Slocum, R.D. (1991) Tissue and subcellular localization of polyamines and enzymes of polyamine metabolism. *In* Biochemistry and Physiology of Polyamines in Plants. Edited by Slocum, R.D. and Flores, H.E. pp. 93–104. CRC Press, Boca Raton, Florida.

Tavladoraki, P., Rossi, M.N., Saccuti, G., Perez-Amador, M.A., Polticelli, F., Angelini, R. and Federico, R. (2006) Heterologous expression and biochemical characterization of a polyamine oxidase from *Arabidopsis thaliana* involved in polyamine back-conversion. *Plant Physiol*. 141: 1519–1532.

Tavladoraki, P., Schininà, M.E., Cecconi, F., Agostino, S.D., Manera, F., Rea, G., Mariottini, P., Federico, R. and Angelini, R. (1998) Maize polyamine oxidase: primary structure from protein and cDNA sequencing. *FEBS Lett*. 426: 62–66.

Vujcic, S., Diegelman, P., Bacchi, C.J., Kramer, D.L. and Porter, C.W. (2002) Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. *Biochem. J*. 367: 665–675.

Vujcic, S., Liang, P., Diegelman, P., Kramer, D.L. and Porter, C.W. (2003) Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. *Biochem. J*. 370: 19–28.

Walden, R., Cordeiro, A. and Tiburcio, A.F. (1997) Polyamines: small molecules triggering pathways in plant growth and development. *Plant Physiol*. 113: 1009–1013.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M., Rouse, D.T. et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J*. 27: 581–590.

Wu, T., Yankovskaya, V. and McIntire, W.S. (2003) Cloning, sequencing and heterologous expression of the murine peroxisomal flavoprotein, N^1-acetylated polyamine oxidase. *J. Biol. Chem*. 278: 20514–20525.

Yamaguchi, K., Takahashi, Y., Berberich, T., Imai, A., Takahashi, T., Michael, A.J. and Kusano, T. (2007) A protective role for the polyamine spermine against drought stress in *Arabidopsis*. *Biochem. Biophys. Res. Commun*. 352: 486–490.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low temperature, or high-salt stress. *Plant Cell* 6: 251–264.

(Received July 20, 2008; Accepted August 5, 2008)