Scuola doctorale in Biologia (XXV Ciclo)
Sezione "Scineze Biomoleculari e Cellulari"

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Coinvolgimento delle poliammino ossidasi di *Arabidopsis thaliana* nei processi di sviluppo e di difesa delle piante

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ABBREVIATIONS

ABA	Abscisic acid
ACL5	ACAULIS5
ADC	Arginine decarboxylase
ADH	Aldehyde dehydrogenase
Arg	Arginine
ATAO	*Arabidopsis thaliana* copper-containing amine oxidase
AtPAO	*Arabidopsis thaliana* polyamine oxidase
BAP	6-benzylaminopurin
BSAO	Bovine serum amine oxidase
Cad	Cadaverine
CMV	Cucumber mosaic virus
CoA	Coenzyme A
CuAO	Copper-containing amine oxidase
Dap	1,3-diaminopropane
GABA	γ-aminobutyric acid
HDL	Hydrolase
HR	Hypersensitive response
HvPAO	*Hordeum vulgare* polyamine oxidase
JA	Jasmonic acid
MdPAO	*Malus domestica* polyamine oxidase
NO	Nitric oxide
Nor-Spd	Norspermidine
Nor-Spm	Norspermine
NtPAO	*Nicotiana tabacum* polyamine oxidase
ODC	Ornithine decarboxylase
Orn	Ornithine
OsPAO	*Oryza sativa* polyamine oxidase
PAO	Polyamine oxidase
PCD	Programmed cell death
Pro	Proline
Put	Putrescine
SAM	*S*-adenosylmethionine
SAMDC	*S*-adenosylmethionine decarboxylase
SMO	Spermine oxidase
Spd	Spermidine
SPDS	Spermidine synthase
Spm	Spermine
SPMS	Spermine synthase
SSAT	Spd/Spm N^1-acetyltransferase
Ther-Spm	Thermospermine
TPQ	2,4,5-trihydroxyphenylalanine quinone cofactor
TYMV	Turnip yellow mosaic virus
ZmPAO	*Zea mais* polyamine oxidase

ABSTRACT

The polyamines (PAs) putrescine (Put), spermidine (Spd), and spermine (Spm) are small aliphatic polycations found in all living cells. They are involved in several cellular processes and play important roles in morphogenesis, growth, differentiation and senescence. In plants, they are also implicated in defence responses to various biotic and abiotic stresses. PA homeostasis is strictly regulated through anabolic and catabolic processes, but also through conjugation, transport and compartimentalization.

Polyamine oxidases (PAOs) are FAD-dependent enzymes involved in PA catabolism. PAOs from monocotyledonous plants, such as the apoplastic maize PAO (ZmPAO), oxidize spermine (Spm) and spermidine (Spd) to produce 1,3-diaminopropane, H_2O_2 and an aminoaldehyde and are considered involved in a terminal catabolic pathway of PAs. Conversely, animal PAOs and spermine oxidases (SMOs) oxidize Spd, Spm and/or their acetyl-derivatives to produce Put and Spd, respectively, in addition to H_2O_2 and 3-aminopropanal and are thus considered involved in a PA back-conversion pathway.

In *Arabidopsis thaliana*, five *PAO* genes (*AtPAO1-5*) are present with a varying amino acid sequence homology to ZmPAO and subcellular localization (putative cytosolic for AtPAO1 and AtPAO5 and peroxisomal for AtPAO2, AtPAO3 and AtPAO4). Furthermore, following heterologous expression in bacteria it was shown that AtPAO1 oxidizes Spm but not Spd, whereas AtPAO2, AtPAO3 and AtPAO4 oxidize both Spm and Spd. Conversely, AtPAO5 substrate specificity has not been determined so far since production of the recombinant protein in various heterologous systems has not been successful. The four characterized AtPAOs are also active towards the uncommon PAs thermospermine (Ther-Spm) and norspermine (Nor-Spm). In particular, AtPAO1 shows a higher catalytic activity towards Ther-Spm and NorSpm than towards Spm, which suggests that these two uncommon PAs may be the physiological substrates of this enzyme. This is of particular interest because it has been recently shown the existence in *Arabidopsis* of an enzyme able to synthesize Ther-Spm and a loss-of-function mutant for this gene shows a severely dwarfed phenotype. Another important characteristic of the four *Arabidopsis* PAOs is their involvement in a PA back-conversion pathway, producing Spd from Spm and Put from Spd, similarly to the animal PAOs / SMOs and contrary to ZmPAO.

Studies on the tissue- and organ-specific expression pattern of *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO5* using *AtPAO::GFP-GUS* transgenic Arabidopsis plants showed some distinct expression patterns for each one of

the four *AtPAO*s, such as in the transition region between cell division and elongation zones of roots and anther tapetum for *AtPAO1*, in columella, stipules and pollen for *AtPAO2*, in hypocotyls and roots, stipules, columella, trichomes, guard cells and pollen for *AtPAO3*, and in the vascular system of roots and hypocotyls for *AtPAO5*. These studies also evidenced increased expression of *AtPAO1* in roots and of *AtPAO2* in guard cells following treatment with the stress-related plant hormone abscisic acid (ABA).

In the present work, the study on the tissue- and organ-specific expression pattern of the five *AtPAOs* was completed analysing *AtPAO4* promoter activity. In particular, histochemical GUS staining of *AtPAO4::GFP-GUS* transgenic Arabidopsis plants evidenced that *AtPAO4* is expressed in the roots (from the meristematic/elongation transition region up to the hypocotyl–root junction site), in the guard cells, in the base of very young and completely closed flower buds, in anther tapetum and in mature pollen grains. These data together with data from promoter analysis of the other four AtPAOs indicate distinct physiological roles for the various AtPAOs during seedling growth and flower development and suggest functional diversity inside the AtPAO gene family.

To determine the physiological roles of the various *AtPAOs*, loss-of-function T-DNA insertional mutants (*atpao1*, *atpao2*, *atpao3*, *atpao4* and *atpao5*) have been previously obtained from the NASC collection of Arabidopsis seeds and homozygous mutant lines have been selected. In the present study, double (*atpao2/atpao4* and *atpao3/atpao4*, *atpao3/atpao2*) and triple (*atpao2/atpao4/atpao3)* mutants for the peroxisomal *AtPAOs* as well as the double *atpao1/atpao5* mutant for the two *AtPAOs* with predicted cytosolic localization were also obtained through sexual crossings.

The *atpao1* single mutant was analyzed for the levels of the common PAs Put, Spd and Spm as well as of the uncommon PA Ther-Spm, evidencing no statistically significant variation comparing to the wild-type plants. This may be due either to gene redundancy or to the activation of homeostatic mechanisms and may exclude the possibility that Ther-Spm is the physiological substrate of AtPAO1. The *atpao1* single mutant was also analyzed for germination and growth rate under physiological and stress conditions, but also in these cases no variation was observed as compared to the wild-type plants. Similar studies on the *atpao1*/*atpao5* double mutant are in progress.

Analysis of PA levels in the single and triple mutants for the three peroxisomal *AtPAOs* showed some alterations in the *atpao2/atpao4/atpao3* triple mutant. Furthermore, since all three *AtPAO2*, *AtPAO3* and *AtPAO4* are highly expressed in the guard cells, specialized cells surrounding stomata pores, stomata movements were evaluated evidencing reduced

ABA- and PA-mediated stomata closure in the corresponding mutant plants as compared to wild-type plants. Furthermore, the *atpao2/atpao4/atpao3* triple mutant appeared more tolerant to dehydration and ABA treatment. Altogether, these data suggest the involvement of the three peroxisomal AtPAOs in the ABA-mediated signaling network. On the other hand, germination and growth rate of the *atpao2/atpao4/atpao3* triple mutant in the absence of sucrose was shown to be delayed comparing to the wild-type plants. The underlying mechanisms in these phenotypical alterations in the *atpao2/atpao4/atpao3* triple mutant are currently under investigation.

In the present study, it was also possible to express AtPAO5 in *35S::AtPAO5-6His* transgenic Arabidopsis plants, to partially purify the corresponding recombinant protein and to determine substrate specificity and reaction products. In particular, it was shown that AtPAO5 has indeed PAO activity, catalyzing the oxidation of Spm, N^1-acetyl-Spm, Ther-Spm and Nor-Spm through a PA back-conversion pathway. Furthermore, confocal analysis of *35S::GFP-AtPAO5* and 35S::AtPAO5-GFP transgenic Arabidopsis plants indicated that AtPAO5 is a cytoplasmic protein undergoing proteasomal control. It was also shown cytokinin-inducible expression of *AtPAO5* as well as AtPAO5 involvement in the control of xylogenesis by cytokinins. Experiments are in progress to determine the physiological significance of AtPAO5 regulation by the proteasomal pathway as well as to unravel the mechanisms by which AtPAO5 is involved in the cytokinin-mediated pathways.

This study represents the starting point to understand the distinct physiological roles of the different PA catabolic pathways in plants during development and defense responses which may permit the application of biotechnological strategies to transfer increased yield and stress-tolerance traits to crops of agronomical relevance.

INTRODUCTION:

Polyamines: General characteristics

Polyamines (PAs) can be considered as one of the oldest group of substances known in biochemistry (Cona *et al*., 2006). They are small aliphatic polycation molecules having variable hydrocarbon chains and two or more primary and secondary amino groups. The di-amine putrescine (Put), the tri-amine spermidine (Spd) and the tetra-amine spermine (Spm) (Fig. 1) are the most common PAs in eukaryotes (Galston and Sawhney, 1990). In addition, other PAs, such as 1,3-diaminopropane (Dap), cadaverine (Cad), thermospermine (Ther-Spm), norspermidine (Nor-Spd) and norspermine (Nor-Spm) (Fig. 1) are found in many organisms as minor components of the cellular PA pool and are referred to as uncommon PAs (Tavladoraki *et al*., 2011).

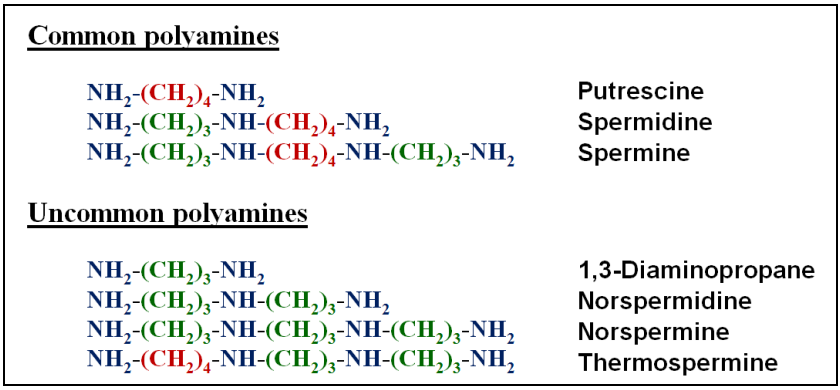

Fig. 1. Structures of common and uncommon PAs

PAs may be present in a free soluble form, but also bound to macromolecules such as proteins and nucleic acids or conjugated to phenolic compounds (Bakhanashvili *et al*., 2005; Groppa and Benavides, 2008). In particular, in plants, PAs can be further conjugated to hydroxycinnamic acid forming hydroxycinnamic acid amides (HCAAs; Bagni and Tassoni, 2001). Indeed, coumaroyl-Put, feruloyl-Put, coumaroylagmatine, dicoumaroyl-Spd, diferuloyl-Spd, and diferuloyl-Spm are present in a wide range of plant species (Martin-Tanguy, 1997; Grienenberger *et al*., 2009; Luo *et al*., 2009). Although these compounds were discovered many years ago, their physiological role(s) remain largely

unknown. HCAAs have been associated with a wide range of growth and developmental processes, including cell division, flowering, cell-wall cross-linking, as well as responses to environmental challenge (Bouchereau *et al*., 1999; Luo *et al*., 2009; Bassard *et al*., 2010). Furthermore, data suggesting turnover and translocation of PA conjugates as well as interconversion between free and conjugated precursors have been reported (Bassard *et al*., 2010).

The overall intracellular concentration of PAs is in the range of several hundred micromolars to a few millimolars and is tightly regulated, as higher levels of PAs are toxic to cells and lead to cell death. PA homeostasis in plants correlates with several important physiological functions, including the control of the N:C balance (Mattoo *et al*., 2006; Moschou *et al*., 2012), stress responses (Alcázar *et al*., 2011), xylem differentiation (Muñiz *et al*., 2008; Tisi *et al*., 2011), pollen tube growth (Wu *et al*., 2010), membrane fluidity, and protein regulation(Baron and Stasolla, 2008; Takahashi and Kakehi, 2010). The intracellular pool of free PAs depends not only on its synthesis, but also on conjugation, transport, degradation and back-conversion (Tiburcio *et al*., 1997; Angelini *et al*., 2010; Moschou *et al*., 2012).

Polyamine biosynthesis

The pathways of PA biosynthesis have been established for many organisms (Bagni and Tassoni, 2001; Wallace *et al*., 2003) . The general mechanism of biosynthesis is conserved from bacteria to animals and plants (Tabor and Tabor, 1984) and begins from the synthesis of the precursor Put, followed by successive additions of aminopropyl groups to produce Spd and Spm (Fig. 2). It has been suggested that plants have acquired a part of the PA biosynthetic pathway from an ancestral cyanobacterial precursor of the chloroplast (Illingworth *et al*., 2003). Therefore, it can be assumed that this is an ancient metabolic route, which is also present in all organisms (Minguet *et al*., 2008).

Put is formed directly by the decarboxylation of ornithine (Orn), via ornithine decarboxylase (ODC; EC 4.1.1.17), or indirectly from arginine (Arg) by arginine decarboxylase (ADC; EC 4.1.1.19) via agmatine (Agm) (Tabor and Tabor, 1985). The biosynthesis of Put from Arg requires the activity of three consecutive enzymes: ADC, agmatine iminohydrolase (AIH; EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.5; Alcázar *et al*., 2011).

In *Arabidopsis thaliana*, Put is produced exclusively through the ADC pathway, since no ODC gene has been identified in the sequenced genome

of this plant and the corresponding enzyme activity has not been detected (Hanfrey *et al*., 2001; Alcázar *et al*., 2010b). In particular, two different genes encoding ADC (*ADC1* and *ADC2*) have been described (Soyka and Heyer, 1999). Although *ADC1* and *ADC2* show 80% homology in amino acid sequence to each other, they exhibit a different expression pattern: *ADC1* is expressed in all tissues, whereas *ADC2* is mainly expressed in cauline leaves and siliques, and is induced by different abiotic stresses (Soyka and Heyer, 1999; Perez-amador *et al*., 2002; Urano *et al*., 2003). In animals, Put is mainly synthesized through the ODC pathway, the ADC pathway being just a minor pathway in specific mammalian tissues (Gilad *et al*., 1996) (Fig. 2). In bacteria, in addition to ADC and ODC, another enzyme is present involved in Put biosynthesis, agmatinase, which directly produces Put from agmatine. Furthermore, in bacteria Spm is not synthesized, since no *SPMS* gene is present (Wortham *et al*., 2007).

Put is converted into Spd and Spm through two sequential reactions catalyzed by two closely related but distinct enzymes, the Spd synthase (SPDS; EC 2.5.1.16) and Spm synthase (SPMS; EC 2.5.1.22), respectively, which add aminopropyl groups. These aminopropyl groups are donated by decarboxylated S-adenosylmethionine (dcSAM), which is formed by decarboxylation of S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC; EC 4.1.50) (Fig. 2; Alcázar *et al*., 2011). Spd can be also converted to Ther-Spm by Ther-Spm synthase (TSPMS or ACL5) which add an aminopropyl group at the N^1-(aminopropyl) end of Spd, differently from SPMS which adds the aminopropyl group at the N^8-(aminobutyl) end of Spd. In Arabidopsis, Spd synthase is encoded by two genes (*SPDS1* and *SPDS2*) whereas SPMS and TSPMS are encoded by single genes (Hanzawa *et al*., 2002; Knott *et al*., 2007). SPDS1 shows high sequence similarity to SPDS2 (82.7% amino acid identity), whereas SPMS shows only 56% identity with both SPDS1 and SPDS2, respectively. Exon structure is conserved between SPDS1, SPDS2, and SPMS whereas *TSPMS* has a completely different genomic organization. SPMS interacts with SPDS1 and SPDS2, to form ‘‘metabolon’’ complexes, while TSPMS does not interact with SPDS (Panicot *et al*., 2002).

The Arabidopsis genome carries at least four genes coding for SAMDCs (*SAMDC1-4*) (Urano *et al*., 2004). They have an unusually long 5’-UTR where two uORFs are well conserved (Franceschetti *et al*., 2001) which control the PA levels. The first uORFs called tiny uORFs which are distal to the 5’ end are 3–4 codons long, while the second one termed small uORFs consists of 50–54 codons. The small uORF-encoded peptide is responsible for translational repression of the main ORF under conditions of excess PA concentration; while the tiny uORF is required for induced translation of the

main ORF during conditions of low PA concentration (Hanfrey *et al*., 2002; 2005). In Arabidopsis, the sequences of the uORFs of SAMDC1 and SAMDC2 are highly conserved, while the uORFs of SAMDC3 and SAMDC4 are not complete which affects their expression (Urano *et al*., 2003).

SAMDC has an important role in the regulation of PA homeostasis in all organisms. Particularly in plants, it is considered to be the rate-limiting enzyme for the synthesis of Spd and Spm(Kusano *et al*., 2008). The activity of SAMDC is positively regulated by Put and negatively regulated by Spd and Spm, thus making the cellular levels of dcSAM responsive to the demands of the PA biosynthetic pathway (Pegg, 1986; Kameji and Pegg, 1987; Xiong *et al*., 1997).

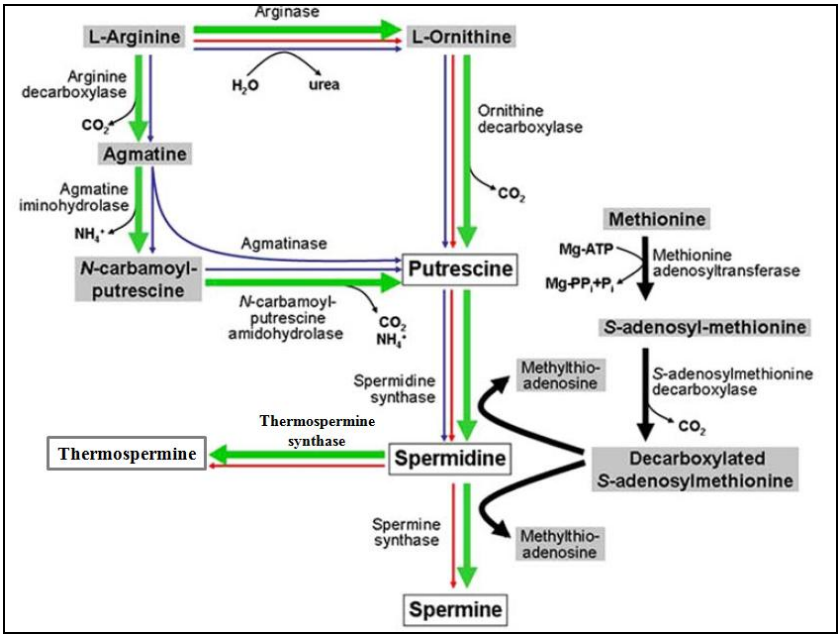

Fig. 2. PA biosynthetic pathways. Plant pathway is indicated by green bold arrows. Blue and red arrows indicate bacterial and animal pathways, respectively. Figure Modified from Kusano *et al*., 2008.

Polyamine catabolism

Two classes of amine oxidases are implicated in PA catabolism, the copper-containing amine oxidases (CuAOs) and the FAD-dependent amine oxidases (PAOs) (Tavladoraki *et al*., 2011).

Copper-containing amine oxidases

CuAOs are homodimeric enzymes; each subunit of 70-90 kD contains a copper ion and a 2,4,5-trihydroxyphenylalanine quinone cofactor generated by a post-translational autocatalytic modification (Medda *et al*., 1997; Angelini *et al*., 2010). They catalyze the oxidation of Put and Cad at their primary amino groups. The reaction products from Put are H_2O_2, NH_4^+ and 4-aminobutanal (Fig. 3). The latter spontaneously cyclises to generate Δ^1-pyrroline and can be further converted to γ-aminobutyric acid (GABA) by an aldehyde dehydrogenase. GABA is subsequently transaminated and oxidized to succinic acid, which is incorporated into the Kreb's cycle, ensuring the recycling of carbon and nitrogen from Put (Tavladoraki *et al*., 2011; Moschou *et al*., 2012) CuAOs are also able to oxidize Spd and Spm, although with a lower affinity than Put, producing 4-aza-8-amino-octan-1-al and 4,9-diaza-dodecan-1,12 dialdehyde, respectively, in addition to H_2O_2, and NH_4^+. In particular, the activities of pea CuAO (PsAO;Tipping and McPherson, 1995) with Put, Spd and Spm are at a ratio of 100:35:0.3, while those of the lentil enzyme at a ratio of 100:42:20 (Sebela *et al*., 2001). Only the animal serum CuAOs, such as bovine serum amine oxidase (BSAO), oxidize preferentially Spd and Spm. The two aminoladehydes produced from CuAO-mediated Spd and Spm oxidation may undergo spontaneous degradation, when not previously further oxidized by aldehyde dehydrogenases, forming Put and Spd, respectively, and the highly toxic aldehyde acrolein (Fig. 3; (Tavladoraki *et al*., 2012).

Plant CuAOs occur at high levels in the extracellular space of several *Fabaceae* species (Federico and Angelini, 1991; Cona *et al*., 2006), reaching levels as high as 2 to 10 U/gfw in etiolated young seedlings (Rea *et al*., 1998). These CuAOs have a quite high catalytic activity, the k_{cat} with the best substrate being about 260 s^{-1}, conversely to the animal CuAOs which have a lower catalytic activity; for example the k_{cat} of the bovine serum CuAOs is 2 s^{-1} (Pietrangeli *et al*., 2007). In *A. thaliana*, seven *CuAOs* have been identified by database search. Only one of them (At4g14940; *AtCuAO1*) was partially characterized and is active with Put and Spd (Møller and McPherson, 1998). *In silico* analysis indicate that all

Arabidopsis CuAOs are targeted to the secretory pathway but their final subcellular sorting has still to be determined.

In plants, a class of CuAOs is also present which preferentially oxidize *N*-methyl-Put although they oxidize also Put and Cad. In particular, in *Nicotiana tabacum*, two *N*-methyl-Put oxidase genes (*NtMPO1* and *NtMPO2*) are present (Heim *et al*., 2007; Katoh *et al*., 2007) which are specifically expressed in the roots and are up-regulated by the plant hormone jasmonate (JA). The two *NtMPO*s share essential structural motifs with the other CuAOs and have high sequence homology to *AtCuAO1* and to *PsAO* (Heim *et al*., 2007; Katoh *et al*., 2007). A putative *N*-methyl-Put oxidase is also present in Arabidopsis (At2g42490; *AtMPO*), which has a high sequence homology with *NtMPO1* and NtMPO2. Phylogenetic tree shows that *NtMPO1*, *NtMPO2* and *AtMPO* form a distinct clade and are separated from PsAO, CuAO1 and the other Arabidopsis CuAO-like proteins (Katoh *et al*., 2007; Moschou *et al*., 2012) . In contrast to the CuAOs, NtMPO1, NtMPO2 and *AtMPO* are predicted to be localized to peroxisomes (Heim *et al*., 2007). The oxidation of *N*-methyl-Put by this class of CuAOs produces 4-methylaminobutanal, which spontaneously cyclises to give rise to the *N*-methylpyrrolinium cation, a precursor of the pyridine and tropane alkaloids, thus driving the flow of N away from PA biosynthesis towards alkaloids (Fig. 3).

The expression of some plant *CuAO*s has been shown to be modulated during development, pathogen attack, wound healing and salt stress. Plant hormones, for example JA and abscisic acid (ABA), were also shown to regulate expression of plant *CuAO*s (Møller and McPherson, 1998; Cona *et al*., 2006; Quinet *et al*., 2010; Toumi *et al*., 2010). Moreover, CuAO activity is higher, and increases to a greater extent upon infection, in chickpea cultivars resistant to the fungus *Ascochyta rabiei* compared with the susceptible ones was shown to be strongly impaired by in vivo CuAO inhibition (Angelini *et al*., 1993). Moreover, infection of Arabidopsis plants with nematodes also induces differential expression of *AtCuAO1* (Møller and McPherson, 1998).

Polyamine oxidases

PAOs are monomers of 50-60 kDa bearing a non-covalently bound FAD molecule (Tavladoraki *et al*., 1998; Binda *et al*., 1999). They catalyze the oxidation of Spm, Spd and/or their acetylated derivatives at the secondary amino groups. PAO reaction products depend on the mode of substrate oxidation, which in turn depends on the mode of substrate binding inside the catalytic site resulting in the oxidation of a different carbon atom. On

the basis of the reaction products, PAOs can be classified in two families; those which terminally oxidize PAs and those catalyzing PA back-conversion. PAOs of the first family have been until now detected only in plants and bacteria. From the plant species, they are present at high quantities in particular tissues of plants belonging to *Gramineae*, such as maize (*Zea mays*), barley (*Hordeum vulgare*), oat (*Avena sativa*), wheat (*Triticum aestivum*) and rye (*Secale cereale*) (Federico *et al*., 1989; Federico and Angelini, 1991; Sebela *et al*., 2001; Stránská *et al*., 2007; Maiale *et al*., 2008; Angelini *et al*., 2010). In particular, in maize three genes (*ZmPAO1*, *ZmPAO2* and *ZmPAO3*) have been identified encoding identical proteins (ZmPAO; (Cervelli *et al*., 2000), while in barley two genes (*HvPAO1* and *HvPAO2*) have been cloned (Cervelli *et al*., 2001). The PAOs of this family oxidize the carbon at the *endo*-side of the N^4 of Spd and Spm, producing 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, in addition to 1,3-diaminopropane (Dap) and H_2O_2 (Fig. 3). The aminoaldehydes produced in the reaction spontaneously cyclise to Δ^1-pyrroline and 1,5-diazabicyclononane, respectively (Federico and Angelini, 1991; Sebela *et al*., 2001), while Dap can be converted to β-alanine by a Dap-aminotransferase, reported in bacteria but not yet in plants, and an aminoaldehyde dehydrogenase (AMADH; Fig. 3). β-Alanine in turn might be metabolized to the osmoprotectant β-alanine betaine by β-alanine *N*-methyltransferase (Fig. 3). Dap is also a precursor of the uncommon polyamines Nor-Spd and Nor-Spm (Fig. 3) which in plants are associated with stress tolerance (Cona *et al*., 2006). These PAOs have a cleavable *N*-terminal signal peptide which targets them to the apoplast. Only the barley PAO isoform HvPAO2, which has also a signal peptide for secretion, is localized to the vacuoles (Cervelli *et al*., 2004). The pH optima for the oxidation of the substrates vary among different species, but for most of the enzymes and for both substrates they are in the range of 5.5 to 6.8 (Federico and Angelini, 1991), which probably reflects their extracellular localization. Only HvPAO2, shows two different pH optima for the two substrates (5.5 for Spm and 8.0 for Spd; Cervelli *et al*., 2001). The so far characterized PAOs from the *Gramineae* are almost equally active with Spd and Spm. Furthermore, these PAOs are characterized by high specific activity (k_{cat} in the range of 50-100 s^{-1}) and affinity (K_m in the range of 1-10 μM) for the two PAs, which may correlate to the low PA levels in the cellular compartment of enzyme accumulation. ZmPAO also cleaves N^1-acetylSpd, N^1-acetylSpm and N^8-acetylSpd at the same C atom site and at the same optimal pH as it does with non-acetylated Spd and Spm (Federico *et al*., 1996). However, the enzyme is quickly inactivated during the reaction (Federico *et al*., 1996). ZmPAO is additionally active with Nor-

Spm and Ther-Spm, though with k_{cat} values 10- to 30-fold lower than those towards Spm (Tavladoraki *et al*., 2006; Fincato *et al*., 2011). It has been demonstrated that Oat PAO is also active with Nor-Spd (Maiale *et al.*, 2008).

The PAOs catalyzing back-conversion of PAs have been so far detected in animals, yeasts and in plants. Animal PAOs and yeast *Saccharomyces cerevisiae* Spm-oxidase (Fms1) oxidize N^1-acetyl-Spm, N^1-acetyl-Spd, and N^1, N^{12}-bis-acetyl-Spm at the carbon on the *exo*-side of N^4-nitrogen to produce Spd, Put, and N^1-acetyl-Spd, respectively, in addition to 3-acetamidopropanal and H_2O_2 with a pH optimal of around 8.0 (Landry and Sternglanz, 2003; Vujcic *et al*., 2003; Wu *et al*., 2003; Cona *et al*., 2006). In this catabolic pathway, PA acetylation is catalysed by the tightly regulated Spd/Spm N^1-acetyltransferase (SSAT), which is the rate-limiting enzyme of this pathway (Wallace *et al*., 2003). Similarly, animal Spm oxidases (SMOs) and Fms1 oxidize Spm at the carbon on the exo-side of N^4-nitrogen to produce Spd, 3-aminopropanal and H_2O_2 with a pH optimal of around 8.0 (Wang *et al*., 2001; Vujcic *et al*., 2002; Cervelli *et al*., 2003; Landry and Sternglanz, 2003). 3-Aminopropanal and 3-acetamidopropanal can be further metabolized by an aminoaldehyde dehydrogenase (AMADH) to form β-alanine and *N*-acetyl-β-alanine, respectively which may be converted to the toxic acrolein (Fig. 3). The best so far characterized plant PAOs involved in PA back-conversion are those of Arabidopsis.

Arabidopsis polyamine oxidases

In *A. thaliana*, five PAO genes are present: *AtPAO1* (*At5g13700*), *AtPAO2* (*At2g43020*), *AtPAO3* (*At3g59050*), *AtPAO4* (*At1g65840*) and *AtPAO5* (*At4g29720*). *AtPAO1*, which has a predicted cytosolic localization, shares with the extracellular *ZmPAO* a 45% homology at the amino acid level and a similar intron/exon organization (Tavladoraki *et al*., 2006). AtPAO1 oxidizes Spm but not Spd (Table 1), differently from ZmPAO but similarly to the animal SMO. It oxidises also the uncommon PAs Ther-Spm and Nor-Spm (Tavladoraki *et al*., 2006) with high efficiency which suggests that these two PAs may be its physiological substrates. In contrast, AtPAO1 has a low catalytic activity with N^1-acetyl-Spm (Table 1). *AtPAO2*, *AtPAO3*, and *AtPAO4* display low sequence homology (23%-24% homology) with *ZmPAO* and the other two *AtPAOs*, but a high sequence homology to each other (85% between *AtPAO2* and *AtPAO3*, 58% between *AtPAO2* and *AtPAO4*, 50% between *AtPAO3* and *AtPAO4*). Furthermore, *AtPAO2*, *AtPAO3* and *AtPAO4* have a very similar intron/exon organization Indeed, these genes bear eight introns at highly conserved positions (Fig. 8).

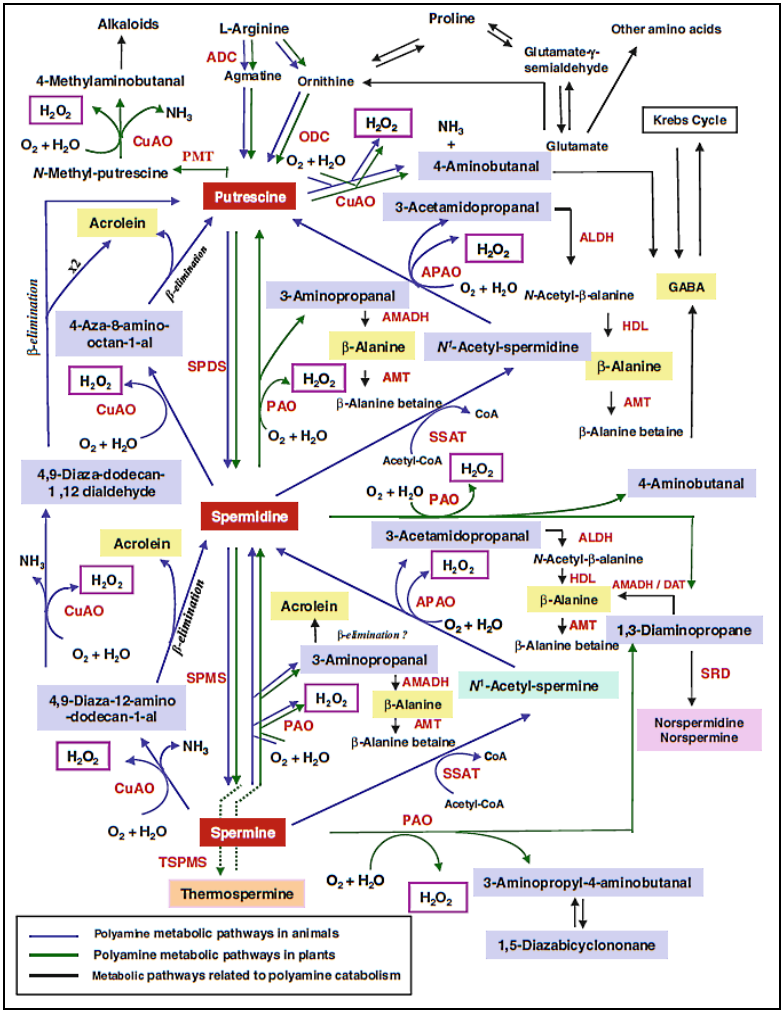

Fig. 3. Schematic representation of PA catabolic pathways and related matabolites in animals and plants. Green arrows indicate PA catabolic pathways in plants, blue arrows indicate PA catabolic pathways in animals and black arrows indicate metabolic pathways related to PA catabolism. ALDH aldehyde dehydrogenase, ADC arginine decarboxylase, AMADH aminoaldehyde dehydrogenase, AMT β-alanine *N*-methyltransferase, DAT 1,3-diaminopropane-aminotransferase, GABA, HDL *N*-acetyl-β-alanine amidohydrolase, ODC ornithine decarboxylase, PMT putrescine *N*-methyltransferase, SSAT spermidine-spermine N^1-acetyltransferase, SPDS spermidine synthase, SPMS spermine synthase, SRD Schiff-base reductase/decarboxylase, TSPMS thermospermine synthase. From Tavladoraki *et al.*, 2012.

This, together with the elevated sequence homology to each other, suggest that these three Arabidopsis genes are recent derivatives from a common ancestor, thus forming a distinct PAO subfamily (*AtPAO2–AtPAO4* subfamily). Interestingly, all the three members of this subfamily have a peroxisomal localization(Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008a). AtPAO2, AtPAO3 and AtPAO4 oxidize both Spd and Spm (Takahashi *et al*., 2010; Fincato *et al*., 2011). In particular, while AtPAO2 is equally active with Spm and Spd, AtPAO3 is 2-fold more active with Spd than with Spm and AtPAO4 is 10-fold more active with Spm than with Spd. AtPAO2, AtPAO3 and AtPAO oxidize also Nor-Spm and Ther-Spm though less efficienly than Spd and/or Spm (Table 1). Furthermore, the catalytic activity of all three peroxisomal AtPAOs towards N^1-acetyl-Spm is very low (Table 1). The fifth Arabidopsis PAO gene (*AtPAO5*) has low sequence homology with the other four AtPAOs and a predicted cytosolic localization. However, information about its catalytic properties has not been still obtained.

AtPAO1, AtPAO2, AtPAO3 and AtPAO4 have different optimum pH (7.0–8.0) than the extracellular ZmPAO (optimum pH of 6.0; Polticelli *et al*., 2005). In particular, the optimum pH for AtPAO1 catalytic activity is 8, for AtPAO2 and AtPAO3 catalytic activity 7.5 and for AtPAO4 8.0 towards Spd, and is 7.0 towards Spm (Tavladoraki *et al*., 2006; Moschou *et al*., 2008b; Fincato *et al*., 2011). These differences in optimum pH among the various enzymes may reflect differences in subcellular localization and/or physiological role(s).

	AtPAO1	AtPAO2	AtPAO3	AtPAO4	ZmPAO	SMO	APAO
	kcat (s-1)						
Spd	-	4.6 ± 1.5	3.4 ± 1.4	0.1 ± 0.03	50.2 ± 6.3	-	-
Spm	2.7 ± 0.3	4.2 ± 1.2	1.7 ± 0.5	4.6 ± 1.0	32.9 ± 1.1	3.9 ± 0.5	0.175 ± 0.005
Nor-Spm	6.9 ± 1.3	2.9 ± 0.8	1.1 ± 0.2	0.45 ± 0.1	5.5 ± 0.1	-	ND
Ther-Spm	5.7 ± 1.1	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.04	9.8 ± 1.0	-	ND
N^1-AcSpm	0.2 ± 0.4	0.8 ± 0.2	0.02	0.01 ± 0.004	ND	0.1 ± 0.01	8.0 ± 0.8

Table 1. Catalytic activity of recombinant AtPAOs, ZmPAO SMO and APAO. Data were taken from Cervelli *et al*., 2003; Wu *et al*., 2003; Polticelli *et al*., 2005 and from Fincato *et al*., 2011. ND: not determined.

Analysis of the AtPAO reaction products evidenced that all characterized *A. thaliana* PAOs are involved in PA back-conversion (Tavladoraki *et al.*, 2006; Kamada-Nobusada *et al.*, 2008; Fincato *et al.*, 2011), similarly to the animal PAOs/SMOs and in contrast to the extracellular PAOs from monocotyledonous plants characterized thus far, which are involved in a terminal PA catabolic pathway (Fig. 3). In this regard, the information so far available allows to propose the terminal catabolic pathway of PAs as specifically active in the extracellular compartments, while the PA back-conversion pathway as mostly intracellular (Fincato *et al.*, 2011).

Analysis of promoter activity for *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO5* using *AtPAO::β-glucuronidase (GUS)* Arabidopsis transgenic plants evidenced distinct expression patterns during seedling and flower development (Fincato *et al.*, 2012). In particular, *AtPAO1* is highly expressed in the transition region between the meristematic and the elongation zone of the root (Fig. 4a, b), while *AtPAO2* and *AtPAO3* are expressed in the root cap (Fig. 4h, n). Interestingly, at the root cap differences exist between *AtPAO2* and *AtPAO3*, although they belong to the same PAO subfamily (Fincato *et al.*, 2011). Indeed, while *AtPAO2* is expressed only near the quiescent center and columella initials, *AtPAO3* is expressed in lateral root cap and in the whole columella (Fig. 4h, n). Furthermore, while all four genes are expressed in the maturation zone of the roots, *AtPAO5* is specifically expressed in the vascular system of this zone (Fig. 4t, u), the other three being present both in vascular and cortical tissues (Fig. 4c, i, o)

In hypocotyls, *AtPAO2* is expressed along the whole organ but only at the early developmental stages of the seedlings (Fig. g), *AtPAO3* is expressed only in the region adjacent to the hypocotyl-root junction site (Fig.4m) and *AtPAO5* is highly and specifically expressed in the vascular system of the whole hypocotyl (Fig. 4s), whereas there is no expression of *AtPAO1* (Fig. 4a). As far as the shoot apex is concerned, *AtPAO1* and *AtPAO2* are expressed in both the shoot apical meristem (SAM) and the stipules (Fig. 4d, e, j, k), *AtPAO3* is expressed only in the stipules (Fig. 4p, q), while *AtPAO5* is not expressed stipules but expressed in SAM (Fig. 4v, w). In addition, while *AtPAO1*- and *AtPAO5*-related GUS-staining is observed in the young cotyledons (Fig. 4f, w), both *AtPAO2*- and *AtPAO3*-related GUS staining are absent in this organ (Fig. 4k, q), *AtPAO2* being expressed only at the cotyledonary tips (Fig. 4k, l). Specific expression pattern for each *AtPAO* gene is also observed both in the newly emerging leaves and the expanded ones. Indeed, while *AtPAO1*, *AtPAO2* and *AtPAO3* are characterized by a quite localized expression pattern, *AtPAO5* shows a

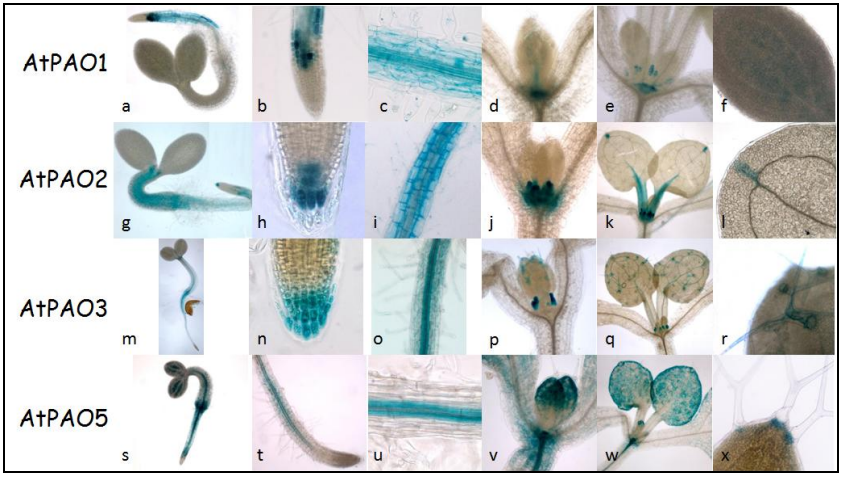

Fig. 4. Histochemical GUS staining of seedlings from *AtPAO::GUS* transgenic Arabidopsis plants for *AtPAO1, AtPAO2, AtPAO3, AtPAO5.* Modified from Fincato *et al.*, 2012.

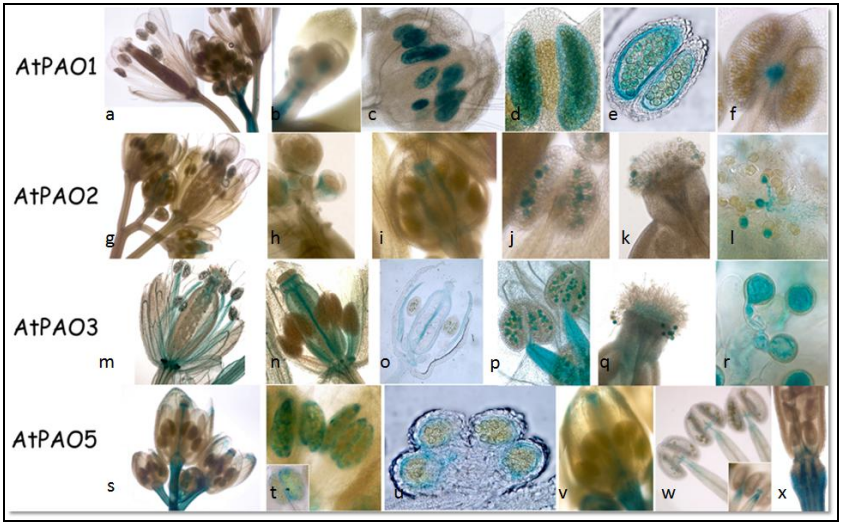

Fig. 5. Histochemical GUS staining of flowers from *AtPAO::GUS* transgenic Arabidopsis plants for *AtPAO1, AtPAO2, AtPAO3, AtPAO5.* Modified from Fincato *et al*., 2012.

rather diffused pattern (Fig. 4v). More specifically, *AtPAO1* and *AtPAO2* are expressed in the leaf hydathodes (data not shown for *AtPAO1* and Fig. 4k for *AtPAO2*) and *AtPAO3* in the guard cells (Fig. 4q, 6) and the trichomes (Fig. 4r). Staining of trichomes is also observed in the *AtPAO5::GUS* transgenic plants which is however restricted to the base of the trichomes (Fig. 4x), differently from the *AtPAO3*-related staining which is present throughout the whole area of the trichomes.

Distinct expression pattern of *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO5* is also additionally evident in inflorescences. In particular, *AtPAO1* seems to be specifically expressed in the microspores and the tapetum (Fig. 5c, d, e) while *AtPAO2* and *AtPAO3* are specifically expressed in pistils (Fig. 5h, n) and pollen grains (Fig. 5j, p). Interestingly, expression of both *AtPAO2* and *AtPAO3* in pollen persisted during pollination and pollen tube growth (Fig. 5k, l, q, r). *AtPAO5* exhibits an overlapping expression pattern with that of *AtPAO1* and *AtPAO2* but with some differences. In particular, although *AtPAO5* is initially expressed in anther tapetal cells (Fig. 5u) and then in the anther-filament junction site similarly to *AtPAO1*. *AtPAO5* is also expressed in the upper part of the filament (Fig. 5w), in sepals and in petals (Fig. 5s) where *AtPAO1* expression is not found. Furthermore, while both *AtPAO1* and *AtPAO5* are expressed in receptacles at the early stages of flower development (Fig. 5a, s), *AtPAO5* expression in receptacles is observed also at the level of the siliques (Fig. 5x). Similarly to *AtPAO2* and *AtPAO3*, *AtPAO5* is also expressed in pistils but only in the stigma and the septum and not in the ovary wall (Fig. 5v) as do *AtPAO2* and *AtPAO3* (Fig. 5i, o). Furthermore, *AtPAO5* is not expressed in pollen grains, in contrast to *AtPAO2* and *AtPAO3*. All these data together support different physiological role(s) of each of the members of the *AtPAO* gene family.

Physiological roles of polyamines

Since PAs are protonated at physiological pH, they have the capability to interact with negatively charged macromolecules, such as DNA, RNA, proteins and phospholipids thus altering the physical and chemical properties of numerous cellular components, stabilizing nucleic acid structures and modulating enzyme activities (Galston and Sawhney, 1990). In this way PAs are involved in the regulation of several fundamental cellular processes, including DNA replication, regulation of gene expression, RNA modification, translation, cell proliferation, cell cycle regulation, ion-channel regulation, modulation of cell signaling, membrane stabilization (Kusano *et al*., 2008; Tavladoraki *et al*., 2011). However, PAs are associated with several cellular processes not only through their

interaction with anionic macromolecules, but also through their metabolic products (Alcázar *et al*., 2010b).

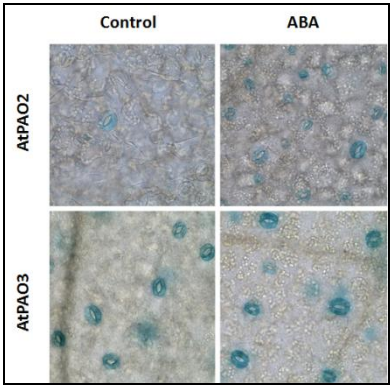

Fig. 6. ABA-inducible expression of *AtPAO2* in guard cells. *AtPAO2::GUS* and *AtPAO3::GUS* transgenic Arabidopsis plants were treated or not with 10 µM ABA for 4 h and then analyzed for GUS activity. From Fincato *et al*., 2012.

In animals, PAs play an important role in cell differentiation and proliferation. Indeed, mice with specific inactivation of *SPMS* have severe developmental defects (Pegg and Michael, 2010). PA synthesis is down-regulated as cells become senescent in many tissues of adults. Administration of Spd markedly extends the longevity of yeast, flies and worms and human immune cells (Eisenberg *et al.*, 2009). On the other hand, dysregulated PA metabolism has been associated with neoplastic transformation and cancer cell growth (Pegg and Feith, 2007). PAs affect numerous processes in carcinogenesis. In fact, PAs are often present at increased concentration in both tumor cell cultures and solid tumors, as determined in breast and colon cancer (Heby and Persson, 1990), while PA depletion leads to inhibition of tumor growth (Averill-Bates *et al*., 2005). It has been demonstrated that PAs can also induce programmed cell death (PCD) in various animal cell types (Wallace *et al*., 2003; Igarashi and Kashiwagi, 2010), thus indicating a bivalent function for these molecules, promoting both cell growth and cell death, likely depending on their concentration and other developmental and environmental signals (Wallace *et al*., 2003, Toninello *et al*., 2006).

In plants, PAs have been suggested to play important roles in regulation of cell proliferation, somatic embryogenesis, differentiation and morphogenesis (Kusano *et al*., 2007, 2008), dormancy breaking of tubers and in seed germination, development of flowers and fruits (Kusano *et al*.,

2007) and senescence (Takahashi *et al*., 2010). Indeed, *A. thaliana* double mutant for *ADC1* and *ADC2*, which cannot produce PAs, died at the embryo stage (Urano *et al*., 2005) while embryo development of Arabidopsis double mutant for *SPDS1* and *SPDS2* is arrested at the heart stage indicating a requirement for Spd during the course of embryogenesis (Imai *et al*., 2004a). On the other hand, it has been demonstrated that organisms deficient in Spm are viable, but show different degrees of dysfunction. This indicates that Spm, although not essential, must also play very important roles in growth and development (Imai *et al*., 2004b; Alcázar *et al*., 2010). Furthermore, Ther-Spm has been shown to be involved in the regulation of vascular differentiation. Indeed, *acl5* mutants of *A. thaliana* which do not synthetise Ther-Spm shows a severely dwarfed phenotype with over-proliferation of xylem tissues (Vera-Sirera *et al*., 2010) . Exogenous application of Ther-spm but not Spm to the *acl5* mutant partially rescues plant phenotype (Kakehi *et al*., 2008).

In plants, PAs are also known to enhance plant tolerance to environmental stresses such as salinity, chilling, drought, potassium deficiency (Martin-Tanguy, 2001; Alcázar *et al*., 2010a), and defence signalling against pathogens (Walters, 2003). Indeed, exogenous applications of PAs have frequently been shown to affect plant growth and response against various stress factors (Groppa *et al*., 2007; Kusano *et al*., 2008; Vera-Sirera *et al*., 2010). Genetic studies using either transgenic plants overexpressing PA biosynthetic genes or loss-of-function mutants support their protective role in plant response to abiotic stress (Alcázar *et al*., 2006; Kusano *et al*., 2008; Gill and Tuteja, 2010), and provide a major advance in the understanding of PA functions. In addition, analysis of expression profiles of PA biosynthetic genes evidenced stress–responsiveness for several of them (Table 2). It has been shown that drought, dehydration, salt and ABA treatments (Urano *et al*., 2003; Alcázar *et al*., 2006) induce *ADC2* expression. In particular, following cold treatment (Table.2), induced expression of *ADC* genes was in parallel with the increase in Put levels and constant or even decreased levels of free Spd and Spm (Cuevas *et al*., 2008) while, other studies showed that higher levels of Spd and Spm and lower levels of free Put could improve the adaptability of plants to salt stress (Duan *et al*., 2008). Furthermore, significant accumulation of SAMDC2 mRNA is observed in Arabidopsis under stress conditions (Urano *et al*., 2003). Overexpression of *SAMDC* has been shown to enhance tolerance to different abiotic stress in different transgenic plants. Indeed, it enhances tolerance to salt stress in *Oryza sativa* (Roy and Wu, 2002), *Nicotiana tabacum* (Waie and Rajam, 2003), *Pyrus communis* L. (He *et al*., 2008) and *Malus sylvestris* (Table. 2; Hao *et al*.,

2005). It enhances also tolerance to drought and fungal wilts (caused by *Verticillium dahliae* and *Fusarium oxysporum*) in *N. tabacum* (Waie and Rajam, 2003), to high temperature in *Lycopersicon esculentum* (Cheng *et al*., 2009) and to cold in *M. sylvestris* (Table. 2; Hao *et al*., 2005) . On the other hand, *spms* Knock-out mutant appears to be more sensitive to drought stress than the wild-type plants (Yamaguchi *et al*., 2007). This phenotype is believed be related to the fact that inward potassium currents across the plasma membrane of guard-cells are blocked by intracellular PAs (Liu *et al*., 2000). It has been also evidenced that *acl5/spms* double mutant is hypersensitive to high levels of KCl but not to high levels of $MgCl_2$ and mannitol (Yamaguchi *et al*., 2006).

On the other hand, PAs play a role as mediators in defence signalling against plant pathogens (Takahashi *et al*., 2003). In particular, 'Spm signalling pathway' involves transport of Spm in the apoplast, upregulation of a subset of defence-related genes, such as those encoding pathogenesis-related proteins and mitogen-activated protein kinases, and a type of programmed cell death (PCD) known as the hypersensitive response. This response is triggered by Spm-derived H_2O_2, produced through the action of PAOs localized in the apoplast (Cona *et al*., 2006; Kusano *et al*., 2008; Moschou *et al*., 2008c).

Physiological roles of polyamine catabolism

In animals, PA catabolism contributes to important physiopathological processes such as cell proliferation and differentiation, apoptosis, amine detoxification and cell signalling through both regulation of PA levels and their oxidatively deaminated reaction products, mainly H_2O_2, aminoaldehydes or dialdehyde and acrolein (Sharmin *et al*., 2001; Averill-Bates *et al*., 2008; Agostinelli *et al*., 2009). As evidenced by the complex role of PAs in cell growth and proliferation, optimal PA levels are necessary for mammalian health. In fact, an important difference between normal and tumor cells is PA content. To ensure optimal PA levels, PA homeostasis is tightly regulated at various steps of PA metabolism and transport (Wallace *et al*., 2003), thus becoming difficult to be perturbed through inhibition of a single biosynthetic or catabolic reaction. Despite this tight control, it has been shown that an altered PA catabolism can cause changes in PA homeostasis. Indeed, constitutive or inducible over-expression of SSAT in animal cells brought a substantial reduction in Spd and Spm pools as well as a large increase in Put and N^1-acetyl-Spd intracellular levels and export of acetylated PAs (Jänne *et al*., 2005; Zahedi *et al*., 2007). Furthermore, over-expression of SMO in mouse neuroblastoma cells and HEK293 cells caused

a statistically significant decrease in Spm levels and an increase in Put levels (Vujcic *et al*., 2002; Amendola *et al*., 2005; Zahedi *et al*., 2007). Notably, the changes in PA levels through PA catabolism were often accompanied by increased DNA damage and changes in cell proliferation (Zahedi *et al*., 2007). These data suggest that PA catabolism has an important role in controlling PA content and thus can be used as a therapeutic target for several diseases.

The other catabolic product, H_2O_2, which can get converted into the highly reactive hydroxyl radical through Fenton-like-catalysis (Fig. 7), is able either to impair cell growth and proliferation or to regulate signal transduction and gene expression, depending on its concentration. Indeed, it has been demonstrated that, in human breast cancer cells, the SMO-derived H_2O_2 in response to treatment with the PA analogue bis(ethyl)norspermine (BENSpm) is cytotoxic (Fig. 7a; Pledgie *et al*., 2005; Casero and Pegg, 2009). Furthermore, the H_2O_2 produced by purified BSAO and Spm exogenously supplied to human colon adenocarcinoma and melanoma cells has been also shown to cause cytotoxicity (Calcabrini *et al*., 2002; Agostinelli *et al*., 2009). However, it is still an open question whether or not H_2O_2, formed by PA catabolism, is always pathologic, or has a role in cell signalling (Wang and Casero, 2006). The aminoaldehydes produced through PA catabolism have been shown to be cytotoxic on animal cells (Fig. 7a), probably due to the inhibition of nucleic acid and protein synthesis (Nocera *et al*., 2003; Wallace *et al*., 2003). Indeed, it has been reported that 3-aminopropanal and acrolein produced from PA catabolism (Fig. 3) are intimately involved in cell damage during ischemia in rats (Igarashi and Kashiwagi, 2010). It was also observed that renal failure patients had increased levels of SMO activity and both free and protein conjugated acrolein (PC-Acro). Furthermore, PC-Acro increased at the locus of infarction after induction of stroke in mice (Igarashi and Kashiwagi, 2010). Moreover, acrolein has been shown to have an inhibitory effect on cell growth. In particular, it has been determined that the toxicity of acrolein on cells in culture medium containing fetal bovine serum with amine oxidase activity is greater than that caused by H_2O_2 (Sharmin *et al*., 2001). However, it was observed that acrolein is not formed under normal conditions, likely due to the fact that PAs mainly exist as RNA-PA complexes, rather than as free molecules. It was therefore hypothesized that when cells are damaged, PAs are released from RNA and acrolein is produced from PA catabolism, especially from Spm by SMO, so that the aldehyde might be used as a biochemical marker for pathologies involving cell damage (Igarashi and Kashiwagi, 2010; Saiki *et al*., 2011).

Gene		Gene function	Gene source	Transgenic plant	Overexpression	Production	Tolerance	Refence
ADC/ODC	ADC	ADC is responsible for the biosynthesis of diamine Put from arginine	*Avena sativa*	*Oryza sativa L.*	Inducible		Salt	Roy and Wu 2001
	ADC		*Datura stramonium*	*Oryza sativa*		Defective in Put	Drought (PEG8000)	Capell et al., 2004
	ADC1, ADC2 (mutants defective in putrescine biosynthesis)		*Arabidopsis thaliana*	*Arabidopsis thaliana*		Defective in Put	Freesing tolerance and cold aclimatation	Cuevas et al., 2008
	ADC		*Avena sativa*	*Oryza sativa*	Inducible	Over production of Put	Salt	Roy and Wu 2010
	ODC		*Mus musculus*	*Nicotiana tabacum*	Constitutive	Overproduction of Put	Salt	Kumria and Rajam 2002
SAMDC	SAMDC	SAMDC is a key enzyme involved in the biosynthesis of the PAs, viz. Spd & Spm	*Tritordeum ascherson*	*Nicotiana tabacum*	Inducible	Overproduction of Spd and Spm	Salt	Roy and Wu 2002
	SAMDC		*Tritordeum ascherson*	*Oryza sativa*	Inducible	Overproduction of Spd and Spm	Salt	Roy and Wu 2002
	SAMDC		*Homo sapiens*	*Nicotiana tabacum*	Constitutive	Overproduction of Put and Spd	Salinity, drought and fungal wilts stress tolerance	Waie and Rajam, 2003
	SAMDC2		*Malus sylvestris*	*Malus sylvestris*			Cold and salt stress	Hao et al., 2005
	SAMDC		*Dianthus caryophyllus*	*Nicotiana tabacum*	Constitutive	Overproduction of Put,Spd and Spm	Broad spectrum	Wi et al., 2006
	SAMDC1		*Arabidopsis Thaliana*	*Arabidopsis thaliana*	Constitutive	Overproduction of Spm	Broad spectrum	Alcazar et al., 2006b
	SAMDC		*Saccharomyces cerevisiae*	*Lycopersicon esculentum*	Constitutive	Overproduction of Spd and Spm	High temperature stress	Cheng et al., 2009
SPDS	SPDS	SPDS converts Put into spermidine	*Cucurbita ficifolia*	*Arabidopsis thaliana*	Constitutive	Overproduction of Spd	Chilling, freezing, salinity, hyperosmosis, drought and paraquat stress tolerance	Kasukabe et al., 2004
	MdSPDS1		*Malus sylvestris*	*Pyrus Communis L. Ballad*	Constitutive	Overproduction of Spd	Salt and Manitol stress tolerance	He et al., 2008
	SPDS		*Malus sylvestris*	*Pyrus Communis*	Constitutive	Overproduction of Spd	Salt, Manitol and heavy metal stress tolerance	Wen et al., 2008

Table 2. Transgenic plants engineered to synthesize PAs for enhanced abiotic stress tolerance. Modified from Gill and Tuteja, 2010 and Alcázar *et al*., 2010

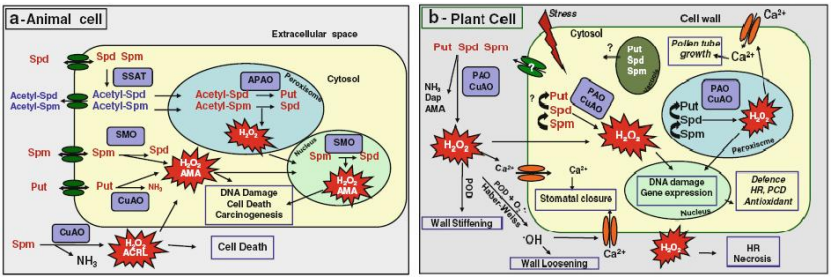

Fig. 7. Physiological roles of PA catabolism in animals and plants. (a) PA catabolism in animals. (b) PA catabolism in plants. AMA, aminoaldehyde; ACRL, acrolein; Dap, *1,3* diaminopropane; HR, hypersensitive response; PCD, programmed cell death; POD, peroxidase. From Tavladoraki *et al*., 2012.

Also in plants, PA catabolism has been shown to have important roles in plant development and stress responses through both regulation of PA levels and their reaction products. Although a key role of the PA biosynthetic pathways in PA homeostasis has been highlighted, recent evidences suggest that the PA catabolic pathways equally play an important role in the regulation of PA levels. In particular, it has been shown that increased PA levels are accompanied by a concomitant increase in their catabolism (Bhatnagar *et al*., 2002). Furthermore, recently, it has been shown that it is possible to induce changes in the levels of specific PAs through manipulation of the catabolic pathways (Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008a; Fincato *et al*., 2011). A tight regulation of PA levels in plants is very important not only because PAs have a direct role in several physiopathological processes as discussed above, but also because PA metabolism has a central role in cellular metabolism (Mattoo *et al*., 2010; Mohapatra *et al*., 2010).

Similarly to animals, PA catabolism in plants plays an important role through the production of H_2O_2 (Fig. 7b), which is necessary for several plant developmental processes. In plants, H_2O_2 produced via apoplastic degradation of PAs drives peroxidase-mediated oxidative cross-linking of structural cell wall components contributing to cell-wall strengthening during development and under stress conditions, such as wound-healing and pathogen attack (Fig. 7b; Cona *et al*., 2006; Angelini *et al*., 2008, 2010). H_2O_2 has also been identified as an important second messenger in signal transduction networks. Indeed, in *A. thaliana* H_2O_2 produced by PAO-mediated Spd oxidation triggers the opening of hyperpolarization-activated Ca^{2+}-permeable channels in pollen, thereby regulating pollen tube growth (Wu *et al*., 2010), a process important for sexual plant reproduction. Plant

PA catabolism is also involved in the regulation of gene expression as shown in AtPAO4-deficient Arabidopsis mutants altered in the expression of genes related to abiotic stress responses and flavonoid and/ or lignin metabolism (Kamada-Nobusada *et al*., 2008). It has been reported that PA catabolism is involved in the regulation of gene expression also under stress conditions (Fig. 7b). H_2O_2 produced by PA catabolism has been also proposed to activate PCD associated with developmental differentiation. Indeed, the presence of an *A. thaliana* CuAO (ATAO1; (Moller *et al*., 1998) and ZmPAO in developing tracheary elements and root cap cells suggests their involvement in PCD which both cell types eventually undergo (Cona *et al*., 2006). H_2O_2 produced by PA catabolism has been shown to induce PCD also as a defence response to abiotic and biotic stresses.

PA catabolism contributes also to the formation of GABA, an important cellular metabolite which is also synthesized by cytosolic glutamate decarboxylase (Yu and Sun, 2007). GABA is rapidly produced in plants in response to biotic and abiotic stresses (Petrivalský *et al*., 2007; Dittami *et al*., 2011). Furthermore, PA catabolism contributes to the formation of β-alanine, which in turn can be further converted in plants to the osmoprotectant β-alanine betaine (Fig. 3). Dap is also a precursor of the uncommon PAs Nor-Spd and Nor-Spm which in plants are associated with stress tolerance (Cona *et al*., 2006). However, the exact contribution of PA catabolism to plant development and defence responses through production of these metabolites has still to be evaluated. Further studies are necessary to verify whether, similar to what is reported in animals, aminoaldehydes derived from PA catabolism in plants have cytotoxic activity and whether acrolein is also formed.

In the yeast *Saccharomyces cerevisiae*, it was shown that β-alanine produced from Spm oxidation by the spermine oxidase FMS1 is necessary for the production of pantothenic acid (vitamin B5), a metabolic precursor to coenzyme A (CoA) which is a cofactor of a large number of metabolic enzymes (White *et al*., 2001). Indeed, overexpression of *FMS1* caused excess of pantothenic acid to be excreted into the medium, whereas deletion mutants required β-alanine or pantothenic acid for growth. Conversely, in bacteria, the β-alanine necessary for pantothenic acid production was shown to be derived by the decarboxylation of L-aspartate. The difference between yeast and bacteria in β-alanine biosynthesis questions as to how other organisms, such as fungi and plants, make β-alanine. At the present time in the public sequence data bases there are over a dozen identifiable aspartate-1-decarboxylase genes from different prokaryotic species, whereas this enzyme does not appear to be present in eukaryotic species.

Plant polyamine catabolism under abiotic and biotic stress conditions

Numerous studies in different plant species have shown that PA catabolism contribute to plant defence responses to several biotic and abiotic stresses. However, this contribution has been mainly shown for extracellular PA catabolic enzymes and interestingly it is linked to PA transport to the apoplast where only limiting amounts of PAs are present under normal growth conditions (Moschou *et al*., 2008a; Kusano *et al*., 2008; Takahashi *et al*., 2010). Stress-related factors that have been shown to induce PA transport in the apoplast are: incompatible and compatible plant-pathogen interactions, salt stress and treatment with the stress-related hormone ABA (Yoda *et al*., 2003; Yoda *et al*., 2006; Marina *et al*., 2008; Moschou *et al*., 2009; Toumi *et al*., 2010). This suggests that PA catabolism in the apoplast is a general defence response against several stresses.

Several data based on the use of PAO-specific inhibitors and transgenic plants evidenced that PA catabolism in the apoplast contributes to stress defence responses through H_2O_2 production. Indeed, it has been shown that the H_2O_2 produced by PA catabolism in the apoplast contributes to the second phase of ROS production during TMV-induced HR, a plant response which is developed during an incompatible plant-pathogen interaction and consists of rapid ROS production, PCD and induction of defence responses aiming to restrict pathogen expansion (Yoda *et al*., 2003). Similar approaches showed that H_2O_2 produced by PA catabolism in the apoplast contributes to the synthesis of the ROS that accumulate under abiotic stress conditions (Moschou *et al*., 2008a) or following treatment with ABA, an hormone which plays a crucial role in plant responses to abiotic stresses (Xue *et al*., 2009; Toumi *et al*., 2010). The H_2O_2 produced by PA catabolism in the apoplast under stress conditions and/or the apoplastic PAs themselves trigger a downstream signal cascade pathway leading to increased expression of specific genes, such as of superoxide dismutase, ascorbate peroxidase, pathogenesis-related proteins, protein kinases, transcriptional factors and several other stress responsive genes (Yamakawa *et al*., 1998; Moschou *et al*., 2008a; Moschou *et al*., 2009; Xue *et al*., 2009). Interestingly, exogenous application of Spm to tobacco leaves, which mimics the apoplastic accumulation of PAs upon an incompatible plant pathogen interaction, increased expression of HR marker genes (Kusano *et al*., 2008). Furthermore, ABA-inducible generation of H_2O_2 by Put catabolism in the apoplast of guard cells signals stomatal closure through a mechanism involving Ca^{2+} as a second messenger (An *et al*., 2008).

The H_2O_2 produced by PA catabolism in the apoplast upon stress may also lead to PCD. In particular, it has been shown that accumulation and further oxidation of free PAs in the apoplast induce PCD during tobacco defence against infection by microorganisms with diverse pathogenesis strategies, i.e. microorganisms establishing host and non-host incompatible interactions, such as TMV and *Pseudomonas cichorii* in tobacco, *Pseudomonas syringae* in Arabidopsis and *Magnaporthe grisea* in rice (Yoda *et al*., 2003, 2006, 2009). PCD was shown to be induced by oxidation of extracellular Spd also under abiotic stress conditions. Indeed, under salt stress conditions the levels of H_2O_2 and PCD were higher in transgenic plants over-expressing the apoplastic ZmPAO than in the wild-type plants (Moschou *et al*., 2008a). Accumulation and further oxidation of free PAs in the apoplast has been also shown to enhance necrotic cell death, and thus increase disease severity, following infection of *N. tabacum* plants with the necrotrophic pathogen *Sclerotinia sclerotiorum*, an effect that was blocked by PAO- and CuAO–specific inhibitors (Marina *et al*., 2008). Interestingly, when the biotrophic bacterial pathogens *Pseudomonas viridiflava*, *Pseudomonas syringae pv tabaci* or hemibiotrophic pathogen oomycete *Phytophthora parasitica* var *nicotianae* were tested in *N. tabacum* host plants PA oxidation in the apoplast strongly decreased bacterial growth *in planta* and caused a reduction in the oocyte induced necrosis (Marina *et al*., 2008; Moschou *et al*., 2009). These data suggest that increased PA catabolism in the apoplast may have opposing effect against pathogens with different pathogenic strategies

Although the data described above strongly support the contribution of the apoplastic amine oxidases involved in the terminal catabolism of PA to plant defence responses, more studies are still necessary to determine in detail the concerned mechanism(s), to comprehend in depth the pleitrophic effects of the PA catabolic pathways and to unravel co-interacting metabolic and signalling pathways. More studies are also necessary to understand the contribution of the newly identified PA back-conversion pathways to plant defence responses.

RESULTS

Studies on the physiological roles of *AtPAO1*

Polyamine levels in atpao1 loss-of-function mutant

It has been recently shown that recombinant AtPAO1 has high catalytic activity towards Spm but not at all towards Spd (Tavladoraki *et al*., 2006). Recombinant AtPAO1 has elevated catalytic activity also towards the uncommon PA Therm-Spm (Tavladoraki *et al*., 2006), which is of particular interest because it has been recently shown the existence in *Arabidopsis* of an enzyme (ACL5) able to synthesize Therm-Spm and the loss-of-function mutant *acl5* shows a severely dwarfed phenotype (Rambla *et al*., 2010; Vera-Sirera *et al*., 2010). To determine whether AtPAO1 is involved in the regulation of PA levels, an Arabidopsis insertional T-DNA knockout mutant for *AtPAO1* (*atpao1*), recently obtained from the SAIL (Syngenta Arabidopsis Insertion Library) collection of Arabidopsis seeds (Fig. 8) and characterized (Dr L. Pomettini, graduation thesis), was analyzed for PA levels. Since the classical methods for PA determination, based on high-performance liquid chromatography or thin-layer chromatography, do not distinguish between Therm-Spm and Spm being isomers, this analysis was also performed by a gas chromatography–mass spectrometry method (Rambla *et al*., 2010) in collaboration with Prof. Juan Carbonell (Universidad Politécnica de Valencia-CSIC, Spain). Results obtained both by HPLC (data not shown) and gas chromatography–mass spectrometry (Fig. 9) showed no significant difference in the levels of the common PAs Put, Spd and Spm as well as in the level of Therm-Spm between *atpao1* and wild-type seedlings. Since it has been recently shown that *AtPAO1* is highly expressed in roots (Fig. 4; Fincato *et al*., 2012), PA levels were also analysed in this specific organ, but also in this case no statistically significant difference was observed between *atpao1* mutant and wild-type plants (data not shown). Similarly, HPLC analyses did not evidence altered PA levels in flowers and leaves of *atpao1* mutant (data not shown). The lack of differences in PA levels between *atpao1* mutant and wild-type Arabidopsis plants may be due either to gene redundancy and/or to activation of homeostatic mechanisms and may exclude the possibility that Ther-Spm is the physiological substrate of AtPAO1.

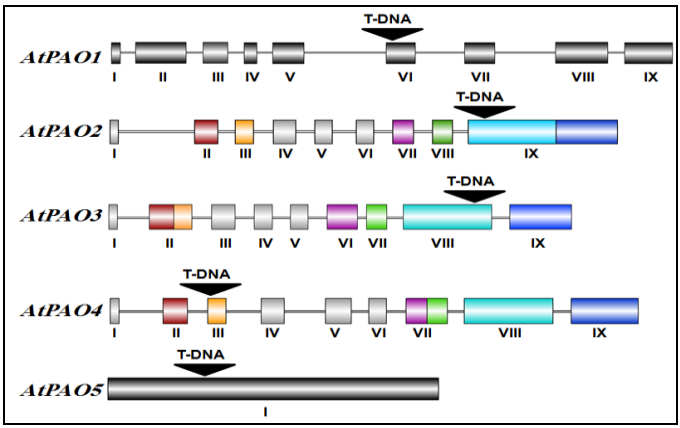

Fig. 8. Schematic representation of the *AtPAO* gene structures with T-DNA insertion sites in the corresponding *atpao1*, *atpao2*, *atpao3*, *atpao4* and *atpao5* mutants. Black triangles indicate the T-DNA insertion site. The black lines represent introns and boxes represent exons. Exons are numbered in Roman numerals. Colored and light grey boxes indicate shared and unshared exons, respectively. Dark grey boxes are unshared exons. Gene analyses were done using FGENESH available on Softberry website and protein domain structures were done using DOG 2.0 software.

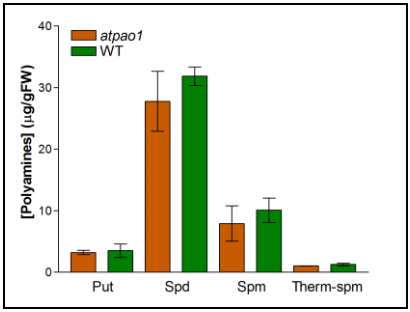

Fig. 9. Polyamine content in *atpao1* mutant. PA levels of *atpao1* and wild-type seedlings were determined by gas chromatography-mass spectrophotometry. Statistical analysis was performed by one way ANOVA test ($p < 0.001$). Bars indicate standard error.

To evaluate whether homeostatic mechanisms have been activated in the *atpao1* mutant, the expression levels of biosynthetic genes (*ADC1*, *ADC2*, *SAMDC*) were examined in this mutant by semi-quantitative RT-PCR but no difference in respect to the wild-type plants was evident (Fig. 10).

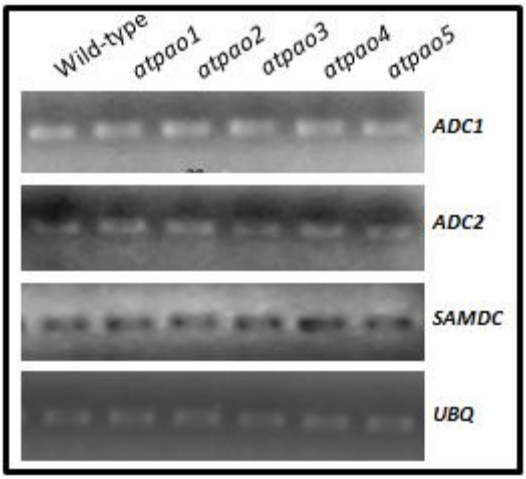

Fig. 10. Expression levels of PA biosynthetic enzymes in *atpao* single mutants under physiological growth conditions. The expression levels of PA biosynthetic enzymes (*ADC1*, *ADC2* and *SAMDC*) were analyzed in whole seedlings of *atpao* single mutants and wild-type *Arabidopsis* plants by semi-quantitative RT-PCR using gene-specific primers. Expression of *UBQ5* was used as loading control. Results were taken at the exponential phase (at 28 cycles for *ADC1* and *UBQ5*, at 25 cycles for *ADC2*, and at 23 for *SAMDC*).

Physiological studies on atpao1 mutant

It has been recently shown that *AtPAO1* is highly expressed in the transition zone between the meristematic and elongation region of the roots (Fig. 4; Fincato *et al*., 2012). To examine whether this enzyme is involved in root development at this region, the number of cortex cells in a cell file extending from the quiescent center to the first elongated cell as well as the length of the same region (Fig. 11C) were determined as a measurement of meristem size. Data evidenced no statistically significant difference in the size of the meristematic region between *atpao1* and wild-type plants both under physiological growth conditions and following treatment with 100 mM NaCl or 5 µM ABA (Fig. 11A, B).

The *atpao1* single mutant was also analyzed for germination and growth rate under physiological and stress conditions, such as NaCl, ABA and JA. However, no variation was observed as compared to the wild-type plants (data not shown).

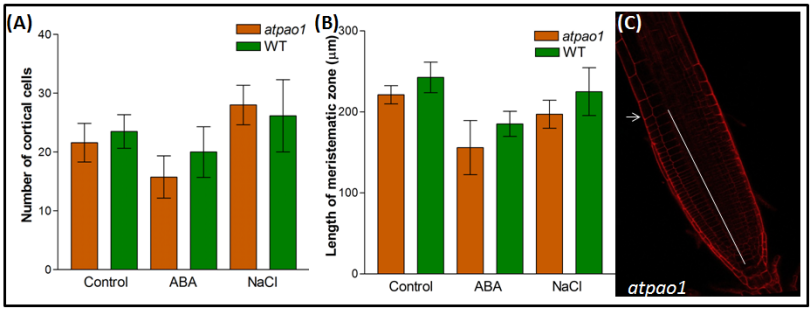

Fig. 11. Root meristematic zone of *atpao1* mutant. The length of root meristematic zone (A) and the number of cortical cells along the root meristematic zone (B) of *atpao1* mutant and wild-type (WT) *Arabidopsis* plants were determined under physiological growth conditions and following 1 week treatment with 5 µM ABA or 100 mM NaCl. To visualize cells, roots were treated with propidium iodide before analysis under confocal microscopy. Statistical analysis was done using two-tailed T-test ($p<0.05$). Bars indicate standard error.

Studies on the physiological roles of *AtPAO2-4* gene family

The existing studies on the various members of the *AtPAO* gene family evidenced important differences among them and in respect to the other plant PAOs characterized so far in substrate specificity, reaction products (Fincato *et al*., 2011), subcellular localization (Kamada-Nobusada *et al.*, 2008; Moschou *et al*., 2008b) and space–temporal expression pattern (Takahashi *et al*., 2010; Fincato *et al*., 2012;). Interestingly, differences in substrate specificity and expression pattern exist even among AtPAO2, AtPAO3 and AtPAO4 which, on the basis of the similar gene structure, the high sequence homology and the same subcellular localization, are considered to be derivatives from a common ancestor and thus to form a distinct PAO subfamily (*AtPAO2-4* subfamily; Fincato *et al*., 2011, 2012). In particular, while AtPAO2 is equally active with Spm and Spd, AtPAO3 is 2-fold less active with Spm than with Spd and AtPAO4 is 10-fold less active with Spd than with Spm (Table 1). On the other hand, while in roots *AtPAO2* is expressed only near the quiescent center and columella initials, *AtPAO3* is expressed in lateral root cap and the whole columella (Fincato *et al*., 2012). Furthermore, while *AtPAO3* is constitutively expressed in the guard cells, *AtPAO2* presents ABA-inducible expression in these cells (Fig.6; Fincato *et al*., 2012).

Since the differences in catalytic properties, subcellular localization and expression pattern may reflect differences in physiological roles, in the present work it was considered very important to complete the studies on *AtPAO* tissue- and organ–specific expression pattern analyzing promoter activity also for *AtPAO4*, the only *AtPAO* for which such information has not been available so far.

AtPAO4 space-temporal expression pattern

To study *AtPAO4* promoter activity, promoter region of 2,900 bp including the 5'-UTR was amplified and inserted upstream of the *GFP–GUS* fusion gene in pKGWFS7 binary vector (Karimi *et al*., 2002) using Gateway technology. The resulting *AtPAO4::GFP-GUS* construct was used to transform *A. thaliana* wild-type plants by the *Agrobacterium tumefaciens*-mediated floral dip transformation method. Several transgenic lines were obtained through selection by kanamycin resistance and PCR analysis (data not shown). Eight *AtPAO4::GFP-GUS* transgenic lines were analyzed by histochemical GUS staining at various developmental stages. Only highly reproducible results were taken into consideration and are reported below.

AtPAO4-related GUS staining was observed in roots, from the meristem/elongation transition region up to the hypocotyl–root junction site similarly to AtPAO2 and AtPAO3 (Fig. 12a). A part of the meristematic region, but no part of the root cap, was also stained differently from AtPAO2 and AtPAO3 (Fig. 12b, c). This *AtPAO4*-related GUS staining in the roots was present in all the analyzed developmental stages (3 to 15 days after germination) and already appeared after 15-30 min of GUS staining. Lateral roots display the same expression pattern as the main root (Fig. 12d). In 5- to 8-day-old seedlings, *AtPAO4*-related GUS staining appeared in cotyledonary tips, leaf (Fig. 12e, f), shoot apex, newly emerging leaves (Fig. 12e, g) and stipules (Fig. 12g). No GUS activity was identified in hypocotyls at any developmental stage, similarly to AtPAO3 but differently from AtPAO2 which is transiently expressed in hypocotyls at early developmental stages (Fig. 12a). *AtPAO4*-related GUS staining was also observed in guard cells of cotyledons (Fig. 12h), stems (Fig. 12o) and petals (Fig. 12i), similarly to the ABA-inducible expression of *AtPAO2* and the constitutive expression of *AtPAO3* in these cells. A strong *AtPAO4*-related GUS signal was also observed at the base of very young and completely closed flower buds similarly to other *AtPAOs* (Fig 10i). As buds developed, GUS staining appeared in anthers, probably in the tapetum (Fig. 12j, k), and in pistil walls (Fig. 12m). In mature flowers, a strong *AtPAO4*-related GUS

signal in pollen grains was detected (Fig. 12n). As in the case of *AtPAO2* and *AtPAO3*, pollen staining persisted during pollination and pollen tube growth (Fig. 12l, p). Thus, this study evidenced common expression patterns among the three members of the AtPAO2-4 subfamily (such as expression in guard cells and pollen grains), but also distinct patterns, as for example in roots and hypocotyls.

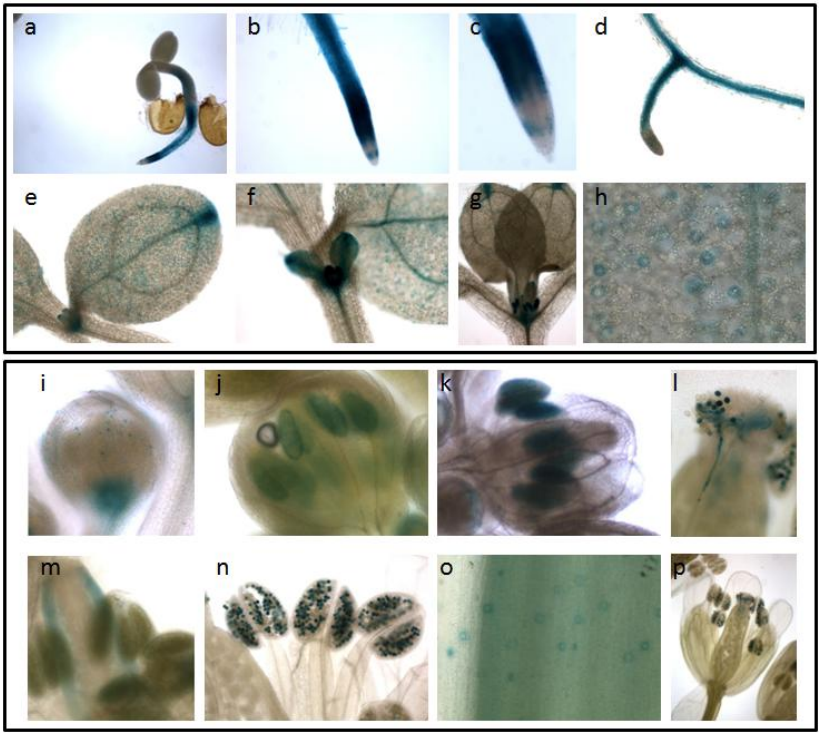

Fig. 12. *AtPAO4* promoter activity during plant growth. Histochemical GUS staining of *AtPAO4::GFP-GUS* transgenic plants in seedlings (a-h) and in inflorescences (i-p).

Characterization of single and multiple mutants for the peroxisomal AtPAO2-4 subfamily.

To determine the physiological roles of the three peroxisomal *AtPAOs*, T-DNA insertional knockout mutants for each one have been recently obtained from the SALK collection of Arabidopsis seeds (Fig. 8) and homozygous plants for the T-DNA insertion have been selected (Dr L. Pomettini, graduation thesis). In the present study, double (*atpao24*,

atpao34 and *atpao32*) and triple (*atpao243)* mutants were also obtained by sexual crossing of the single mutants, considering the common expression patterns of the three peroxisomal genes and the possibility of gene redundancy which may hide the effect of each gene. Following characterization by PCR and RT-PCR (data not shown), the mutants were used for physiological studies.

Polyamine content in double and triple mutants for AtPAO2-4 gene family

The *atpao2*, *atpa3* and *atpao4* single mutants, as well as the *atpao24* and *atpao34* double mutants were analyzed for PA content by HPLC analysis to verify whether the three peroxisomal *AtPAOs* are involved in PA homeostasis. This analysis showed no statistically significant difference in the levels of Put, Spd and Spm between these mutants and the wild-type plants (data not shown). No change was also shown in *ADC1*, *ADC2* and *SAMDC* expression levels in the *atpao2*, *atpao3* and *atpao4* single mutants (Fig. 10). Conversely, a significant increase in Spd levels was evidenced in the *atpao243* triple mutant as compared to wild-type plants (Fig. 13). These data are in agreement with the high catalytic activity of AtPAO2 and AtPAO3 towards Spd and indicate that the three peroxisomal AtPAOs act redundantly to control PA homeostasis.

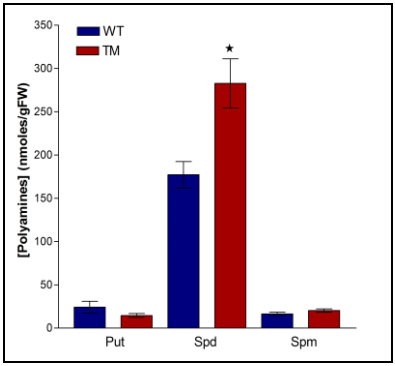

Fig. 13. Polyamine content in *atpao243* triple mutant. PA levels were determined in 14-day old *atpao243* and wild-type (WT) seedlings by HPLC analysis. Experiments were repeated 3 times with similar results with three independent replicates in each experiment. Statistical analysis was performed by one way ANOVA test ($p < 0.001$). Bars indicate standard error.

Stomatal closure in mutants for AtPAO2-4 gene family

The histochemical GUS analysis of the *AtPAO::GFP-GUS* transgenic plants demonstrated that all three peroxisomal *AtPAOs* are expressed in guard cells. In particular, *AtPAO2* presents ABA-inducible expression in guard cells (Fig. 6; Fincato *et al*., 2012),while *AtPAO3* (Fig. 6; Fincato *et al*., 2012).and *AtPAO4* (Fig. 12h, o) are constitutively expressed in these cells.

The guard cells are located in the leaf epidermis and pairs of guard cells surround and form stomatal pores, which regulate CO_2 influx from the atmosphere into leaves for photosynthetic carbon fixation. Stomatal guard cells also regulate water loss of plants via transpiration to the atmosphere. Signal transduction mechanisms in guard cells integrate a multitude of different stimuli to modulate stomatal apertures. Stomata open in response to light and close in response to drought stress, elevated CO_2, ozone and low humidity. In response to drought, plants synthesize the hormone ABA that triggers closing of stomatal pores through a complex signalling network which among others involves cytosolic calcium increases, phospholipids, phospholipid kinases, phospholipid lipases, protein dephosporylation, ROS and NO.

The expression of AtPAO2, AtPAO3 and AtPAO4 in the guard cells, together with the fact that the expression of these genes is regulated by ABA (Moschou *et al*., 2008b; Fincato *et al*., 2012), led us to hypothesize involvement of the *AtPAO2-4* subfamily in the control of stomata movement. To verify this hypothesis, experiments were conducted aiming to determine stomatal closure of *atpao* knockout mutants following treatment with ABA, a phytohormone which plays a central role in the control of stomata movements. Our results showed that, after ABA treatment, *atpao2*, *atpao3* and *atpao4* single knockout mutants display a reduced stomatal closure as compared to wild-type plants (Fig. 14; Fincato's PhD thesis). Interestingly, the reduced stomata closure observed in the single mutants was even more pronounced in the double *atpao24* and *atpao34* mutants and the triple *atpao243* mutant, the last one presenting the highest variation in stomata movement in respect to the wild-type plants (Fig. 14). These data suggest the involvement of the peroxisomal *AtPAOs* in the ABA-mediated control of guard cells.

The *atpao243* triple mutant was also analyzed for stomata movement following various stress treatments known to induce stomata closing to protect plants from pathogen entrance and water loss (Melotto *et al*., 2006; Desikan *et al*., 2008; Zhao *et al*., 2011). In particular, stomata movement was determined following treatment with the microbial elicitors flagellin

(its derived peptide flg22; Boller and Felix, 2009) and Elongation Factor Tu (its derived peptide elf18; Kunze *et al*., 2004). On the other hand, we analysed stomata closure also following treatment with polyethylene glycol and salt (NaCl). However, no statistically significant difference in stomata closure between *atpao243* triple mutant and wild-type plants was observed following these treatments (data not shown).

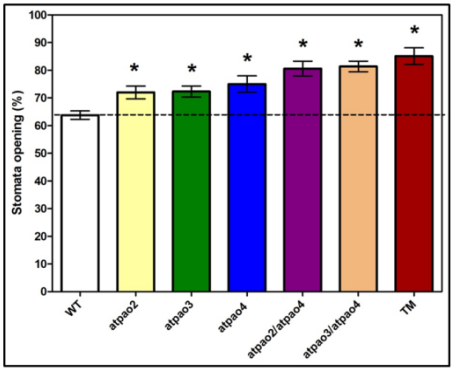

Fig. 14. Stomatal closure in single, double and triple knockout mutants for *AtPAO2-4* gene family following ABA treatement. Seedlings from *atpao2*, *atpao3* and *atpao4* single mutants, *atpao24* and *atpao34* double mutants, as well as *atpao243* triple mutants (TM) and wild-type (WT) plants were treated with 50 μM ABA for 1 h. Stomatal apertures (width/length) were measured (n>60) and percentages of opening relative to the corresponding untreated controls were calculated. Experiments were repeated 4 times with similar results and mean values from all experiments are presented. Asterisks indicate values statistically different from wild-type plants by one-way ANOVA test ($p<0.001$). Bars indicate standard error.

In an attempt to understand the mechanism by which the peroxisomal *AtPAOs* are involved in the ABA-mediated control of guard cells, the *atpao243* mutant was also analysed for stomata movement following treatment with exogenous PAs. Results evidenced that the common PAs Put, Spd and Spm induce stomata closure both in the triple mutant and the wild-type plants (Fig. 15A). However, the triple mutants appeared less responsive to each one of the three PAs than the wild-type plants (Fig. 15A). Furthermore, the level of PA-mediated stomata closure was similar to the ABA-mediated closure, whereas treatment with both Spd and ABA did not further alter the level of stomata movement in respect to the single treatments (Fig. 15B). These data may suggest that the reduced stomata closure mediated by ABA is not due to the increased Spd levels in the triple mutant. More studies are still necessary to determine the underlying

mechanisms in the different effect of ABA and PAs on stomata closure in the triple mutant and the wild-type plants.

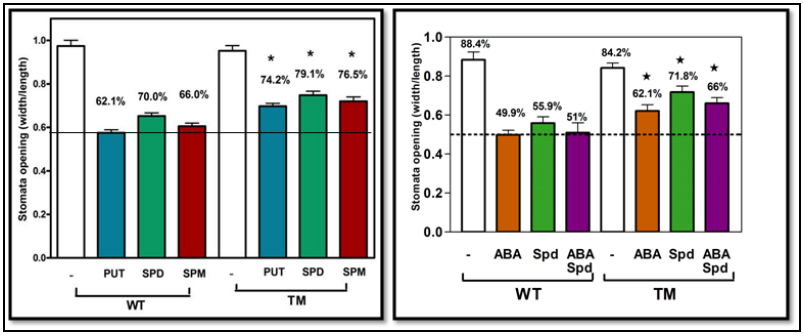

Fig. 15. Stomatal movement in *atpao243* mutant following treatment with exogenous PAs and/or ABA. Seedlings from atpao*243* mutant (TM) and wild-type (WT) plants were treated with 1mM of Put, Spd or Spm and/or 50 µM ABA for 1 h. Width and length of stomata pores were measured and stomatal apertures were expressed as width/length. Numbers in parentheses indicate percentages of opening relative to the corresponding untreated controls. Experiments were repeated 3 times with similar results and representative experiments are shown. Asterisks indicate values statistically different from wild-type plants by one-way ANOVA test ($p<0.001$). Bars indicate standard error.

Water loss in atpao243 triple knockout mutant

Since in terrestrial plants, 95% of water loss occurs through transpiration from stomata (Schroeder *et al.*, 2001) and considering the reduced stomatal closure shown by *atpao243* triple mutants following ABA treatment, experiments were performed to determine the rate of water loss in *atpao243* triple mutants and wild-type plants. However, results showed no apparent difference in water loss rate between the *atpao243* mutant and the wild-type plants (Fig. 16).

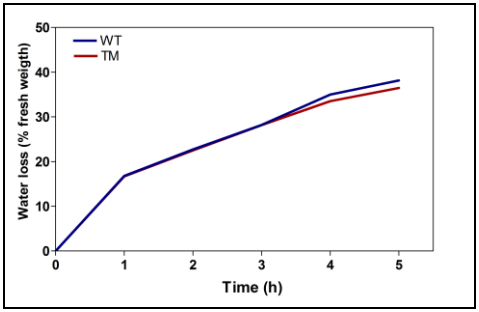

Fig. 16. Rate of water loss in *atpao243 triple* mutant (TM) and wild-type (WT) plants. One month-old plants separated from roots were weighed at various time intervals and the loss of fresh weight (% of initial fresh weight) was used to indicate water loss. Experiment was repeated 3 times (n=6) with similar results and a representative experiment is shown. Error bars are not shown for reason of clarity.

Effect of dehydration on atpao243 triple mutant growth

Since dehydration induces ABA-mediated stomata closure and considering that the *atpao243* mutant plants display reduced stomata closure in response to ABA, the *atpao243* triple mutant was also tested for tolerance/susceptibility to dehydration. In particular, 10-day old seedlings were left for 30 min on dry (dehydration) or wet (control) paper and then left to grow onto ½ MS containing agar plates. Root length was measured every 3 days and expressed as percent of the initial root length. In the absence of dehydration, no significant difference in root elongation was observed between the triple mutant and the wild-type plants (Fig. 17). Conversely, following dehydration, which inhibits root elongation, the triple mutant displayed reduced inhibition in root elongation as compared to the wild-type plants (Fig. 17). These data suggest that the *atpao243* triple mutant is more tolerant to dehydration than the wild-type plants, contrary to what was hypothesized considering the reduced ABA-mediated stomata closure observed in the triple mutant.

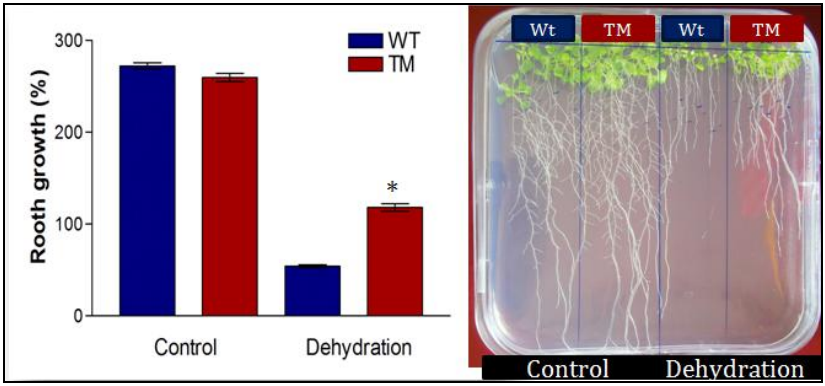

Fig. 17. Root length of *atpao243* triple mutant after dehydration. Triple mutant *atpao243* and wild-type Arabidopsis plants were put on dry (dehydration) or wet (control) paper for 30 min and then transferred on agar plates containing ½ MS medium, 0.5% sucrose. (A) Root length was measured every three days and data collected after 2 weeks for control plants and 3 weeks for dehydrated ones are shown. Values are percentages of root length in respect to initial length. Experiment was repeated 3 times with similar results and a representative experiment is shown. Asterisks indicate values statistically different from the corresponding wild-type plants by one-way ANOVA test ($p<0.001$). Bars indicate standard error. (B) A picture from a representative plate is shown.

Growth of atpao243 triple mutants in the presence of ABA

ABA has also an effect on seedling growth via a combination of limited cell extensibility and inhibited cell division due to arrest at the G1 phase of the cell cycle which may involve protein phosphorylation

In this study, the growth rate of *atpao243* mutant in the presence of the stress-related hormone ABA was also examined. In detail, plants were first grown for one week under physiological conditions and then transferred into medium supplied or not with 5 and 10 μM ABA. In the absence of ABA, no significant difference in seedling growth and root elongation was observed between the triple mutant and the wild-type plants (Fig. 18). In the presence of ABA, seedling growth was inhibited both in triple mutant and the wild-type plant (Fig. 18). However, in the presence of ABA the triple mutant showed increased root elongation comparing to the wild-type plants, while no difference between them in the growth of the aerial part was evident (Fig. 18). These results indicate that the *atpao243* triple mutant is more tolerant to ABA treatment than the wild-type plants and suggest that the *AtPAO2-4* gene family is involved in the ABA-mediated signalling which controls root elongation.

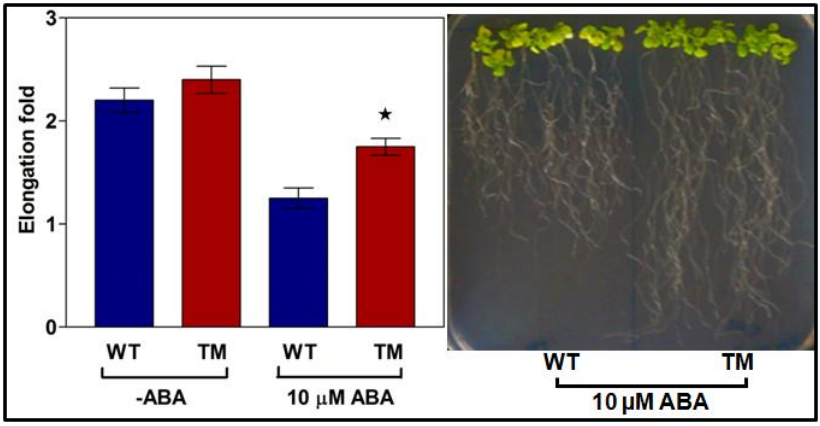

Fig. 18. Root elongation of *atpao243* triple mutant in the presence of ABA. (A) Root elongation (expressed as fold in respect to the initial length) of wild-type plants (WT) and *atpao243* triple mutant (TM) under physiological growth conditions (-ABA) or in the presence of 5 μM and 10 μM ABA is shown. Asterisks indicate values statistically different from the corresponding WT plants by one-way ANOVA test ($p<0.001$). Bars indicate standard error. (B) A picture of a representative plate with plants grown in the presence of 5 μM ABA. Experiments were repeated 5 times with similar results and a representative experiment is shown.

Germination of atpao243 triple mutants in the presence of sucrose

In the present study, the germination rate of the *atpao243* triple mutant was also examined under both physiological and nutrient-starved conditions. In particular, the germination rate was analyzed in plates containing ½ MS salts supplied (physiological condition) or not (nutrient-starved condition) with 2% (w/v) sucrose. Under both growth conditions, the triple mutant presented significantly lower germination rate and delayed growth as compared to the wild-type plants (Fig. 19). Indeed, the number of non germinated *atpao243* seeds was higher than that for the wild-type seeds (Fig. 19). Furthermore, the number of *atpao243* plants with delayed growth was higher than that of the wild-type plants, whereas the number of *atpao243* plants with normal growth was smaller than that of the wild-type plants (Fig. 19). Interestingly, the latter differences were more pronounced in the absence of sucrose than in the presence of sucrose, since seedling establishment of the *atpao243* mutant was notably impaired in the absence of sucrose (the plants with a delayed phenotype prevailing to those with

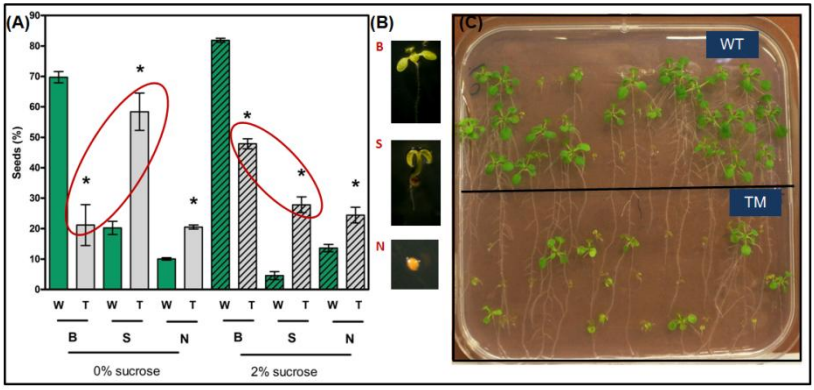

Fig. 19. Germination and growth rate of *atpao243* triple mutant under physiological and nutrient-starved conditions. (A) Percentage of germinated (b and s) and non germinated seeds (n) from *atpao2/atpao34* (T) and wild-type (W) plants one week after sawing is shown. Percentage of plants with normal (b) and delayed (s) growth is also shown. Germination and growth rate was analyzed under physiological (2% sucrose) and nutrient-starved (0% sucrose) conditions. The experiment was repeated 5 times with similar results and a representative experiment is shown. Asterisks indicate values statistically different from the corresponding W plants by one-way ANOVA test ($p<0.001$). Bars indicate standard error. (B) Images of a non germinated seed (n), a plant with delayed growth (s) and a plant with normal growth (b). (C) Photo of a representative plate with seeds germinated in the absence of sucrose.

normal growth), while the number of wild-type plants with delayed growth was always low (Fig. 19). In contrast, germination was not further compromised in the absence of sucrose both for *atpao243* mutant and wild-type plants (Fig. 19). These results indicate that the *atpao243* mutant, but not the wild-type plants, requires sucrose supplementation in the heretotrophic growth phase of the plant. The metabolic alterations in *atpao243* mutant leading to such difference from the wild-type plants are currently under investigation through complementation experiments. One possibility is that *atpao243* mutant is defective in β-alanine and pantothenic acid metabolism, similar to *S cerevisiae* mutants in which the *FMS1* gene encoding for a Spm oxidase is deleted (White *et al*., 2001), and thus in Coenzyme A biosynthesis, fatty acid β-oxidation and utilization of storage lipids for seedling establishment.

Studies on *AtPAO5* physiological roles

AtPAO5 sequence analysis

Since several attempts to functionally express *AtPAO5* in various heterologous systems have been unsuccessful (Fincato *et al*., 2011), *AtPAO5* sequence was analyzed to determine whether *AtPAO5* is indeed a functional *PAO* orthologous. Analysis of the genomic sequence evidenced that *AtPAO5* gene bears no intron differently from the other *AtPAOs* and the *ZmPAO* which have 8 introns (Fincato *et al*., 2011). Furthermore, ClutstalW alignment of several PAO sequences showed that the two regions of high similarity, one near the *N*-termini and the other one near *C*-termini, involved in FAD binding (Wu *et al*., 2003), are highly conserved in AtPAO5 (Fig. 20) indicating that AtPAO5 is indeed a flavoprotein similarly to the other plant and animal PAOs. Furthermore, this sequence alignment evidenced that *AtPAO5* has a higher sequence homology with *MmSMO* and *MmAPAO* (31%) than with *AtPAO1-4* and *ZmPAO* which have low sequence homology (Fig. 20) with the two animal enzymes. The elevated sequence homology of AtPAO5 with MmSMO and MmAPAO extends not only in the FAD-binding domains but also in the catalytic site. In particular, the MmSMO residues of catalytic site His82, Tyr482, Ser527, Thr528 and Lys367 (Tavladoraki *et al*., 2011) are conserved in AtPAO5, (Fig. 20). Among these, His82 has an important role in substrate binding (Tavladoraki *et al*., 2011;Adachi *et al*., 2012; Tormos *et al*., 2012) and is conserved in most PAOs involved in PA back-conversion, as for example MmAPAO, yeast FMS1, AtPAO2, AtPAO3 and AtPAO4 (Fig. 20). By contrast, His82

residue is substituted by a Glu residue in ZmPAO, HvPAO1 and HvPAO2, enzymes involved in the terminal catabolism of PAs.

Most of the amino acid AtPAO5 has differs highly both from the other four AtPAOs and the extracellular ZmPAO. On the other hand, analysis of the AtPAO5 amino acid sequence by PSORT did not reveal the presence of any known targeting sequence to a specific subcellular compartment, thus suggesting cytosolic localization similarly to AtPAO1 (Tavladoraki *et al.*, 2006) but differently from AtPAO2-4 which have a peroxisomal localization (Kamada-Nobusada *et al.*, 2008; Moschou *et al.*, 2008b) and the ZmPAO which has an extracellular localization (Cona *et al.*, 2006; Angelini *et al.*, 2010).

The analysis of the AtPAO5 amino acid sequence also evidenced the presence in AtPAO5 of four additional domains non present in other plant and animal PAOs (Fig. 20). Instead, the MmSMO extra domain involved in nuclear targeting of the μ splicing variant of MmSMO (nuclear domain A; Cervelli *et al.*, 2004; Bianchi *et al.*, 2005) is absent in AtPAO5 (Fig. 20). In AtPAO5, two putative PEST motifs for protein degradation are also present, as shown by sequence analysis using the PESTFIND program (*http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind*). In particular, a domain with high probability to be recognized as a PEST motif is found near the N-terminus of the protein (region 76-96 aa), while a second one with lower probability is found in one of the AtPAO5 extra domains near the C-terminus of the protein (region 420-435 aa; Fig. 20).

Phylogenetic relationship of AtPAO5 with other plant PAOs

A phylogenetic analysis was performed to obtain information about the distribution of AtPAO5 orthologous in the plant taxa. In this analysis all the available amino acid sequences of plant PAOs collected through Blast searches using the amino acid sequences of the five AtPAOs and the ZmPAO sequence were taken into consideration. In particular, ten *Glycine max* PAOs (GmPAOs), five *Hordeum vulgare* PAOs (HvPAOs), four Zea mays PAOs (ZmPAOs), five *Ricinus communis* PAOs (RcPAOs), four *Medicago truncatula* PAOs (MtPAOs), one *Amaranthus hypochondriacus* PAO (AhPAO1), one *Malus domestica* PAO (MdPAO1), one *Nicotiana tabacum* PAO (NtPAO1), one *Physcomitrella patens* PAO (PpPAO1), five *Sorgum bicolor* PAOs (SbPAOs), seven *Vitis vinifera* PAOs (VvPAOs), seven *Populus trichocarpa* PAOs (PtPAOs), one *Brassica juncea* PAO (BjPAO1), nine *Oryza sativa* PAOs (OsPAOs). In addition the sequences of mouse SMO (MmSMO) and APAO (MmAPAO) were included in this study.

Fig. 20. Alignment of amino acid sequences of *AtPAO1*, *AtPAO2*, *AtPAO5*, *ZmPAO1*, *MmSMO* and *MmAPAO*. The alignment was obtained with the program CLUSTALW2. The numbering of amino acid residues is shown on the right. In ZmPAO the peptide signal is underlined, and the numbering starts from the first amino acid residue of the mature protein. The amino acid residues of the catalytic site of ZmPAO, as well as the corresponding residues conserved in the other PAO are marked with red letters while those of MmSMO in violet. Identical amino acids in respect to the sequence of AtPAO5 are highlighted in gray. The red boxes enclose the PEST motifs. The white characters identify the amino acids present in AtPAO5 extra domains (highlighted in blue) and MmSMO (highlighted in green). The sequences for peroxisomal localization of AtPAO2 and MmAPAO are marked in green. The *AtPAO2* was chosen as the representative member of the subfamily *AtPAO2-4*.

This analysis showed that AtPAO5 orthologues are widely distributed among the various plant species (Fig. 21). In particular, this analysis showed that plant PAOs are divided into four clades, as it was also proposed by Ono *et al*., (2012). Clade I includes AtPAO1-like PAOs, Clade II comprises PAOs similar to the extracellular ZmPAO1 involved in PA terminal catabolism, all of them bearing a putative signal peptide for protein targeting to the secretory pathway, Clade III includes the AtPAO5-like PAOs and clade IV includes the AtPAO2,3,4-like PAOs with peroxisomal localization. This analysis evidenced that AtPAO1-like PAOs of clade I are present in all the dicotyledonous plants taken into consideration (except from *A. hypochondriacus*, the genome sequencing of which is not complete) but in none of the monocotyledonous plants (*Z. mays*, *O. sativa*, *H. vulgare* and *S. bicolor*). ZmPAO-like PAOs are present in all the monocotyledous plants analyzed but only in some of the dicotyledonous plants. Indeed, *A. thaliana*, *G. max*, *M. truncatula* and *N. tabacum* seem to miss ZmPAO-like PAOs. Thus, while some dicotyledonous plants have both ZmPAO1-like and AtPAO1-like PAOs others, in particular those belonging to the Brassicacee and Fabacee, have only AtPAO1-like PAOs.

Purification of recombinant AtPAO5 from Arabidopsis transgenic plants

To examine AtPAO5 catalytic activity and since ectopic expression of this protein in heterologous systems (bacteria and yeast) has been previously shown impossible, in the present study AtPAO5 expression in homologous system was attempted. In particular *35S::AtPAO5-6His* Arabidopsis transgenic plants with the sequence of the 6-His tag at the 3'-end of the *AtPAO5* cDNA were obtained. Among the various transgenic lines selected by kanamycin resistance the one with the highest expression levels was identified by semi-quantitative RT-PCR analysis and Western blot analysis (transgenic line #3; Fig. S1, Fig. S2). This transgenic line was used to attempt purification of the recombinant protein through affinity chromatography. In fact, using these plants it became possible to purify a small amount of recombinant AtPAO5 which had indeed PAO activity obtaining a yield of $3x10^{-4}$U/gfw. However, despite an enrichment of about 100–fold (Fig. S2), the purification of the recombinant protein resulted partial, as electrophoretic and spectrophotometric analysis of the final product showed (data not shown). Since various attempts to increase the purity of the recombinant protein were not successful, the enriched AtPAO5 preparations were used to obtain information about substrate specificity, optimum pH and reaction products of the recombinant protein. Furthermore,

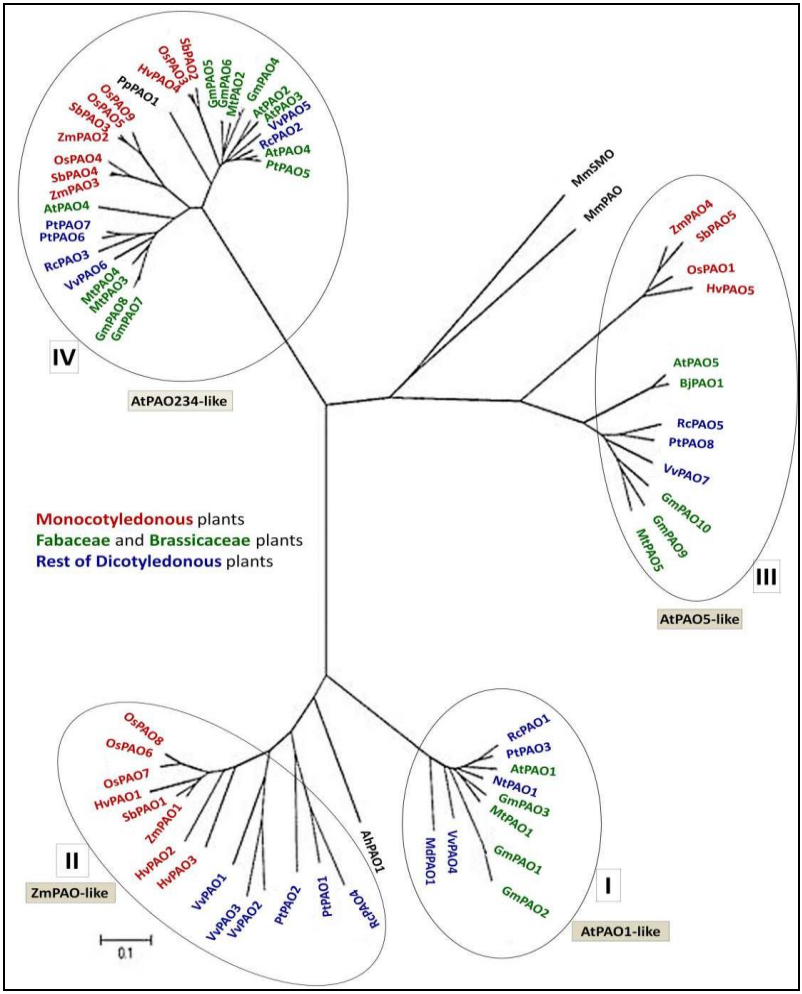

Fig. 21. Phylogenetic relationships of AtPAO5 with other plant PAOs. AtPAO: *Arabidopsis thaliana* PAO; GmPAO: *Glucine max* PAO, HvPAO: *Hordeum vulgare* PAO; ZmPAO: *Zea mays* PAO; RcPAO: *Riccinus communis* PAO; MtPAO: *Medicago truncatula* PAO; AmPAO: *Amaranthus hypochondriacus* PAO; MdPAO: *Malus domestica* PAO; NtPAO1: *Nicotiana tabacum* PAO; PpPAO: *Physcomitrella patens* PAO; SbPAOs: *Sorgum bicolor* PAO; VvPAO: *Vitis vinifera* PAO; PtPAO: *Populus trichocarpa* PAO; BjPAO: *Brassica juncea* PAO; OsPAOs: *Oryza sativa* PAO; MmSMO: *Mus musculus SMO*; MmAPAO; *Mus musculus* APAO. Phylogenetic analysis was performed with MEGA5 software.

a similar purification protocol applied to wild-type plants did not result to a preparation bearing PAO activity, indicating that the PAO activity present in the enriched AtPAO5 preparation was specific of the recombinant protein.

Substrate specificity of recombinant AtPAO5

Enzymatic activity assays evidenced that recombinant AtPAO5 is active towards Spm, N^1-Acetyl-Spm, Therm-Spm, and Nor-Spm (Spm> N^1-Acetyl-Spm>Therm-Spm>Nor-Spm; Fig. 22) with a pH optimum of 8.0. Instead, AtPAO5 is not active towards Spd, similarly to AtPAO1 and MmSMO (Tavladoraki *et al.*, 2006; Fincato *et al.*, 2011). Interestingly, the catalytic activity of AtPAO5 with N^1-Acetyl-Spm is only 50% lower than that with Spm, thus being the only plant PAO until now characterized with a good activity with this PA. This is consistent with the high sequence homology of *AtPAO5* to the animal peroxisomal *PAOs*, which oxidize preferentially, acetylated PAs. As it has been shown, all other characterized plant PAOs have a much lower catalytic activity with the acetylated PAs than with the non acetylated PAs. Indeed, N^1-acetyl-Spm is 5- to 170-fold poorer substrate for AtPAO1-4 (Tavladoraki *et al.*, 2006; Fincato *et al.*, 2011) and a non competitive inhibitor of ZmPAO (Federico *et al.*, 1990).

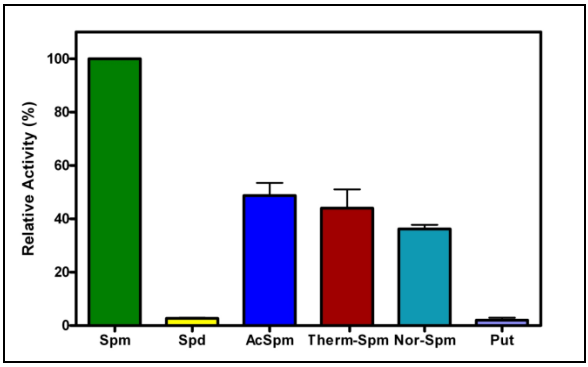

Fig. 22. Substrate specificity of recombinant AtPAO5 partially purified from *35S::AtPAO5-6His* transgenic Arabidopsis plants. The enzymatic activity assays were carried out in Tris-HCl 50 mM at pH 8.0 .and relative activities in respect to the activity with Spm were determined. Purification procedure and catalytic activity determination were repeated three times and mean values are shown. Bars indicate standard error. AcSpm: N^1-Acetyl-Spm.

Reaction products of recombinant AtPAO5

Analysis of the AtPAO5 reaction products using Spm and N^1-acetyl-Spm as substrate performed both by HPLC (Fig. 23) and TLC (Fig. S3) showed the production of Spd from both substrates and not all production of Dap. This indicates that AtPAO5, similarly to the other four AtPAOs, is involved in PA back-conversion, indicating the absence of PAO involved in the terminal catabolism of PAs in Arabidopsis.

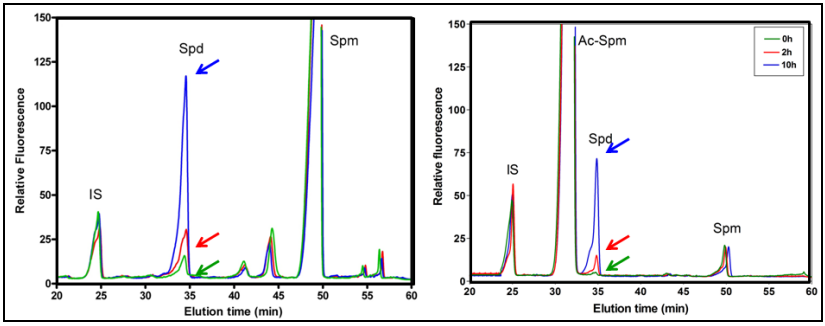

Fig. 23 Analysis of AtPAO5 reaction products from Spm and N^1-acetyl-Spm by HPLC. Recombinant AtPAO5 protein was partially purified from *35S::AtPAO5-6His* transgenic Arabidopsis plants. Reactions were allowed to proceed for 0, 2 and 10 h and reactions products were analyzed by high performance liquid chromatography following dansylation. IS: 1,7 diaminoheptane used as an internal standard.

Subcellular localization of AtPAO5

To confirm the cytosolic localization of AtPAO5, transgenic Arabidopsis plants expressing translational fusions between AtPAO5 and GFP were generated. To rule out mistargeting due to the presence of the reporter, GFP was appended either at the N- or C-terminus of AtPAO5 (*35S::GFP-AtPAO5* and *35S::AtPAO5-GFP* transgenic plants, respectively). The various *35S::GFP-AtPAO5* and *35S::AtPAO5-GFP* transgenic plants were analyzed for expression levels of transgenes by semi-quantitative RT-PCR and Western blot analysis (Fig. S4). Comparative analysis of the best expressors among the various independent *35S::GFP-AtPAO5* and *35S::AtPAO5-GFP* transgenic lines (Fig. S4. Transgenic lines A3 and B6, respectively) showed higher expression levels of the transgene in the *35S::GFP-AtPAO5* transgenic plants as compared to those in the *35S::AtPAO5-GFP* transgenic plants both at the RNA and the protein level. In particular, it was calculated that the *GFP-AtPAO5* transcript level in

transgenic line A3 was 3-fold higher than that of the *AtPAO5-GFP* transcript in the transgenic line B6, out of a 10-fold difference at the corresponding protein levels (Fig. S4), suggesting either a higher translational efficiency or protein stability of the recombinant *GFP-AtPAO5* in respect to the recombinant *AtPAO5-GFP*.

The *35S::GFP-AtPAO5* and *35S::AtPAO5-GFP* transgenic plants were analyzed for the subcellular distribution of the GFP fluorescence using confocal microscopy. Analysis of *35S::AtPAO5-GFP* protoplasts and leaves showed the presence of intracellular GFP-related fluorescent bodies of variable dimension (0.7μm-1.5μm), number (1 to 5) and shape (Fig. 24a, b, e, f). These protein bodies did not present co-localization with chloroplasts and mitochondria (Fig. 25). Furthermore, association of these protein bodies with peroxisomes can be excluded because of their number which was always low, conversely to the high number of peroxisomes present in the plants at the developmental stage in which the confocal analysis of the transgenic plants expressing the AtPAO5 fusion proteins was performed. Furthermore the *AtPAO5-GFP* related protein bodies did not display the characteristic intracellular movement of peroxisomes and often did not present a canonical spherical shape (Mano *et al.*, 2002).

In the case of *35S::GFP-AtPAO5* leaves and protoplasts, a GFP-related fluorescence was observed mainly at the cell periphery (Fig. 24c, d, g, h) . This distribution of the fluorescence may represent *GFP-AtPAO5* localization in the cell wall, plasma membrane, tonoplast or the thin layer of the cytoplasm that is between the plasma membrane and the tonoplast considering that plant cells typically contain a very large vacuole that accounts for most of the cell volume. However, the presence of the *GFP-AtPAO5* related fluorescence in the freshly prepared protoplasts as well as the detachment of the GFP-AtPAO5 fluorescence from the cell wall together with the plasma membrane caused by plasmolysis (Fig. 26) permit to exclude cell-wall localization. Plasma membrane localization can be also excluded since the GFP-AtPAO5 related fluorescence did not completely overlap with the fluorescence of the membrane-specific marker FM4-64 (Fig. 26). Furthermore, the often diffused distribution (Fig. 24c) of the GFP-AtPAO5 fluorescence as well as its presence in cytoplasmic strands (Fig. 24d) excludes localization to the tonoplast and suggests cytosolic localization. This is in agreement with the absence of any known targeting motif as shown by PSORT analysis. Indeed, the distribution of the *GFP-AtPAO5*-related fluorescence resembles that of the free GFP which is detected as a thick, patchy line circumventing the cell as well as in cytoplasmic strands and nuclei (Bloch *et al*., 2005). On the other hand, the

GFP-AtPAO5 fluorescence sometimes accumulated in fluorescent bodies (Fig. 24c) similarly to the AtPAO5-GFP fluorescence.

Effect of the proteasomal inhibitorMG132 on AtPAO5 accumulation

To determine the nature of the protein bodies in which the AtPAO5-GFP and the GFP-AtPAO5 fluorescence are associated and considering that putative PEST motifs for protein target to the proteasomal machinery were identified in the AtPAO5 sequence, *35S::AtPAO5-GFP* and *35S::GFP-AtPAO5* seedlings and protoplasts were treated with the proteasomal inhibitor MG132 and then observed in the confocal microscope (Fig. 27). MG132 is a potent and selective inhibitor of chymotrypsin-like proteolytic activity of the proteasome with no effect on earlier steps of the proteasomal pathway. In this way, MG132 treatment causes an increase in the accumulation levels of the proteins marked for degradation by the proteasome complex (Lee and Goldberg, 1998). Treatment of both leaves and protoplast from *35S::AtPAO5-GFP* transgenic plants with MG132 induced an increase in the number of the cells presenting fluorescent protein bodies. MG132 caused increase in the number of the fluorescent bodies also in the *35S::GFP-AtPAO5* transgenic plants (Fig. 27h) but not in the *35S::GFP* transgenic plants (data not shown). Similar results were obtained using the proteasomal inhibitor MG115 (data not shown) which has the same mode of action as MG132.

The increase in the GFP-AtPAO5 and AtPAO5-GFP protein bodies caused by the two proteasomal inhibitors was further confirmed by Western blot analysis of protein extracts from seedlings treated or not with MG132 (Fig. 28). Indeed, this analysis showed an increase in the amount of the two fusion proteins in the corresponding transgenic plants following treatment with the proteasomal inhibitor as compared the untreated transgenic plants (Fig. 28A). The MG132 treatment induces an increase also in the levels of the AtPAO5-6His recombinant protein in the corresponding *35S::AtPAO5-6His* transgenic plants (Fig. 28B) indicating that the effect of the MG132 on the accumulation levels of the recombinant proteins is AtPAO5-specific and GFP-independent. In contrast, MG132 induced no change in the amount of the corresponding mRNAs (data not shown), thus suggesting that the increase in the levels of the recombinant proteins might be due to protein stabilization. Altogether, these data suggest that AtPAO5 is a cytosolic protein found under the control of the proteasome and that the observed fluorescent protein bodies represent aggregated forms of the protein resulting from the proteasomal pathway.

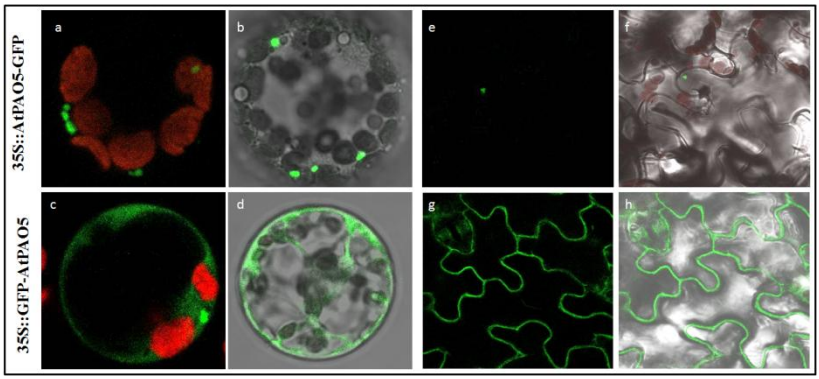

Fig. 24. Subcellular distribution of GFP-AtPAO5 in transgenic plants *35S::GFP-AtPAO5* and *3::AtPAO5-GFP*. Analysis of protoplasts by confocal microscopy (images a, b, c and d) and leaves (images e, f, g and h) of transgenic plants *35S::GFP-AtPAO5* and *35s::AtPAO5-GFP*. The fluorescence of GFP is shown in green. The autofluorescence of the chlorophyll is shown in red.

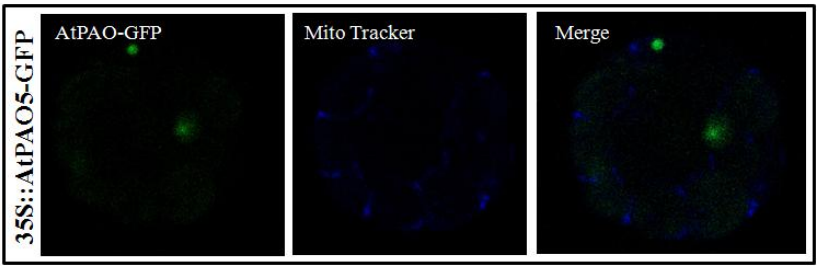

Fig. 25. The AtPAO5-GFP related fluorescent bodies do not co-localize with mitochondta. The GFP fluorescence is shown in green, while that of the specific dye of mitochondria (MitoTraker; Invitrogen) is shown in blue.

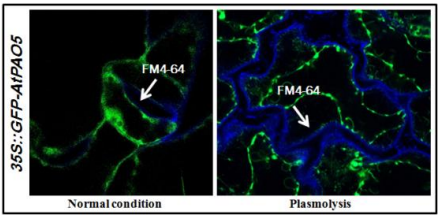

Fig. 26. Colocalization of membrane specific dye FM4-64 and GFP related AtPAO5 protein distribution in transgenic plants *35S::GFP-AtPAO5*. Colocalization under normal and plasmolysis conditions. Fluorescence of GFP is in green while the fluorescence of FM4-64 is in blue.

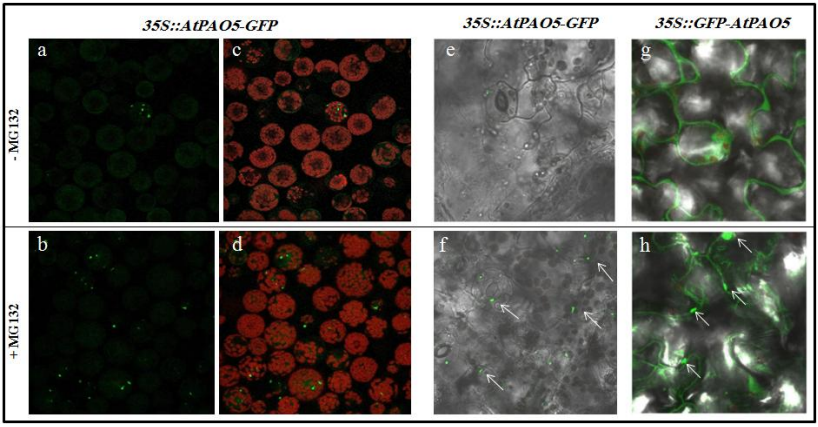

Fig. 27. Effect of proteasome inhibitor MG132 on the distribution of GFP fluorescence in protoplasts and leaves from *35S::AtPAO5-GFP* and *35S::GFP-AtPAO5* transgenic plants. The Protoplasts (A) and leaves (B) were treated with 0.4% DMSO without MG132 (-MG132; images a, c, e and g) with 40 µM MG132 (+ MG132; images b, d, f and h) for 16 hours. Fluorescence of GFP is shown in green while the autofluorescence of chlorophyll is in red in the merged images (c, d, g nd h).

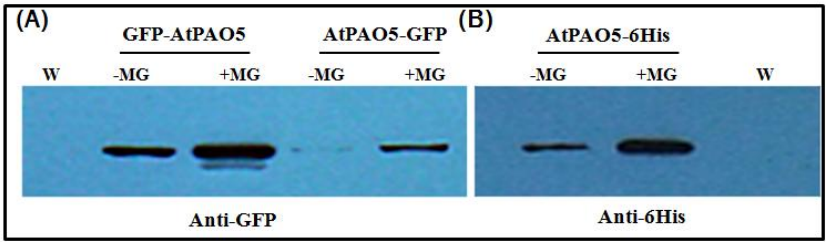

Fig. 28. Effect of proteasome inhibitor MG132 on the accumulation of AtPAO5. Protein extracts of transgenic plants *35::GFP-AtPAO5*, *35S::AtPAO5-GFP* and *35S::AtPAO5-6His* treated with 40 µM MG132 or 0.4% DMSO for 16 hours were analyzed by Western blot*ting* using an anti-GFP (A) or anti-6His (B). W: wild-type Arabidopsis plants. The experiment was repeated three times with similar results.

Polyamine levels in AtPAO5 over-expressing Arabidopsis plants and atpao5 knock out mutant

To determine whether AtPAO5 over-expression in Arabidopsis alters PA homeostasis, the *35S::AtPAO5-6His* transgenic plants were analyzed for PA content by HPLC. In parallel, PA content was determined in a knock out mutant for AtPAO5 (*atpao5*; Fig. 29). In seedlings, the results showed that the *35S::AtPAO5-6His* transgenic plants have reduced levels of Spm and increased levels of Spd as compared to the wild-type plants (Fig. 29A), in agreement with the high catalytic activity of AtPAO5 towards Spm but not towards Spd (Fig. 22). Put levels appeared also increased (Fig. 29A), which may be due to the catalytic activity of the other AtPAOs or to the activation of other homeostatic mechanisms. In the case of the *atpao5* mutant, increased Spm levels and decreased Spd levels were observed in respect to the wild-type plants when leaves and roots were analyzed (Fig. 29B, C), whereas no statistically significant difference was evident when whole seedlings were analyzed (Fig. 29A). Studies are currently in progress to determine the levels of Therm-Spm and N^1-acetyl-Spm in the *35S::AtPAO5-6His* and the *atpao5* plants.

Effect of cytokinins on Arabidopsis root growth and differentiation

Cytokins are plant hormones implicated in several aspects of plant growth and development and in the responses to various biotic and abiotic environmental cues. They also play crucial and pleiotropic roles in vascular development including enhancement of procambial/cambial proliferation and suppression of protoxylem vessel formation (Mähönen *et al*., 2006; Matsumoto-Kitano *et al*., 2008).

Microarray data retrieved from the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi Winter *et al*., 2007) showed that AtPAO5 expression is induced by zeatin. To confirm these data, qRT-PCR experiments were performed and results demonstrated 23- and 2.5-fold increase in the levels of AtPAO5 transcript after treatment for 2h with 1 μM 6-benzylaminopurine (BAP) or zeatin , respectively (Fig. 30A, B).. Interestingly, this increase in the AtPAO5 expression levels was followed by a sharp decrease (Fig. 37).

To determine whether AtPAO5 is involved in cytokin-mediated signaling, the effect of BAP on root growth and differentiation was analysed in *35S::AtPAO5-6His* and *atpao5* plants. Particular attention was given in the xylem differentiation since histochemical analysis of *AtPAO5::GFP-GUS* transgenic plants evidenced *AtPAO5* expression in the vascular system

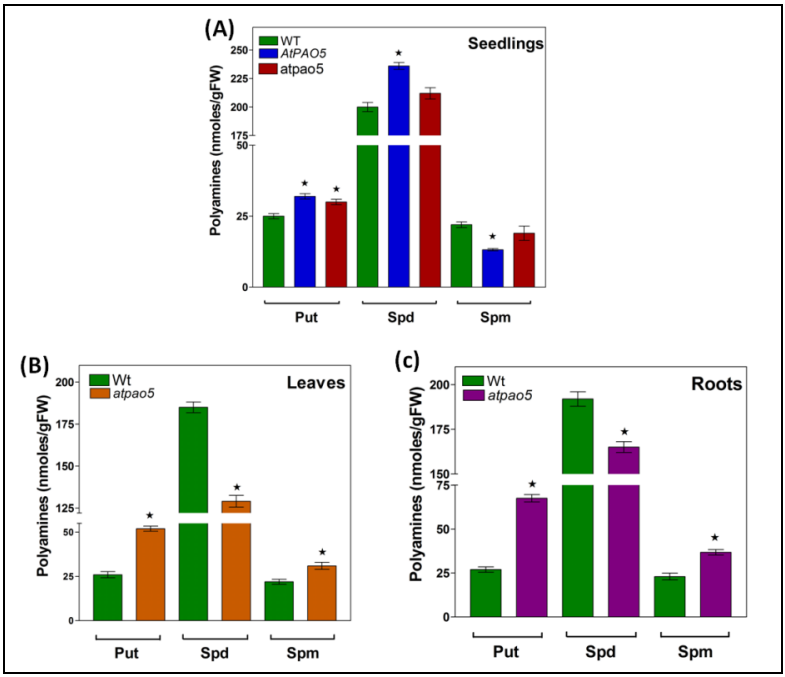

Fig. 29. Polyamine content in *atpao5* mutant and *35S::GFP-AtPAO5* transgenic plants. PA levels were determined in seedlings, roots and leaves of *35S::GFP-AtPAO5*, *atpao5* and wild-type seedlings by HPLC analysis. Experiments were repeated 3 times with similar results. Statistical analysis was performed by one way ANOVA test ($p < 0.001$). Bars indicate standard error.

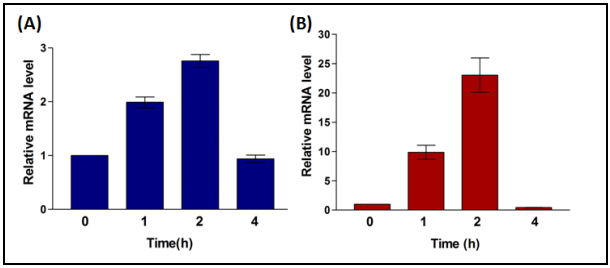

Fig. 30. **Effect of cytokinins on AtPAO5 expression levels.** Fifteen day-old Arabidopsis seedlings were treated with 1 µM 6-benzylaminopurine (BAP) (A) or 1 µM Zeatin. (B) for various time intervals. AtPAO5 expression levels were determined by qRT-PCR using gene-specific primers.The analysis was repeared three times and means values are shown. Error bars represent standard deviation.

of roots, the hypocotyls and stems (Fig. 4s, t, u). In detail, *35S::AtPAO5-6His atpao5* and wild-type plants were first grown vertically in solid ½ MS medium, 0.5% sucrose for one week and then transferred onto a new medium supplied or not with 1 µM BAP. Root length and the distance from the xylem initiation to the quiescent center were measured at various time intervals after transfer (Fig. 31). Results showed that 1 µM BAP impairs both root elongation and xylogenesis. In particular, while BAP inhibits root elongation at the same extent in all three plants, the BAP effect on xylem initiation resulted reduced in the *35S::AtPAO5-6His* plants and increased in the *atpao5* plants (Fig. 31). Similar results were obtained following treatment with zeatin (data not shown). These results suggest that AtPAO5 is involved in cytokin-mediated xylogenesis. Experiments are still necessary to make clear the underlying mechanisms.

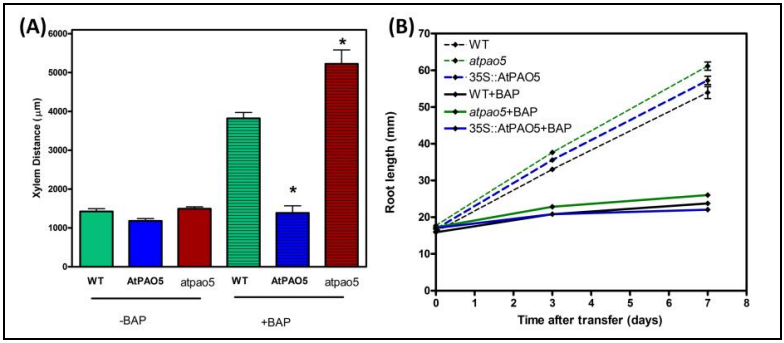

Fig. 31. Effect of 6-benzylaminopurine (BAP) on root growth and root vascular differentiation in *35S::AtPAO5-6His* transgenic plants and *atpao5* mutants. The distance of the first protoxylem cells with secondary cell-wall thichening from the quiescent center (xylem distance) was measured under confocal microscopy after 7 days of treatment or not with 1µM BAP of 7 day-old plants initially grown under physiological conditions (A). Root elongation of the same plants was also measured at various time intervals (B). Asterisks indicate values statistically different from wild-type plants by one-way ANOVA test ($p<0.001$).

DISCUSSION

PA levels in the *atpao* mutants and *AtPAO* over-expressing plants

In the *atpao1* mutant no difference in Put, Spd, Spm and Therm-Spm levels was observed which may indicate that AtPAO1 does not contribute in homeostasis of the bulk amount of PAs. This may due to the low expression levels of AtPAO1 (as it was observed by RT-PCR analysis of whole seedlings; data not shown) and to the highly tissue-specific expression pattern (meristematic/elongation transition zone of the roots, shoot apical meristem and anther tapetal cells (Fig. 4, 5; Fincato *et al*., 2012). It is also possible that Therm-Spm is not the physiological substrate of this enzyme. However, gene redundancy cannot be excluded and in this regard it is important to determine PA levels in the *atpao1/atpao5* double mutant, which has been very recently obtained and is currently under investigation. This is because both AtPAO1 and AtPAO5 are cytosolic enzymes which oxidize Spm and Therm-Spm. Furthermore, it has also to be taken into consideration that AtPAO1 oxidizes bound forms of PAs.

The *atpao243* triple mutant presents increased levels of Spd as compared the wild-type plants, whereas no change in PA levels was observed in the corresponding single or double mutants (Fig. 13). These data indicate that the three peroxisomal AtPAOs act redundantly in controlling PA homeostasis.

Analysis of the free PA levels in the *35S::AtPAO5-6His* and *atpao5* plants evidenced altered levels in respect to the wild-type plants (Fig. 29). These data indicate that both the native and the recombinant AtPAO5 proteins are functionally expressed to control PA homeostasis, despite the fact that AtPAO5 is under the control of the proteasome.

AtPAO physiological roles

The physiological studies undergone so far on AtPAO1 using the *atpao1* mutant did not permit to evidence any metabolic pathway in which AtPAO1 is involved. Since this may be due to gene redundancy, physiological studies on the double *atpao1/atpao5* double mutant are in progress. Furthermore, to avoid the activation of adaptive mechanisms which may hide physiological alterations in the *atpao1* mutant, different experimental strategies are going to be adopted, such as inducible gene-silencing or

inducible over-expression for *AtPAO1* gene. Studies on the regulatory mechanisms controlling AtPAO1 expression levels may contribute to determine the AtPAO1 physiological roles. On other hand, more physiological processes have still to be examined using the *atpao1* single mutant.

The physiological studies on the *atpao2*, *atpao3* and *atpao4* single, double and triple mutants showed that the three peroxisomal AtPAOs are involved in the ABA-mediated control of stomata closure. Indeed, it has been shown that following treatment with ABA, the various mutants for *AtPAO2-4* gene family display a reduced stomata closure as compared to the wild-type plants. However, the fact that *atpao*243 triple mutant is not completely impaired in ABA-mediated stomata closure suggests that also other enzymes are involved in this very important process for plant survival. Indeed, in *A. thaliana* two plasma membrane-associated NADPH oxidases (AtrbohD and AtrbohF) and a CuAO have been shown to be implicated in ABA-induced stomatal closure in Arabidopsis and *Vicia faba*, respectively (Kwak *et al*., 2003; An *et al*., 2008). Further studies are necessary to determine the mechanism(s) through which the *AtPAO2-4* gene family is involved in the control of stomata movement. A role through H_2O_2 production may be proposed since reactive oxygen species (ROS) are important second messengers in the ABA signaling network. However, a role of the peroxisomal AtPAOs in the ABA-inducible modulation of stomata movement through regulation of PA levels is also possible. Indeed, it has been shown that PAs modulate stomata aperture through a direct effect on the voltage-dependent inward K^+ channels in the plasma membrane of the guard cells (Liu *et al*., 2000).

The triple mutant appears more tolerant to the ABA-mediated inhibition of root elongation than the wild-type plants. These data, together with the reduced ABA-mediated stomata closure as compared to the wild-type plants, indicate that the *atpao243* triple mutant displays reduced responsiveness to ABA and suggest that the *AtPAO2-4* gene family is involved in a general ABA signaling network. However, it is difficult to explain the higher tolerance of the triple mutant to dehydration, considering that dehydration induces ABA-mediated stomata closure to protect plant from water loss a process which is impaired in the triple mutant. It is possible that the dehydration-induced inhibition of root growth is independent of stomata opening. It may be due to a direct effect of endogenous ABA on root growth, similarly to the effect of exogenous ABA, thus explaining the increased root elongation in the *atpao243* mutant which appears less responsive to ABA.

The results presented here indicate that the *atpao243* mutant displays a delay in seedling establishment as compared to the wild-type plants, a delay which becomes more pronounced in the absence of sucrose. The metabolic alterations in *atpao243* mutant leading to such difference from the wild-type plants are not known yet. One possibility is that *atpao243* mutant is defective in β-alanine and pantothenic acid metabolism, similarly to *S. cerevisiae* mutants in which the *FMS1* gene encoding for a Spm oxidase is deleted (White *et al*., 2001). Impairement in pantothenic acid biosynthesis compromises CoA biosynthesis, fatty acid β-oxidation and thus utilization of storage lipids for seedling establishment in oilseed plants, such as Arabidopsis (Rubio *et al*., 2006). Experiments are currently in progress to verify this hypothesis which may be of great interest taking into account that CoA biosynthesis appears to be a sensitive step in plants under salt stress (Yonamine *et al*., 2004). Indeed, an improved knowledge of this pathway might help our understanding of how plants cope with abiotic stresses.

AtPAO5 expression is induced by the cytokinins zeatin and BAP. Furthermore, it was shown that ectopic expression of *AtPAO5* reduced the BAP effect on root xylem differentiation, but not on root length. Conversely, knock-out mutation of *AtPAO5* increased the BAP effect on xylem differentiation. These results suggest that AtPAO5 is involved in the cytokinin-mediated control of xylogenesis, acting as a negative regulator. Several aspects of the underlying mechanisms are still unclear and experiments are in progress to answer important questions raised from the present study. As for example, how constitutive expression of *AtPAO5* interferes with a BAP-inducible effect on a specifcc developmental program, such as xylem differentiation? Do cytokinins affect AtPAO5 mRNA and/or protein stability? Is AtPAO5 involved in other BAP-mediated signalling pathways?

AtPAO5 substrate specificity, reaction products and post-translational regulation

The recent characterization of the catalytic properties of four out of five AtPAOs (AtPAO1, AtPAO2, AtPAO3 and AtPAO4) evidenced important differences in substrate specificity among them (Tavladoraki *et al*., 2006; Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008b; Fincato *et al*., 2011). It has been also demonstrated that these four AtPAOs are involved in PA back-conversion, differently from the other plant PAOs so far characterized (i.e. the extracellular PAOs from monocotyledonous plants) which are involved in the terminal catabolism of PAs but similarly to the

animal orthologues. In the present study, after several unsuccessful attempts in the past, it became possible to partially characterize recombinant AtPAO5. In particular, in this study recombinant AtPAO5 was expressed in a homologous system (i.e. in *A. thaliana* plants) under the control of a strong and constitutive promoter. Following partially purification it was shown that recombinant AtPAO5 oxidizes Spm, but not Spd, similarly to AtPAO1 and MmSMO (Cervelli *et al*., 2003; Tavladoraki *et al*., 2006). Recombinant AtPAO5 presents activity also towards N^1-acetyl-Spm differently from the other plant PAOs so far characterized. Analysis of acetylated PAs in the *35S::AtPAO5-6His* and *35S::GFP-AtPAO5* transgenic plants as well as in the *atpao5* mutant will permit to determine whether N^1-acetyl-Spm is the physiological substrate of this enzyme and to study the physiological role of the acetylated PAs in plants, which is completely unknown due to the fact that no information exists for the plant enzymes involved in their metabolism. Recombinant AtPAO5 is also active with Therm-Spm, similarly to AtPAO1. This is important considering that Therm-Spm has an important role in vascular system development in Arabidopsis (Clay and Nelson, 2005; Kakehi *et al*., 2008; Kakehi *et al*., 2010; Rambla *et al*., 2010) and AtPAO5 is specifically expressed in the vascular system of the roots, the hypocotyls and the stem (Fincato *et al*., 2012). Determination of Therm-Spm levels in *35S::AtPAO5-6His*, and *atpao5* plants may permit to make clear whether AtPAO5 is indeed involved in Therm-Spm metabolism.

Analysis of the AtPAO5 reaction products showed that AtPAO5, similarly to the other four AtPAOs, is involved in PA back-conversion, thus indicating that no PAO involved in the terminal catabolism of PAs is present in Arabidopsis, differently from maize, barley and rice plants in which both the PA back-conversion pathway and the terminal catabolic pathway are present (Fig. 21; Fincato *et al*., 2011; Ono *et al*., 2012; Tavladoraki *et al*., 2012). Further studies are necessary for a detailed understanding of the specific physiological roles of these two PA catabolic pathways in plants.

Data from confocal and Western blot analyses of *35S::GFP-AtPAO5*, *35S::AtPAO5-GFP* and *35S::AtPAO5-6His* transgenic plants treated with the proteasomal inhibitor MG132 suggest that AtPAO5 is a cytosolic protein regulated at the post-translational levels by proteasome. This is the first time that such a regulatory mechanism is proposed for a PAO. As regard the PA metabolic pathways, a similar regulatory mechanism has been shown only for animal ODC (Pegg, 2006) and SSAT (Coleman and Pegg, 2001). The physiological relevance of the proteasome-mediated regulation of AtPAO5 expression has still to be determined. However, this

finding suggests an important physiological role for AtPAO5 taking into consideration that the post-translational control is a characteristic of proteins that carry out critical cellular functions. It is likely that a cellular rapid-response system is in place that limits either polyamine excess or insufficiency and controls changes in availability in response to demand.

In conclusion, the data presented herein suggest specific physiological roles for the various members of the *A. thaliana* PAO gene family, in line with previous results from biochemical and expression studies (Fincato *et al*., 2011; Fincato *et al*., 2012).

This study gives a further contribution towards understanding the involvement of the various PA catabolic pathways in plant development and defense responses which may permit the application of biotechnological strategies to transfer increased yield and stress-tolerance traits to crops of agronomical relevance.

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SUPPLEMENTARY DATA

Primer	Sequence
AtPAO4 Real for	**5'- CTTCTGTTATTGTGATTGTAGTGG**
AtPAO4 Real rev	**5'-GTATAATGGGAGCTAAGGGATTCT**
AtPAO5 Real for	**5'- GAGAGTGAGTATCAGATGTTTCCAG**
*AtPAO5*Real rev	**5'- AGCACACCTAAAGAGACAGTAACAA**
Over*AtPAO5*-His for	**5'-ATGGCGAAGAAAGCAAGAATTGTTATAATCG**
Ove*AtPAO5*-His rev	**5'-CTAGTGGTGGTGGTGGTGGTGTCCTCC**
*AtPAO5*His rev	**5'-CTAGTGGTGGTGGTGGTGGTGTCCTCCAAAATTAC**
ADC1 for	**5'- TCTTAAACACCGAGCCGAGGAG**
ADC1 rev	**5'- GCCACCAGCTGCAGCTTCATC**
ADC2 for	**5'- TGGACAAGAATGGCAGTGGAGG**
ADC2 Rev	**5'- GCTCATGCTGCATTGCTCGGA**
SAMDC For	**5'- CAGCTGGTGACAAGGGTTCTCTC**
SAMDC Rev	**5'- GGCGATCCACAGTACTTACCAAGC**
UBQ5 for	**5'- GGA AGA AGA AGA CTT ACA CC**
UBQ5 rev	**5'- AGT CCA CAC TTA CCA CAG TA**
UBC21 for	**5'- CTGCGACTCAGGGAATCTTCTAA**
UBC21 rev	**5'- TTGTGCCATTGAATTGAACCC**
*AtPAO1*for	**5'-CTCACATGGAAATATCTTGGACTAC**
*AtPAO1*rev	**5'-AGAGAAGATCAGATTGGAGAACAC**
*AtPAO2*for5	**5'-GAAGTCTCTCTGTTTTCATCTTTCTCTCTC**
*AtPAO2*rev5	**5'-CGAAATCGAAGATGAAAAAATCAGGC**
*AtPAO3*for5	**5'-CTGTGGAAGAAACAGATAAGAGAAAATCGCA**
*AtPAO3*rev5	**5'-GCGATAAAGGATCTAACGATTTCTGCTC**
*AtPAO4*for5	**5'-GATAAGAAGAAGAATTCGTTTCCAGATAATC**
*AtPAO4*rev5	**5'-TTCCTTGAAGAACAGACATGTCATTGGCAGTT**
AtPAO1-LP1	**5'-GAACGACCAAAAAGATGCATC**
AtPAO1-RP1	**5'-TACCGCCTCAAACTAAACCAG**
AtPAO 2-LP2	**5'-GCACTTTTGCAAGCTTGGTTTC**
AtPAO2-RP2	**5'-CTAGTAAACGATAACTGATGGAGT**
AtPAO3-RP3bcd	**5'-GTTAATTGAAACATTCACAGGAAGAACTG**
AtPAO3-LP3 bcd	**5'-CTAACAATGTTCCCATATGGAGGTG**
AtPAO4-LP4	**5'-ATGTCACTGTTCCCCATAAGAG**
AtPAO4-RP4	**5' GGATGGAGGCGTGGTTTGCTG**
AtPAO5-LP5	**5'-GTGGAAGCCCTGTTTATAGAATCG**
AtPAO5-RP5	**5'-GCTTCACTTCATTACTCTGCCAC**
LBa1	**5'-GATGGTTCACGTAGTGGGCCATCGC**
Lb2	**5'-CTTCCTATTATATCTTCCCAAATTAC**
LB1-pAC161	**5'-CGATCGTGAAGTTTCTCATCTAAGCC**
AtPAO5-loc-for1	**5'-ATGGCGAAGAAAGCAAGAATTGTTATAATC**
AtPAO5-locA-rev1	**5'- TCA AAA ATT ACA TTT GTA ATG CTT GAG AAG**
EGFP for	**5'- GGTGAGCAAGGGCGAGGAGCTGTTC**
EGFP rev	**5'- GTCGTCCTTGAAGAAGATGGTGCGCTC**

Table S1. Sequences of primers used for T-DNA insertional mutant analysis, AtPAO4 promoter as well as for semi-quantitative and quantitative RT-PCR analyses.

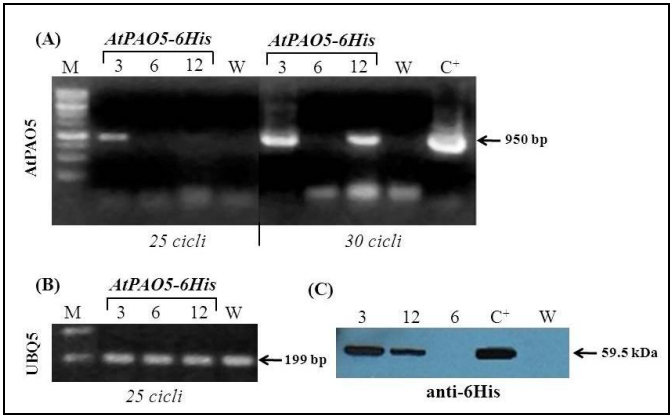

Fig. S1. *AtPAO5* expression analysis in transgenic plants *35S::AtPAO5-6His*. Semiquantitative RT-PCR (A and B) and Western blot analysis (C) evidenced the expression of recombinant AtPAO5 in three independent transgenic lines (lines 3, 6 and 12). (A) Total RNA extracted from rosette leaves of *35S::AtPAO5-6His* plants was analyzed by semiquantitative RT-PCR using the oligonucleotides PAO5 Real for and OverAtPAO5-His rev, specific for the AtPAO5 and the 6-His sequence, respectively. C+: PCR from plasmid *35S::AtPAO5-6His*-pK2GW7. (B) Semiquantitative RT-PCR analysis performed with ubiquitin (UBQ5)-specific oligonucleotides as a loading control. M: molecular marker (1 kb DNA Gene RulerTM Ladder, Fermentas). (C) Protein extracts from leaves were analyzed by Western blotting using an anti-6His antibody. C^+: Recombinant ZmPAO-6His (Polticelli *et al.*, 2005), W: wild-type Arabidopsis plants.

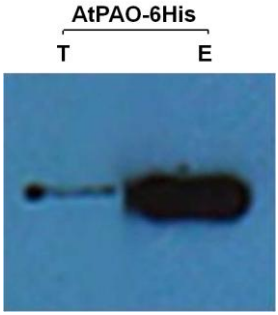

Fig. S2. Western blot Analysis of recombinant AtPAO5 purified from transgenic Arabidopsis plants. Recombinant AtPAO5-6His proteins were purified from transgenic plants *35S :: AtPAO5-6His* (line 3) through affinity chromatography. T: Total extract of proteins from transgenic *35S :: AtPAO5-6His*, E: elution fraction of the affinity column. Anti-6His was used as antibody.

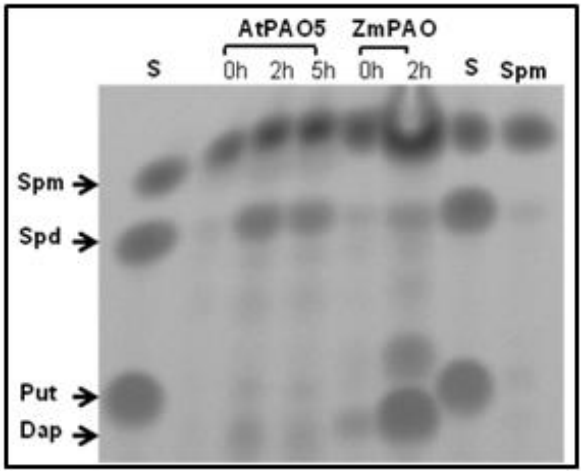

Fig. S3. Analysis of AtPAO5 reaction products from Spm by TLC. Enzymatic reactions for were stopped at 0h, 2h and 5h with 5% PCA and products were analysed by thin layer chromatography following dansylation. ZmPAO reaction products were also analysed in parallel as a control. S: Mixture of Spm, Spd and Put.

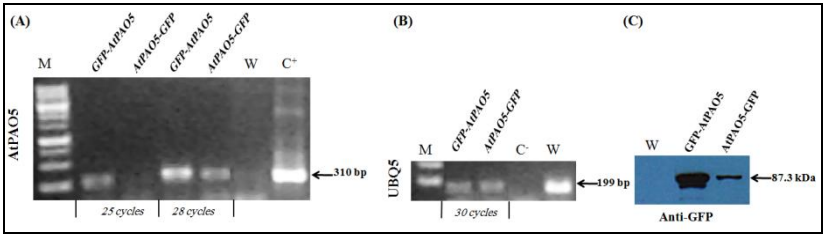

Fig. S4. Expression analysis of AtPAO5 in *35S::AtPAO5-GFP* and *35S::GFP-AtPAO5* transgenic plants. Semiquantitative RT-PCR (A and B) and Western blot analysis (C) of *35S::GFP-AtPAO5* (line A3) and *35S::GFP-AtPAO5* (line B6) transgenic plant. (A) Total RNA extracted from rosette leaves of *35S::GFP-AtPAO5* and *35S::GFP-AtPAO5* transgenic plants was analyzed by semiquantitative RT-PCR using GFP-specific oligonucleotides (EGFPfor/EGFPrev). (B) Semiquantitative RT-PCR analysis performed with UBQ5-specific oligonucleotides as a loading control C+: PCR from plasmid *35S::GFP-AtPAO5-pK7WGF2*; C^-: negative control of the PCR reaction. (C) Protein extracts from rosette leaves were analyzed by Western blot using an anti-GFP antibody. W: RNA or protein extract derived from wild-type plants. M: molecular marker (1 kb DNA Gene RulerTM Ladder, Fermentas).