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Advances in the involvement of polyamine oxidases in *Arabidopsis thaliana* cellular metabolism

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Index

ABBREVIATIONS

ABA	Abscisic acid
ACL5-ACAULIS5	Thermospermine synthase
ADC	Arginine decarboxylase
Agm	Agmatine
Arg	Arginine
ATHB8	ARABIDOPSIS THALIANA HOMEOBOX8
b-HLH	Basic helix-loop-helix
BAP	6-benzylaminopurine
Cad	Cadaverine
CuAO	Copper-containing amine oxidases
Dap	1,3diaminopropane
DMTU	*N*,*N*^{1}-dimethylthiourea
EDTA	Ethylenediaminetetraacetic acid
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride
MES	2-(N-morpholino)ethanesulfonic acid
$MgCl_2$	Magnesium chloride
MmAPAO	Murine APAO
MmSMO	Murine SMO
Nor-Spd	Norspermidine
Nor-Spm	Norspermine
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
PASAC51	Polyamine
PCD	Programmed cell death
PHB	PHABULOSA
PIN1	PIN-FORMED1
Put	Putrescine
ROS	Reactive oxygen species
SAC51	SUPPRESSOR OF ACAULIS 5
SAM	S-adenosyl methionine
SAMDC	S-adenosylmethionine decarboxylase
SMO	Spermine oxidase

Spd	Spermidine
SPDS	Spermidine synthase
SPMS	Spermine synthase
SSAT	Spd/Spm N^1-acetyltransferase
Therm-Spm	Thermospermine
ZmPAO	*Zea mays* polyamine oxidase

ABSTRACT

In plants, the polyamines putrescine, spermidine (Spd), spermine (Spm) and thermospermine (Therm-Spm) are involved in the regulation of several cellular processes, such cell proliferation and differentiation, programmed cell-death, and defense responses. In particular, Therm-Spm, a structural isomer of Spm, is important for normal growth and development, since it is involved in the control of xylem differentiation having an auxin antagonizing effect.

In *Arabidopsis thaliana*, five polyamines oxidases (AtPAO1–AtPAO5) are present involved in polyamine catabolism. AtPAO1 and AtPAO5 are cytosolic enzymes catalyzing the back-conversion of Spm and Therm-Spm to Spd. AtPAO5 is also able to oxidize N^1-acetyl-Spm. Conversely, the other three members of the Arabidopsis PAO family, AtPAO2, AtPAO3 and AtPAO4, have a peroxisomal localization and are able to oxidize both Spd and Spm, but not Therm-Spm. Furthermore, based on the high sequence homology and the similar gene structure the three peroxisomal *AtPAOs* are considered recent derivatives of a common ancestor gene and thus to form a distinct subfamily. Studies on the tissue- and organ-specific expression pattern of the five *AtPAOs* using *AtPAO::GFP-GUS* Arabidopsis transgenic plants, showed common, but also distinct expression patterns for each one of the five *AtPAOs*. Characteristic expression patterns of *AtPAO1* is the transition region between the meristematic and the elongation zone of the root and the anther tapetum, of *AtPAO2*, *AtPAO3* and *AtPAO4* the guard cells and pollen grains, while of *AtPAO5* the vascular tissue and the anther tapetum.

In the present study, quantitative Real-time RT-PCR (qRT-PCR) analysis evidenced that *AtPAO1*, *AtPAO2* and *AtPAO3* expression levels are modulated by the stress related hormones abscisic acid (ABA) and methyl Jasmonate (MeJA), as well as by stress conditions (NaCl treatment and drought), while on the contrary, the expression levels of *AtPAO4* and AtPAO5 remain invariable under these conditions.

Recent studies have shown that *atpao2*, *atpao3* and *atpao4* insertional mutants display reduced stomatal closure, as compared to wild-type plants, in response to ABA. Interestingly, the reduced stomata closure observed in the single mutants was even more pronounced in the double *atpao2atpao4* and *atpao3atpao4* mutants and the triple *atpao2atpao3atpao4* (*atpao234)* mutants, the last one presenting the highest variation in stomata movement in respect to the wild-type plants. This suggests the involvement of all three peroxisomal *AtPAOs* in the ABA-mediated control of guard

cells in a synergistic way. Here, further studies were performed using the *atpo234* triple mutant and *AtPAO3* over-expressing Arabidopsis transgenic plants (*AtPAO3* transgenic plants) to better understand the contribution of the *AtPAO234* gene subfamily in the control of stomata movement. It was shown that *atpao234* triple mutant, but not the *AtPAO3* transgenic plants, displays reduced stomata closure not only in response to ABA but also to MeJA, H_2O_2 and polyamines, as compared the wild-type plants. The underlying mechanisms in the reduced stomata closure exhibited by the *atpao234* plants are not clear so far. However, reduced production of H_2O_2, which is an important second messenger in the ABA signaling network, as well as variations in polyamine levels, which play an important role in the modulation stomata movement, probably do not contribute to this effect. Noteworthy, the reduced responsiveness of the *atpao234* triple mutant to the stress-related stimuli leading to stomata closure, which should contribute to reduce water loss through transpiration is not accompanied by increased tolerance to abiotic stress conditions. The lack of such a correlation has still to be analyzed.

It was recently shown that AtPAO5 is involved in the control of plant growth. Indeed, two *atpao5* mutants and an *AtPAO5* over-expressing Arabidopsis transgenic lines (*AtPAO5* transgenic plants) present developmental differences from the wild-type plants. In particular, the *atpao5* mutants produce longer and thicker flowering stems, while the *AtPAO5* plants produce thinner and shorter stems as compared to the wild-type plants. These phenotypical alterations of *atpao5* mutants and *AtPAO5* transgenic plants were attributed to *AtPAO5*-mediated changes in Therm-Spm homeostasis and are accompanied by changes in the expression level of genes involved in auxin and cytokinin signaling, suggesting that AtPAO5 interferes with cytokinin and auxin signaling pathways. In the present study, to further investigate on the auxin signaling in the *AtPAO5* and *atpao5* plants, sexual crossings of these plants with *DR5::GUS* transgenic plants, *DR5* being an artificial auxin–regulated promoter, were performed and analyzed. It was shown that the *AtPAO5* present increased *DR5::GUS* expression levels in the root meristematic region, whereas *atpao5* decreased as compared the *DR5::GUS* expression levels in wild-type roots. It was further shown that the *AtPAO5* and *atpao5* plants present differences from wild-type plants regarding xylem differentiation throughout the entire plant, with *AtPAO5* plants presenting an increased number of xylem vessel elements and *atpao5* mutants a decreased number compared with wild-type plants. Furthermore, it was shown that the *atpao5* plants exhibit increased

tolerance to salt stress and drought, as well as decreased stomata closure in response to stress-related conditions.

In conclusion, the presented data evidence an important role of the three peroxisomal AtPAOs in the control of stress-mediated stomata closure. It was also shown a tightly controlled interplay between *AtPAO5*, Therm-Spm, auxin and cytokinins necessary for proper xylem differentiation and plant growth. *AtPAO5* most probably contributes to this regulatory network participating in the feedback mechanisms which control Therm-Spm levels. On the other hand, *AtPAO5* participates in the control of stomata closure and plant tolerance to salt and drought stress.

RIASSUNTO

Nelle piante, le poliammine putrescina, spermidina (Spd), spermina (Spm) e termospermina (Therm-Spm) sono coinvolte nella regolazione di diversi processi cellulari, come la proliferazione e il differenziamento cellulare, la morte cellulare programmata e le reazioni di difesa. In particolar modo, la Therm-Spm, un isomero strutturale della Spm, è importante per la crescita e lo sviluppo delle piante, in quanto è implicata nel controllo del differenziamento dello xilema avendo un effetto antagonista all'auxina

In *Arabidopsis thaliana*, sono presenti cinque poliamminossidasi (AtPAO1-AtPAO5) coinvolte nel catabolismo delle poliammine. AtPAO1 e AtPAO5 sono enzimi citosolici che catalizzano la conversione di Spm e Therm-Spm a Spd. AtPAO5 è anche in grado di ossidare l'N^1-acetil-Spm. Al contrario, gli altri tre membri della famiglia di PAO di Arabidopsis, AtPAO2, AtPAO3 e AtPAO4, hanno una localizzazione perossisomale e sono in grado di ossidare sia Spd che Spm, ma non Therm-Spm. Inoltre, sulla base della forte omologia di sequenza e della simile struttura genica, le tre PAO perossisomali sembra derivino da un comune gene ancestrale e per questo vengono considerate membri di una distinta sottofamiglia. Studi d'analisi di espressione tessuto-specifica delle cinque *AtPAO* che si sono avvalsi dell'uso di piante transgeniche *AtPAO :: GFP-GUS* di Arabidopsis hanno mostrato simili e anche distinti *pattern* d'espressione per ciascuna delle cinque *AtPAOs*. I *pattern* di espressione caratteristici di *AtPAO1* sono la regione di transizione tra la zona meristematica e la zona di allungamento della radice e il tappeto nelle antere, per le *AtPAO2*, *AtPAO3* e *AtPAO4* le cellule di guardia e i granuli pollinici e per l' *AtPAO5* il tessuto vascolare e il tappeto nelle antere.

Nel presente studio, l'analisi quantitativa RT-PCR (qRT-PCR) ha evidenziato che i livelli di espressione di *AtPAO1*, *AtPAO2* e *AtPAO3* sono regolati dagli ormoni acido abscissico (ABA) e metil giasmonato (MeJA), come anche dallo stress salino e idrico. Contrariamente, i livelli di espressione di AtPAO4 e AtPAO5, rimangono invariati sotto queste condizioni.

Recenti studi hanno dimostrato che mutanti inserzionali di *atpao2*, *atpao3*, e *atpao4* mostrano una riduzione della chiusura stomatica rispetto alle piante *wild-type* in risposta al trattamento con ABA. È interessante notare che la riduzione della chiusura stomatica osservata nei singoli mutanti è ancor più pronunciata nei doppi mutanti *atpao2atpao4* e *atpao3atpao4* e nel triplo mutante *atpao2atpao3atpao4* (*atpao234*),

presentando quest'ultimo un tasso di apertura stomatica più elevato rispetto alle piante *wild-type*. Ciò suggerisce un coinvolgimento di tutte e tre le PAO perossisomali nel controllo ABA-mediato delle cellule di guardia in modo sinergico. Nel presente lavoro, sono stati effettuati ulteriori studi per comprendere meglio il contributo della sottofamiglia delle *PAO* perossisomali nel controllo del movimento stomatico utilizzando il triplo mutante *atpao234* e piante transgeniche che sovraesprimono *AtPAO3* (piante transgeniche *AtPAO3*). È stato dimostrato che il triplo mutante *atpao234*, ma non le piante transgeniche *AtPAO3*, presenta una riduzione della chiusura stomatica rispetto alle piante *wild-type* non solo in risposta all'ABA ma anche a MeJA, il H_2O_2 e al trattamento con le poliammine.I meccanismi delle ridotta chiusura stomatica mostrati dal mutante *atpao234* non sono ancora chiari. Tuttavia, una ridotta produzione di H_2O_2, che è un importante messaggero secondario nella via di segnalazione dell'ABA, nonché le variazioni dei livelli delle poliammine, che svolgono un ruolo importante nella modulazione del movimento stomatico, probabilmente non contribuiscono a provocare questo effetto. Da notare, la ridotta risposta del triplo mutante *atpao234* agli stimoli correlati allo stress che inducono la chiusura stomatica, che dovrebbe contribuire a ridurre la perdita di acqua attraverso la traspirazione, non è accompagnata da una maggiore tolleranza delle piante ae condizioni di stress abiotico. La mancanza di tale correlazione deve ancora essere analizzata.

Recentemente, è stato dimostrato che *AtPAO5* è coinvolto nel controllo della crescita delle piante. Infatti, due mutanti *atpao5* di Arabidopsis e piante trasgeniche che che sovraesprimono *AtPAO5* (piante transgeniche *AtPAO5*) presentano differenze di sviluppo dalle piante di *wild-type*. In particolare, i mutanti *atpao5* producono steli più lunghi e più spessi, mentre le piante transgeniche *AtPAO5* producono steli più sottili e più corti rispetto alle piante *wild-type*. Queste alterazioni fenotipiche dei mutanti *atpao5* e delle piante transgeniche *AtPAO5* sono state attribuite a cambiamenti mediati da *AtPAO5* nell'omeostasi della Therm-Spm e sono accompagnati da cambiamenti nel livello di espressione dei geni coinvolti nella segnalazione dell' auxina e delle citochinine suggerendo che *AtPAO5* interferisca con le vie di segnalazione delle citochinine e dell'auxina. Nel presente studio, per studiare la segnalazione di auxina e citochinine nelle piante transgeniche *AtPAO5* e i mutanti *atpao5*, sono state ottenuti ed analizzati degli incroci di queste piante con piante transgeniche *DR5:::GUS*, *DR5* essendoun promotore artificiale inducibile da auxina. È stato dimostrato che le piante transgeniche AtPAO5 presentano alti livelli di espressione *DR5::GUS* nella regione meristematica della radice, mentre i

mutanti *atpao5* più bassi rispetto ai livelli di espressione *DR5 :: GUS* della radice del *wild-type*. E 'stato inoltre dimostrato che le piante *AtPAO5* e *atpao5* presentano differenze dal *wild-type* per quanto riguarda il differenziamento dello xilema attraverso l'intera pianta; le piante *AtPAO5* presentano un aumento del numero di cellule xilematiche mentre i mutanti *atpao5* un numero ridotto rispetto alle piante *wild-type*. Inoltre, è stato dimostrato che le piante *atpao5* presentano una maggiore tolleranza allo stress salino e allo stress idrico, nonché una diminuzione della chiusura stomatica in risposta a condizioni relative allo stress.

In conclusione, i dati presentati in questo lavoro evidenziano un ruolo importante delle tre *AtPAO* perossisomali nel controllo della chiusura stomatica legata allo stress. E' stata anche dimostrata l'esistenza di un finemente regolato sistema di interazioni tra *AtPAO5*, Therm-Spm, auxina e citochinine necessaria per un corretto differenziamento dello xilema e un'appropriata crescita della pianta.AtPAO5 probabilmente contribuisce a tale sistema partecipando ai meccanismi di controllo a *feedback* che regolano i livelli di Therm-Spm. Inoltre, *AtPAO5* contribuisce al controllo della chiusura stomatica e della tolleranza delle piante allo stress salino e idrico.

1. INTRODUCTION

1.1 The Polyamines

The polyamines are low-molecular-weight polycations with aliphatic structure having hydrocarbon chains of variable length and two or more primary and secondary amino groups. The most common polyamines, the diamine putrescine (Put), the triamine spermidine (Spd), and the tetramine spermine (Spm) are founded in all living organisms from: bacteria to animals, from chromista to fungi and plants (Hussain *et al*., 2011) and involved in several important physiological processes. In addition to these three polyamines, other polyamines, such as 1,3-diaminopropane (Dap), cadaverine (Cad), thermospermine (Therm-Spm), norspermidine (Nor-Spd) and norspermine (Nor-Spm) (Fig. 1) are found in many organisms as minor components of the cellular polyamine pool and are referred to as uncommon polyamines (Tavladoraki *et al*., 2011). The positively charged amino groups in polyamines are critical for their biological effects as they allow polyamines to interact with cellular components with negative charge, such as DNA, RNA, and phospholipids (Moinard *et al*., 2005) thus altering their physical and chemical properties.

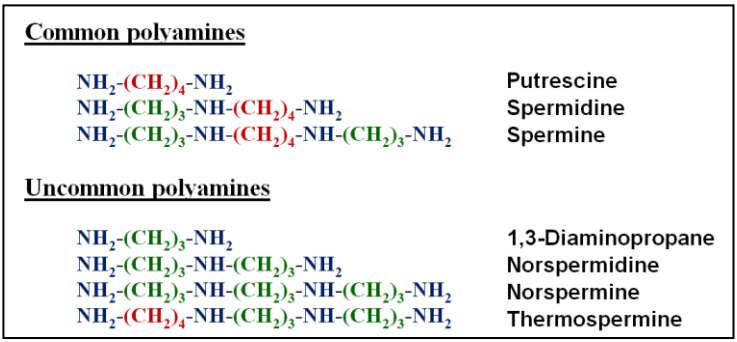

Fig. 1. Common and uncommon polyamines.

Polyamines regulate several physiological processes in both eukaryotic and prokaryotic cells, not only through their interaction with anionic macromolecules, but also through their metabolic products (Igarashi and Kashiwagi, 2000; Alcázar and Tiburcio, 2014). Polyamines are involved in the control of embryogenesis, signal transduction and membrane stabilization, maintenance of DNA structure, RNA processing, regulation of translation and modulation of enzyme activities and

programmed cell death (PCD) (Igarashi and Kashiwagi, 2000; Seiler and Raul, 2005; Kusano *et al*., 2008; Alcázar and Tiburcio, 2014). In bacteria polyamines have also an essential role in biofilm formation (Patel *et al*., 2006). A key role of Spd to provide the 4-aminobutyl group for the conversion of a conserved lysine residue of the translation factor elF5A to the unsual amino acid hypusine, an important process for several aspects of the cellular biology was also shown (Park *et al*., 2010; Miller-Fleming *et al*., 2015; Belda-Palazón *et al*., 2016).

In plants, polyamines play an important role in growth and development, in the regulation of flower initiation, leaf senescence and fruit development and ripening (Galston *et al*., 1990). In addition to their function in plant development, polyamines play a role in stress responses because their levels in plant cells increase under a number of environmental stress conditions (Pottosin and Shabala, 2014), this increase being often beneficiary for plant performance following stress. In particular, polyamines modulate plant defense responses to diverse environmental stresses, which include metal toxicity, oxidative stress, drought, salinity, chilling and heat stress and wounding (Bouchereau *et al*., 1999; Tavladoraki *et al*., 2012; Takano *et al*., 2012; Alcázar and Tiburcio, 2014; Jiménez-Bremont *et al*., 2014; Minocha *et al*., 2014; Tiburcio, *et al*., 2014). The detailed mechanisms of how polyamines exert their protective function are not clear. Among various possibilities is that they can act as molecular chaperons, protecting membranes and biomolecules, due to their ability to bind to negatively charged surfaces. Additionally, polyamines may act as reactive oxygen species (ROS) and free-radical scavengers and activate the antioxidant enzyme machinery, reducing oxidative stress-induced membrane injury and electrolyte leak. The protective function of polyamines under environmental stresses may be associated to their essential role in the regulation plant membrane transport inhibiting ion channels in plant vacuoles and plasma membranes (Pottosin *et al*., 2014). On the other hand, plants polyamines play a role as mediators in defence signalling against pathogens (Takahashi and Kakehi, 2010). In particular, the 'Spm signalling pathway' involves Spm transport to the extracellular space, upregulation of a subset of defence-related genes, such as those encoding pathogenesis-related proteins and mitogen-activated protein kinases, and a type of PCD known as the hypersensitive response. This response is triggered by Spm-derived H_2O_2, produced through polyamine catabolism in the apoplast (Cona *et al*., 2006; Kusano *et al*., 2008; Moschou *et al*., 2008b). Moreover, polyamines, in particular Put, are additionally

required for the synthesis of tropane and nicotine alkaloids in plants (Tavladoraki *et al*., 2012; 2016).

Polyamine levels are finely regulated through complex homeostatic pathways involving polyamine biosynthesis, catabolism, conjugation, transport, and uptake which are well coordinated through feed-back mechanisms (Martin-Tanguy, 1997, Tiburcio *et al*., 1997; Angelini *et al*., 2010; Moschou *et al*., 2012).

1.2 Thermospermine

Therm-Spm, a structural isomer of Spm, was first detected in the thermophilic bacterium *Thermus thermophilus*, and recently was identified in the diatom *Thalassiosira pseudonana* and in *Arabidopsis* (Knott *et al.*, 2007). Genome analyses in many organisms suggest that it may be present in most plants, but not in animals and fungi (Minguet *et al*., 2008). Several recent studies support an important role of Therm-Spm in in several different processes during xylem differentiation, including lignin biosynthesis, cell-wall formation, and auxin/cytokinin signaling (Ge *et al*., 2006; Cui *et al*., 2010; Vera-Sirera *et al*., 2010). Indeed, in *Arabidopsis* disruption of thermospermine synthase (*ACAULIS5*; *ACL5*) gene, which is expressed specifically in procambial cells and xylem precursor cells during vascular differentiation (Clay and Nelson, 2005; Muñiz *et al*., 2008), caused impaired stem elongation, incomplete secondary cell-wall formation and early expression of xylem cell-death markers, and consequently early vessel cell death and over-proliferation of xylem vessels and thicker veins compared with the wild type plants, suggesting that Therm-Spm has a protective role against premature xylem maturation and cell death (Hanzawa *et al*., 1997; Hanzawa *et al*., 2000; Clay and Nelson, 2005; Kakehi *et al*., 2008; Muñiz *et al*., 2008). Conversely, increased *ACL5* expression levels or exogenously supplied Therm-Spm suppressed xylem differentiation (Kakehi *et al*., 2010; Milhinhos *et al*., 2013; Baima *et al*., 2014). Also, mutation of the *Arabidopsis BUD2/SAMDC4* gene, one of the four *Arabidopsis S-adenosylmethionine decarboxylase* (*SAMDC)* genes involved in polyamine biosynthesis, which has been shown to be down-regulated by Therm-Spm and has been proposed to predominantly mediate Therm-Spm synthesis (Kakehi *et al*., 2010; Kim *et al*., 2014), produced plants with vascular defects similar to those of *acl5* (Ge *et al*., 2006).

Recently, many studies have evidenced the regulation of *ACL5* and *BUD2/SAMDC4* by auxin and Therm-spm. Indeed *ACL5*, together with *BUD2/SAMDC4*, is positively regulated by auxin (Hanzawa *et al*., 2000; Cui *et al*., 2010), and negatively regulated by exogenous Therm-Spm

(Kakehi *et al*., 2010) through a feedback mechanism involving the regulation of the basic helix-loop-helix (b-HLH) transcription factor *SUPPRESSOR OF ACAULIS 51* (*SAC51*) at the translational level (Imai *et al*., 2006). In turn, SAC51, as well as SAC51-like proteins heterodimerizes with LHW, thus competing TMO5/LHW interactions and preventing activation of TMO5/LHW target genes to suppress the over-proliferation caused by excess TMO5/LHW activity (Katayama *et al*., 2015; Vera-Sirera *et al*., 2015). It has been also shown that Therm-Spm and auxin have opposite action in the control of xylem differentiation (Yoshimoto *et al*., 2012). Indeed, increased Therm-Spm levels delay xylem differentiation by negatively affecting the expression of auxin-regulated transcription factors belonging to *HD–ZIP III* gene family and key auxin signaling genes resulting in alteration of auxin-mediated processes (Yoshimoto *et al*., 2012; Milhinhos *et al*., 2013; Baima *et al*., 2014). In this regulatory mechanism, which involves a well-controlled feedback circuit operating to fine-tune formation and differentiation of xylem, the *ATHB8* transcription factor of the *HD–ZIP III* gene family has an important role directly regulating *ACL5* expression (Baima *et al*., 2014).

Therm-Spm metabolism has been also shown to be involved in the plant defence responses to pathogens (Sagor *et al*., 2012; Marina *et al*., 2013).

1.3 Polyamine biosynthesis

The plant polyamine biosynthetic pathway has been extensively studied (Kusano *et al*., 2008; Vera-Sirera *et al*., 2010; Pegg and Casero, 2011; Gupta *et al*., 2013) and differs from that of animals in that it involves two precursors, L-ornithine and L-arginine, to generate Put, while only ornithine is used in animals. In particular, in plants Put is produced either directly by ornithine decarboxylase (ODC) or in three steps by arginine decarboxylase (ADC) (Fig. 2).

Put is then converted into Spd by Spd synthase (SPDS), with the addition of an aminopropyl moiety donated by decarboxylated S-adenosylmethionine (dcSAM). dcSAM is synthesized from methionine via two sequential reactions that are catalyzed by methionine adenosyltransferase and S-adenosylmethionine decarboxylase (SAMDC), respectively. Spd is then converted into Spm or Therm-Spm, again using dcSAM as an aminopropyl donor, in a reaction catalyzed by Spm synthase (SPMS) and ACL5, respectively. SAMDC has an important role in the regulation of polyamine homeostasis, particularly in plants where it is considered to be the rate-limiting enzyme for the synthesis of Spd and Spm

(Kusano *et al.*, 2008). The activity of SAMDC in fact 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 polyamine biosynthetic pathway (Xiong *et al.*, 1997).

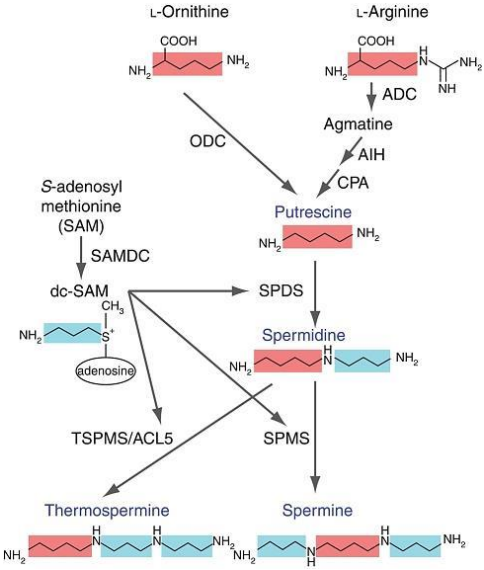

Fig. 2. Biosynthetic pathways of polyamines. ADC, Arginine decarboxylase; **CPA**, N-carbamoylputrescine amidohydrolase; **ODC**, Ornithine decarboxylic acid; **SAM**, S-adenosylmethionine; **SAMDC**, S-adenosylmethionine decarboxylase; **SPDS**, Spermidine synthase; **SPMS**, Spermine synthase; **TSPMS**, Thermospermine synthase; **ACL5**, *ACAULIS5*. (Figure taken from Takahashi and Kakehi, 2010).

In the sequenced genome of the model plant *Arabidopsis thaliana* no gene encoding ODC was found, suggesting that in this species Put may be produce only via the ADC pathway (Hanfrey *et al.*, 2001; Liu *et al.*, 2015). In particular, in *Arabidopsis* two *ADC* (*ADC1* and *ADC2*) genes 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). Moreover, in *Arabidopsis* two *SPDS* (*SPDS1* and *SPDS2*) genes are present, whereas SPMS and ACL5 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. Intron/exon structure is conserved in *SPDS1*, *SPDS2*, and *SPMS*, whereas *ACL5* has a completely different genomic organization. Interestingly, SPMS interacts with SPDS1 and SPDS2 to form ‘‘metabolon’’ complexes, while ACL5 does not interact with SPDS (Panicot *et al*., 2002).

1.4 Polyamine catabolism

Polyamine catabolism is mediated by two classes of enzymes, the copper-containing amine oxidases (CuAOs) and the FAD-dependent polyamine oxidases (PAOs). CuAOs and PAOs, abundant in the apoplast of Fabaceae and Gramineae, respectively, have long been considered characteristic of these two plant families, leaving polyamine catabolism uncovered in other plant families and underestimating their potential contribution in other subcellular compartments. More recently, a number of CuAOs and PAOs have been detected in several taxa and the occurrence of polyamine catabolism in intracellular compartments has been demonstrated (Cona *et al*., 2006; Angelini *et al*., 2010; Tavladoraki *et al*., 2012).

CuAOs are homodimeric enzymes that oxidize mainly Put and Cad, and less efficiently Spd and Spm at the primary amino groups, producing ammonia, H_2O_2 and an aminoaldehyde, are thus considered involved in PA terminal catabolism. CuAOs with catalytic activity towards *N*-methyl-Put, which is involved in nicotine biosynthesis, as well as towards monoamines, such as 2-phenylethylamine, tyramine and tryptamine, have been identified (Tavladoraki *et al*., 2016). In *Arabidopsis thaliana*, ten *CuAO* genes are present among which only eight encoding for putative functional CuAOs with either apoplastic, cytosolic or peroxisomal localization (Angelini *et al*., 2010; Tavladoraki *et al*., 2016).

PAOs catalyze the oxidative deamination of Spm, Spd and/or their acetylated derivatives at the secondary amino group. The chemical identity of PAO reaction products depends on the enzyme source and the mode of substrate oxidation. In particular, PAOs can be classified in two families according to the mode of substrate oxidation: the PAOs oxidizing the carbon at the *endo*-side of the N^4-nitrogen of Spd and Spm to produce 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, in addition to Dap and H_2O_2 (*endo*-mode of oxidation) and the PAOs oxidizing the carbon at the *exo*-side of the N^4-nitrogen of Spd and Spm with the production of Spd from Spm and Put from Spd, in addition to 3-aminopropanal and H_2O_2 (*exo*-mode of oxidation) (Fig. 3) (Tavladoraki *et al*., 2016). PAOs with the *endo*-mode of substrate oxidation are present at

high quantities in plants belonging to Gramineae (Cona *et al*., 2006; Angelini *et al*., 2010; Tavladoraki *et al*., 2012). In maize (*Zea mays*), three genes have been identified encoding identical apoplastic proteins (ZmPAO1; Cervelli *et al*., 2000), while in barley two genes (HvPAO1 and HvPAO2) have been cloned (Cervelli *et al*., 2001; Cona *et al*., 2006; Angelini *et al*., 2010; Tavladoraki *et al*., 2016). PAOs with the *exo*-mode of substrate oxidation have been first characterized in yeast and animals. In particular, the mammalian PAOs with a peroxisomal localization (APAOs) oxidize mainly the acetylated forms of Spm and Spd. In this catabolic pathway, polyamine acetylation is obtained by the tightly regulated Spd/Spm N^1-acetyltransferase (SSAT), which is the rate-limiting enzyme of this pathway (Wallace *et al*., 2003). In addition, the mammalian spermine oxidases, which have cytosolic/nuclear localization (SMO), preferentially oxidize the free form of Spm (Wang *et al*., 2001, Vujcic *et al.*, 2002, Cervelli *et al*., 2003). Interestingly, 3-aminopropanal and 3-acetamidopropanal produced by SMO or APAO can be further metabolized first to β-alanine or *N*-acetyl-β-alanine, respectively, by an aminoaldehyde dehydrogenase and then to the toxic compound acrolein, which animals has an important regulatory role (Tavladoraki *et al*., 2012).

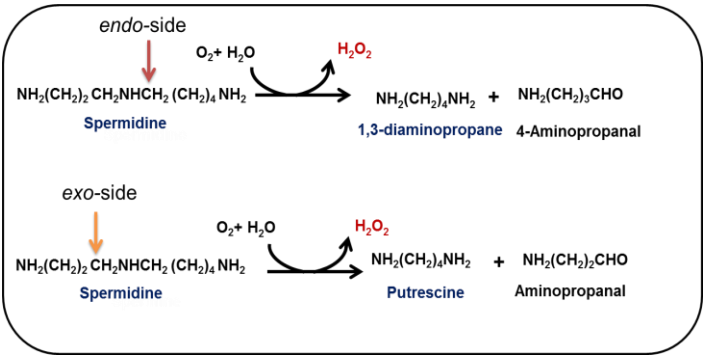

Fig. 3. Modes of substrate oxidation by PAOs

1.5 *Arabidopsis thaliana* Polyamine Oxidases

The *Arabidopsis* genome contains five *PAO* genes, termed AtPAO1 (At5g13700), *AtPAO2* (At2g43020), *AtPAO3* (At3g59050), *AtPAO4* (At1g65840) and *AtPAO5* (At4g29720) (Fig. 4; Tavladoraki *et al*., 2006). *Arabidopsis* PAOs present some common characteristics to each other, but also important differences in gene structure, substrate specificity,

subcellular localization, and expression pattern. These differences may reflect differences in physiological roles (Fincato *et al.*, 2011).

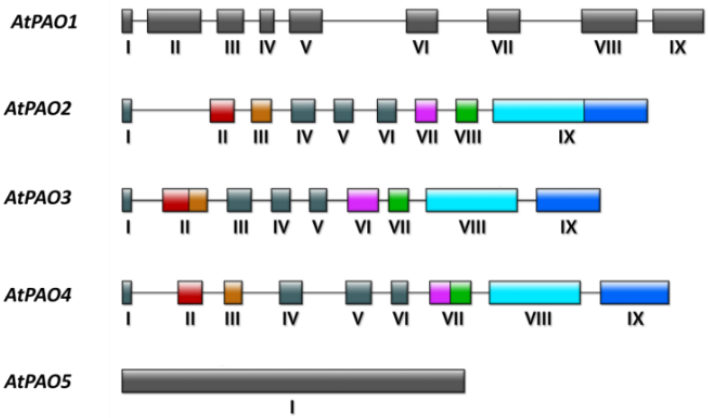

Fig. 4. Schematic representation of the exon/intron organization of *AtPAO* genes. Introns are represented by lines and exons by boxes. Exons are numbered in Roman numerals. Open and filled boxes indicate shared and unshared exons among the various *AtPAO* genes, respectively. Stripes and stipples show shared exon domains which are found either joined to each other or separated by the presence of an intron, according to the specific gene considered. Exons and introns are drown in scale (modify from Fincato *et al*., 2011)

AtPAO1 has a cytosolic localization and shares with the extracellular ZmPAO1 a 45% homology at the amino acid level as well as a similar intron/exon organization (Tavladoraki *et al*., 2006). Furthermore, AtPAO1 oxidizes Spm but not Spd, differently from ZmPAO1 but similarly to the animal SMOs. AtPAO1 oxidises also the uncommon polyamines Therm-Spm and Nor-Spm with higher efficiency than Spm, which suggests that these two polyamines may be the physiological substrates of AtPAO1 (Table 1; Fincato *et al*., 2011). In contrast, AtPAO1 has a low catalytic activity with N^1-acetyl-Spm. AtPAO2, AtPAO3, and AtPAO4 display low sequence homology (23%-24% sequence identity at the amino acid level) with ZmPAO1 and the other two AtPAOs, but a high sequence homology to each other (85% amino acid sequence identity between AtPAO2 and AtPAO3, 58% between AtPAO2 and AtPAO4, and 50% between AtPAO3 and AtPAO4). Moreover, AtPAO2, AtPAO3 and AtPAO4 have a peroxisomal localization and are able to oxidize both Spd and Spm, but not Therm-Spm (Moschou *et al.*, 2008c; Kamada-Nobusada *et al.*, 2008; Fincato *et al.*, 2011; Ono *et al.*, 2012). In particular, 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; Fincato *et al*., 2011). Furthermore, *AtPAO2*, *AtPAO3* and *AtPAO4* genes have a very similar intron/exon organization to each other, with eight introns at highly conserved positions, which is different from that of *AtPAO1* and *AtPAO5*.

	AtPAO1	AtPAO2	AtPAO3	AtPAO4	AtPAO5	ZmPAO1	MmSMO	MmAPAO
	k_{cat} (s^{-1})							
Spd	-	4.6	3.4	0.1	-	50.2	-	-
Spm	2.7	4.2	1.7	4.6	1.9	32.9	3.9	0.175
Nor-Spm	6.9	2.9	1.1	0.45	-	5.5	-	ND
Ther-Spm	5.7	0.4	0.5	0.1	1.7	9.8	-	ND
N′-acetyl-Spm	-	-	-	-	6.2	-	-	-

Table 1. Catalytic activity of recombinant AtPAOs, ZmPAO1, as well as of murine SMO and APAO. Data were taken from Cervelli *et al*., 2003, Wu *et al*., 2003, Polticelli *et al*., 2005, Fincato *et al*., 2011 and Ahou *et al*., 2014. ND: not determined.

These similarities suggest that *AtPAO2*, *AtPAO3* and *AtPAO4* genes are recent derivatives from a common ancestor, thus forming a distinct PAO subfamily (*AtPAO234* subfamily). This is further supported by phylogenetic studies performed to obtain information about the distribution of AtPAO orthologous in the plant taxa (Ono *et al*., 2012; Ahou A, PhD thesis). This analysis showed that plant PAOs are divided into four clades (clade I to IV) including AtPAO1-, ZmPAO1-, AtPAO5- and AtPAO2,3,4-like PAOs, respectively (Fig. 5). Interestingly, clade I PAOs result specific for dicotyledonous plants, while clade II PAOs specific for the monocotyledonous plants (Fig. 5).

AtPAO5 has low amino acid sequence homology with the other AtPAOs and ZmPAO1 (19–23%) and a higher sequence homology with murine SMO (MmSMO) and murine APAO (MmAPAO) (31% sequence identity; Ahou *et al*., 2014). Furthermore, differently from the other *AtPAO* genes and *ZmPAO1*, *AtPAO5* gene bears no intron (Fig. 4; Fincato *et al*., 2011). AtPAO5 is a cytosolic enzyme regulated at the post-translational level by the proteasome (Ahou *et al*., 2014). It catalyzes the conversion of Spm and Therm-Spm to Spd. AtPAO5 is also able to oxidize N^1-acetyl-Spm with a higher catalytic efficiency than Spm, and Therm-Spm (Ahou *et al*., 2014). In this way, AtPAO5 represents the first plant enzyme characterized so far involved in polyamine catabolism with a good activity with

acetylated polyamines. (Tavladoraki *et al.*, 2006; Moschou *et al.*, 2008b; Fincato *et al.*, 2011). Furthermore, AtPAO5 has been classified as a dehydrogenase rather than as an oxidase, since it has been shown that O_2 is a poor electron acceptor in the reaction catalyzed by this enzyme (Ahou *et al.*, 2014). Only in bacteria has the existence of spermidine dehydrogenases been reported so far, which have an important role in the utilization of polyamines as carbon and nitrogen source (Tabor and Kellog, 1970; , 1992; Dasu *et al*., 2006).

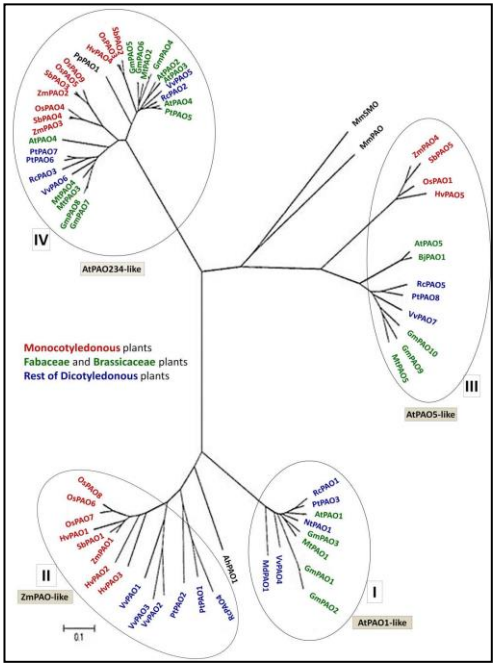

Fig. 5. Phylogenetic relationships of AtPAOs with other plant PAOs. AtPAO: *Arabidopsis thaliana* PAO; GmPAO: *Glucine max* PAO, HvPAO: *Hordeum vulgare* PAO; ZmPAO: *Zea mays* PAO; RcPAO: *Riccinus communis* PAO; MtPAO: *Medicago truncatula* PAO; AmPAO: *Amaranthus hypochondriacus* PAO; MdPAO: *Malus 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; M*us* m*usculus* APAO. From Ahou A, PhD thesis.

The function of AtPAO5 as a dehydrogenase suggest that AtPAO5 has a role in polyamine homeostasis rather than in H_2O_2 production, in contrast to the other PAOs so far characterized, as for example ZmPAO1, for which involvement in important physiological processes through H_2O_2 production has been shown (Cona *et al*., 2006; Angelini *et al*., 2010; Tisi *et al*., 2011).

Noteworthy, analysis of reaction products evidenced that all five AtPAOs present an *exo*-mode of substrate oxidation (Tavladoraki *et al*., 2006; Kamada-Nobusada *et al*., 2008; Fincato *et al*., 2011; Tavladoraki *et al*., 2016), similarly to the animal APAOs/SMOs and in contrast to the extracellular PAOs from monocotyledonous plants characterized thus far.

Analysis of promoter activity using *AtPAO::β-glucuronidase* (*GUS*) *Arabidopsis* transgenic plants evidenced distinct expression patterns during growth in seedling and flower development which may reflect a distinct physiological role (Fincato *et al*., 2012; Ahou, PhD thesis; Ahou *et al*., 2014). In particular, *AtPAO1* is mainly expressed in the transition region between the meristematic and the elongation zone of roots and anther tapetum (more specifically illustrated in the caption of figures Fig. 6; Fig. 7; Fincato *et al*., 2012). Interestingly, the *AtPAO1* expression in the meristematic/elongation transition zone of the roots has an unilateral distribution and is increased following treatment with the stress-related hormone abscisic acid (ABA) (fig. 8 and . 9; Fincato *et al*., 2012). *AtPAO2* and *AtPAO3* and *AtPAO4* are specifically expressed in the root cap, guard cells and pollen grains (more specifically illustrated in the caption of figures 6 and 7; Fincato *et al*., 2012; Ahou A, PhD thesis). In the guard cells, *AtPAO3* and *AtPAO4* are constitutively expressed, while *AtPAO2* in an ABA-inducible way (Fig. 9; Fincato *et al*., 2012; Ahou A, PhD thesis). In roots *AtPAO2* expression involves all tissues, while *AtPAO3* involves epidermis, cortex, pericycle and the vascular system but not endodermis (Fig. 8; Fincato et al., 2012).

AtPAO5 is expressed in roots, hypocotyls, cotyledons, stems and anther tapetum (more specifically illustrated in the caption of figures Fig. 6; Fig. 7; Fincato *et al*., 2012). In roots, *AtPAO5* expression is extended from the site where the spiral secondary cell-wall thickenings of the protoxylem elements first become evident up to the hypocotyl-root junction involving the vascular system (xylem, phloem, and procambial/cambial cells) and pericycle (Fig. 8). In stems, *AtPAO5* is specifically expressed in the cortex and the xylem vessels (Fig. 8). AtPAO5 is up-regulated by cytokinins, auxin and Therm-Spm specifically in the roots involving the root vasculature and,

in the case of cytokinins, in the root meristematic region (Fig. 10; Ahou *et al*., 2014; Alabdallah *et al*., 2017).

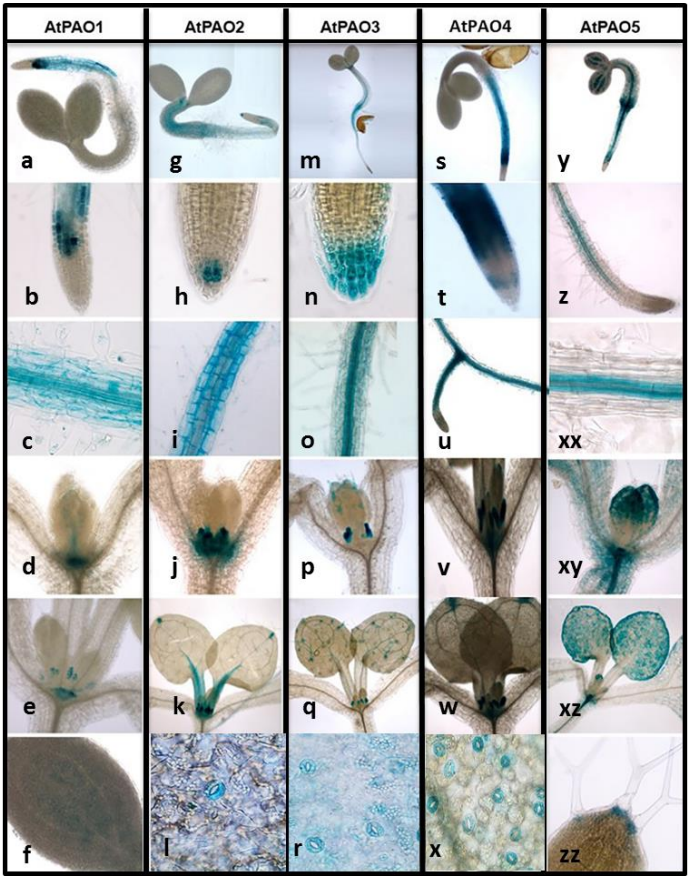

Fig. 6. *AtPAO* promoter activity studies using *AtPAO::GUS* transgenic Arabidopsis plants. Histochemical GUS staining during plant growth. *AtPAO1* is expressed in the transition region between the meristematic and the elongation zone of the roots (a, b), root maturation zone (a), lateral roots (c), shoot apex (d-e) and stipules (e). *AtPAO2* is expressed in root cap of columella (g, h), root elongation zone (i), shoot apex (j) and petioles (k). *AtPAO3* is expressed in root cap of columella (m-n), root elongation zone (o), stipules (p) and trichomes (q). *AtPAO4* is expressed in roots (from the meristematic /elongation transition region (s, t) up to the hypocotyl–root junction site (v) hydathodes (w). *AtPAO5* is expressed in the cotyledons (y), hypocotyles (xy-xz), vascular tissue of root (xx), shoot apex (xy) and trichomes (zz).

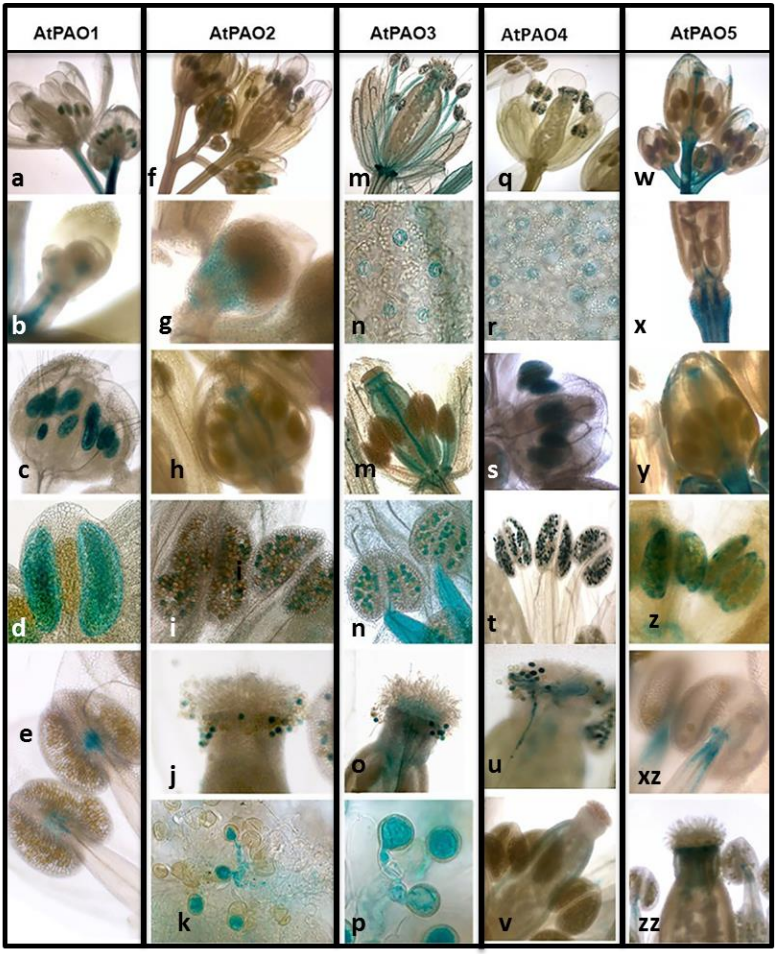

Fig. 7. *AtPAO* promoter activity studies using *AtPAO::GUS* transgenic Arabidopsis plants. Histochemical GUS staining during inflorescence development. *AtPAO1* is expressed in stems (a), and anthers (b-e). *AtPAO2* is expressed in pistil walls (f-h) and pollen grains (i-k); *AtPAO3* is expressed in pistil walls (l, m) and pollen grains(n-o-p); *AtPAO4* is expressed in flower buds (q), in anther tapetum (s) and in mature pollen grains (t-v); *AtPAO5* is expressed in anthers (z, xz), receptacle (w, x) and stamens (zz).

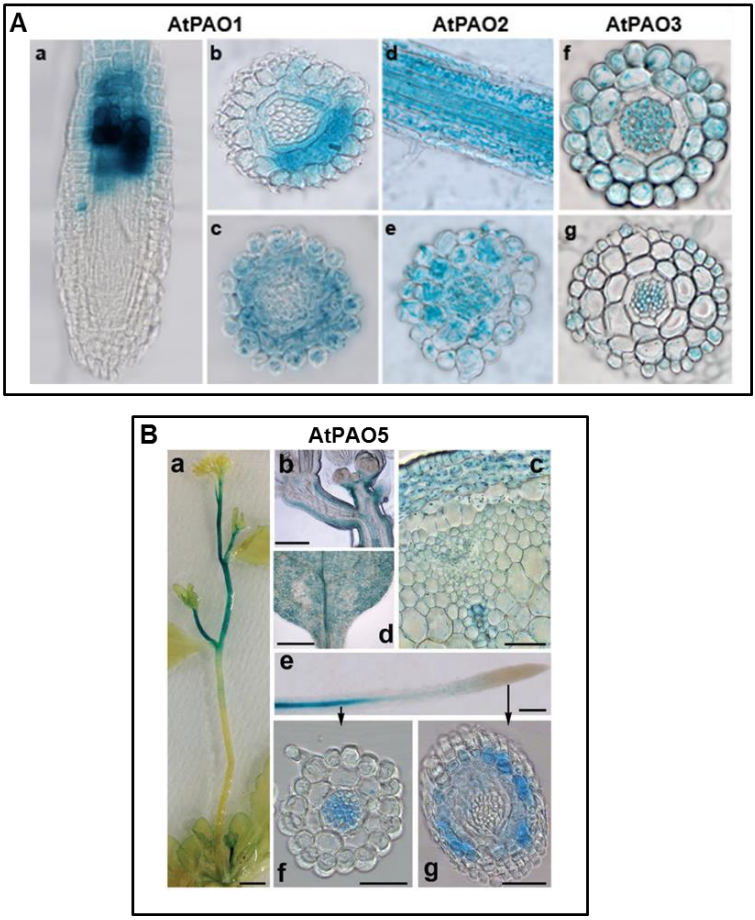

Fig. 8. *AtPAO* promoter activity studies using *AtPAO::GUS* transgenic Arabidopsis plants. A) *AtPAO1*, *AtPAO2* and *AtPAO3* promoter activity studies in roots. Longitudinal (a, d) and transversal (b, c, e–g) sections of roots (a–f) and hypocotyls (g) obtained after GUS staining are shown Modified from Fincato *et al*., 2012. B) *AtPAO5* promoter activity in stems (a–c), leaves (d), and roots (e–g) as determined by histochemical GUS staining of *AtPAO5::GFP-GUS* transgenic plants. Longitudinal (b) and transverse (c) sections of stems and transverse sections of roots (f, g) are also shown. Arrows indicate the position of the section. Scale bars represent 50 μm (c, f, g), 200 μm (e), 500 μm (b, d), and 5 mm (a). From Alabdallah *et al*., 2017).

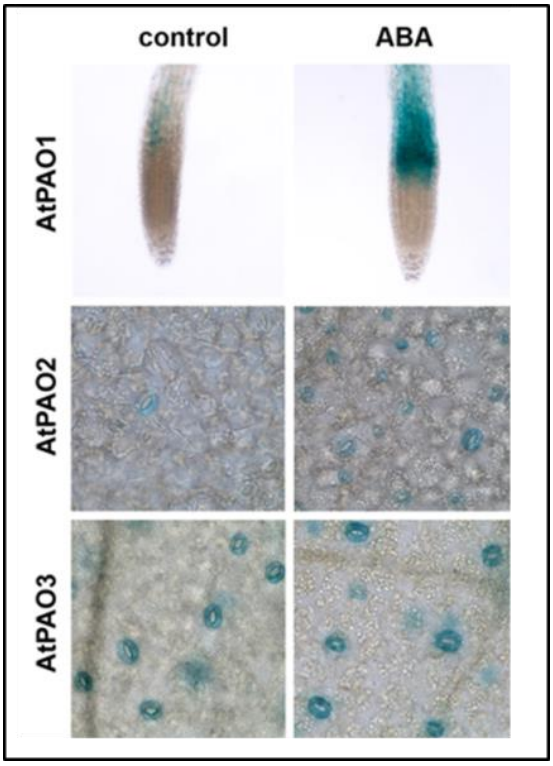

Fig. 9. ABA-inducible expression of *AtPAO1* and *AtPAO2*. *AtPAO::GUS* transgenic *Arabidopsis* plants were treated or not with 10 μM ABA for 4 h and then analyzed for GUS activity. From Fincato *et al*., 2012.

1.6 Physiological roles of polyamine catabolism

In animals, polyamine catabolism contributes to important physiogical and pathological processes, such as cell proliferation and differentiation, apoptosis, amine detoxification and cell signalling through both regulation of polyamine levels and their catabolic products, mainly H_2O_2, aminoaldehydes and acrolein (Tavladoraki *et al*., 2011). As evidenced by the complex role of polyamines in cell growth and proliferation, optimal polyamine levels are necessary for mammalian health. In fact, an important difference between normal and tumor cells is polyamine content. Despite the tightly controlled polyamine homeostasis, it was shown that an altered polyamine catabolism can cause changes in

polyamine content. Indeed, constitutive or inducible over-expression of SSAT in animal cells caused 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 polyamines (Jänne *et al*., 2004; 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 polyamine levels through polyamine catabolism are often accompanied by increased DNA damage and changes in cell proliferation (Zahedi *et al*., 2007). These data suggest that polyamine catabolism has an important role in controlling polyamine content and thus can be used as a therapeutic target for several diseases. On the other hand, the H_2O_2 produced through polyamine catabolism is able either to impair cell growth and proliferation or to regulate signal transduction and gene expression, depending on its concentration (Pledgie *et al*., 2005; Casero and Pegg, 2009; Wang and Casero, 2006). Furthermore, aminoaldehydes and acrolein produced through polyamine catabolism are cytotoxic to animal cells (Igarashi and Kashiwagi, 2010), probably due to the inhibition of nucleic acid and protein synthesis (Nocera *et al*., 2003; Wallace *et al*., 2003).

Also in plants, polyamine catabolism has important roles in plant development and stress responses through regulation of polyamine levels and their reaction products (Fig. 10). Indeed, recent evidences suggest that the polyamine catabolic pathways play an important role in control of polyamine levels (Bhatnagar *et al*., 2002; Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008a; Fincato *et al*., 2011). Furthermore, polyamine-mediated production of H_2O_2 is necessary for several plant developmental and deference processes (Fig. 10). In particular, H_2O_2 produced via apoplastic degradation of polyamines drives peroxidase-mediated oxidative cross-linking of structural cell-wall components, contributing to cell-wall strengthening during development (Fig. 11). It is also associated with PCD events taking place during developmental differentiation (Fig. 10).
Indeed, the presence of an apoplastic *A. thaliana* CuAO (ATAO1; Møller *et al*., 1998) and of ZmPAO1 in developing treachery 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 polyamine catabolism in the apoplast contributes to ROS production and PCD responses also under stress conditions, such as wound-healing, pathogen attack, and abiotic stress conditions or following treatment with ABA, an hormone which plays a crucial role in plant responses to abiotic stresses (Cona *et al*., 2006;

Angelini *et al*., 2008, 2010; Moschou *et al*., 2008a,b; Xue *et al*., 2009; Toumi *et al*., 2010).

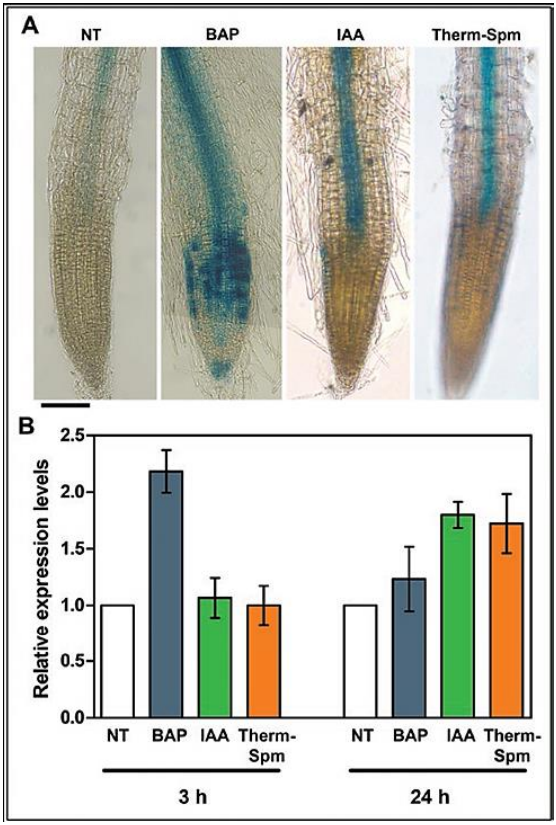

Fig. 10. *AtPAO5* up-regulation by cytokinin, auxin, and Therm-Spm. (A) Histochemical GUS staining of 7-d-old *AtPAO5::GFP-GUS* Arabidopsis transgenic seedlings treated with 1 μM BAP, 1 μM IAA, or 100 μM Therm-Spm for 24 h. NT indicates no-treatment control. The scale bar indicates 100 μm. (B) Quantitative RT-PCR analysis of *AtPAO5* expression levels in whole seedlings of 10-d-old wild-type plants treated with 1 μM BAP, 1 μM IAA, or 100 μM Therm-Spm for 3 h or 24 h. Mean values ±SE of relative quantitation from two independent experiments are shown. From Alabdallah *et al*., 2017.

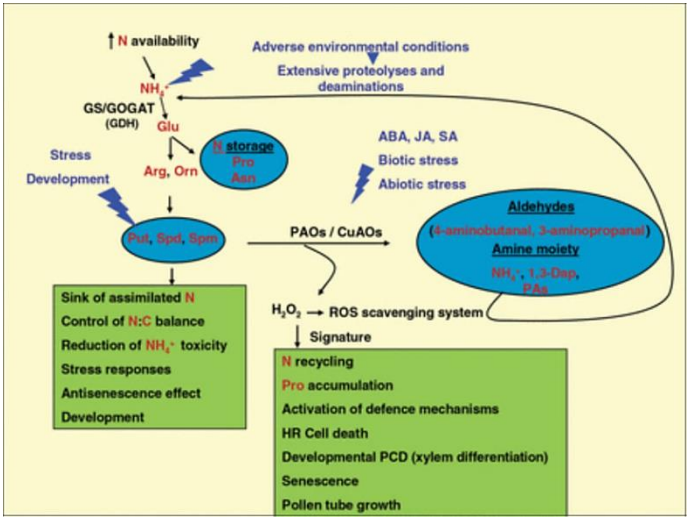

Fig. 11. Physiological roles of polyamine metabolism in plants. Polyamine homeostasis in plants is correlated with several important physiological functions, such as control of the N:C balance, development, and stress responses. Catabolism of polyamines represents a major node of the N and C recycling pathway, besides being linked to several biological processes. Polyamine derived H_2O_2 plays an important role in cell-wall maturation and stress-induced stiffening, in signalling of stomatal opening, as well as in PCD during plant development and defence mechanisms. ABA, abscissic acid; CuAOs, copper amine oxidases; 1,3-Dap, 1,3-diaminopropane; GOGAT, glutamate synthase; GS, glutamine synthetase; GDH, glutamate dehydrogenase; JA, jasmonic acid; PAs, polyamines; PAOs, polyamine oxidases; Put, putrescine; ROS, reactive oxygen species; SA, salicylic acid; Spd, spermidine; Spm, spermine. From Moschou *et al*., 2012.

Interestingly, these events are linked to polyamine transport to the apoplast where only limiting amounts of polyamines are present under normal growth conditions (Tavladoraki *et al*., 2004; Yoda *et al*., 2006; Moschou *et al*., 2008b; Kusano *et al*., 2008; Takahashi and Kakehi, 2010). H_2O_2 production by polyamine catabolism was also 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 (Fig. 12; Wu *et al*., 2010), a process important for sexual plant reproduction. Plant polyamine 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).

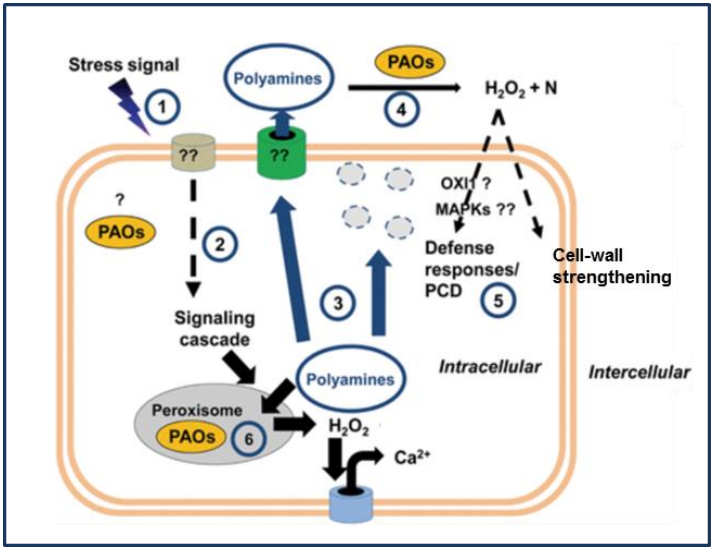

Fig. 12. Model for possible functions of polyamine oxidation in plants. (1) A stress signal is perceived. (2) Signal is transduced by various cellular pathways. (3) The transduction pathway leads to increase of intracellular polyamine levels, and polyamines are secreted. (4) Polyamine are oxidized by apoplastic PAOs to produce H_2O_2. (5) H_2O_2 may direct further responses, through different cascades, which may include mitogen-activated protein kinases, or the Oxidative Signal Inducible 1 (OXI1) pathway (Rentel *et al*., 2004). Depending on the levels of H_2O_2 produced, PCD is induced (high levels) or H_2O_2 is efficiently scavenged (low levels), a process which most probably will modulate defence responses. (6) In peroxisomes, polyamines are back-converted with the simultaneous production of H_2O_2 and other nitrogenous molecules. H_2O_2 produced from this pathway can activate Ca^{2+}-permeable channels (Wu *et al*., 2010). Figure modified from Moschou *et al*., 2012.

In plants, polyamine catabolism may also contribute to the formation of γ-aminobutyric acid (GABA), an important cellular metabolite, which is rapidly produced in response to biotic and abiotic stresses (Yu and Sun, 2007; Petrivalský *et al*., 2007; Dittami *et al*., 2011). Polyamine catabolism contributes also to the formation of β-alanine, which in turn can be further converted in plants to the osmoprotectant β-alanine betaine. Furthermore, as shown in the yeast *Saccharomyces cerevisiae*, β-alanine produced from Spm oxidation may be 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). Dap, a reaction product of the PAOs with *endo*-mode of substrate oxidation, is a precursor of the uncommon polyamine Nor-Spd and Nor-Spm which in plants are associated with stress tolerance (Cona *et al*., 2006).

1.7 *AtPAO5* physiological roles

Recent studies using two loss-of-function mutants, *atpao5-1* and *atpao5-2* lacking *AtPAO5* expression and *35S::AtPAO5-6His transgenic Arabidopsis plants* (*AtPAO5-1;* Ahou *et al*., 2014) with 70-fold higher *AtPAO5* expression levels than the endogenous gene (Supplementary Fig. S1; Ahou *et al*., 2014; Alabdallah *et al*., 2017) have shown that *AtPAO5* contributes to Spm, Therm-Spm and N^1-acetyl-Spm homeostasis (Ahou *et al*., 2014; Alabdallah *et al*., 2017). In particular, it was shown that Spm, Therm-Spm, and N^1-acetyl-Spm levels are decreased in *AtPAO5-1* plants and increased in *atpao5-1* and *atpao5-2* ones, as compared with wild-type seedlings (Ahou *et al*., 2014; Alabdallah *et al*., 2017) indicating that the three polyamines are *in vivo* substrates of AtPAO5. Interestingly, it was further shown that *AtPAO5* participates in the feedback mechanism controlling the expression of *ACL5* and *SAMDC4*, two genes involved in Therm-Spm biosynthesis. Indeed, these genes are up-regulated in the *AtPAO5-1* transgenic plants and down-regulated in the *atpao5* mutants, as compared the wild-type plants (Alabdallah *et al*., 2017).

AtPAO5 is also specifically involved in the cytokinin-mediated control of root protoxylem differentiation, with this specific signalling pathway being impaired in the presence of increased *AtPAO5* expression levels and enhanced in the absence of *AtPAO5* expression. In particular, it was shown that the cytokinin-mediated inhibition of root protoxylem differentiation is less pronounced in *AtPAO5-1* plants and more pronounced in *atpao5-1* and *atpao5-2* mutants than in wild-type plants (Fig. 13).

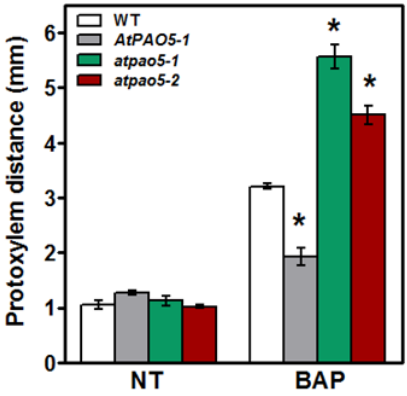

Fig. 13. **Cytokinin-mediated protoxylem differentiation in *AtPAO5-1* and *atpao5* seedlings.** Distances of the first protoxylem cells from the quiescent center (protoxylem distance) were measured after 5 d of treatment with 1 μM of the cytokinin 6-benzylaminopurine (BAP). NT indicates untreated control. Aasterisks denote statistically significant differences from the corresponding wild-type (WT) seedlings. From Alabdallah *et al*., 2017.

In agreement with these data, genes related to the cytokinin signalling pathways are differently expressed in the various genotypes, as are genes related to the auxin signalling and xylem differentiation pathways (Fig. 14; Alabdallah *et al*., 2017). Altogether these studies suggest that both the cytokinin and the auxin signalling pathways are perturbed in the *atpao5* mutants and *AtPAO5-1* transgenic plants as compared the wild-type plants. In particular, data are compatible with increased auxin and decreased cytokinin signaling in *AtPAO5-1* plants and, conversely, decreased auxin and increased cytokinin signalling in *atpao5-1* mutants pathways (Fig. 14; Alabdallah *et al*., 2017). *AtPAO5* is also involved in the control of root and stem growth. Indeed, the *atpao5*-1 and *atpao5-2* mutants produce longer and thicker stems than wild-type plants, while *AtPAO5-1* produce shorter and thinner stems leading to a semi-dwarf phenotype with reduced apical dominance (Fig. 15). On the contrary, the stems of *AtPAO5-2* plants with only fold higher *AtPAO5* expression levels than the endogenous gene (Supplementary Fig. S1; Alabdallah *et al*., 2017) do not present phenotypical differences from wild-type plants (Fig. 15 A). In a similar way, *atpao5-1* and *atpao5-2* plants present longer roots than wild-type plants, while *AtPAO5-1* transgenic plants shorter ones (Fig. 15 B).

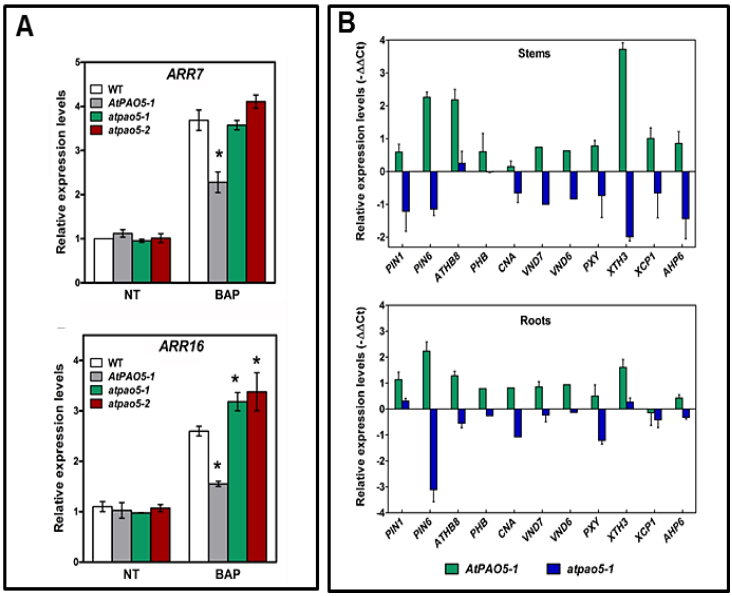

Fig. 14. Relative expression levels of auxin- and cytokinin-related genes in *AtPAO5-1* and *atpao5-1* plants. (A) Regulation of cytokinin-related genes the cytokinin 6-benzylaminopurine (BAP) in *AtPAO5-1*, *atpao5-1* plants and wild-type (WT) plants. Asterisks denote statistically significant differences from the corresponding WT plants. (B) Relative expression levels of auxin- and cytokinin-related genes in stems and roots of *AtPAO5-1* and *atpao5-1* plants. From Alabdallah *et al*., 2017.

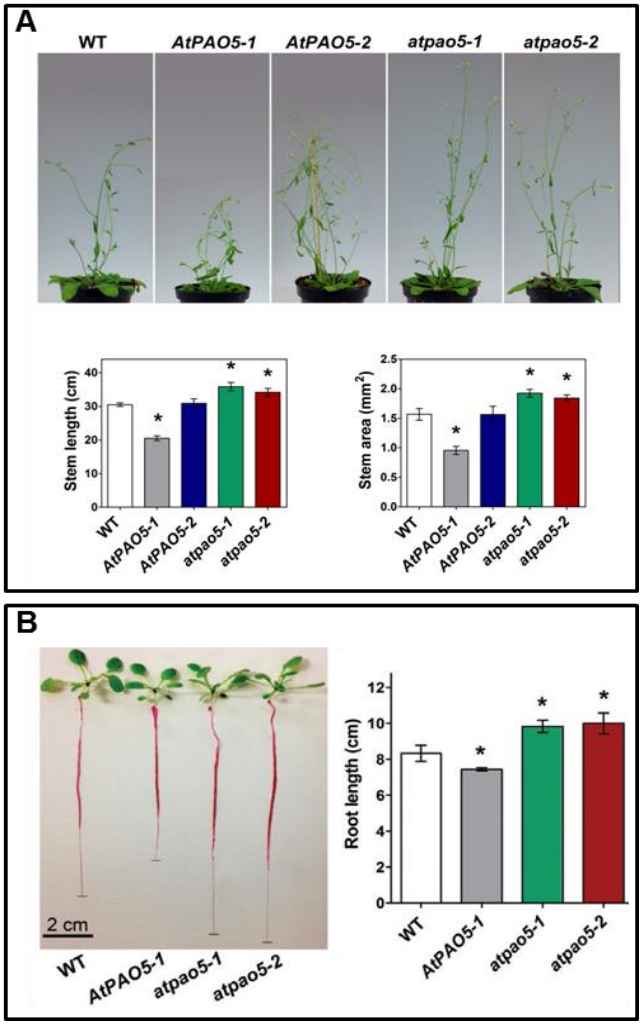

Fig. 15. Phenotype of *AtPAO5* and *atpao5* transgenic plants. Stem (A) and root (B) phenotype of *AtPAO5*, *atpao5*, and wild-type (WT) plants. Roots were stained externally with fuchsin to help visualization. Asterisks indicate statistically significant differences from WT plants (P<0.05). Figure modified from Alabdallah *et al*., 2017

1.8 Polyamine metabolism and stomata movement

Stomatal pores are formed by pairs of guard cells and serve as major gateways for both CO_2 influx into plants from the atmosphere and the transpirational water loss of plants (Kim *et al*., 2010). Regulation of stomatal movement is critical for plant growth and adaptation to environmental stresses. The opening and closing of stomatal pores is mediated by turgor and volume changes in guard cells (Kim *et al*., 2010). ABA, which plays a central role in the control of stomata movements, promotes stomatal closing under dehydration stress, whereas stomatal opening is induced by light, including blue and red light. Blue light activates the plasma membrane H^+-ATPase, hyperpolarizes the membrane potential with simultaneous apoplast acidification and drives K^+ uptake through voltage-gated K^+ channels, which further results in a decrease of water potential and subsequent water uptake in guard cells. The elevated turgor then increases guard cell volume, and widens the stomatal aperture. Apart from the movement of ions, starch metabolism in the guard cell might also be involved. As the accumulation of positively charged K^+ ions in guard cells must be compensated by anions, malate, the main anion accumulated in guard cells in most plant species during stomatal opening, is synthesized. Malate can be produced through the degradation of starch in guard cells under blue light. How the starch is then metabolized to produce malate in guard cells remains unclear.

Generation of ROS is a downstream step in stomata closure induced by the drought hormone ABA (Kim *et al*., 2010). ROS are also involved in the regulation of stomata aperture by ethylene (Desikan *et al*., 2006), methyl jasmonate (Munemasa *et al*., 2007), and salicylic acid (Mori *et al*., 2001). Generation of H_2O_2 during ABA-induced stomata closure causes the activation of inward-rectifying Ca^{2+}-influx channel and the transient rise of cytosolic Ca^{2+} (Kim *et al*., 2010). Intracellular Ca^{2+} increases further affect plasma membrane transporters, facilitating stomata closure and preventing stomata re-opening. Specific protein kinases, phosphatidic acid, phosphatidyl-inositol-3-phosphate, inositol-3-phosphate, inositol-6-phosphate, sphingolipids, phospholipase C, and the reactive nitrogen species nitric oxide (NO) are also important ABA signalling intermediates in guard cells (Desikan *et al*., 2004; Kim *et al*., 2010).

Plants respond to stress through many mechanisms, stomatal regulation being one of the most studied. Among the ion channels in guard cells, the inward K^+ channel (I_{Kin}), outward K^+ channel (I_{Kout}), and anion channels in the plasma membrane are well characterized by patch-clamp studies. Some of these stress factors, including drought, high salt, and air

pollutants, are also the factors that elevate polyamine levels and it has been suggested that elevated polyamine levels may be related to stomatal regulation. Furthermore, it was shown that all natural polyamines, including Spd, Spm, Cad and Put, mimic stress conditions in blocking stomatal opening and inducing stomatal closure and that the I_{Kin} channels in guard cells are inhibited by these polyamines (Fig. 16; Liu *et al*., 2000). Recently, it was also shown that the acetylated form of Dap modulates plasma membrane electrical and ion transport properties, and thus stomata movement, in an opposite way to that of ABA (Jammes *et al*., 2014).

Polyamine catabolism plays important role in the control of stomata aperture (Fig. 16; Pottosin *et al*., 2014). Stomatal closure induced by ABA in *Vicia faba* and by ethylene in *Arabidopsis* involves induction of CuAO and PAO activity, respectively, as a source of H_2O_2 (An *et al*., 2008; Hou *et al*., 2013; Qu *et al*., 2014). It has also been demonstrated that the activities of CuAO and NADPH oxidase contribute to ABA induced H_2O_2 production independently and that both routes converge at the level of H_2O_2-induced Ca^{2+} influx (An *et al*., 2008).

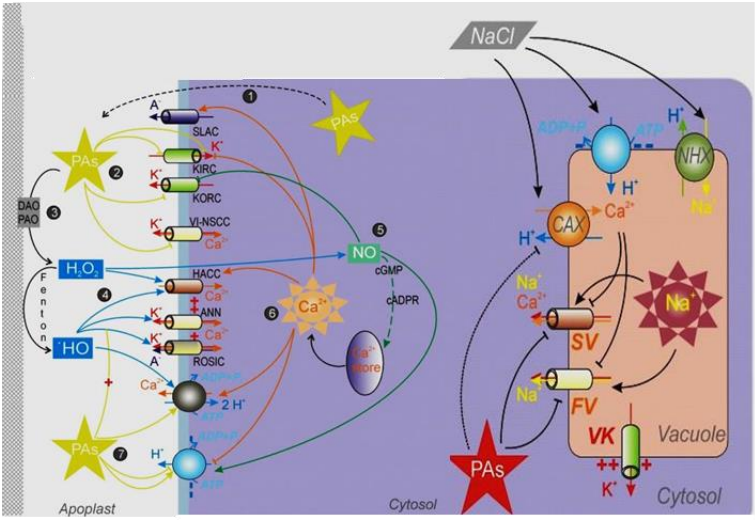

Fig. 16. Regulation of ion transport across the plasma membrane (PM) and the vacuolar membranes by polyamines and their catabolites. (1)Polyamines (PAs) are exported from the cytosol to the apoplast against the electrochemical gradient. (2) PAs inhibit inward-rectifying (KIRC) and outward rectifying (KORC) K^+ and non-selective voltage-independent cation (VI-NSCC) channels. (3)PAs oxidation by diamine (DAO) and/or polyamine (PAO) oxidases generates H_2O_2 in the apoplastic space. There H_2O_2 can be converted to •OH. (4) H_2O_2 and •OH activate a variety of non-selective Ca^{2+}-permeable channels, including hyperpolarization-activated Ca^{2+} influx channel (HACC), annexin-formed channel (ANN), and non-selective voltage-independent conductance (ROSIC). (5)H_2O_2 released during PA catabolism, causes a rapid NO generation. In its turn, NO inhibits KORC and induces the intracellular Ca^{2+} release. (6)Several PM channels, including slow anion channel (SLAC) and pumps, are regulated by cytosolic Ca^{2+}. (7)PAs potentiate the ROSIC activation, activate the PM Ca^{2+}-ATPase and alter the activity of the PM H^+-ATPase. Intracellular PAs and vacuolar Ca^{2+} may act as alternative regulators of vacuolar cation channels. At high salinity, efficient vacuolar Na^+ sequestration is critical for the salt tolerance. This requires the increased Na^+/H^+ antiport activity and a decrease of Na^+ leaks through non-selective fast (FV) and slow (SV) activating vacuolar cation channels. The block by PAs would abolish the FV-mediated current, and strongly suppress the SV current. Continuous operation of the K+-selective channels (VK), weakly sensitive to PAs, acts as a shunt conductance for the electrogenic H^+-pump, which fuels the active Na^+-uptake, and contributes to the recuperation of the salt-induced cytosolic K^+ loss. Modified from Pottosin and Shabala, (2014).

AIM OF THE WORK

Plant PAOs are characterized by a broad variability in substrate specificity, catalytic mechanism, subcellular localization and expression pattern which suggests variability also in physiological roles. PAOs contribute to different physiological processes through regulation of polyamine levels and/or reaction products. Extracellular PAOs have been shown to play crucial roles during plant growth under physiological and stress conditions, giving rise to increased apoplastic H_2O_2, which in turn signals cell-wall development, xylem differentiation and defense responses.

The aim of the present work is to study the contribution of the five members of the *Arabidopsis thaliana PAO* gene family in developmental and defense processes. This study is focus on the three peroxisomal *AtPAOs (AtPAO2, AtPAO3* and *AtPAO4)*, which are regulated by stress-related factors, are highly expressed in guard cells and are involved in the control of stomata movement, as evidenced by preliminary data. In particular, this works further investigates the contribution of the three peroxisomal *AtPAOs* to stomata closure and to plant defense responses under salt-stress and drought conditions. This study is also focused on the cytosolic polyamine oxidase/dehydrogensase 5 (AtPAO5) for which a role in the control of plant growth has been shown. An important aim of the present work is to determine how *AtPAO5* interferes with plant growth and to examine whether *AtPAO5* interferes with plant growth also under stress conditions. Important tools of the present work are loss-of-function mutants and Arabidopsis transgenic plants ectopically expressed various AtPAOs. This study will allow to further comprehend the functional complexity characterizing polyamine metabolism.

2 RESULTS

2.1 Expression studies on *AtPAO* gene family

To study the contribution of the *AtPAO* gene family and in particular of the *AtPAO234* gene subfamily to the plant stress responses, the expression levels of the five members of the *AtPAO* gene family were analysed following treatment with the stress related hormones ABA and Methyl Jasmonate (MeJA), as well as under stress conditions (NaCl treatment and drought) by quantitative Real-time RT-PCR (qRT-PCR). This analysis evidenced that *AtPAO1* expression levels are modulated by these treatments (Fig. 17).

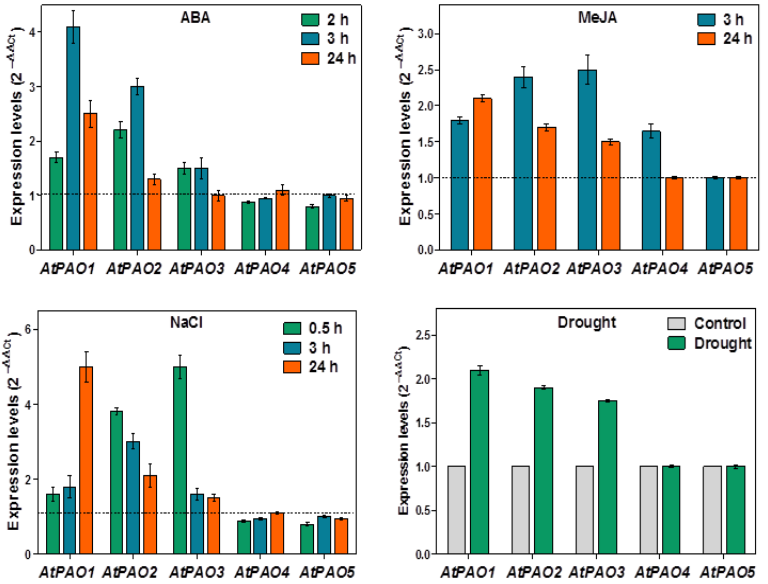

Fig. 17. Regulation of *AtPAO1*, *AtPAO2*, *AtPAO3*, *AtPAO4* and *AtPAO5* expression levels. Quantitative RT-PCR analysis of *AtPAO* expression levels was performed in whole seedlings of 10-day-old wild-type plants grown *in vitro* treated with 50 µM ABA, 50 µM MeJA or 100 mM NaCl for the indicated time periods. Relative expression levels are presented as fold-changes ($2^{-\Delta\Delta Ct}$) in respect to the untreated controls at each time point. *AtPAO* expression levels were also analyzed in plants undergone water withdrawal for 2 h and then left to recover for 1 h (Drought). Dashed lines, added to facilitate visualization of differences) show the baseline expression levels of the untreated controls. Mean values ± SE of relative quantitation from two independent experiments are shown.

AtPAO2 and *AtPAO3* expression levels are also regulated by ABA, JA, NaCl and drought, while on the contrary, the expression levels of *AtPAO4*, which forms together with *AtPAO2* and *AtPAO3* a distinct PAO group (*AtPAO234* subfamily), remain invariable under these conditions. This analysis additionally showed that *AtPAO5* expression levels are not modulated by ABA, JA, NaCl and drought treatments. These data are in part in agreement with recent studies showing ABA-inducible expression of *AtPAO1* in the transition region between the meristematic and the elongation zone of roots and of *AtPAO2* in the guard cells (Fig. 9; Fincato *et al*., 2012).

2.2 Studies on the physiological roles of the *AtPAO234* gene subfamily

2.2.1 Contribution of *AtPAO234* gene family to stomata movement

Despite some important differences among the three members of the *AtPAO234* gene subfamily regarding substrate specificity, expression pattern (specific expression pattern in columella) and regulation of gene expression (constitutive expression in the case of *AtPAO4* and stress inducible in the case of *AtPAO2* and *AtPAO3*), the common origin of *AtPAO2*, *AtPAO3* and *AtPAO4* and some similar characteristics (peroxisomal localization, catalytic mechanism and expression in guard cells and pollen grains) suggest similar physiological roles. Indeed, recent studies in our laboratory have shown that *atpao2*, *atpao3*, *atpao4* insertional mutants display reduced stomatal closure as compared to wild-type plants in response to ABA treatment (Fincato P, PhD thesis; Ahou A, PhD thesis). Interestingly, the reduced stomata closure observed in the single mutants was even more pronounced in the double *atpao2atpao4* and *atpao3atpao4* double mutants and the triple *atpao2atpao3atpao4* (*atpao234)* mutant, the last one presenting the highest variation in stomata movement in respect to the wild-type plants suggesting the involvement of all three peroxisomal *AtPAOs* in the ABA-mediated control of guard cells in a synergistic way (Ahou A, PhD thesis).

Here, more studies were performed to analyze the contribution of the *AtPAO234* gene subfamily in the regulation of stomata movement using the *atpao234* triple mutant, which present decrease in Put levels and increased Spd levels compared to the wild-type plants (Fig. 18). Parallel studies have been performed using *Arabidopsis* transgenic plants ectopically expressing

high levels of *AtPAO3* (*AtPAO3over*) and presenting increased levels of Put (Fincato *et al*., 2011).

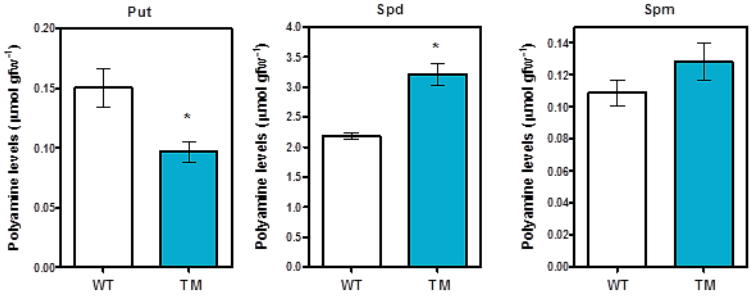

Fig. 18. Polyamine content in *atpao234* triple mutant plants. Polyamine levels were determined in 10-day-old *atpao243* (TM) and wild-type (WT) seedlings by HPLC analysis. The analysis was repeated three times obtaining similar results and a representative experiment is shown. Bars indicate standard error of three independent replicates. Asterisk denote statistically significant differences from the WT plants. Statistical analysis was performed by one way ANOVA test ($p < 0.001$).

These studies confirmed that the *atpao234* mutant plants respond less in the ABA-mediated stomata closure than the wild-type plants and further showed that the *AtPAO3over* plants do not display statistically significant differences in ABA-mediated stomata movement compared to the wild-type plants (Fig. 19). This may due to low activity of the *35SCaMV* promoter in guard cells.

Similarly to ABA, the volatile phytohormone methyl jasmonate (MeJA), which regulates various physiological processes, including pollen maturation, and responses to wounding and pathogen attack (Liechti and Farmer, 2002; Turner *et al*., 2002), plays also a role in stomatal closure. Indeed, jasmonate-induced stomatal closure has been observed in several plant species (Munemasa *et al*., 2011a). Interestingly, interaction between MeJA and ABA signaling pathways was shown in guard cells (Munemasa *et al*., 2011b). Considering the importance of MeJA in the control of stomata closure, the *atpao234* mutants as well as the *AtPAO3over* plants transgenic plants were analyzed for stomata movement also following treatment with MeJA. Results showed that *atpao234* mutant plants respond less in the MeJA-mediated stomata closure than the wild-type plants, whereas *AtPAO3over* plants do not display statistically significant differences from wild-type plants in MeJA-mediated stomata movement (Fig. 19). Similar results have been obtained following treatment with NaCl or following water withdrawal (Fig. 20).

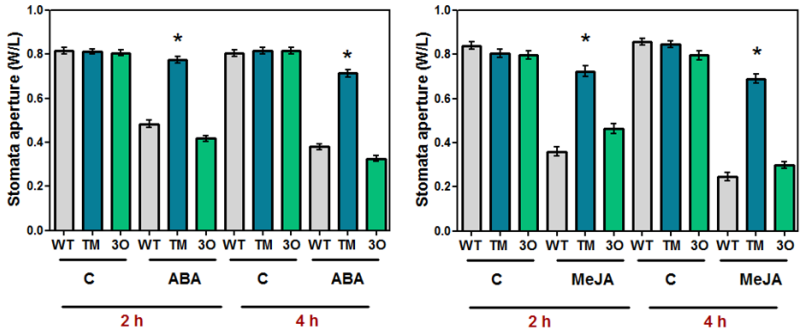

Fig. 19. Stomata movements in *atpao234* triple mutants and *35S::AtPAO3* transgenic Arabidopsis plants ectopically expressing AtPAO3. Stomatal movements were determined in *atpao243* triple mutant (TM), *35S::AtPAO3* transgenic plants (3O) and wild-type (WT), 7 days old plants grown *in vitro*, following treatment with 50 µM abscisic acid (ABA) or methyl Jasmonate (MeJA) for 2 h (ABA) or 4 h. Width (W) and length (L) of stomata pores were measured from images of stomata using a digital ruler (ImageJ) and stomata aperture (width/length) was calculated. Bars indicate standard error (n>60). Experiments were repeated more than 3 times and representative experiments are shown. Asterisks indicate values statistically different from the corresponding WT plants by one-way ANOVA test ($p<0.001$). C: untreated control.

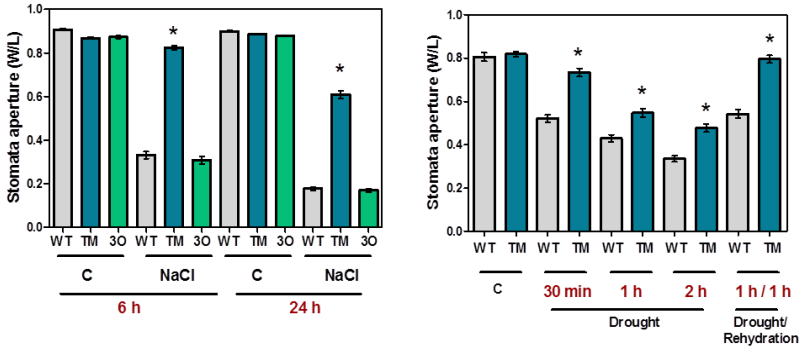

Fig. 20. Stomata movements in *atpao234* triple mutants and *35S::AtPAO3* transgenic Arabidopsis plants ectopically expressing AtPAO3. Stomatal movements were determined in *atpao234* triple mutants (TM), *35S::AtPAO3* transgenic plants (3O) and wild-type (WT), 7 days old plants grown *in vitro*, following treatment with 150 mM NaCl for 6 h or 24 h. Stomatal movements were also determined in plants undergone medium withdrawal for 30 min, 1 h or 2h (drought), as well as in plants left to recover for 1 h in medium after 1 h of medium withdrawal. Width (W) and length (L) of stomata pores were measured from images of stomata using a digital ruler (ImageJ) and stomata aperture (width/length) was calculated. Bars indicate standard error (n>60). Experiments were repeated more than 3 times and a representative experiment is shown. Asterisks indicate values statistically different from the corresponding WT plants by one-way ANOVA test ($p<0.001$). C: untreated control.

In the latter case, rewatering induces stomata reopening with the same efficiency in both wild-type and *atpao234* plants, considering that the differences in stomata aperture between wild-type and *atpao234* plants after water withdrawal persist also after rewatering (Fig. 20).This suggests that *atpao234* plants respond differently from the wild-type plants in stomata movement mainly under conditions which induce stomata closure. This may explain the lack of statistically significant differences between wild-type and *atpao234* plants under physiological (control) conditions.

The ABA-induced stomata closure involves several intermediate signaling molecules among which H_2O_2 has a crucial role. Indeed, addition of *N*,*N*[1]-dimethylthiourea (DMTU), H_2O_2 scavenger inhibited ABA-mediated stomata closure in wild-type plants (Fig. 21). To determine whether the reduced stomata closure exhibited by the *atpao234* mutant plants is due to reduced production of H_2O_2, stomata closure was also determined in these plants following treatment with H_2O_2 alone or together with ABA. As shown in Fig. 21, *atpao234* mutants present reduced response not only to ABA, but also to H_2O_2.

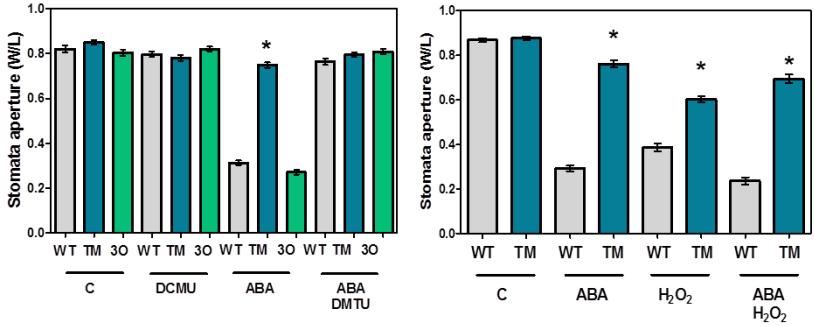

Fig. 21. Stomata movements of *atpao234* mutants and *35S::AtPAO3* transgenic Arabidopsis plants ectopically expressing *AtPAO3*. Stomatal movements were determined in *atpao234* triple mutants (TM), *35S::AtPAO3* transgenic plants (3O) and wild-type (WT) plants following treatment with 50 μM abscisic acid (ABA) and/or 100 μM *N*,*N*'-dimethylthiourea (DMTU) and/or 250 μM H_2O_2 for 4 h. Stomata aperture is expressed as width/length of stomata pores. Bars indicate standard error (n>60). Experiments were repeated 3 times and a representative experiment is shown. Asterisks indicate values statistically different from the corresponding WT plants by one-way ANOVA test ($p<0.001$). C: untreated control.

Furthermore, the addition of H_2O_2 together with ABA had an additive effect on stomata closure in both the *atpao234* mutants and the wild-type plants, without however restoring the *atpao234* defects in the ABA-mediated closure. Indeed, the *atpao234* mutants showed reduced stomata closure compared to the wild-type plants also under these

conditions. These data suggest that the reduced responsiveness of the *atpao234* mutants to ABA-mediated stomata closure is not due to reduced production of H_2O_2.

To understand whether the reduced responsiveness of *atpao234* guard cells to ABA, MeJA compared to the wild-type plants, salt stress and drought is due to altered levels of polyamines, stomata movement in *atpao234* mutants and wild-type plants was analyzed following treatment with polyamines. This analysis showed that all polyamines tested (Put, Spd, Spm, Dap and Nor-Spm) induce stomata closure in wild-type plants with only a very limited effect in *atpao234* stomata (Fig. 22). Furthermore, treatment with both polyamines and ABA had an additive effect in stomata closure both in *atpao234* mutants and wild-type plants, the *atpao234* mutants responding always less that the wild-type plants in these treatments (Fig. 22). These data indicate that the reduced responsiveness of the *atpao234* mutants to ABA-mediated stomata closure is not due to altered polyamine levels. Furthermore, altogether these studies suggest that the reduced stomata movements presented by the *atpao234* mutant plants are not specific to the ABA or MeJA signaling pathways, being observed under other stimuli inducing stomata closure (H_2O_2 and polyamines).

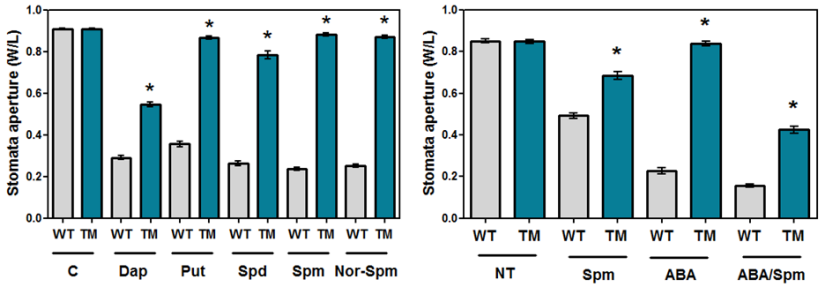

Fig. 22. Effect of poyamines on stomata movement in *atpao234* Arabidopsis mutants. Stomatal movements were determined in *atpao234* triple mutants (TM) and wild-type (WT) plants following treatement with 20 mM abscisic acid (ABA) and/or 1 mM putrescine (Put), spermidine (Spd), spermine (Spm), norspermine (Nor-Spm) or 1,3 diaminopropane (Dap) for 5 h. Stomata aperture is expressed as width/length of stomata pores. Bars indicate standard error (n>60). Experiments were repeated 3 times and a representative experiment is shown. Asterisks indicate values statistically different from the corresponding WT plants by one-way ANOVA test ($p<0.001$). C: untreated control.

2.2.2 Effect of ABA and NaCl on the growth of *atpao234* mutants

Since the reduced stomatal closure exhibited by the *atpao234* mutants following ABA, MeJA, polyamine and salt treatments, as well as dehydration may be correlated to altered defense responses under stress conditions, studies were performed to analyze growth of *atpao234* and wild-type plants in the presence of ABA or NaCl.

In the absence of ABA, no significant difference in seedling growth (data not shown) and root elongation (Fig. 23) was observed between *atpao234* mutant and the wild-type plants (Fig. 23). In the presence of ABA, seedling growth was inhibited both in *atpao234* mutant plants and the wild-type plants. In particular, in the presence of ABA the *atpao234* mutant plants showed a small, but statistically significant difference, in root elongation from the wild-type plants (Fig. 23), while no difference between the two genotypes in the growth of the aerial part was evident (data not shown).

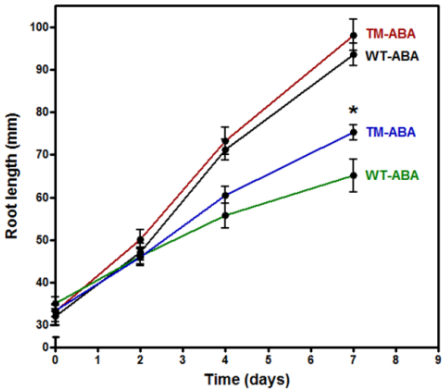

Fig. 23. Effect of abscisic acid (ABA) on root growth of *atpao234* Arabidopsis mutants. Wild-type (WT) and *atpao234* (TM) seedlings of 5 days were transferred onto plates containing (+ABA) or not (-ABA) 10 μM ABA. Root length was measured at various time intervals after transferring. Bars indicate standard error. Asterisks indicate values statistically different from the corresponding wild-type (WT) plants by one-way ANOVA test ($p<0.001$).

Furthermore, in the presence of NaCl, inhibition of root growth (Fig. 24A) and level of leaf chlorosis (Fig. 24B) in *atpao234* seedlings were similar to those of wild-type plants. In addition, analysis of salt tolerance in plants growing on soil or a hydroponic system did not evidence differences in the growth and survival among *atpao234*, *AtPAO3over* and wild-type plants (data not shown). These data suggest that the *atpao234* and the *AtPAO3over* plants do not present altered tolerance to salt stress conditions in respect to the wild-type plants, in agreement with recently published studies (Sagor *et al*., 2016). The response of the *atpao234* and *AtPAO3over* plants under drought stress conditions has still to be analyzed to further assess the contribution of *AtPAO234* gene family to abiotic stress conditions.

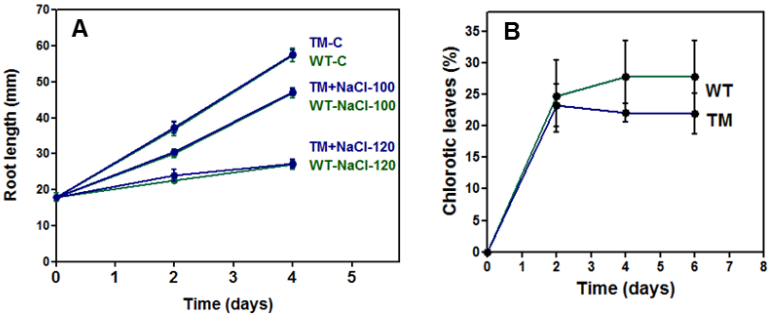

Fig. 24. Root growth and leaf chlorosis of *atpao234* Arabidopsis mutants growing under salt stress conditions. Wild-type (WT) and *atpao234* (TM) seedlings of 5 days were transferred onto plates containing 100 mM (A, B) or 120 mM (A) NaCl. Root length **(A)** and number of chlorotic leaves **(B)** were measured at various time intervals after transferring. Bars indicate standard deviation. C: untreated control.

2.3 Studies on the *AtPAO5* physiological roles

2.3.1 The contribution of *AtPAO5* in the control of xylem differentiation

Recent data have shown that AtPAO5 is involved in the control of root and stem growth. Indeed, two *AtPAO5* loss-of-function mutants (*atpao5*-1 and *atpao5-2* mutants) produce longer and thicker stems than wild-type plants, while *AtPAO5* overexpressing transgenic Arabidopsis plants (*AtPAO5-1*) with 70-fold (*AtPAO5-1*; Supplementary Fig. S1; Ahou *et al*., 2014; Alabdallah *et al*., 2017) higher expression levels than the endogenous gene, produce shorter and thinner stems leading to a semi-dwarf phenotype with reduced apical dominance (Fig. 15; Alabdallah *et al*., 2017). In a similar way, *atpao5-1* and *atpao5-2* plants present longer roots than wild-type plants, while *AtPAO5-1* transgenic plants shorter ones (Fig. 15; Alabdallah *et al*., 2017). To understand the differences in inflorescence and root development among *atpao5* mutant plants, *AtPAO5* overexpressing plants, and wild-type plants at the cellular level, stem and root anatomy was examined by analyzing transverse sections. These analyses showed that *AtPAO5-1* homozygous plants undergo excessive primary xylem differentiation. Indeed, they present a much higher number of large-diameter, thick-walled metaxylem vessels than wild-type plants, and display a highly reduced secondary growth (Fig. 25; Fig. 26). Similarly, hemizygous *AtPAO5-1* plants present an increased number of metaxylem vessels compared to wild-type plants, although to a lesser degree than the corresponding homozygous plants (Fig. 25; Fig. 26). In contrast, *AtPAO5* overexpressing transgenic Arabidopsis plants (*AtPAO5-1*) with 4-fold higher expression levels than the endogenous gene (*AtPAO5-2*; Supplementary Fig. S1) do not present changes in xylem differentiation. Furthermore, the *atpao5-1* and *atpao5-2* mutant plants have a lower number of metaxylem vessels and a more extensive secondary growth than wild-type plants (Fig. 25; Fig. 26). These differences in xylem differentiation were observed both in young inflorescences before the appearance of differences in stem length and in later developmental stages. Similar differences in xylem differentiation between *AtPAO5* overexpressing plants, *atpao5* mutants and wild-type plants were observed also in roots and hypocotyls (Fig. 25).

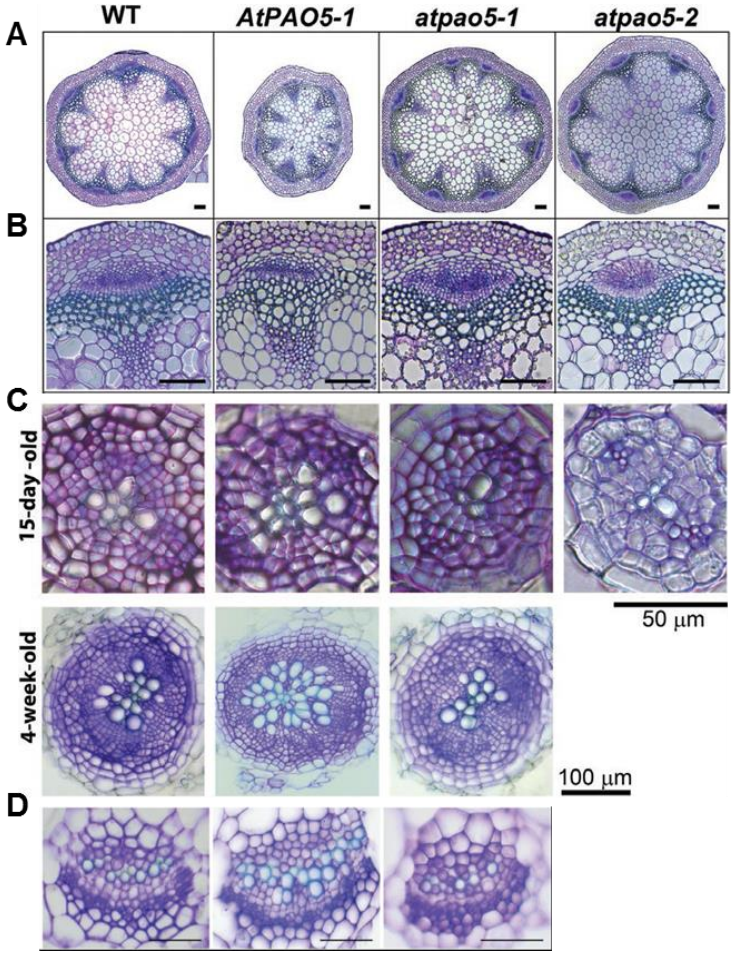

Fig. 25. Histological analysis of inflorescence stems, roots and petioles of *AtPAO5-1* and *atpao5* plants. (A) Transverse sections of the second internodes at the basal end of inflorescence stems stained with toluidine blue. (B)Vascular bundles of inflorescence stems as observed from transverse sections stained with toluidine blue. (C) Root transverse sections of 15-day-old plants grown *in vitro* (upper row) and 4-week-old plants grown in hydroponic cultures (lower row). (D) Transverse sections of petioles stained with toluidine blue. Black bars in D indicate 50 μm.

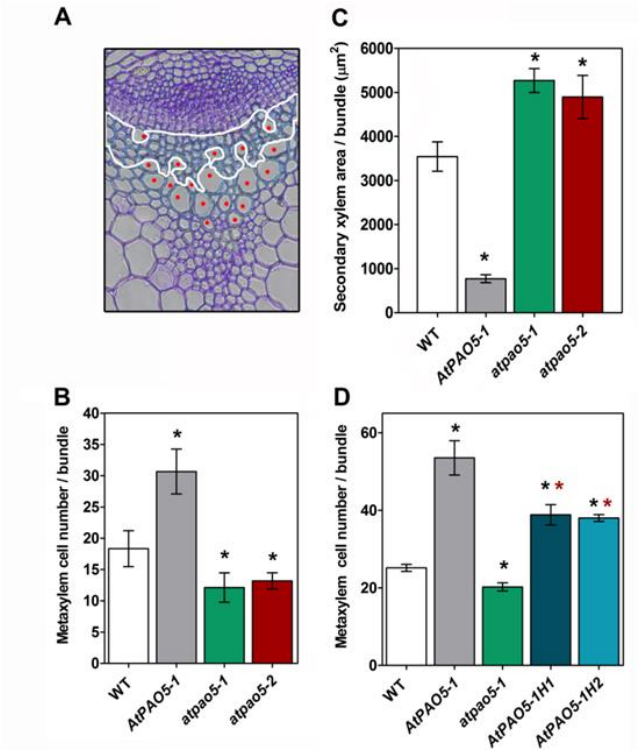

Fig. 26. Stem section analysis of *AtPAO5-1*, *atpao5-1*, *atpao5-2* and wild-type (WT) plants. (A) An example of quantification of the number of metaxylem cells and the size of secondary xylem area in a stem transverse section stained with toluidine blue. Measurements were performed following image acquisition through ImageJ software. (B; D) Number of metaxylem vessels in stem bundles of *atpao5-1* and *atpao5-2* mutants, homozygous *AtPAO5-1* transgenic plants, two heterozygous *AtPAO5-1* transgenic plants (*AtPAO5-1H1*, *AtPAO5-1H2*; Supplementary Fig. S2), and WT plants. (C) Size of secondary xylem in bundles of *AtPAO5-1*, *atpao5-1*, *atpao5-2*, and WT stems. Numbers are mean values ±SE. Black and red asterisks indicate statistically significant differences from WT and *AtPAO5-1* plants ($P<0.05$; one-way ANOVA test). Representative experiments are shown.

The phenotypical differences among the various genotypes are probably due to differences in the levels of Therm-Spm that is involved in the control of xylem differentiation. Indeed, *AtPAO5-1* transgenic plants and the two *atpao5* mutants present differences in Therm-spm levels, together with differences in Spm and N^1-acetyl-Spm, from the wild-type plants (Ahou *et al*., 2014; Alabdallah *et al*., 2017). Furthermore, the shorter stems together with the altered stem anatomy observed in *AtPAO5-1* plants

recall the *acl5* and *bud2/samdc4* mutants with reduced levels of Therm-Spm which also display an increased number of primary xylem vessels and weak production of secondary xylem (Hanzawa *et al*., 2000; Clay and Nelson, 2005; Ge *et al*., 2006; Kakehi *et al*., 2008; Muniz *et al*., 2008; Cui *et al*., 2010). To better comprehend the underlying mechanism in *AtPAO5* contribution to xylem differentiation, stem growth and xylem differentiation of *AtPAO5-1* and *atpao5-1* plants were compared to those of *acl5* mutants and *ACL5* overexpressing transgenic plants (*ACL5::ACL5-GFP*; Baima *et al*., 2014) growing under the same conditions This analysis showed that *acl5* mutants present more severe phenotypical alterations than *AtPAO5-1* transgenic plants. In particular, *acl5* stems are much shorter than those of *AtPAO5-1* transgenic plants, which in turn have shorter stems than those of the wild-type plants (Fig. 27).

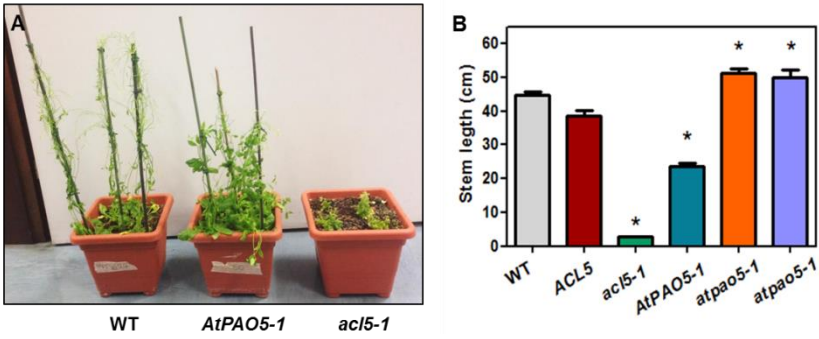

Fig. 27. Phenotype of plants with modulated *AtPAO5* and *ACL5* expression levels. (A) Inflorescences of *AtPAO5-1*, *acl5-1*, (Baima *et al*., 2014) and wild-type (WT) plants. (B) *AtPAO5-1*, atpao5-1, atpao5-2, *acl5-1*, *ACL5::ACL5-GUS (ACL5)* (Baima *et al*., 2014) and wild-type (WT) plants were grown under the same conditions for 6 weeks and stem lengths were measured. Numbers are mean values ±SE. Asterisks denote statistically significant differences from WT plants (P<0.05; one-way ANOVA test). Representative experiment is shown. The *acl5-1* and *ACL5::ACL5-GUS* plants were a kind gift from Dr. Simona Baima.

Furthermore, stem section analysis showed that *acl5* plants present more extensive proliferation of xylem vessels than the *AtPAO5-1* plants (Fig. 28). Conversely, the *ACL5* plants present a lower number of xylem vessels than the wild-type similarly to those of the *atpao5-1* plants (Fig. 28). These data further suggest that the different phenotype of the AtPAO5-1 and *atpao5-1* plants from that of the wild-type plants is probably due to differences in Therm-Spm levels. The differences between *AtPAO5-1* and

acl5 plants in the severity of the phenotypical alteration can be explained by the fact that the reduction of Therm-Spm levels in *AtPAO5-1* plants is based on the AtPAO5 catalytic reaction, whereas in *acl5* mutants it is based on the impairment of Therm-Spm biosynthesis. However, the contribution of N^1-acetyl-Spm to these differences in xylem differentiation has still to be analyzed.

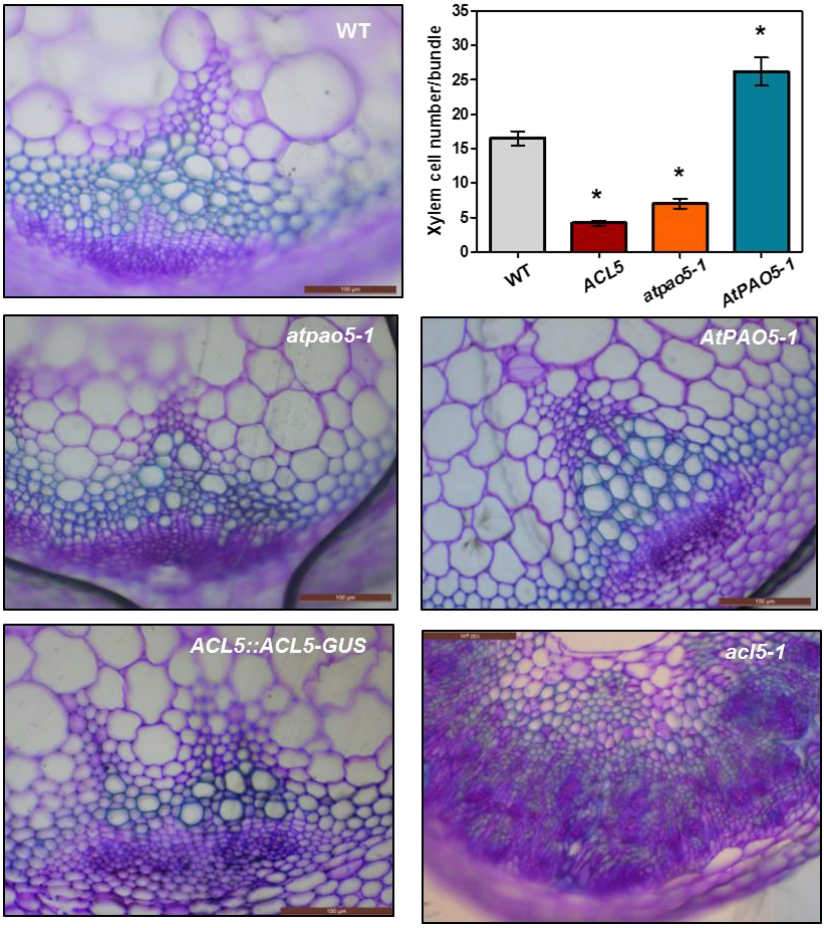

Fig. 28. Vascular bundles of *AtPAO5-1*, *atpao5-1*, *acl5-1*, *ACL5::ACL5-GUS* and wild-type (WT) plants. Transverse sections of the second internodes at the basal end of inflorescence stems of 6-week-old plants stained with toluidine blue The number of metaxylem vessels in stem bundles were measured. Numbers are mean values ±SE. Asterisks denote statistically significant differences from WT plants ($P<0.05$; one-way ANOVA test). *ACL5::ACL5-GUS* and *acl5-1* plants were a kind gift from Dr. Simona Baima.

2.3.2 Effect of exogenous Therm-Spm and Nor-Spm on xylem differentiation in *AtPAO5-1* and *atpao5* plants

To further verify whether the altered phenotype of *AtPAO5-1* and *atpao5* plants is due to altered Therm-Spm levels, the different genotypes were grown in the presence of Therm-Spm or Nor-Spm, the latter having been shown to functionally substitute for Therm-Spm (Kakehi *et al*., 2010). Therm-Spm treatment reduced the number of metaxylem vessels in wild-type and *atpao5-1* roots, but not in *AtPAO5-1* roots (Fig. 29 A). Furthermore, Nor-Spm treatment in plants grown in a hydroponic system reduced the number of metaxylem vessels in both wild-type and *AtPAO5-1* stems (Fig. 29B).

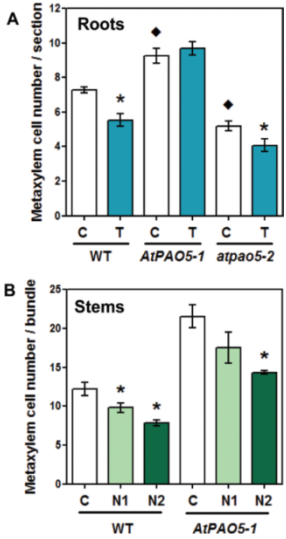

Fig. 29. Modulation of xylem differentiation by Therm-Spm and Nor-Spm. Number of metaxylem vessels in roots **(A)** and stems **(B)** following treatment with Therm-Spm and Nor-Spm, respectively. For root sections, 5-d-old seedlings were transferred either onto agar plates containing 100 μM Therm-Spm (T) or onto no-treatment control plates (C) and left to grow for 10 days more. For stem sections, plants grown in a hydroponic system were treated at the beginning of bolting with 20 μM (N1) or or 40 μM (N2) of Nor-Spm for 10 days. C indicates untreated controls. Mean values ±SE are shown. Asterisks denote statistically significant differences from the corresponding untreated controls and diamonds indicate differences from the untreated wild-type (WT) control plants.

2.3.3 Analysis of auxin distribution in *AtPAO5-1* and *atpao5-1* plants

Recent data have shown that both the cytokinin and the auxin signalling pathways are perturbed in the *atpao5* mutants and *AtPAO5-1* transgenic plants as compared the wild-type plants. In particular, data are compatible with increased auxin and decreased cytokinin signaling in *AtPAO5-1* plants and, conversely, decreased auxin and increased cytokinin signalling in *atpao5-1* mutants. Indeed, auxin- and cytokinin related genes are differentially expressed in *AtPAO5-1, atpao5-1 and wild-type* plants. To investigate whether *AtPAO5* indeed interferes with auxin distribution and response, *AtPAO5-1* and *atpao5-1* plants were crossed with a *DR5::GUS* marker line, *DR5* being a synthetic auxin responsive promoter (Chen *et al*., 2013). Histochemical GUS staining of these plants showed increased *DR5::GUS* expression in the root meristematic region of the *AtPAO5-1* roots and decreased in *atpao5*-1 roots as compared the *DR5::GUS* expression in wild-type plants (Fig. 30). These data are consistent with increased auxin signaling in *AtPAO5-1* plants and decreased in *atpao5-1* mutants. However, no reproducible difference in *DR5::GUS* expression levels among *AtPAO5-1*, *atpao5*-1 and wild-type plants in other organs was observed.

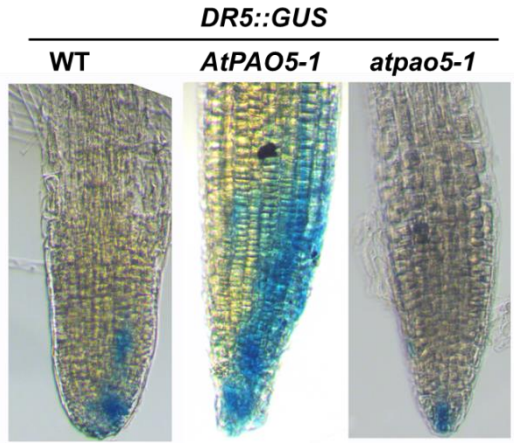

Fig. 30. Effect of *AtPAO5* expression levels on auxin distribution. Histochemical GUS staining of *AtPAO5-1, atpao5-1* and wild-type (WT) plants expressing *DR5::GUS. AtPAO5-1* and *atpao5-1* plants were crossed with *DR5::GUS* plants (a kind gift of Dr. Sabrina Sabatini).

2.3.4 Analysis of AtPAO5 contribution to plant defence responses to salt and drought stress

Polyamines were shown to contribute to plant defense responses to biotic and abiotic stresses (Marina *et al*., 2013; Kusano *et al*., 2008; Takahashi *et al*., 2010; Alcázar *et al*., 2006; Groppa and Benavides, 2008; Gupta *et al*., 2016). To deteremine whether the *AtPAO5-1* transgenic plants, and the *atpao5* mutants with altered levels of Spm, Therm-Spm and N^1-acetyl–Spm respond differently from the wild-type plants to abiotic stresses, the growth of these plants was examined under conditions of salt stress. In particular, plants grown on soil or on a hydroponic system have been treated with increasing amounts of NaCl (Fig. 31). After 24 days (growth on soil) or 10 days (growth on hydroponic system) of NaCl treatment, the *atpao5-1* and *atpao5-2* mutants presented a lower number of yellow leaves than the wild-type plants while conversely the *AtPAO5-1* plants a higher number (Fig. 31). These data indicate that the *atpao5-1* mutants are more tolerant to salt stress than the wild-type plants and the *AtPAO5-1* plants more sensitive than the wild-type plants. Very similar results have been obtained under drought stress conditions (Fig. 32). These data are in agreement with recently published studies (Sagor *et al*., 2016; Zarza *et al*., 2017).

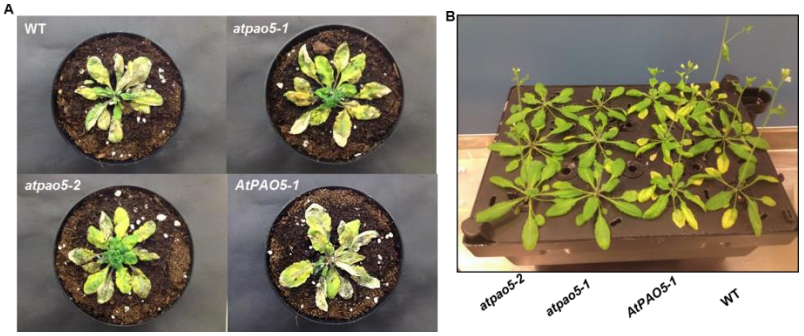

Fig. 31. Salt stress response of plants with altered *AtPAO5* expression levels. Plants grown on soil **(A)** or in a hydroponic system **(B)** for 25 days under standard conditions were treated with gradually increasing concentrations of NaCl (50 mM to 150 mM). The plants were photographed after 24 days (A) or 10 days (B) of NaCl treatment. Representative experiments of three replicates are shown.

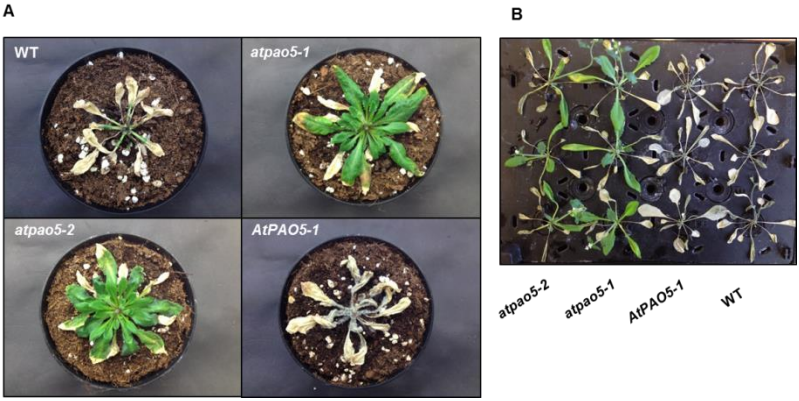

Fig. 32. Response to drought of plants with altered *AtPAO5* expression levels. (A) Water was withhold for 20 days from 30-day-old plants previously grown on soil pots under well-watered conditions. (B) Water was withdrawn for 40 h in 30-day-old plants grown in a hydroponic system. Following re-watering, plants were left to recover for 3 days and then photographed. The experiments have been repeated three times.

2.3.5 Contribution of AtPAO5 to stomata movement

Since polyamines have an important role in stomata movement, to investigate whether *AtPAO5* contributes to the plant defence responses, the different plant genotypes with altered *AtPAO5* expression levels, and thus altered polyamine homeostasis, were examined for stomata movement. These studies showed that the *atpao5* mutant plants respond less in the ABA-mediated stomata closure than the wild-type plants, while no statistically significant difference between *AtPAO5-1* and wild-type plants was observed in ABA-mediated stomata closure (Fig. 33). The *atpao5* mutant plants, but not the *AtPAO5-1* plants, differently respond also to the salt-, drought-, MeJA-, H_2O_2-, and polyamine-mediated stomata closure from the wild-type plants (Fig. 33; Fig. 34). These data indicate that *AtPAO5*, similarly to the peroxisomal *AtPAO* gene family (*AtPAO2*, *AtPAO3* and *AtPAO4*), is involved in the control of the stress-related stomata closure despite the fact that it is not expressed in the guard cells. Differently from the *atpao5* mutant plants, *atpao1* mutants do not present differences in ABA- and salt- mediated stomata movement from the wild-type plants (Fig. 33), while the double *atpao1atpao5* (*atpao15*) mutant respond in a similar way to that of *atpao5* mutants (Fig. 33).

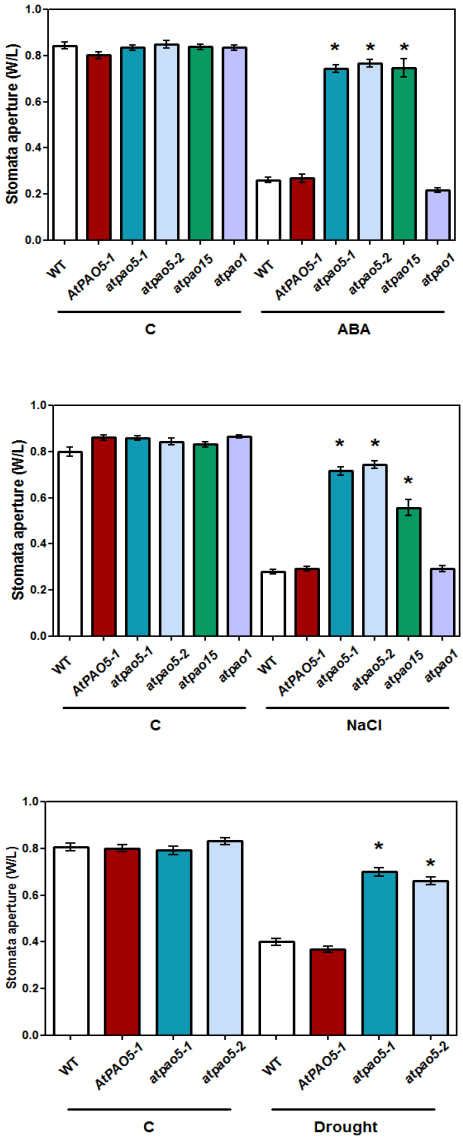

Fig. 33. Stomata movements in *atpao5* and *atpao1* single mutants, *atpao5atpao1* (*atpao15*) double mutants and in *AtPAO5-1* transgenic Arabidopsis plants. Stomatal movements were determined following treatment with 20 mM abscisic acid (ABA) for 4 h or 150 mM NaCl for 24 h, as well as following medium withdrawal for 1 h (drought). Width (W) and length (L) of stomata pores were measured from images of stomata using a digital ruler (ImageJ) and stomata apertures (width/length) were calculated. Bars indicate standard error (n>60). The different experiments were repeated for more than 3 times and representative experiments are shown. Asterisks indicate values statistically different from the corresponding wild-type (WT) plants by one-way ANOVA test ($p<0.001$). C: untreated control.

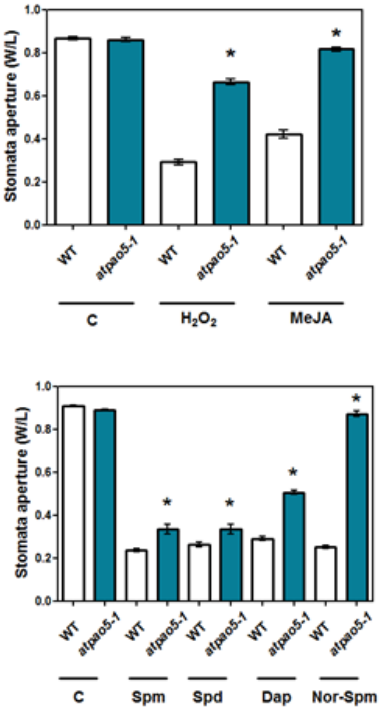

Fig. 34. Stomata movements in *atpao5* mutants. Stomatal movements were determined following treatment with 250 µM H_2O_2 or 50 µM methyl jasmonate (MeJA) for 4 h. Stomatal movements were also determined following treatment with 1 mM of putrescine (Put), spermidine (Spd), spermine (Spm), norspermine (Nor-Spm) or 1,3 diaminopropane (Dap) for 5 h. Stomata aperture is expressed as width/length of stomata pores. Bars indicate standard error (n>60). The different experiments were repeated more than 3 times and representative experiments are shown. Asterisks indicate values statistically different from the corresponding wild-type (WT) plants by one-way ANOVA test ($p<0.001$). C: untreated control.

3 DISCUSSION

The physiological studies on the *atpao234* triple mutant showed that the three peroxisomal AtPAOs are involved in the control of ABA-, MeJA-, H_2O_2 and polyamine-mediated stomata closure. Indeed, it was shown that the *atpao234* triple mutant displays reduced stomata closure in response to ABA, MeJA, H_2O_2 and polyamines, as compared the wild-type plants. Conversely, the *atpao234* mutant plants respond to the factors inducing stomata opening (as for example light/opening solution and rewatering following water withdrawal) as efficiently as the wild-type plants. This suggests that the *atpao234* plants respond differently from the wild-type plants in stomata movement mainly under conditions which induce stomata closure. The underlying mechanisms in the reduced stomata closure exhibited by the *atpao234* mutant are not clear so far. Reduced production of H_2O_2, which is an important second messenger in the ABA signaling network, as well as variations in polyamine levels, which play an important role in the modulation stomata movement through a direct effect on the voltage-dependent inward K^+ channels in the plasma membrane of the guard cells (Liu *et al.,* 2000), probably do not contribute to this effect, considering that the *atpao234* mutant respond differently from the wild-type plants also in the H_2O_2- and polyamine-mediated stomata closure. However, a dose-dependent effect of the altered H_2O_2 and/or polyamine levels in the reduced responsiveness of the *atpao234* guard cells cannot be excluded. On the other hand, it should be noted that, despite the reduced responsiveness of the *atpao234* triple mutant to the stress-related stimuli leading to stomata closure, which should reduce water loss through transpiration, the *atpao234* plants do not display altered tolerance to abiotic stress conditions in respect to the wild-type plants. The lack of such correlation has still to be analyzed.

Recent studies showed that *AtPAO5* contributes to Spm, Therm-Spm and N^1-acetyl-Spm homeostasis and that it participates in the feedback mechanisms controlling *ACL5* and *SAMDC4* expression (Ahou *et al.,* 2014; Alabdallah *et al.,* 2017). It was further shown that *AtPAO5* interferes with the cytokinin/auxin interplay, the cytokinin and the auxin signalling pathways being perturbed in the *atpao5* mutants and *AtPAO5-1* transgenic plants as compared the wild-type plants. Indeed, the *AtPAO5-1* and *atpao5*

plants present altered expression levels of genes implicated in auxin and cytokinin signaling (Fig. 14; Alabdallah *et al*., 2017). Consistently with these data, the present study showed increased *DR5::GUS* expression levels in the meristematic region of *AtPAO5-1* roots and decreased in *atpao5-1* roots as compared the *DR5::GUS* expression levels in wild-type roots (Fig. 30).

Previous studies showed that *AtPAO5* is also involved in the control of root and stem growth. Indeed, the *atpao5-1* and *atpao5-2* mutants produce longer and thicker stems than wild-type plants, while *AtPAO5-1* produce shorter and thinner stems leading to a semi-dwarf phenotype with reduced apical dominance (Fig. 15; Alabdallah *et al*., 2017). In a similar way, *atpao5-1* and *atpao5-2* plants present longer roots than wild-type plants, while *AtPAO5-1* transgenic plants shorter ones (Fig. 15). In the present study, it is shown that the *AtPAO5-1* and *atpao5* plants present differences from wild-type plants regarding primary xylem development in roots and stems, with *AtPAO5-1* plants presenting an increased number of metaxylem vessel elements and *atpao5* mutants a decreased number compared with wild-type plants (Fig. 26). In agreement with these changes, a number of genes involved in the regulation of xylem differentiation (*ATHB8*, *PHB*, *CNA*, *VND6*, *VND7*) and cell-wall formation (*XTH3*) are up-regulated in *AtPAO5-1* plants and down-regulated in *atpao5-1* mutants, both in stems and roots (Alabdallah *et al*., 2017). Differently from what takes place at the primary xylem, *AtPAO5-1* plants exhibit reduced production of secondary xylem in stems compared with wild-type plants, while atpao5 mutants exhibit increased production of secondary xylem. The reasons for this difference between primary and secondary xylem differentiation in *AtPAO5-1* and *atpao5* plants are still unclear, but it may be due to altered timing and/or extent of cell-death of xylem precursor cells, as shown for acl5 mutants (Muñiz *et al.,* 2008) which also display an increased number of primary xylem vessels and weak production of secondary xylem. In agreement with this, *XCP1*, a marker of xylem differentiation associated with programmed cell-death (Lucas *et al.,* 2013), is up-regulated in *AtPAO5-1* plants and *acl5* mutants and *et al.,* 2017). These data suggest mis-regulation of xylem maturation in *AtPAO5-1* and *atpao5-1* plants, thus not allowing proper secondary growth and organ extension.

The altered phenotype of *AtPAO5-1* with regards to stem length and vascularization (Fig. 15; Fig. 25; Fig. 26) is similar to that of *acl5* and *bud2/samdc4* mutants, which also present a dwarf phenotype and excessive xylem differentiation (Fig. 27; Clay and Nelson, 2005; Kakehi *et al*., 2008; Muñiz *et al.,* 2008; Cui *et al.,* 2010). These data, together with the participation of *ACL5*, *SAMDC4*, and *AtPAO5* in the feedback mechanisms controlling Therm-Spm levels (Alabdallah *et al.,* 2017), indicate that the phenotypical and the anatomical alterations of *AtPAO5-1* plants and *bud2/samdc4* mutants are mainly due to reduced Therm-Spm levels. In agreement with this, exogenous Therm-Spm or Nor-Spm caused a decrease in the number of xylem cells (Fig. 29). Furthermore, the atpao5-1 and *atpao5-2* mutants with increased Therm- Spm levels present a long-stem phenotype (Fig. 15) and reduced number of xylem vessels (Fig. 25; Fig. 26). However, a contribution of N^1-acetyl-Spm to the phenotypical alterations of the plants with modified *AtPAO5* expression cannot be excluded so far.

The *AtPAO5-1* and *atpao5* plants present altered xylem differentiation not only in stems but also in roots, and petioles, thus suggesting that *AtPAO5* interferes with this process throughout the entire plant, consistently with the altered expression levels of auxin- and xylem-related genes both in whole seedlings and in specific organs (i.e. roots and stems; Alabdallah *et al.,* 2017). It should, however, be noted that no difference was observed among *AtPAO5-1*, *atpao5*, and wild-type plants with regards to meristem size, hypocotyl length, leaf size, and flower development, which suggests that *AtPAO5* involvement in auxin and cytokinin signaling is specifically restricted to the xylem, probably due to local disruption of Therm-Spm and auxin homeostasis. This is in contrast to the long-hypocotyl phenotype of *acl5* plants, which was attributed to an increase in auxin signaling at very early stages of xylem cell differentiation, when the procambial cells start to elongate, giving rise to an increased length of the organ (Baima *et al.*, 2014). On the other hand, differently from the *AtPAO5-1* plants, *bud2/samdc4* mutants are hypersensitive to cytokinins with regards to root elongation and callus growth and have increased levels of cytokinins (Cui *et al.,* 2010). These differences among *AtPAO5-1*, *acl5*, and *bud2/samdc4* plants can be explained by possible temporal, hormone-dependent and tissue-specific differences in Therm- Spm and/or auxin

and/or cytokinin homeostasis in these plants. It also has to be considered that reduction of Therm- Spm levels in *AtPAO5-1* plants is based on the *AtPAO5* catalytic reaction, whereas in *acl5* and *bud2/samdc4* mutants it is based on the impairment of Therm-Spm biosynthesis. These differences may also explain the fact that acl5 plants present more severe phenotypical alterations regarding stem length and stem xylem differentiation as compared the *AtPAO5-1* plants (Fig. 27).

Recently some important pieces of information have been obtained regarding the mechanism through which Therm-Spm interferes with the auxin signaling pathway. In particular, it has been shown that the control of auxin on *ACL5* and *BUD2/SAMDC4* expression is directly mediated by ATHB8, a transcription factor involved in auxin signaling, through recognition of cis regulatory element on the promoter regions (Baima *et al.*, 2014). In turn, *ACL5* negatively regulates *ATHB8* as well as other auxin signaling genes through its enzymatic product Therm-Spm (present study; Kakehi *et al.*, 2010; Vera-Sirera *et al.*, 2010; Takano *et al.*, 2012; Yoshimoto *et al.*, 2012; Milhinhos *et al.*, 2013; Baima *et al.*, 2014; Tong *et al.*, 2014). Furthermore, it has been shown that Therm-Spm exerts its feedback inhibitory effect on auxin signaling through a positive effect on the translation and the stability of the *SAC51* mRNA, which is under the control of uORF-mediated translation repression through the action of some ribosomal proteins (Imai *et al.*, 2006; Jorgensen and Dorantes-Acosta 2012; Yoshimoto 2012; Kakehi *et al.*, 2015). This is consistent with the fact that cellular polyamines generally interact with RNA molecules and differentially affect their translation (Hanfrey *et al.*, 2005; Igarashi and Kashiwagi, 2010; Ivanov *et al.*, 2010; Miller-Fleming *et al.*, 2015). However, it is still unknown how *SAC51* negatively regulates auxin signaling. In agreement with the effect of Therm-Spm on the control of *SAC51* expression levels mainly at the translational levels (Kakehi *et al.*, 2015, Imai *et al.*, 2006), the *AtPAO5-1* and *atpao5* plants do not present statistically significant differences in the *SAC51* transcript levels as compared with the wild-type plants.

In conclusion, the data presented here further support a tightly controlled interplay among Therm-Spm, auxin, and cytokinins necessary for proper xylem differentiation and plant growth. *AtPAO5* contributes to this

regulatory network by participating in the feedback mechanism that controls Therm-Spm levels. This mechanism seems to operate specifically at the xylem level as a safeguard mechanism against the damaging and/or inhibitory effect of Therm-Spm on xylem, against the inhibitory effect of cytokinins on protoxylem differentiation, and against an excessive xylem proliferation by decreased levels of Therm-Spm and increased levels of auxin and cytokinins that would negatively affect organ expansion and plant growth (Yoshimoto *et al*., 2012a). Indeed, *AtPAO5* is up-regulated by both auxin and BAP specifically at the xylem level (Fig. 10; Alabdallah *et al*., 2017). It is probably because of this regulatory role of *AtPAO5* on appropriate xylem differentiation that it has activity as a dehydrogenase rather than as an oxidase, thus avoiding excessive H_2O_2 production, which can damage cellular components. It may be for the same reason that *AtPAO5* expression is regulated at the post-transcriptional level by the proteasome-dependent degradation pathway (Ahou *et al*., 2014), which is a critical element of many plant hormone signaling pathways and developmental processes, such as xylem differentiation (Han *et al*., 2012; Li *et al*., 2013; Kurepa *et al*., 2013).

Although *AtPAO5* is not up-regulated by stress-related conditions, as the present study shows, it appears to be additionally involved in the plant defense responses to abiotic stresses. Indeed, the *atpao5* mutants appear more tolerant to salt and drought stress than the *AtPAO5-1* and wild-type plants (Fig. 31; Fig. 32). The existing data do not permit to understand whether *AtPAO5* is involved in plant defense responses contributing to polyamine homeostasis and/or controlling xylem differentiation. Furthermore, *AtPAO5* appears to be involved in the control of stomata movement, the atpao5 mutants responding less to ABA-, MeJA-, H_2O_2-, NaCl-, drought- and polyamine-mediated stomata closure than the wild-type plants. The mechanisms though which *AtPAO5* controls stomata movement and plant defense responses are still unknown. Furthermore, it is still unknown whether a correlation exists between the reduced xylem differentiation, the increased stress tolerance and the reduced responsiveness of the guard cells to stress–related stimuli.

4 MATERIAL AND METHODS

4.1 Plant material

All experiments were performed with Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia. Two T-DNA insertional mutants of *AtPAO5* (At4g29720) previously characterized (Ahou *et al*., 2014; Albdalah O, PhD thesis; Alabdallah *et al*., 2017) were used: *atpao5-1* (allele SAIL_664_A11.v1; Alonso *et al*., 2003) and *atpao5-2* (SALK_053093; Sessions *et al*., 2002). Furthermore, two homozygous *35S::AtPAO5-6His* (*AtPAO5-1* and *AtPAO5-2*) were used (Ahou *et al*., 2014; Alabdallah *et al*., 2017). Hemizygous *AtPAO5-1* transgenic plants (*AtPAO5-1H1*, *AtPAO5-1H2*) were additionally obtained in the present work by sexual crossing with wild type plants. *ACL5* overexpressing transgenic plants and *acl5* mutants (Baima *et al*., 2014) were a kind gift from Dr. S. Baima. Moreover, *AtPAO5-1*, and *atpao5-1* plants crossed with a *DR5::GUS* marker line were obtained in the present study. The *atpao234* triple mutant was obtained previously (Ahou A, PhD thesis) by sexual crossing of the single mutant plants (*atpao2*: SALK_046281.36.95; *atpao3*: SALK_079055.48.35, *atpao4*: SALK_133599.45.55). Arabidopsis transgenic plants ectopically expressing high levels of *AtPAO3* (*AtPAO3over*) were obtained previously as described in Fincato *et al*., 2011. Moreover *AtPAO1* gene was obtained from alleles SAIL_882_A11.v2 (Dr. Abdellah Ahou Ph.D. thesis).

4.2 Plant growth conditions

Arabidopsis plants were grown in a growth chamber at a temperature of 23°C under long-day conditions (16 h of light and 8 h of dark) and 55% relative humidity. For *in vitro* growth, Arabidopsis seeds were first sterilized (1 min wash with 70% ethanol, rinse with water, 10 min treatment with 2.5% sodium hypochlorite, wash with excess of water) and stratified for 48 h at 4 °C, Seeds were then put on plates containing, half-strength Murashige and Skoog basal medium with Gamborg's vitamins and 0.5% (w/v) sucrose (½MS) together with 0.8% plant-agar. Plates are placed horizontally or vertically in the growth chamber.

For soil growth, seeds were first germinated on Jiffy-7® Peat Pellets after 48 h of stratification at 4°CTen-day-old seedlings were transferred on soil/perlite (3:1) containing pots.

For hydroponic growth, sterilized and stratified seeds were placed in rectangular boxes(Araponics©) containing water and oligoelements (1:1000 Araponics©).
For qRT-PCR analysis following hormone or salt treatment, 5-day-old seedlings grown on ½MS agar plates were transferred in ½MS liquid medium and were grown for 5 days more. After addition of fresh medium, seedlings were treated with 50 µM ABA, 50 µM MeJA or 100 mM NaCl for various time intervals. For qRT-PCR analysis following drought stress plants undergone water withdrawal for 2 h and then left to recover for 1 h. Following treatment, seedlings were frozen with liquid nitrogen and conserved at -80°C until further use.

4.3 Measurement of stomata aperture

Arabidopsis seedling of 7 days grown *in vitro* on ½MS agar plates were transferred in opening solution containing 10 mM KCl, 10 mM MES, pH 6.15 for around 2 h. Following stomata opening, seedlings were treated with 10 or 100 µM ABA, 50 µM MeJA, 0.25 mM or 1 mM H2O2, 150 NaCl, 1 mM Put, spd, spm, Nor-Spm and 1,3 Dap, 100 µM *N*,*N*[1]-dimethylthiourea (DMTU) for various time periods (2 to 24 h). For drought stress, seedlings, after incubation in opening solution were kept dehydrated into the growth chamber for for various time periods (30 min, 1 h and 2 h). Seedling undergone medium withdrawal for 1 h were also left to recover in medium for 1 h. Seedling were fixed with 1% glutaraldehyde, 10 mM sodium phosphate pH 7.0, 5 mM $MgCl_2$, 5 mM EDTA for 30 min and observed under microscope (Zeiss Axioplan 2) Images of a large number of stomata (n>60) were acquired by a Leica DFC420 digital camera. Measurement of stomatal aperture (width/length of stomata pore) was performed using Image J.

4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was isolated using the TRIzol® reagent (INVITROGEN) protocol. To eliminate traces of genomic DNA, RNA samples were treated with RNase-free DNase I (Invitrogen) or RQ1 RNasi-free DNasi (Promega) following the manufacturer's instructions. The first cDNA strand was synthesized from total RNA following the protocol of the ImProm-II Reverse Transcription System (Promega) and using random primers (random hexamers). Each RNA/primer mixture was prepared adding 1 µl (0.5 µg) of random hexamers (25 ng/µl) to 4 µl of total RNA (3 µg of total RNA). The

mixture was incubated at 70°C for 5 min and was then placed on icewater for 5-10 min. For each RNA/primer mixture, a cDNA synthesis mix was prepared containing 4 µl of 5X buffer, 1.2 µl of 25 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 1 µl of Recombinant RNasin® Ribonuclease Inhibitor (2 U/µl) and 1 µl ImProm-II™ Reverse Transcriptase and RNase-free water to a final volume of 15 µl. Each RNA/primer mixture was added to 15 µl of the cDNA synthesis mix and cDNA synthesis was carried out in an iCyclerTM Thermal Cycler (Bio-Rad) with the following parameters: 5 min at 25°C, 1 h at 42°C and 15 min at 70°C. The mixture was stored at -20°C or immediately used for PCR.

4.5 Quantitative RT-PCR analysis

For qRT-PCR analysis, cDNA synthesis and PCR amplification were carried out using GoTaq® 2-Step RT-qPCR System 200 (Promega) according to the manufacturer's protocol. The PCR reactions were run in a Corbett RG6000 (Corbett Life Science, QIAGEN) utilizing the following program: 95°C for 2 min and then 40 cycles of 95°C for 3s and 60°C for 30 s. Reactions were performed in triplicate and mean values ±SD were calculated. Only values with SD $\leq$ 0.2 were taken into consideration. At least two independent biological replicates were performed for each experiment and mean values of relative expression levels from the different biological replicates are shown. Relative expression levels are presented as fold change (2-ΔΔCt). The gene for ubiquitin conjugating enzyme 21 (UBC21; At5g25760) was chosen as a reference gene.

4.6 Plant dehydration and sodium chloride treatment on soil and hydroponics

For dehydration studies in soil, plants were grown for 30 days under well-watered conditions. Afterwards, progressive drought treatment was applied by withholding watering for 20 days. Control plants were continuously grown under well-watered conditions.

For dehydration studies in hydroponic system, plant were grown for 30 days changing the nutrient solution every 5 days. Then, the nutrient solution was removed from the hydroponic pots for 40 h. The plants were re-watered adding nutrient solution and left to recover for 3 days.
For salt stress, plants grown in soil or in hydroponic cultures for 25 days under well-watered conditions were treated every 3 or 4 days with NaCl

solution of gradually increasing concentration starting from 50 mM up to 150 mM. Plants were left to grow until appearance of chlorotic leaves.

4.7 Histology

Transverse section of stem, root and hypocotyls were taken from adult plants of 4 weeks grown in hydroponic culture. Root transverse sections of 15-day-old plants grown *in vitro* were also obtained.
In detail, the various organs were cut and put on 100% ethanol overnight. Then they were transferred on ethanol 70% overnight; tissues were soaked in Technovit 7100 resin (Kulzer) and ethanol (70%):1:1 overnight. After, tissues were placed on little square containers of Technovit 7100 resin 12h and fixed in 1:15 Technovit 7100 hardner: Technovit 7100 resin and installed by glue on wood support kit. Sections (20 µm) were made using a Microm HM330 microtome For histochemical localization of lignin tissues were first stained in a 0.05% (w/v) toluidine blue solution and then observed under light microscope using a Zeiss Axioplan 2 microscope connected to a digital camera (Leica DFC310FX) for image acquisition. Following acquisition, images were used to quantify the number of the large-diameter, thick-walled xylem cells (metaxylem cells) and the area of the secondary xylem through ImageJ software.

4.8 Histochemical GUS Analysis

GUS staining of Arabidopsis plants was performed essentially as described by Jefferson (1987). Briefly, 7-day-old seedlings were gently soaked in 90% (v/v) cold acetone for 1 hr at -20°C for prefixation, rinsed with 50 mM sodium phosphate buffer pH 7.0 and vacuum infiltrated in staining solution [1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 2.5 mM potassium ferrocyanide, 2.5 mM potassium ferricyanide, 0.2% (v/v) Triton X-100, 10 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0]. The reaction was allowed to proceed overnight at 37°C. Reaction was stopped ethanol:acetic acid (3:1) and then with 70% (v/v) ethanol. Photos were acquire using a Zeiss Axioplan 2 microscope connected to a digital camera (Leica DFC310FX).

4.9 Extraction of total free polyamines from Arabidopsis plants

Ten-day-old seedling were homogenized initially with liquid nitrogen and then with cold 5% (v/v) PCA containing 80 µM 1,7-diaminoheptane as an

internal standard (tissue to 5% PCA ratio 1:3). Crude extracts were incubated at 4°C for 18 h and then were clarified by centrifugation. The supernatant was used to analyze total free polyamines by HPLC following derivatization with dansyl chloride.

For dansyl chloride derivatization, 100 ul of saturated sodium carbonate were added to 100 µl of polyamine extract. To this mixture 200 µl of dansyl chloride (7.5 mg/mL in acetone) were added. After 1 min shaking using vortex, the mixture was incubated in the dark for 24 h at 4°C, to allow the dansyl chloride to react with the primary amino groups. The sample was then centrifuged at 15000 x g for 5 min at room temperature. The supernatant, containing dansylated polyamines was recovered and 50 µL of L-proline (100 mg/mL) were added to remove the excess of dansyl chloride. After incubation in the dark at room temperature for 1 h, 250 µL of toluene were added in order to extract the dansylated polyamines. After vigorous shaking for 1 min, the mixture was centrifuged for 5 min. The organic phase was collected and dried using a vacuum speed.
HPLC analysis was performed on a reverse phase C18 column (Spherisorb S5 ODS2, 5-mm particle diameter, 4.6 x 250 mm) using a discontinued solution A (acetonitrile, methanol and water at a ratio of 3:2:5) to solution B (acetonitrile and methanol at a ratio 3:2) gradient (72% solution A for 5 min, 72–36% solution A in 42 min, 36–20% in 3 min, 20–15% in 5 min, at a flow rate of 1.0 mL/min). Eluted peaks were detected by a spectrofluorimeter (excitation 365 nm, emission 510 nm). TLC analysis was performed on silica gel plates (Alugram SIL G⁄ UV254; Macherey-Nagel) using a chloroform- triethylamine (25:2) solvent system.

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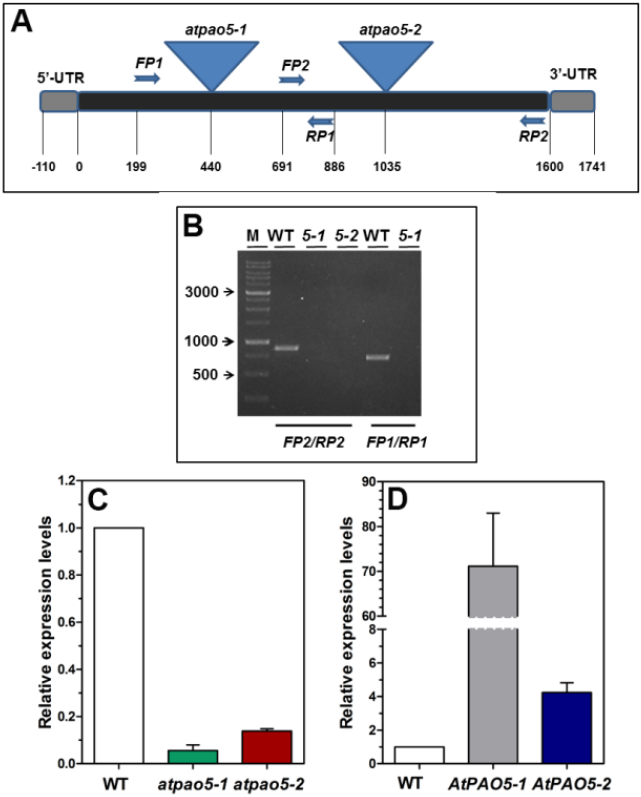
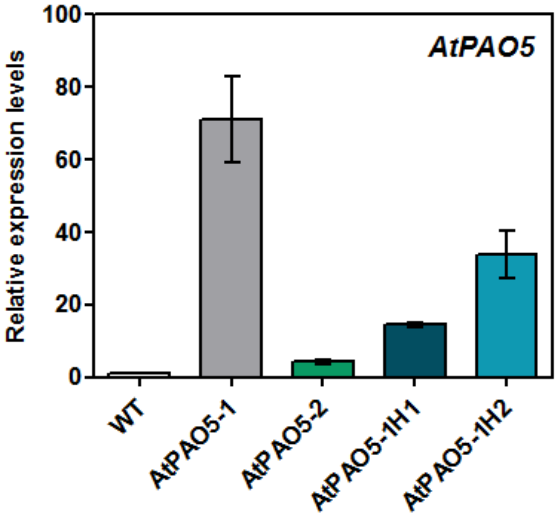
6 SUPPLEMENTARY DATA



Supplementary Fig. S1. *AtPAO5* expression levels in *atpao5* loss-of-function mutants and *35S::AtPAO5-6His Arabidopsis* transgenic plants. (A) Schematic genome structure of *AtPAO5*. Triangles indicate the positions of the two T-DNA insertions and arrows the position of the primers used for mutant characterization. (B) Analysis of selected homozygous lines of *atpao5-1* and *atpao5-2* mutants for *AtPAO5* expression levels by RT-PCR. (C, D) Relative *AtPAO5* expression levels in young seedlings of two *atpao5* mutants (*atpao5-1* and *atpao5-2*), two independent homozygous *35S::AtPAO5-6His Arabidopsis* transgenic lines (*AtPAO5-1* and *AtPAO5-2*), and wild-type (WT) plants. The expression levels were determined by qRT-PCR. Very similar results for *AtPAO5* expression levels were obtained when specific organs (leaves, roots and stems) of *AtPAO5-1* transgenic plants were tested. From Alabdallah *et al*., 2017.



Supplementary Fig. S2. *AtPAO5* expression levels in two *AtPAO5-1* heterozygous plants (*AtPAO5-1H1* and *AtPAO5-1H2)* obtained by sexual crossings of *AtPAO5-1* homozygous plants with wild-type (WT) plants. Relative expression levels were determined by qRT-PCR analysis. Numbers are mean values ± SE of three replicates. From Alabdallah *et al*., 2017.