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XXVIII Ciclo
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Molecular and physiological aspects regarding the Arabidopsis polyamine oxidase gene family

Dr. Osama "Mohammad Ali" Ahmad Alabdallah

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ABBREVIATIONS

ABA	Abscisic acid
ACL5	ACAULIS5; Thermospermine synthase
ADC	Arginine decarboxylase
Agm	Agmatine
AHP6	HISTIDINE PHOSPHOTRANSFER PROTEIN 6
Arg	Arginine
ATHB8	ARABIDOPSIS THALIANA HOMEOBOX8
AtPAO	*Arabidopsis thaliana* polyamine oxidase
BAP	6-benzylaminopurine
b-HLH	Basic helix-loop-helix
Cad	Cadaverine
CNA	CORONA
CuAO	Copper-containing amine oxidases
Dap	1,3diaminopropane
Dc-SAM	Decarboxylated S-adenosylmethionine
H_2O_2	Hydrogen peroxide
HD-ZIP III	III homeo-domain leucine zipper
IAA	Indole-3-acetic acid
MmAPAO	Murine APAO
MmSMO	Murine SMO
MP	MONOPTEROS
Nor-Spd	Norspermidine
Nor-Spm	Norspermine
ODC	Ornithine decarboxylase
PA	Polyamine
PAO	Polyamine oxidase
PAT	Polar auxin transport
PCD	Programmed cell death
PHB	PHABULOSA
PHV	PHAVOLUTA
PIN1	PIN-FORMED1
Put	Putrescine
QC	Quiescent centre
REV	REVOLUTA
ROS	Reactive oxygen species
SAC51	SUPPRESSOR OF ACAULIS 51

SAM	S-adenosyl methionine
SAMDC	S-adenosylmethionine decarboxylase
SCR	SCARECROW
SHR	SHORT ROOT
SMO	Spermine oxidase
Spd	Spermidine
SPDS	Spermidine synthase
Spm	Spermine
SPMS	Spermine synthase
SSAT	Spd/Spm N^1-acetyltransferase
Therm-Spm	Thermospermine
TZ	Transition zone
VND6	VASCULAR-RELATED NAC-DOMAIN6
ZmPAO	*Zea mays* polyamine oxidase

ABSTRACT

In plants, the polyamines (PAs) putrescine (Put), spermidine (Spd), spermine (Spm) and thermospermine (Therm-Spm) are involved in several physiological processes. In particular, Spd is important for survival, while Put and Spm have been implicated in plant responses to drought, high salt stress, wounding and pathogens. Furthermore, Therm-Spm is involved in the control of xylem differentiation having an auxin antagonizing effect. PA oxidases (PAOs) are FAD-dependent enzymes involved in PA catabolism. In *Arabidopsis thaliana*, five *PAO* genes (*AtPAO1–AtPAO5*) have been identified. 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* gene family (AtPAO2, AtPAO3 and AtPAO4) have a peroxisomal localization and are able to oxidize both Spd and Spm, but not Therm-Spm.

To investigate the physiological role(s) of *AtPAO5* during plant growth and development, two *35S::AtPAO5-6His Arabidopsis* transgenic lines that ectopically express *AtPAO5*, one with 70-fold (*AtPAO5-1*) and the other 4-fold (*AtPAO5-2*) higher expression levels than the endogenous gene, were characterized. Parallel studies were also performed with two loss-of-function mutants lacking *AtPAO5* expression (*atpao5-1* and *atpao5-2* mutants). Analysis of PA levels showed decreased levels of Spm, Therm-Spm, and N^1-acetyl-Spm in *AtPAO5-1* seedlings and increased levels in *atpao5-1* and *atpao5-2* whole seedlings, as well as in specific organs (stem and leaves), as compared to the wild-type plants. Instead, the *AtPAO5-2* transgenic line does not present differences in PA levels from the wild-type plants. These data are in agreement with the AtPAO5 substrate specificity *in vitro* and indicate that Spm, Therm-Spm and N^1-acetyl-Spm are the substrates of AtPAO5 also *in vivo*, and that AtPAO5 contributes in a dose-dependent way to PA homeostasis along the entire plant. Additionally, analysis of the expression levels of Therm-Spm biosynthetic genes thermospermine synthase (*ACAULIS5*; *ACL5)* and *S-adenosylmethionine decarboxylase 4* (*SAMDC4*) showed that they are up-regulated in *AtPAO5-1* plants and down-regulated in *atpao5* mutants, but not affected at all in *AtPAO5-2* plants. Instead, no change in *spermine synthase* (*SPMS*) expression levels was observed in any of the *AtPAO5* and *atpao5* plants as compared to the wild-type plants. These data suggest that *AtPAO5* participates in a feedback mechanism controlling Therm-Spm homeostasis.

Phenotypical analyses of *AtPAO5* and *atpao5* plants evidenced some developmental differences in different plant organs (stems, roots, leaves and hypocotyls). In particular, the two *atpao5* mutants produce longer and thicker flowering stems, while conversely the *AtPAO5-1* transgenic plants produce thinner and shorter ones compared to the wild-type plants. Similarly, *AtPAO5-1* transgenic plants present shorter roots with a higher number of lateral roots than the wild-type plants, while *atpao5* mutant plants longer ones. Transverse sections showed that the *AtPAO5-1* transgenic plants undergo excessive xylem differentiation, while the *atpao5* mutants reduced. Furthermore, *AtPAO5-1* and *atpao5* plants present altered vasculature thickness in hypocotyls and leaves as compared with the wild-type plants. Taken together, these phenotypical differences indicate that *AtPAO5* contributes to plant development controlling xylem differentiation, which is consistent with the high expression levels of *AtPAO5* in the vascular system, as shown by histochemical analysis of *AtPAO5::GFP-GUS* transgenic plants.

In the present study it has been also shown that *AtPAO5* expression is up-regulated by cytokinins and Therm-spm, specifically in the roots. Furthermore, cytokinin and Therm-Spm treatment differently affected xylem differentiation in the roots of the *AtPAO5-1*, *atpao5* and wild-type plants, suggesting that *AtPAO5* is involved in the cytokinin and Therm-Spm-mediated control of root xylem differentiation. To understand the mechanism(s) through which *AtPAO5* is involved in vascular system differentiation and considering that both auxin and cytokinin have an important role in these processes, the expression of some cytokinin-, auxin- and xylem-related genes were analyzed in *AtPAO5-1*, *atpao5* and wild-type plants by qRT-PCR. The results showed that several auxin- and xylem-related genes are up-regulated in *AtPAO5-1* plants and down-regulated in *atpao5* mutants compared to the wild type plants. Moreover, some cytokinin-related genes were differently regulated in *AtPAO5-1*, *atpao5* and wild-type plants following cytokinin treatment. These data altogether suggest altered auxin and cytokinin signaling, together with altered xylem differentiation, in the *atpao5* mutants and the *AtPAO5-1* transgenic plants comparing with the wild-type plants. To further investigate on the auxin and cytokinin signaling in the *AtPAO5-1* and *atpao5* plants, sexual crossings of these plants with *DR5::GUS* transgenic plants, *DR5* being an artificial auxin–regulated promoter, are in progress.

Since *AtPAO1* presents some similarities to *AtPAO5*, the *atpao1* single mutant and the *atpao1*/*atpao5* double mutant (*DM15*) were additionally analyzed. Data evidenced that *AtPAO1* participates, together

with *AtPAO5*, to Spm and Therm-Spm homeostasis, as well as to the control of stem and root development, with *AtPAO5* playing however a major role in these processes.

Several studies evidenced an important role of PAs in plant defense responses to biotic and abiotic stresses. To determine whether *AtPAO1* and *AtPAO5* contribute to these processes, the *AtPAO5-1* transgenic plants, as well as the *atpao5* and *DM15* mutants were observed under drought and salt stress. Preliminary results indicate that the *atpao5* and *DM15* mutants are more tolerant to both salt and drought stress as compared to the wild-type plants, the *AtPAO5-1* plants appearing more sensitive to these stresses. Experiments are still in progress to further understand the role of the *AtPAO1* and *AtPAO5*, as well as the other members of the *AtPAO* gene family, under conditions of environmental stress.

In conclusion, our studies further support a tightly controlled interplay between Therm-Spm, auxin and cytokinins necessary for proper xylem differentiation and plant growth. *AtPAO5* and *AtPAO1* redundantly contribute to this regulatory network participating in the feedback mechanisms which control Therm-Spm levels. On the other hand, *AtPAO5*, together with *AtPAO1*, participate in the control of plant responses to salt and drought stress.

INTRODUCTION

1. Polyamines

1.1. General characteristics and physiological roles

Polyamines (PAs) are small organic molecules found in all living organisms (Cohen, 1998) and they can be considered as one of the oldest group of substances known in biochemistry (Galston, 1991). The diamine putrescine (Put), the triamine spermidine (Spd) and the tetramine spermine (Spm) constitute the most common PAs in eukaryotes (Galston and Sawhney, 1990). In addition, other PAs, such as 1,3-diaminopropane (Dap), cadaverine (Cad), thermospermine (Therm-Spm), norspermidine (Nor-Spd) and norspermine (Nor-Spm) (Table 1) are found in many organisms as minor components of the cellular PA pool and are referred to as uncommon PAs (Tavladoraki *et al*., 2011). PAs occur in the free form or as conjugates bound to phenolic acids and other low molecular weight compounds or to macromolecules, such as proteins and nucleic acids (Kusano *et al*., 2008).

PAs are involved in the regulation of a diverse range of vital cellular processes in both eukaryotic and prokaryotic cells, including cell proliferation, signal transduction, membrane stabilization, maintenance of DNA structure, RNA processing, regulation of translation, modulation of enzyme activities (Cohen, 1998; Wang *et al*., 2003; Kusano *et al*., 2008). In animals, they are additionally involved in the control of programmed cell death (PCD), particularly apoptosis (Seiler & Raul, 2005; Groppa and Benavides, 2008; Alcázar *et al.*, 2010), while in bacteria an essential role for PAs in biofilm formation and adaptation to various stresses has been demonstrated (Lee *et al.*, 2009; Morimoto *et al.*, 2010).

In plants, PAs have been suggested to play important roles including the control of the N:C balance (Mattoo *et al*., 2006; Moschou *et al*., 2012), xylem differentiation (Muñiz *et al*., 2008; Tisi *et al*., 2011), membrane fluidity, protein regulation (Baron and Stasolla, 2008), morphogenesis, growth, embryogenesis, organ development, leaf senescence, abiotic and biotic stress response (Alcázar *et al*., 2006; Groppa *et al*., 2007; Kusano *et al*., 2008). Indeed, Spd is important for survival in that the *Arabidopsis spds1/spds2* double mutant for the two genes encoding for spermidine synthase (SPDS) shows embryonic lethality (Imai *et al*., 2004). Furthermore, Put and Spm have been implicated in plant responses to drought, high salt stress, wounding and pathogens (Tavladoraki *et al*., 2012; Takano *et al*.,

Name	Structure
Diamines	
1,3-Diaminopropane	$NH_2(CH_2)_3NH_2$
Putrescine	$NH_2(CH_2)_4NH_2$
Cadaverine	$NH_2(CH_2)_5NH_2$
Triamines	
Spermidine	$NH_2(CH2)_3\ NH(CH_2)_4NH_2$
Homospermidine	$NH_2(CH2)_4\ NH(CH_2)_4NH_2$
Norspermidine	$NH_2(CH2)_3\ NH(CH_2)_3NH_2$
Tetraamines	
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
Homospermine	$NH_2(CH_2)_3NH(CH_2)_4\ NH(CH_2)_4NH_2$
Norspermine	$NH_2(CH_2)_3NH(CH_2)_3\ NH(CH_2)_3NH_2$
Thermospermine	$NH_2(CH_2)_3NH(CH_2)_3\ NH(CH_2)_4NH_2$

Table 1. Common and uncommon PAs

2012; Jiménez-Bremont *et al*., 2014; Minocha *et al*., 2014). Put is also required for the synthesis of tropane and nicotine alkaloids in plants (Tavladoraki *et al*., 2012). Therm-Spm, which is a structural isomer of Spm and was first detected in the thermophilic bacterium *Thermus thermophilus*, was recently identified in plants (Knott *et al*., 2007; Naka *et al*., 2010; Rambla *et al*., 2010). 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 controlling xylem differentiation. Indeed, disruption of the Therm-Spm biosynthetic gene, *thermospermine synthase* (*ACAULIS5*; *ACL5*) gene, in *Arabidopsis*, 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, over-proliferation of xylem vessels and thicker veins in hypocotyls, leaves and inflorescence stems (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 PA 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). 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).

To adjust the overall intracellular concentration of PAs to the levels required by the physiological state of the cells, various organisms have evolved complex homeostatic mechanisms involving PA biosynthesis, catabolism, conjugation, transport, and uptake (Martin-Tanguy, 1997, Tiburcio *et al*., 1997; Angelini *et al*., 2010; Moschou *et al*., 2012).

1.2. Polyamine metabolism

1.2.1. Polyamine biosynthesis

In animals and most plants, Put is synthesized directly by decarboxylation of ornithine via the enzyme ornithine decarboxylase (ODC) or indirectly from arginine (Arg) by arginine decarboxylase (ADC) via agmatine (Agm) (Tabor and Tabor, 1985). In *Arabidopsis thaliana*, Put is produced by the ADC pathway, since no ODC gene has been identified in the sequenced genome of this plant and the corresponding enzyme activity has not been detected (Hanfrey *et al*., 2001; Alcázar *et al*., 2010). In bacteria, in addition to ADC and ODC, another enzyme is present involved in Put biosynthesis, agmatinase, which directly produces Put from agmatine (Wortham *et al*., 2007). After Put synthesis, next biosynthetic steps require the activity of SAMDC to produce from decarboxylation of S-adenosyl methionine (SAM), decarboxylated S-adenosylmethionine (dc-SAM) which acts as a donor of aminopropyl groups in the successive reactions catalyzed by SPDS, spermine synthase (SPMS) and ACL5 for the synthesis of Spd, Spm and Therm-Spm, respectively (Fig. 1). The Arabidopsis genome carries together with the four *SAMDCs* (Urano *et al*. 2003), two *ADC* genes (Soyka and Heyer, 1999), two *SPDS* genes (*SPDS1*and *SPDS2*), a single *SPMS* gene and a single *ACL5* gene (Hanzawa *et al*., 2002; Knott *et al*., 2007).

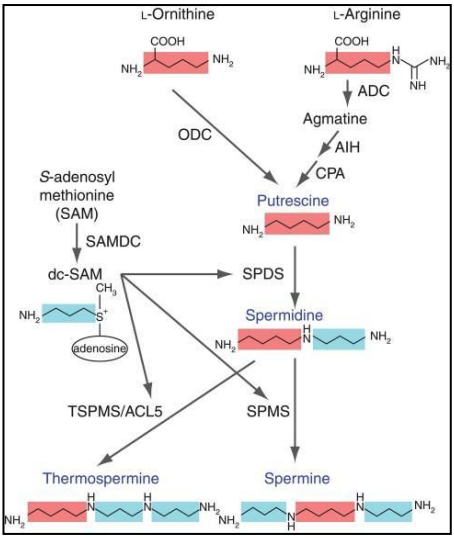

Fig. 1. PA biosynthetic pathways. Figure taken from Takahashi and Kakehi, 2010.

1.2.2. Polyamine catabolism

PA catabolism contributes greatly to PA homeostasis and is involved in several physiological processes (Angelini *et al.*, 2010). Copper-containing amine oxidases (CuAO) oxidize Put to 4-aminobutanal with concomitant production of NH_3 and H_2O_2 in a terminal catabolic pathway (Tavladoraki *et al.*, 2012; Planas-Portell *et al.*, 2013). The flavin-containing amine oxidases (polyamine oxidases; PAOs) catalyze the oxidation of the free form and/or the acetylated derivatives of Spm, Spd and Therm-Spm either through a terminal catabolic pathway or through a PA back-conversion pathway (Fig. 2). The PAOs involved in the terminal catabolic pathway, among which the extracellular *Zea mays* PAO1 (ZmPAO1) is the best characterized (Tavladoraki *et al*., 2006; Fincato *et al*., 2011), oxidize the *endo*-side of the N^4-nitrogen of the free forms of Spd and Spm with the production of 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, together with 1,3-diaminopropane and H_2O_2. Conversely the PAOs participating in the PA back-conversion pathway oxidize the *exo*-side of N^4-nitrogen of Spm or Spd to produce Spd or Put respectively, in addition to 3-aminopropanal and

H_2O_2 (Angelini *et al.*, 2010; Tavladoraki *et al.*, 2012). Among the PAOs involved in PA back-conversion, the animal spermine oxidases (SMOs) which have a cytosolic/nuclear localization and oxidize only the free form of Spm, are the best characterized (Wang *et al.*, 2001; Vujcic *et al.*, 2003; Cervelli *et al.*, 2003; Landry and Sternglanz, 2003). The animal peroxisomal PAOs (Vujcic *et al.*, 2003; Wu *et al.*, 2003; Cona *et al.*, 2006) which preferentially oxidize N^1-acetyl-Spm, N^1-acetyl-Spd, and N^1,N^{12}-bis-acetyl-Spm, whereas the yeast Fms1 oxidizes Spm and N^1-acetyl-Spm (Adachi *et al.*, 2012; Landry and Sternglanz, 2003) are also involved in PA back-conversion. The best so far characterized plant PAOs involved in PA back-conversion are those of Arabidopsis.

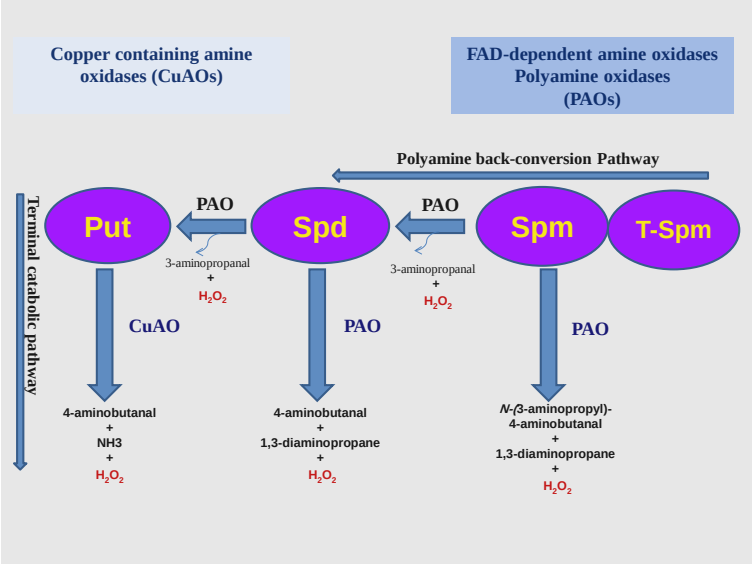

Fig. 2. Polyamine catabolic pathways.

1.2.2.1. *Arabidopsis thaliana PAOs*

In *Arabidopsis thaliana*, there are five *PAO* genes (*AtPAO1-AtPAO5*), which present some common characteristics, but also important differences in gene structure (Fig. 3), substrate specificity, subcellular localization, and expression pattern, differences which may reflect differences in physiological

roles (Fincato *et al.*, 2011). AtPAO1, which has a predicted cytosolic localization, shares with the extracellular ZmPAO1 a 45% homology at the amino acid level and a similar intron/exon organization (Tavladoraki *et al*., 2006). AtPAO1, differently from ZmPAO1, catalyzes the back-conversion of Spm and Therm-Spm to Spd (Tavladoraki *et al*., 2006). Promoter activity studies using *AtPAO1::GFP-GUS* transgenic plants showed that *AtPAO1* is highly expressed in the transition region between the meristematic and the elongation zone of the root. In young seedlings, the expression was shown in cotyledons, shoot apex and newly emerging leaves. Later in development, the expression was also observed at the tips and hydathodes of the cotyledons and expanded leaves respectively (Fincato *et al.*, 2012).

AtPAO2, AtPAO3 and AtPAO4, display low sequence homology (23% - 24% homology) with ZmPAO and the other two AtPAOs, but a high sequence homology to each other. In particular, the amino acid sequence identity between AtPAO2 and AtPAO3 is 85%, while it is 58% between AtPAO2 and AtPAO4 and 50% between AtPAO3 and AtPAO4 (Fincato *et al.*, 2011). Furthermore, *AtPAO2*, *AtPAO3* and *AtPAO4* have a very similar intron/exon organization to each other bearing eight introns at highly conserved positions (Fig. 3). This, together with the elevated sequence homology to each other, suggest that these three Arabidopsis genes are recent derivatives from a common ancestor, thus forming a distinct *PAO* subfamily (*AtPAO2–AtPAO4* subfamily). 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). *AtPAO2*, *AtPAO3* and *AtPAO4* are expressed in the root cap. Interestingly, at the root cap differences exist between *AtPAO2* and *AtPAO3*, although they belong to the same *PAO* subfamily. Indeed, while *AtPAO2* is expressed only near the quiescent center and columella initials, *AtPAO3* is expressed in lateral root cap and in the whole columella. The expression of the three genes were also observed in the different plant organs and cells sharing some common expression pattern. In particular, *AtPAO2*, *AtPAO3* and *AtPAO4* are expressed in the guard cells, which may reflect a distinct physiological role (Fincato *et al*., 2012).

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% ; Ahou *et al*., 2014). Furthermore, differently from the other *AtPAO* genes and *ZmPAO1*, *AtPAO5* gene bears no intron (Fig. 3; Fincato *et al.*, 2011). AtPAO5 is a cytosolic enzyme which is regulated at the post-translational level by the

proteasome (Ahou *et al.*, 2014). Furthermore, AtPAO5 catalyzes the back-conversion of Spm and Therm-Spm to Spd. It is also able to catalyze the back-conversion of N^1- acetyl-Spm to Spd with a k_{cat} higher than with Spm, and Therm-Spm (Ahou *et al.*, 2014). In this way, AtPAO5 represents the first plant enzyme characterized so far involved in PA catabolism with a good activity with acetylated PAs. (Tavladoraki *et al.*, 2006; Moschou *et al.*, 2008b; Fincato *et al.*, 2011). Furthermore, differently from the other animal and plant PAOs so far characterized, 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 with an important role in the utilization of PAs as carbon and nitrogen source been reported so far (Tabor and Kellog, 1970; Hisano *et al.*, 1990, 1992; Dasu *et al.*, 2006). The function of AtPAO5 as a dehydrogenase and its involvement in PA back-conversion suggest that AtPAO5 has a role in PA 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). *AtPAO5* is expressed mainly in the vascular tissue of the roots, hypocotyls and cotyledons. It is also expressed in leaves showing a diffused pattern and in flower buds, in particular in the anthers (Fincato *et al.*, 2012).

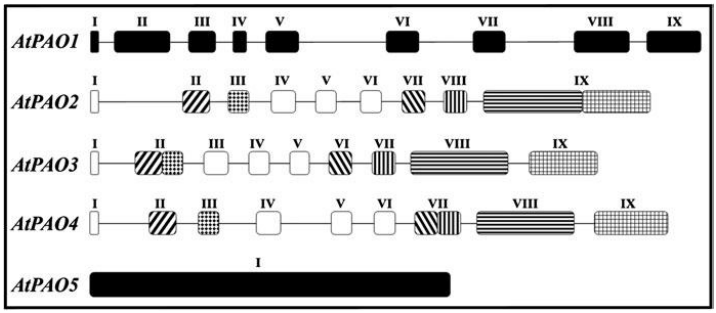

Fig. 3. Schematic representation of the exon/intron organization of *AtPAO* genes. Introns are represented by lines and exons by boxes. Exons are numbered in Roman numerals. Open and filled boxes indicate shared and unshared exons among the various *AtPAO* genes, respectively. Stripes and stipples show 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. Figure taken from Fincato *et al.*, 2011.

2. Auxin, cyokinin, Therm-Spm and their interplay in vascular tissue differentiation

Vascular plants possess a regulatory network to coordinate the different phases of xylem maturation, including secondary cell-wall formation, cell death, and finally autolysis of the cell contents. Auxins and cytokinins are important members of this network interacting antagonistically on many levels.

Recent genetic studies revealed the molecular basis of the xylem differentiation and specification by auxin and cytokinin. One of the earliest expressing transcriptional regulators in the preprocambial cells is the ARABIDOPSIS THALIANA HOMEOBOX8 (ATHB8), a class III homeo-domain leucine zipper (HD-ZIP III) transcription factor family gene. The transcription of ATHB8 is activated directly by auxin responsive transcription factor, MONOPTEROS (MP). ATHB8 subsequently directs the formation of preprocambial cells and induces the expression of PIN-FORMED1 (PIN1) auxin enflux proteins (Scarpella et al., 2006). These processes establish a positive feedback loop of auxin-MP-ATHB8-PIN1-auxin flow which contribute in the initiation and specification of vascular stem cells and in the restriction of procambium precursor cells to continuous and narrow regions (Ohashi-Ito and Fukuda, 2010; Nieminen et al., 2014). Besides ATHB8, the Arabidopsis genome contains four other *HD-ZIP III* genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *CORONA* (*CNA*)/*ATHB15* which were shown to be regulated by SHORT ROOT (SHR), SCARECROW (SCR) and miR165/166 (Kondo et al., 2014). All of them are expressed in vascular tissues in somewhat overlapping fashion, suggesting a likely redundancy in their function. In Arabidopsis root, a loss of all the five HD-ZIP IIIs results in the failure of xylem vessel development (Carlsbecker *et al.*, 2010). On the contrary, over-expression of *ATHB8* causes enhanced xylem differentiation (Baima *et al.*, 2001), indicating that HD-ZIP IIIs are de novo regulators of the xylem vessel formation. LONESOME HIGHWAY (LHW), a bHLH transcriptional activator, regulates the bilateral symmetry of vascular pattern and proliferation of procambium cells in roots. The TARGET OF MONOPTEROS (TMO5) has been found as one of the interactors with LHW and the auxin-dependent TMO5/LHW transcriptional complex triggers several rounds of periclinal cell divisions response to signaling by auxin (Ohashi-Ito and Fukuda, 2010). The NAC domain transcription factors

VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 also play an essential role in xylem vessel differentiation in both Arabidopsis and Populus. Indeed, it has been demonstrated that VND6 and VND7 act as central regulators of metaxylem and protoxylem vessel differentiation, respectively (Kubo *et al.*, 2005; Ohashi-Ito and Fukuda, 2010).

The first step of cytokinin perception requires the binding of cytokinin to a transmembrane histidine kinase receptor. This binding induces, within the receptor, the autophosphorylation of a conserved His residue in its kinase domain. In *A. thaliana*, there are three histidine kinase receptors (AHKs). Following autophosphorylation, the phosphate group is then transferred to a conserved Asp residue within the receiver domain of the AHK protein, and subsequently transferred to a member of the ARABIDOPSIS HIS PHOSPHOTRANSFER PROTEIN (AHP) family. Following transfer to the nucleus, phosphorylated AHPs transfer the phosphate group to the nuclear-localized ARABIDOPSIS RESPONSE REGULATOR (ARR) proteins. AHP6 is an AHP that lacks the conserved histidine residue required for phosphotransfer and therefore acts as a negative regulator of cytokinin signalling by competing with other AHPs (Mähönen *et al*., 2006). The *ARRs* are encoded by a gene family and are classified into type A, type B and type C, depending on their protein domains and cytokinin responsiveness. Type-A ARRs (ARR3–ARR9 and ARR15–ARR17) possess short C-termini, while type-B ARRs (ARR1, ARR2, ARR10–ARR14 and ARR18–ARR21) have longer C-termini, which contain a conserved GARP (GOLDEN2/ARR/Psr1) DNA-binding domain. Type-A ARRs act as negative regulators of cytokinin responses, via an as yet unknown mechanism. Type-B ARRs, which are transcription factors, play a positive role in mediating cytokinin-regulated gene expression. The type-C ARRs are more distantly related to type-A and type-B ARRs. AHPs also mediate the accumulation in the nucleus of other plant-specific cytokinin-responsive genes, the *CYTOKININ RESPONSE FACTORS* (*CRFs*), members of the *A. thaliana APETALA 2* family of transcription factors. In response to cytokinin, activated type-B ARRs and CRFs act together to regulate gene expression of common and unique targets, including type-A *ARRs*. The transcriptional activation of the type-A *ARRs* represents a negative feedback loop required to dampen the response upon high or prolonged signal input. Cytokinins have a dual role during vascular development acting both as a promoter of periclinal cell divisions and an inhibitor of protoxylem differentiation (Mähönen *et al*., 2000; Mähönen *et al*., 2006). *AHP6*, expressed in protoxylem and adjacent pericycle cells,

promotes protoxylem development by negatively regulating cytokinin signaling (Mähönen *et al*., 2006).

AHP6 expression is auxin-dependent, and loss of AHP6 function results in expansion of the expression domains of cytokinin-response genes *ARR5* and *ARR15* from the procambium into the protoxylem position (Mähönen *et al*., 2006; Bishopp *et al*., 2011). Cytokinin signaling, in turn, regulates auxin availability. High cytokinin signalling in the procambium promotes the efflux of auxin from the procambium into the xylem axis by stimulating lateralization of PIN1 protein and by increasing the expression of the laterally localized PIN7, and perhaps also PIN3 (Bishopp *et al*., 2011). This mutually inhibitory interaction between cytokinin and auxin in adjacent locations maintains the bisymmetric vascular pattern in the primary root (Bishopp *et al*., 2011). A connection between the auxin-MP-TMO5/LHW and the cytokinin-AHP6 pathways also exists. Indeed, the TMO5/LHW dimer promotes the expression of the xylem precursor-specific genes LONELY GUY 3 (LOG3) and LOG4, which encode enzymes catalyzing the final reaction step of cytokinin biosynthesis (De Rybel *et al*., 2014; Ohashi-Ito *et al*., 2014). Since TMO5/LHW also promotes the expression of *AHP6*, the protoxylem pre-cursor cells have low cytokinin signaling levels despite being the site of cytokinin synthesis, and therefore display a reduced rate of periclinal cell division. However, cytokinin is able to move from the xylem precursor cells to the neighboring procambial cells where it activates the cytokinin signaling pathway and thus promotes periclinal cell division (Mähönen *et al*., 2006; De Rybel *et al*., 2014; Ohashi-Ito *et al*., 2014).

PAs are implicated in several different processes during xylem differentiation, including cell wall formation, lignin biosynthesis, and auxin–cytokinin signaling (Ge *et al*., 2006; Cui *et al*., 2010; Vera-Sirera *et al*., 2010). In particular, Therm-Spm has important role on xylem differentiation (Muñiz *et al*., 2008). Indeed, *acl5* loss-of-function mutants exhibit incorrect or incomplete secondary cell-wall formation, as well as early expression of xylem cell death markers, and consequently early vessel cell death compared with the wild type, suggesting that Therm-Spm has a protective role against premature xylem maturation and cell death (Muñiz *et al*., 2008). 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).

3. Roles of polyamines under abiotic and biotic stress conditions

Plants are exposed continuously to a variety of adversely changing environmental (abiotic) stresses including salinity, drought, freezing, heat, hypoxia, ozone , heavy metal toxicity and mechanical wounding. Plants are also exposed to pathogen infections (biotic) stress, such as viruses, fungi and bacteria. Several studies from different plant species has shown that PA accumulation increases in response to abiotic and biotic stress (Marina *et al*., 2013; Kusano *et al*., 2008; Takahashi *et al*., 2010; Alcázar *et al*., 2006; Groppa and Benavides, 2008). In particular, transcript profiling by using qRT-PCR has revealed that water stress induces the expression of *ADC2*, *SPDS1* and *SPMS* (Alcázar *et al*., 2006; Phuc., *et al* 2014). It has also shown that cold enhances the expression of *ADC1*, *ADC2* and *SAMDC2* genes while heat shock induces *SPMS* expression (Urano *et al*., 2003; Cuevas *et al*., 2008; Cuevas *et al*., 2009; Sagor *et al*., 2012). Furthermore, recent studies using either transgenic over-expression or loss-of-function mutants support a protective role of PAs under abiotic stresses (Alcázar *et al*., 2006; Kusano *et al*., 2008; Gill and Tuteja, 2010). High Put levels induced by over-expression of homologous *ADC1* enhanced freezing tolerance in Arabidopsis (Altabella *et al*., 2009). Similarly, elevated levels of Put by over-expression of *ADC2* produces drought tolerance in Arabidopsis, which may be related to reduction of water loss by the induction of stomata closure (Alcázar *et al*., 2010). Moreover, *acl5/spms* Arabidopsis double mutants that do not produce Spm

are hypersensitive to salt and drought stresses, and the phenotype is mitigated by application of exogenous Spm (Kusano *et al*., 2007; Yamaguchi *et al*., 2006). All these observations support the protective role of PAs in plant response to abiotic stress (Alcazar *et al*., 2010).

Abscisic acid (ABA) is a plant hormone that reduces water loss through stomatal pores on the leaf surface. Enhanced biosynthesis of ABA occurs in response to water deficit, resulting in the redistribution and accumulation of ABA in guard cells (Bray, 1997). The expression of some of the PA biosynthetic genes, such as *ADC2*, *SPDS1* and *SPMS* are altered in ABA-deficient (*aba2-3*) and ABA-insensitive (*abi1-1*) mutants subjected to water stress (Alcázar *et al*., 2006). In particular, these three genes display reduced transcriptional induction in the stressed *aba2-3* and *abi1-1* mutants compared to the wild-type plants, indicating that ABA modulates PA metabolism at the transcription level by up-regulating the expression of *ADC2*, *SPDS1* and *SPMS* genes under water stress conditions (Alcázar *et al*., 2006). In addition, Put accumulation in response to drought is also impaired in the *aba2-3* and *abi1-1* mutants compared to wild-type plants. This result is further supported by metabolomic studies showing that PA responses to dehydration are also impaired in loss-of-function mutants for *NCED3*, a key biosynthetic gene of ABA (Urano *et al*., 2009). The ABA signaling pathway in stomata regulation involves many different components such as ABA receptors, G-proteins, protein kinases and phosphatases, transcription factors and secondary messengers, including Ca^{2+}, reactive oxygen species (ROS) and NO (Kuppusamy *et al*., 2009). PAs are also linked with reactive oxygen species (ROS) and NO signaling, since PAs catabolism generates H_2O_2, and also there is evidence that PAs enhance NO production (Tun *et al*., 2006). It has been also reported that Put, Spd and Spm regulate stomatal responses (Liu *et al*., 2000; An *et al*., 2008). which indicates that PAs participates in the hormonal pathways, including ABA regulation of abiotic stress responses (Fig. 4) (Alcazar *et al*., 2010; Yamasaki and Cohen, 2006).

Furthermore, it has been shown that biotic and abiotic stresses induce PA transport in the apoplast (Yoda *et al*., 2003; Yoda *et al*., 2006; Marina *et al*., 2013; Moschou *et al*., 2009; Toumi *et al*., 2010). Stress-related factors that have been shown to induce PA transport in the apoplast are: incompatible and compatible plant pathogen interactions, salt stress and treatment with the stress-related hormone ABA (Yoda *et al*., 2003; Yoda *et al*., 2006; Marina *et al*., 2008; Moschou *et al*., 2009; Toumi *et al*., 2010). This suggests that PA catabolism in the apoplast is a general defence response against several stresses. It has been also shown that H_2O_2 produced

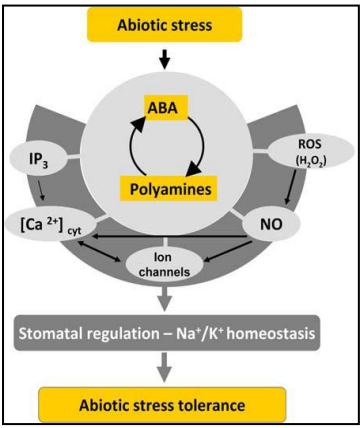

Fig. 4. Scheme represents the integration of PAs with ABA, ROS (H_2O_2), NO, Ca^{2+} homeostasis and ion channel signaling during abiotic stress responses. Figure taken from Alcazar *et al*., 2010.

by PA catabolism in the apoplast contributes to the second phase of ROS production during Tobacco mosaic virus (TMV)-induced HR, a plant response which is developed during an incompatible plant-pathogen interaction and consists of rapid ROS production, PCD and induction of defence responses aiming to restrict pathogen expansion (Yoda *et al*., 2003). Similar approaches showed that H_2O_2 produced by PA catabolism in the apoplast contributes to the synthesis of the ROS that accumulate under abiotic stress conditions (Moschou *et al*., 2008b). PCD was also shown to be induced by oxidation of extracellular Spd also under salt stress.

AIM OF THE WORK

PAOs contribute to various physiological processes through regulation of PA levels and reaction products. PAOs are characterized by a broad variability in substrate specificity, catalytic mechanism and subcellular localization. Extracellular PAOs have been shown to have 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. Conversely, little is known about the physiological roles of intracellular PAOs, such as those of *Arabidopsis thaliana*.

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, with particular focus on the two cytosolic PAOs (AtPAO1 and AtPAO5), which present some similarities to each other, but also important differences, as concerned substrate specificity, catalytic mechanism and tissue-specific expression pattern. Among them, AtPAO5 results of particular interest since it has been shown to be regulated at the post-transcriptional level by proteasome and to function mainly as a dehydrogenase, differently from all other PAOs so far characterized which are classified as oxidases. This study will permit to elucidate the functional complexity characterizing PA metabolism.

RESULTS

1. *AtPAO5* is involved in the control of polyamine homeostasis

To investigate the physiological role(s) of *AtPAO5* during plant growth and development, two *35S::AtPAO5-6His Arabidopsis* transgenic lines that ectopically express *AtPAO5*, one with 70-fold (*AtPAO5-1*; Fig. 5; Ahou *et al*., 2014) and the other 4-fold (*AtPAO5-2*; Fig. 5) higher expression levels than the endogenous gene, were characterized during plant growth and development. Parallel studies were also performed with two loss-of-function mutants, *atpao5-1* (Ahou *et al*., 2014) and *atpao5-2* which lack *AtPAO5* expression (Fig. 5).

The *AtPAO5* transgenic plants and the *atpao5 mutants* were analyzed for PA levels. This analysis has been performed both in whole seedlings and in specific organs in taking into consideration that *AtPAO5* is expressed in all organs (Fig. 6; Fincato *et al*., 2012). Results evidenced decreased levels of Spm, Therm-Spm, and N^1-acetyl-Spm in mature leaves and inflorescence stems of *AtPAO5-1* transgenic plants and increased levels in those of *atpao5-1* and *atpao5-2* plants, as compared to the wild-type ones (Fig. 7). Similar results were obtained in whole seedlings (data not shown). In contrast to the *AtPAO5-1* transgenic plants, the *AtPAO5-2* transgenic line does not present differences in the levels of Spm, Therm-Spm and N^1-acetyl-Spm from those of the wild-type plants (Fig. 7), which suggests a dose-dependent contribution of *AtPAO5* in PA homeostasis. The analysis of the PA levels further evidenced higher Spm and Therm-Spm levels in stems than in leaves, in agreement with previous studies (Fig. 2; Naka *et al*., 2010). This is probably determined by the relative amounts of the anabolic and catabolic enzymes specific for these PAs (such as *ACL5*, *SAMDC4*, *AtPAO1* and *AtPAO5*), considering their similar organ-specific gene expression pattern (Fig. 6A). Contrary to Spm and Therm-Spm, comparable N^1-acetyl-Spm levels are present in stems and leaves (Fig. 7).

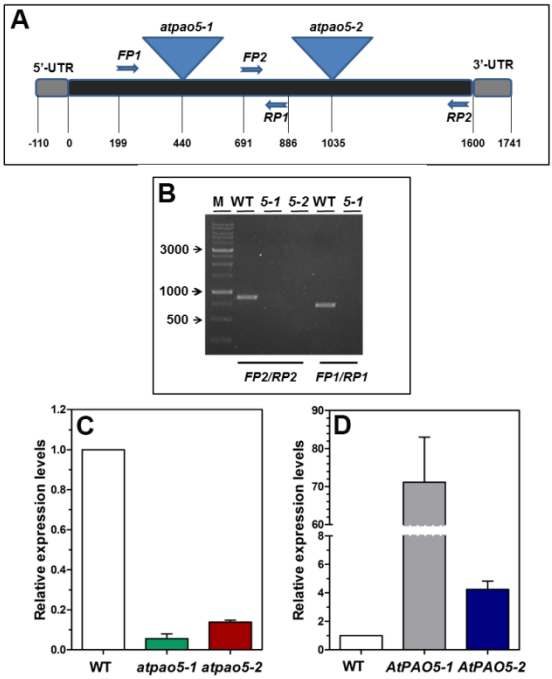

Fig. 5. *AtPAO5* expression levels in *atpao5* loss-of-function mutants and *35S::AtPAO5-6His Arabidopsis* transgenic plants. (A) Schematic genome structure of *AtPAO5*. Triangles indicate the positions of the two T-DNA insertions and arrows the position of the primers used for mutant characterization. (B) Analysis of selected homozygous lines of *atpao5-1* and *atpao5-2* mutants for *AtPAO5* expression levels by RT-PCR. (C and D) Relative *AtPAO5* expression levels in young seedlings of the two *atpao5* mutants (*atpao5-1* and *atpao5-2*), the two independent *35S::AtPAO5-6His Arabidopsis* transgenic lines (*AtPAO5-1* and *AtPAO5-2*) and the 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.

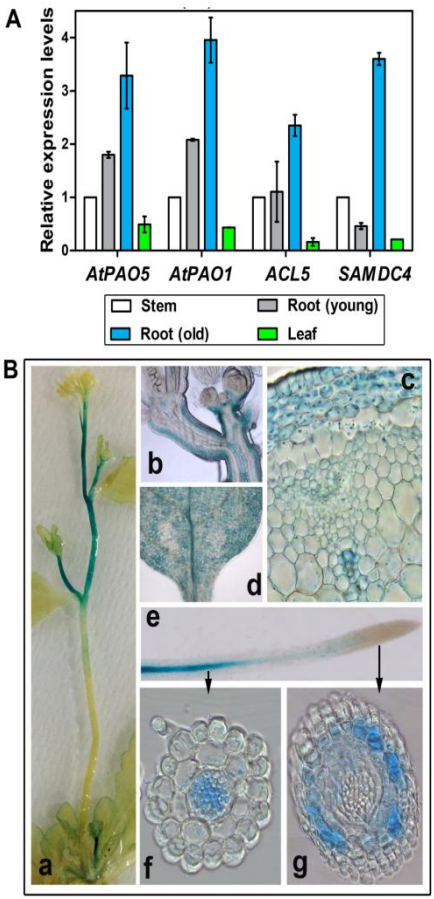

Fig. 6. Expression of *AtPAO1*, *AtPAO5*, *ACL5* and *SAMDC4* in various plant organs. (A) Relative expression levels of *AtPAO1*, *AtPAO5*, *ACL5* and *SAMDC4* in inflorescence stems, leaves and roots of 10-day-old (young) and 3-week-old (old) plants as determined by qRT-PCR. Mean values ± SE of two independent experiments are shown. (B) *AtPAO5* promoter activity in stems (a, b, c), leaves (d) and roots (e, f, g) 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.

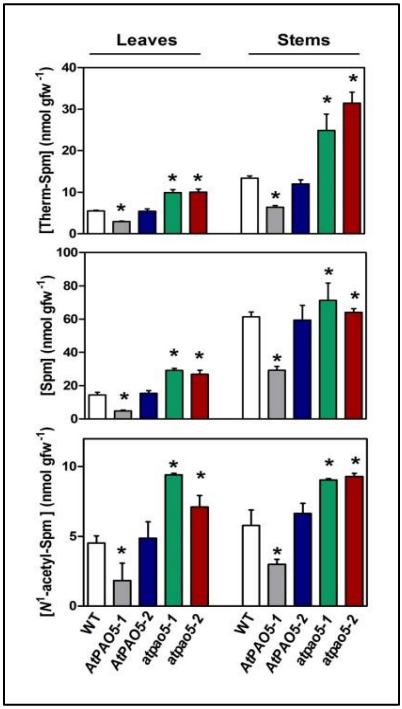

Fig. 7. Spm, Therm-Spm and N^1-acetyl-Spm levels in *AtPAO5* and *atpao5* plants. PA levels were determined in rosette leaves and inflorescence stems of 5-week-old seedlings by GC-MS. The analysis was repeated three times and a representative experiment is shown. Numbers are mean values ± SE of three replicates obtained from the same pooled fresh material. Statistical analysis was performed by one-way ANOVA test ($P<0.05$). Asterisks indicate statistically significant differences from the wild-type plants.

It is well established that the expression levels of *ACL5* and *SAMDC4* are regulated by Therm-Spm levels through a negative feedback loop (Hanzawa *et al*., 2000; Clay and Nelson, 2005; Kakehi *et al*., 2008; Kakehi *et al*., 2010; Baima *et al*., 2014). To address whether such a regulatory mechanism is activated upon modulation of *AtPAO5* expression levels, the *AtPAO5* over-expressing transgenic plants, as well as the *atpao5-1* and *atpao5- 2* mutants were analyzed for *ACL5* and *SAMDC4* expression levels by qRT-PCR. As shown in Fig. 8, *ACL5* and *SAMDC4* genes are up-regulated in *AtPAO5-1* seedlings and down-regulated in the *atpao5-1* and *atpao5-2* ones, but not affected at all in *AtPAO5-2* seedlings with low expression levels of the transgene and unaltered PA content. Instead, no change in *SPMS* expression levels was observed in any of the *AtPAO5* and *atpao5* mutant plants. Similar results were obtained when roots and stems

(Supplemental Fig. S1), as well as leaves and flowers (data not shown), were analyzed.

These data, together with recently published data showing *AtPAO5* up-regulation by Therm-Spm (Ahou *et al*., 2014), suggest that *AtPAO5* participates in the feedback mechanism controlling *ACL5* and *SAMDC4* expression levels, as well as Therm-Spm homeostasis along the entire plant. The activation of this feedback mechanism in *AtPAO5-1* plants may explain the reduction of Spm, Therm-Spm, and N^1-acetyl-Spm levels to only 50% of those in wild-type plants despite the very high expression levels of ectopic *AtPAO5* in the *AtPAO5-1* plants.

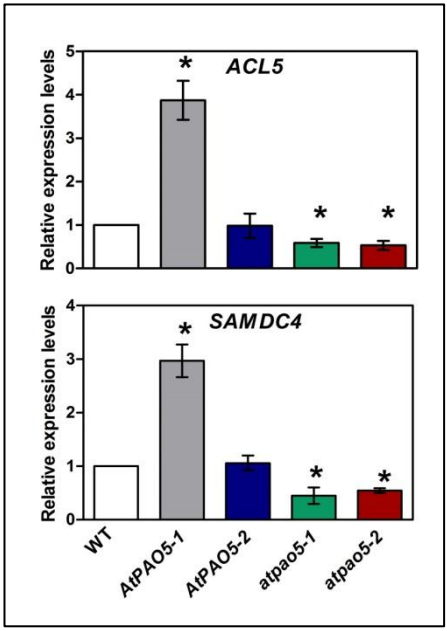

Fig. 8. *ACL5* and *SAMDC4* expression levels in *AtPAO5* and *atpao5* plants. Relative expression levels were determined by qRT-PCR analysis of 10-day-old seedlings. Numbers are mean values ± SE of three independent repetitions of the experiment. Statistical analysis was performed by one-way ANOVA test. Asterisks indicate statistically significant differences from the wild-type (WT) plants.

2. *AtPAO5* is up-regulated by cytokinins and auxin in an organ-specific way

AtPAO5 promoter activity studies using *AtPAO5prom::GFP-GUS* transgenic plants showed that in roots *AtPAO5* expression is extended from the hypocotyl–root junction region throughout the maturation and elongation zones until when the spiral secondary cell wall thickenings of the protoxylem elements first become evident. In particular, the *AtPAO5* expression in the roots is tissue-specific involving the whole vascular system (xylem, phloem and procambial/cambial cells) and pericycle (Fig. 6B, e and f; Fincato *et al*., 2012; Ahou *et al*., 2014), similarly to the tissue-specific expression of *AtPAO5* in hypocotyls (Fincato *et al*., 2012). Occasionally, *AtPAO5* appeared to be expressed also in the root meristematic region, mainly in cortical tissues (Fig. 6B, g). In cotyledons and leaves, *AtPAO5* promoter activity is extended to the entire area (Fincato *et al*., 2012), including veins (Fig. 6B, d). In stems, *AtPAO5*-related staining was specifically present in cortex and xylem vessels (Fig. 6B, b and c), mainly in the upper internodes (Fig. 6B, a). Treatment of *AtPAO5::GFP-GUS* transgenic plants with 6-benzylaminopurine (BAP) increased the intensity of GUS staining in the root vasculature and the meristematic region (Fig. 9A). Instead, no increase in *AtPAO5*-related GUS staining was evident in the above-ground part of the plants. These data indicate that *AtPAO5* expression is under the control of cytokinins specifically in the roots. A root-specific up-regulation of *AtPAO5* was additionally observed following treatment with indole-3-acetic acid (IAA) (Fig. 9A). The up-regulation of *AtPAO5* by cytokinins and auxin was further confirmed by qRT-PCR analysis (Fig. 9B).

3. *AtPAO5* is involved in the cytokinin-mediated control of root xylem differentiation

Cytokinins have a critical role in root elongation, root apical meristematic activity and protoxylem differentiation (Perilli *et al*., 2010; Del Bianco *et al*., 2013; Kondo *et al*., 2014). Indeed, increased cytokinin levels shift the position of the meristematic/elongation transition zone (TZ) more distally, shortening the meristematic region and impairing root elongation, whereas decreased cytokinin levels shift the TZ proximally, producing longer

meristems and roots. Furthermore, cytokinins negatively regulate protoxylem specification (Mähönen *et al*., 2006; Bishopp *et al*., 2011; Perilli *et al*., 2010).

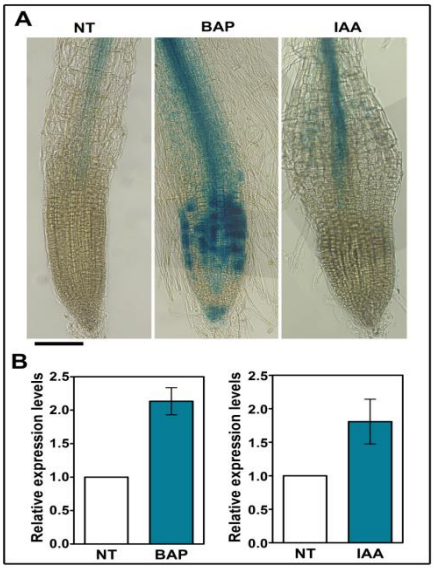

Fig. 9. *AtPAO5* up-regulation by cytokinins and auxin. (A) Histochemical GUS staining of 7-day-old *AtPAO5::GFP-GUS Arabidopsis* transgenic plants treated with 1 μM BAP or 10 μM IAA for 24 h. Bar indicates 100 μm. (B) Quantitative RT-PCR analysis of *AtPAO5* expression levels in whole seedlings (BAP) or roots (IAA) of 10-day-old wild-type plants treated with 5 μM BAP or 10 μM IAA for 3 h. Mean values ± SE of relative quantitation from two independent experiments are shown.

To determine the physiological significance of the root–specific effect of cytokinins on *AtPAO5* expression levels, root elongation rate of the *AtPAO5* over-expressor and mutant plants in the absence and presence of BAP, was compared with that of wild-type plants. However, no difference among the different genotypes was apparent even though BAP impaired root elongation (Fig. 10A). Despite the lack of differences in root elongation rate among the transgenic and the wild-type plants in the presence and the absence of exogenous cytokinins, a detailed analysis of the timing of protoxylem and metaxylem differentiation in roots was performed determining the distance between the first protoxylem and metaxylem elements from the quiescent centre (QC). Root apical meristematic activity was additionally analyzed measuring the distance between TZ and QC. In the

absence of exogenous BAP, no difference was observed in root protoxylem and metaxylem differentiation (Fig. 11), nor in root apical meristematic activity among the *AtPAO5* over-expressor plants, the *atpao5* mutants, or the wild-type plants (Fig. 10 B).

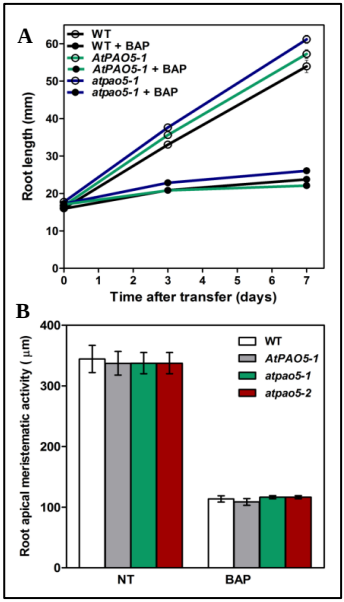

Fig. 10. Effect of cytokinins on root elongation and meristematic activity of *AtPAO5-1* and *atpao5* plants.) Effect of BAP on root elongation. (B) Effect of BAP on root tip length. *AtPAO5-1, atpao5* and wild-type (WT) seedlings of 5 days were transferred onto plates containing 1 µM BAP. Root length was measured at various time intervals after transferring. Root tip length was determined 6 days after transferring to the BAP-containing medium measuring the distance from the quiescent center to the first cells displaying elongation under confocal microscope. NT: not treated control.

Addition of BAP in the growth medium, delayed protoxylem differentiation in all plant genotypes, thus shifting the position of the first protoxylem vessels more proximally than in the mock treated plants. However, this delay was less pronounced in *AtPAO5-1* transgenic plants and more pronounced in *atpao5-1* and *atpao5-2* mutants than in wild-type plants (Fig. 11). Further, BAP treatment highly reduced apical meristematic activity, but at the same level in all genotypes (Fig. 10B). This is despite the fact that BAP increases *AtPAO5* expression levels not only in the root vasculature but also in the meristematic region. BAP treatment additionally caused shortening of the distance from the quiescent center (QC) of the first metaxylem vessels probably due to the reduced apical meristematic activity. The effect, however, appeared genotype-independent. These data suggest that *AtPAO5* is specifically involved in the cytokinin-mediated control of root

protoxylem differentiation, this specific signaling pathway being impaired in the presence of increased *AtPAO5* expression levels and enhanced in the absence of *AtPAO5* expression.

Since vascular system differentiation is controlled also by auxins and considering the organ-specific effect of IAA in *AtPAO5* expression levels, *atpao5-1* and *AtPAO5-1* plants together with wild-type plants were analyzed for root growth (data not shown) and xylem differentiation (Supplemental Fig. S2) in the presence and absence of IAA. This analysis showed that IAA inhibited root growth and reduced the distance of the first protoxylem and metaxylem elements from the QC at the same level in all plant genotypes.

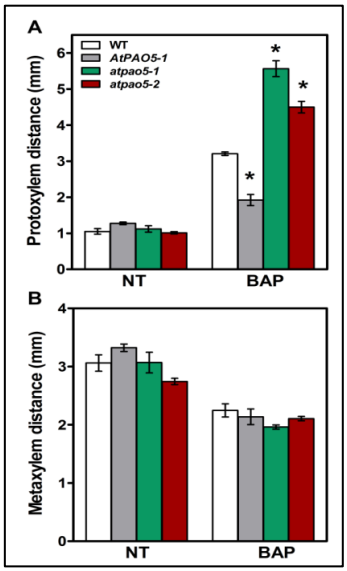

Fig. 11. Cytokinin-mediated xylem differentiation in the *AtPAO5-1* and *atpao5* plants. (A) Distance of the first protoxylem cells from QC (Protoxylem distance). (B) Distance of the first metaxylem elements from QC (Metaxylem distance). Protoxylem and Metaxylem distances were measured after 5 days of treatment or not with 1 μM BAP of 7-day-old plants initially grown under physiological conditions. For protoxylem and metaxylem visualization seedlings were stained with fuchsin and observed under confocal microscope. The analysis was repeated at least three times and a representative experiment is shown. Numbers are mean values ± SE ($n \geq 4$) and asterisks denote statistically significant differences from the corresponding wild-type (WT) plants ($P<0.05$; One-way ANOVA). NT: untreated control.

4. *AtPAO5* is involved in the control of stem and root development

As regards the aerial part of the plants, at early development stages the *atpao5* mutants and the *AtPAO5* over-expressing transgenic plants display the same growth pattern and phenotype as the wild-type plants and only after the transition from vegetative to reproductive growth some differences become evident. In particular, while the *atpao5-1* and *atpao5-2* mutants as well as the *AtPAO5-1* and *AtPAO5-2* transgenic plants initiated vegetative

growth and transition to the inflorescence meristem in the same manner as the wild-type plants, the two *atpao5* mutants produced longer and slightly thicker flowering stems than the wild-type plants, while, conversely, the *AtPAO5-1* transgenic plants produced shorter and thinner flowering stems leading to a semi-dwarf phenotype with reduced apical dominance (Fig. 12). Instead, the stems of the *AtPAO5-2* transgenic plants which have lower transgene expression levels than the *AtPAO5-1* transgenic plants did not show differences in length and thickness compared to the wild-type plants (Fig. 12).

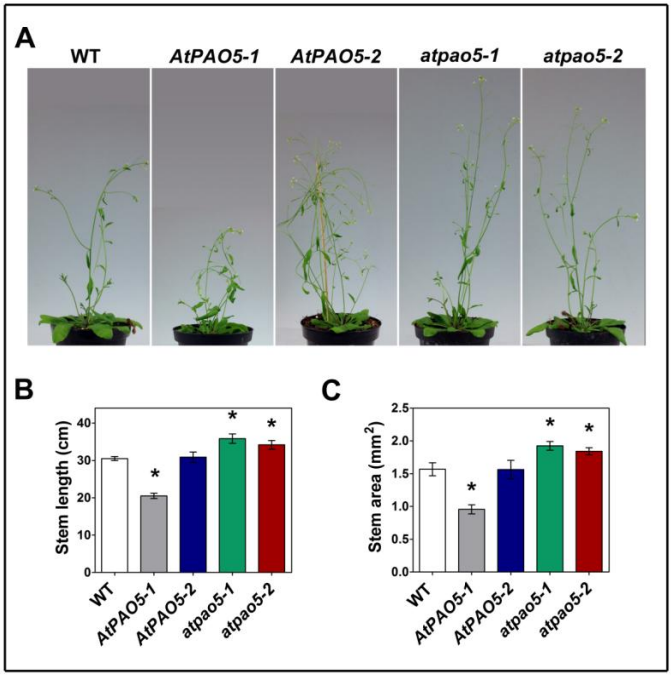

Fig. 12. Phenotype of *AtPAO5* and *atpao5* transgenic plants. (A) Stem phenotype of 5-week-old soil-grown *AtPAO5*, *atpao5* and wild-type (WT) plants. (B) Stem length of 5-week-old *AtPAO5-1*, *atpao5* and WT plants. The analysis has been repeated for more than 6 times (n=4 to 10) with highly reproducible results. (C) Stem thickness of *AtPAO5* and *atpao5* transgenic plants. Stem thickness was determined by measuring the area of transverse sections from the stem basal ends using ImageJ analysis. Numbers are mean values ± SE (n≥4) from a representative experiment. Statistical analysis was performed by one-way ANOVA test and asterisks indicate statistically significant differences compared with WT plants.

To understand these differences in inflorescence development among the *atpao5* mutants, the *AtPAO5* over-expressing transgenic plants and the wild-type plants at the cellular level, the inflorescence anatomy of these plants were examined analyzing transverse sections of the first and second internodes at the basal end of the inflorescence stem (Fig. 13). These analyses showed that the *AtPAO5-1* transgenic plants on the one hand undergo excessive primary xylem differentiation presenting a much higher number of large-diameter, thick-walled metaxylem vessels than the wild-type plants (Fig. 14), while on the other hand display a highly reduced secondary growth (Fig. 13). On the opposite, the *atpao5-1* and *atpao5-2* mutant plants present a slightly lower number of metaxylem vessels and a more extensive secondary growth than the wild-type plants (Fig. 13; Fig. 14). These differences in the xylem development were observed both in young inflorescences before appearance of differences in stem length and in later

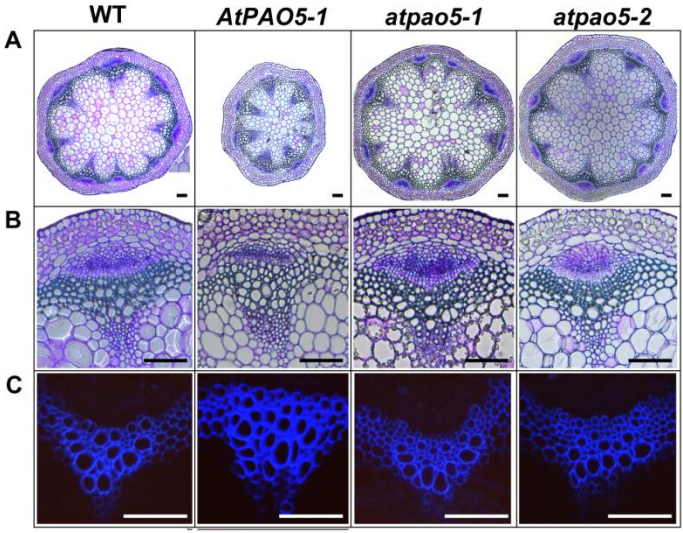

Fig. 13. Histological analysis of inflorescence stems of *AtPAO5-1* and *atpao5* plants. (A) Transverse sections of the second internodes at the basal end of inflorescence stems stained with toluidine blue. (B) Vascular bundles of inflorescence stems as observed from transverse sections stained with toluidine blue. (C) Vascular bundles of inflorescence stems observed under UV light. Black and white bars indicate 100 μm. Similar results were obtained from transverse sections of the first internodes at the basal end of inflorescence stems.

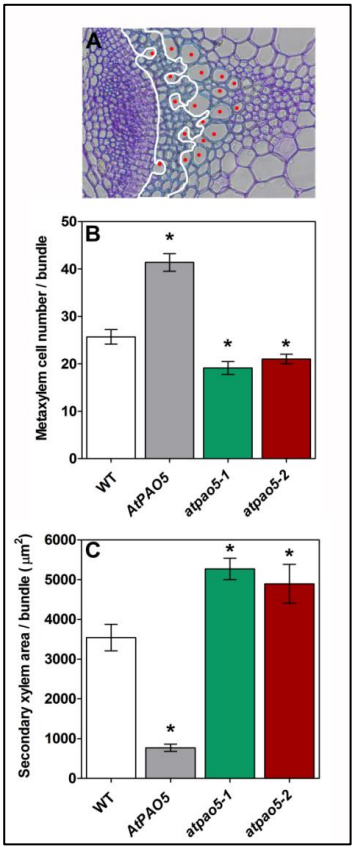

Fig. 14. Number of metaxylem vessels and size of secondary xylem in bundles of *AtPAO5-1*, *atpao5-1*, *atpao5-2* and wild-type (WT) stems. (A) An example of quantification of the number of metaxylem cells and the size of secondary xylem area in a stem transverse section stained with toluidine blue. Measurement of secondary xylem area was performed following image acquisition through ImageJ software. (B) Number of metaxylem vessels in bundles of *AtPAO5-1*, *atpao5-1*, *atpao5-2* and WT stems. (C) Size of secondary xylem in bundles of *AtPAO5-1*, *atpao5-1*, *atpao5-2* and WT stems. Numbers are mean values ±SE. Asterisks indicate statistically significant differences from the wild-type (WT) plants ($P<0.05$; one-way ANOVA test). A representative experiment is shown.

developmental stages. The different degree of xylem differentiation in the stems of the *AtPAO5-1*, *atpao5-1* and *atpao5-2* mutants as compared to the wild-type plants most probably interfered with the normal organ growth.

Additionally to the differences in stem elongation and thickness, the *AtPAO5* and *atpao5* plants present some differences in root length from the wild-type plants in advanced developmental stages. Indeed, despite the lack of differences in root elongation at early developmental stages among the different genotypes, mature *AtPAO5-1* transgenic plants present slightly shorter roots than the wild-type plants, while *atpao5-1* and *atpao5-2* longer ones (Fig. 15 A and B). Furthermore, observation of root transverse sections

at the maturation zone of 15-day-old plants growing *in vitro* evidenced increased number of metaxylem vessel elements in the *AtPAO5-1* transgenic plants as compared with the wild-type plants and decreased in the *atpao5-1* and *atpao5-2* mutants (Fig. 15 C). These data indicate that *AtPAO5* contributes to xylem development both in stems and roots, the effect appearing more pronounced in stems than in roots most likely due to the longer growth period of the stems comparing with the roots. However, a major role of *AtPAO5* in stem than in root xylogenesis cannot be excluded.

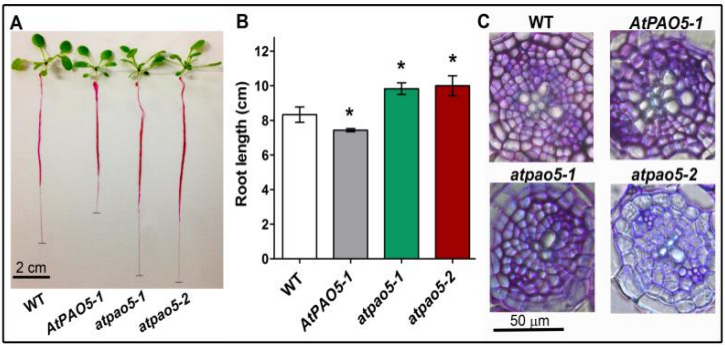

Fig. 15. Root phenotype of *AtPAO5* over-expressing transgenic plants and *atpao5* mutants. (A) Roots from 16-day-old plants grown in hydroponic cultures. To help visualization, roots were stained with fuchsin. (B) Root length of 16-day-old plants grown in hydroponic cultures. Numbers are mean values ± SE (n≥4) from a representative experiment. Statistical analysis was performed by one-way ANOVA test and asterisks indicate statistically significant differences compared to WT plants. (C) Transverse sections of roots stained with toluidine blue. Sections at 2 cm below root-hypocotyl junction were made.

5. *AtPAO5* is involved in the control of lateral roots

A further analysis of root development showed that the *AtPAO5-1* transgenic plants present a higher number of lateral roots than the wild-type plants (Fig. 16B). Instead, no statistically significant difference was evidenced in the number of lateral roots for the *atpao5-1* and *atpao5-2* plants in respect to the wild-type plants (Fig. 16). The addition of Therm-Spm in the growth medium did not alter significantly the number of lateral roots in the different genotypes as compared with the untreated controls, the number of the lateral roots in the *AtPAO5-1* transgenic plants being higher than that of

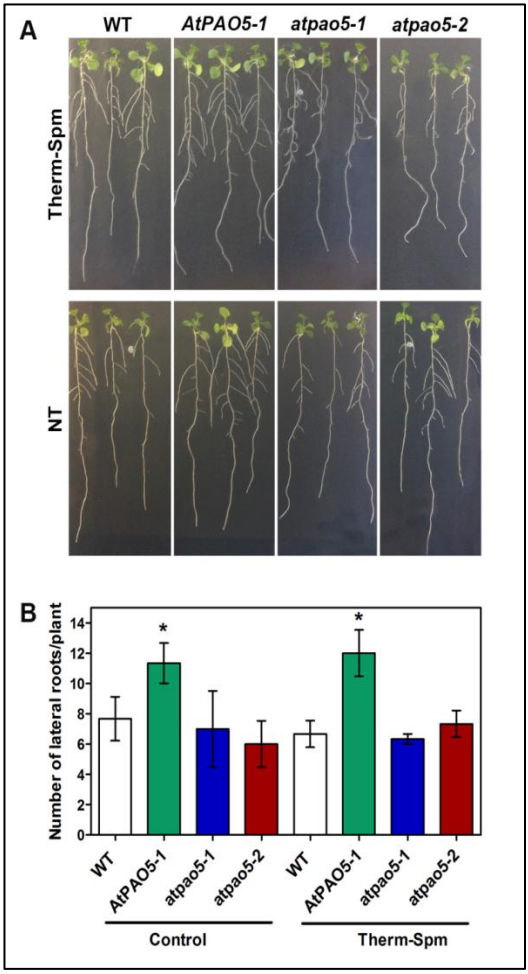

Fig. 16. Modulation of lateral roots by *AtPAO5* ectopic expression. (A) Root growth of *AtPAO5-1*, *atpao5-1*, *atpao5-2* and wild-type (WT) plants in the presence and the absence of 100μM Therm-Spm. (B) Number of lateral roots of *AtPAO5-1*, *atpao5-1*, *atpao5-2* and wild-type (WT) plants grown in the absence or in the presence of 100 μM Therm-Spm. A representative experiment is shown. Bars denote standard errors and asterisks indicate statistically significant differences in respect to the corresponding wild-type (WT) plants ($P<0.05$; One-way ANOVA).

the wild-type plants also under these conditions (Fig. 16). However, in the presenceof Therm-Spm, lateral roots of *atpao5* mutant plants present reduced axial extension comparing with those of *AtPAO5-1* transgenic plants and wild-type plants, the latter plants presenting an intermediate phenotype (Fig. 16A). On the contrary, no difference was observed among the different genotypes, as considered root elongation and growth of the aerial parts of the plants, both in the presence and the absence of Therm-Spm, during the 5- to 8-day period of observation.

The reduced axial extension of the lateral roots may be due to reduced strength of the roots, being thus unable to withstand the forces of stretching that occur during organ expansion inside the agar plates. Indeed, in the presence of Therm-Spm, protoxylem and metaxylem differentiation appeared highly delayed in *atpao5-1* and *atpao5-2* mutants but not in *AtPAO5-1* transgenic plants and the wild-type plants (Fig. 17A). Additionally, in the presence of Therm-Spm, several discontinuities were observed in the protoxylem elements of the main roots in *atpao5-1* and *atpao5-2* mutants, but not in those of *AtPAO5-1* transgenic plants (Fig. 17B). The discontinuities in the protoxylem elements of *atpao5* roots were more prominent in the presence of 100 μM Therm-Spm than in the presence of 50 μM (Fig. 17B). Wild-type roots only occasionally presented discontinuities in protoxylem elements. Contrary to Therm-Spm, Spm affected neither the axial extension of the lateral roots nor disconnected protoxylem elements (data not shown).

These data are consistent on the one hand with the high catalytic activity of AtPAO5 towards Therm-Spm, thus back-converting the available amount of Therm-Spm in the medium to Spd and on the other hand to the opposing effects of Therm-Spm and auxin on xylem differentiation (Yoshimoto *et al*., 2012). It is probably because of this negative effect of Therm-Spm on xylem integrity that prolonged growth of *atpao5* mutants in the presence of Therm-Spm inhibited growth of the aerial part of the plants as recently reported (Kim *et al*., 2014).

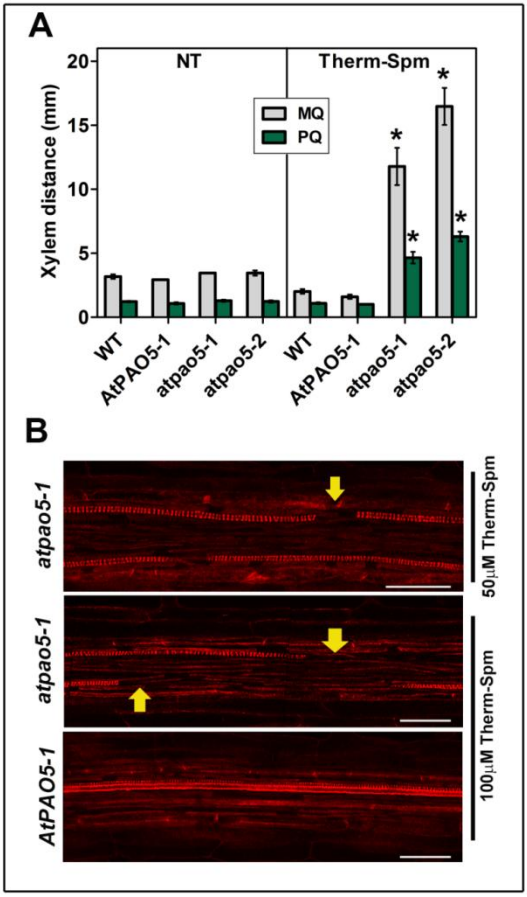

Fig. 17. Modulation of xylem development by Therm-Spm. (A) The distances from QC of the first protoxylem (PQ) and metaxylem (MQ) vessels were measured in 10-day-old seedlings growing for the 5 last days in the presence or the absence of 100 μM Therm-Spm. Mean values ± SE ($n \geq 6$) from a representative experiment are shown. Asterisks indicate statistically significant differences in respect to the corresponding wild-type (WT) plants ($P<0.05$; One-way ANOVA). (B) Protoxylem discontinuities in the maturation zone of roots of *atpao5-1* plants. grown in the presence of 50 μM or 100 μM Therm-Spm. Yellow arrows show regions in *atpao5-1* roots in which metaxylem vessels are missing and protoxylem discontinuity is observed. White bars indicate 50 μm distance.

6. AtPAO5 and *atpao5* plants exhibit altered vasculature thickness in hypocotyls, cotyledons and leaves

To examine whether *AtPAO5* has any relevance in differentiation of vascular system also in leaves and hypocotyls, cell-wall auto-fluorescence was analyzed in *AtPAO5-1*, *atpao5-1* and wild-type plants by dark-field and fluorescence microscopy. This analysis showed that the vasculature of hypocotyls and petioles (Fig. 18), as well as of cotyledons (Supplemental Fig. S3) and leaves (data not shown) in *AtPAO5-1* plants present increased auto-fluorescence as compared with the wild-type plants, while in *atpao5-1* mutant reduced.

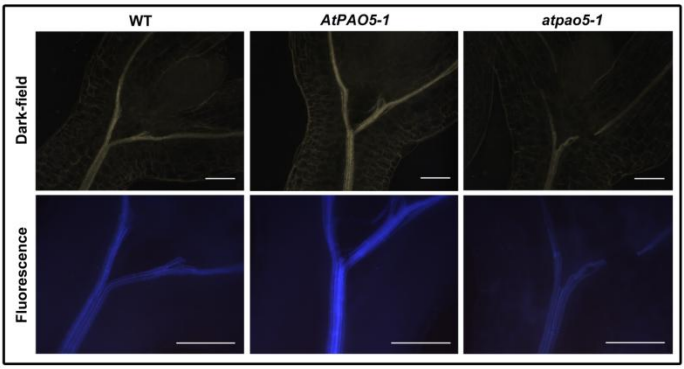

Fig. 18. *AtPAO5* contribution to vasculature formation. Vasculature at the hypocotyl/cotyledon junction zone of *AtPAO5-1*, *atpao5-1* and wild-type (WT) plants as observed by dark-field and fluorescence microscopy. Note the more- and less-prominent autofluorescence in the vasculature of the *AtPAO5-1* and *atpao5-1* plants, respectively, compared with the wild-type plants. Bars indicate 100 μm.

7. Auxin- and xylem differentiation-related genes are differently expressed in *AtPAO5-1* and *atpao5-1* plants

The phenotypical alterations of *atpao5* mutants and *AtPAO5-1* transgenic plants regarding stem length resemble those of the plants with altered cytokinin levels and /or signaling (Zhang *et al*., 2009; Argyros *et al*., 2008; Schoor *et al*., 2011) which is consistent with the altered response to cytokinins at the root xylem differentiation level of the same plants as

compared with the wild-type plants. The *AtPAO5-1* phenotypical alterations also resemble those of the *bud2/samdc4* null mutant with altered cytokinin and auxin response (Ge *et al*., 2006; Cui *et al*., 2010). On the other hand, the shorter stems together with the altered stem anatomy observed in the *AtPAO5-1* transgenic plants recall also the *acl5/kv Arabidopsis* mutants with reduced levels of Therm-Spm (Hanzawa *et al*., 2000; Clay and Nelson, 2005; Kakehi *et al*., 2008; Muñiz *et al*., 2008) in the stem of which the polar auxin transport has been shown to be 66.2% of that in wild-type plants (Clay and Nelson, 2005). Furthermore, the increased number of lateral roots in *AtPAO5-1* plants and the altered vasculature thickness in hypocotyls, cotyledons and leaves of *AtPAO5-1* and *atpao5* plants suggest altered auxin signaling/transport, since several evidences support a decisive role of auxin in lateral root development and leaf vein patterning (Casimiro *et al*., 2001; Vilches-Barro and Maizel, 2015; Sieburth, 1999; Sawchuk *et al*., 2013). To verify whether cytokinin and auxin levels, signaling and/or transport are altered in the *atpao5-1* mutants and the *AtPAO5-1* transgenic plants, seedlings, roots and stems were analyzed for expression levels of some cytokinin- and auxin-related genes. The expression levels of genes regulated by Therm-Spm, as well of genes correlated to xylem differentiation were additionally analysed (Supplemental Table S2). In particular, some of the *Arabidopsis* type-A cytokinin response regulators (*ARR5*, *ARR7*, *ARR15*, *ARR16*), which are up-regulated by cytokinins, were analyzed to investigate on the cytokinin-dependent signaling pathway. The *HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*), which is a negative regulator of cytokinin signaling, plays a role in protoxylem development (Mähönen *et al*., 2006), and is down-regulated by cytokinins (Mähönen *et al*., 2006) and up-regulated by auxins (Bishopp *et al*., 2011; Del Bianco *et al*., 2013), was also chosen for this analysis to obtain information on the cytokinin/auxin interplay. The expression levels of the plasma membrane-localized *PIN1*, which is involved in intercellular auxin transport, and of the endoplasmic-reticulum-localized *PIN6*, which is involved in intracellular auxin transport and which has been shown to be up-regulated by auxin (Cazzonelli *et al*., 2013) and down-regulated by Therm-Spm (Tong *et al*., 2014), were also analyzed. The *VND6* and *VND7* transcription factors of the NAC-domain protein gene family and the *ATHB8*, *PHB*, and *CNA* transcription factors of the *HD-ZIP III* gene family, which are all involved in the regulation of vascular differentiation (Miyashima *et al*., 2013) and have been shown to be down-regulated by Therm-Spm (Kakehi *et al*.,2008; Tong *et al*., 2014) and up-regulated by auxin (Yoshimoto *et al*., 2012), were additionally taken into

consideration. Transcript levels of the *SAC51* gene were also analyzed, since it has been shown to be up-regulated by Therm-Spm (Kakehi *et al*., 2008). The expression levels of *SCARECROW* (*SCR*) transcription factor which is down-regulated by cytokinins were in parallel examined (Carlsbecker *et al*., 2010; Zhang *et al*., 2013; Miyashima *et al*., 2013). Furthermore, the marker of xylem differentiation *XYLEM CYSTEINE PEPTIDASE 1* (*XCP1*; Funk *et al*., 2002; Avci*et al*., 2008), the *Xyloglucan endotransglycosylase/hyrdolase 3* (*XTH3*) gene encoding a cell-wall loosening protein and the *PHLOEM INTERCALATED WITH XYLEM* / *TDIF RECEPTOR* (*PXY*; Fisher and Turner, 2007) encoding a receptor kinase were analyzed since they have been recently shown to be down-regulated by Therm-Spm (Tong *et al*., 2014).

Data from qRT-PCR showed that the expression levels of the auxin- and /or xylem-related genes *PIN1*, *PIN6*, *VND6*, *VND7*, *ATHB8*, *PHB*, *CNA*, *XCP1*, *XTH3* and *PXY* are up-regulated in the *AtPAO5-1* transgenic plants and down-regulated in the *atpao5-1* mutant plants in whole seedlings (Supplemental Fig. S4) and specific organs, such as stems and roots (Fig. 19). Instead, no statistically significant variation was observed in the expression levels of *ARR5*, *ARR7*, *ARR15* and *ARR16* in the *AtPAO5-1* and *atpao5-1* plants comparing with the wild-type plants. Only *AHP6* among the genes related to cytokinin signaling is up-regulated in the *AtPAO5-1* transgenic plants and down-regulated in the *atpao5-1* mutants (Fig. 19; Supplemental Fig. S4). Interestingly, *AHP6* was also shown to be down-regulated by Therm-Spm, while on the contrary *ARR5*, *ARR7* and *ARR15* to be up-regulated (Fig. 20A). On the other hand, no statistically significant variation was observed in the expression levels of *SCR* and *SAC51* in the *AtPAO5-1* and *atpao5-1* plants comparing with the wild-type plants.

Concerning *ARRs*, although they are expressed at the same level in the *AtPAO5-1*, *atpao5* and wild-type plants under physiological conditions, *ARR5*, *ARR7* and *ARR16* are differently up-regulated following treatment with BAP in the different genotypes (Fig. 20). The expression levels of these three *ARRs* are less induced by BAP in the *AtPAO5-1* plants than in the wild-type plants (Fig. 20). Furthermore, *ARR5* and *ARR16* are more induced by BAP in the two *atpao5* mutants than in the wild-type plants (Fig. 20).

These data altogether suggest altered auxin and cytokinin signaling in the *atpao5-1* mutants and the *AtPAO5-1* transgenic plants comparing with the wild-type plants. In particular, these data are compatible with increased auxin and decreased cytokinin signaling in the *AtPAO5-1* transgenic plants and conversely decreased auxin and increased cytokinin signaling in the *atpao5-1* mutant plants.

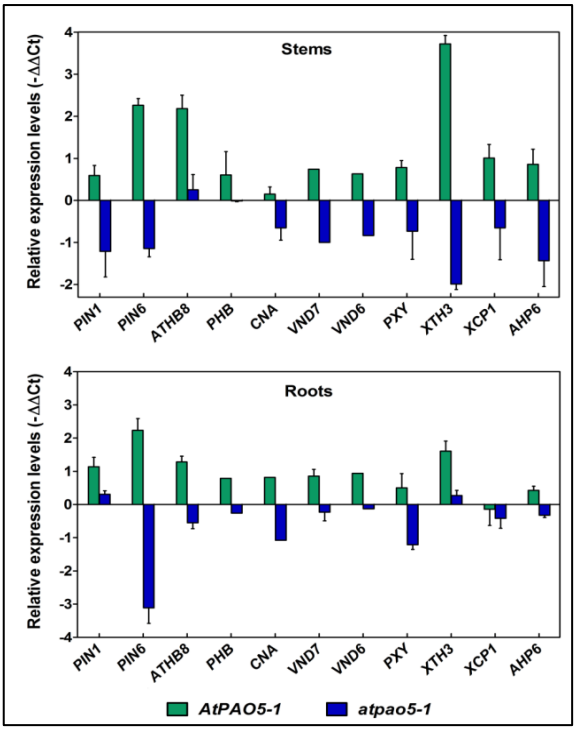

Fig. 19. Relative expression levels of auxin- and cytokinin-related genes in stems and roots of *AtPAO5-1* and *atpao5-1* plants. Relative expression levels were determined by qRT-PCR. Mean values ± SE of three independent biological replicates are shown.

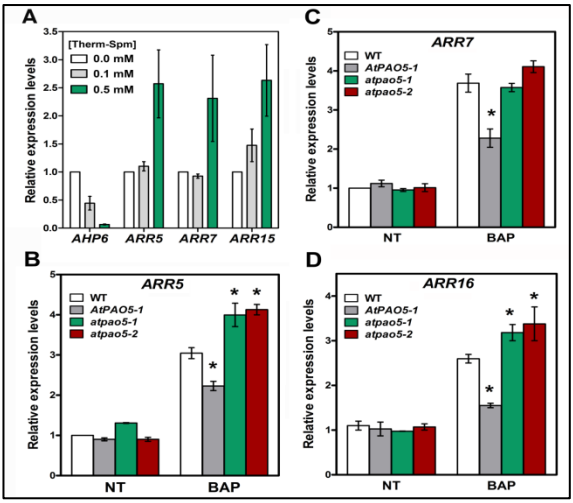

Fig. 20. Regulation of cytokinin-related genes by Therm-Spm and BAP. (A) Regulation of cytokinin-related genes by Therm-Spm. Wild-type seedlings were treated or not (NT) with the indicated concentrations of Therm-Spm for 24 hr. (B-D) Regulation of *ARR5*, *ARR7* and *ARR16* by BAP in the *AtPAO5-1* and *atpao5* plants. *AtPAO5-1*, *atpao5-1*, *atpao5-2* and wild-type (WT) seedling were treated or not (NT) with 5 μM BAP for 3 hr. Numbers are mean values ± SE of at least two biological repetitions and asterisks denote statistically significant differences from the corresponding WT plants (P<0.05; One-way ANOVA).

8. *AtPAO1* contributes together with *AtPAO5* in stem and root development

AtPAO1 presents some similarities to AtPAO5, as for example subcellular localization (cytosolic), substrate specificity (Therm-Spm and Spm) and tissue-specific expression pattern (expression in anther tapetal cells and the anther–filament junction; Fincato *et al.*, 2012). These similarities between AtPAO1 and AtPAO5 permit the suggestion to be made that they have some common physiological roles, although the existing important differences between these two enzymes (i.e. regarding catalytic activity towards N^1-acetyl-Spm and expression in the vascular system) indicate some distinct physiological roles too.

Previous studies showed that *atpao1* mutant plants do not present differences in PA levels, morphology and growth rate in respect to wild-type plants (Ahou A, PhD thesis, 2013). In the present study, to verify whether

AtPAO1 contributes redundantly together with *AtPAO5* in plant development and defense responses, the *atpao1/atpao5* double mutant (*DM15*) was analyzed. Preliminary results showed increased Spm and Therm-Spm levels in *DM15* mutant plants comparing to both the wild-type plants and the *atpao5* single mutants (Fig. 21A). Furthermore, the *DM15* mutant plants, differently from the *atpao1* mutant plants, present lower *ACL5* expression levels than those of the wild-type plants and the *atpao5* mutants (Fig. 21B). These results suggest that AtPAO1, together with AtPAO5, participates in the regulation of *ACL5* gene expression and contributes to PA homeostasis. Phenotypical analysis evidenced that the *DM15* plants present longer stems than those of both the wild-type plants and the *atpao5* single mutants. Conversely, the stem length of the *atpao1* single mutant presents no statistically significant difference in respect to that of the wild-type plants (Fig. 21C). Moreover, *DM15* mutant plants present longer roots than the *atpao5-1*, *AtPAO5-1* and wild-type plants (Fig. 22), a difference which becomes evident even at early developmental stages, differently from *AtPAO5-1* and *atpao5* mutants which present differences in root length comparing to the wild-type plants only at late developmental stages.

Taken altogether these data suggest that *AtPAO1* and *AtPAO5* act redundantly in the control of stem and root development, with *AtPAO5* playing a major role. More studies are still necessary to determine the mechanism of *AtPAO1* contribution to plant growth and development.

9. *AtPAO5*, together with *AtPAO1*, participates in the control of plant responses to salt and drought stress

Several studies have evidenced an important role of PAs in 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). In particular, it has been reported that *spms* mutant, which does not produce Spm, appears to be more sensitive to drought and salt stress than the wild-type plants. Moreover, the *acl5/spms* double mutant was also found hypersensitive to those stresses (Kusano *et al*., 2007; Yamaguchi *et al*., 2006).

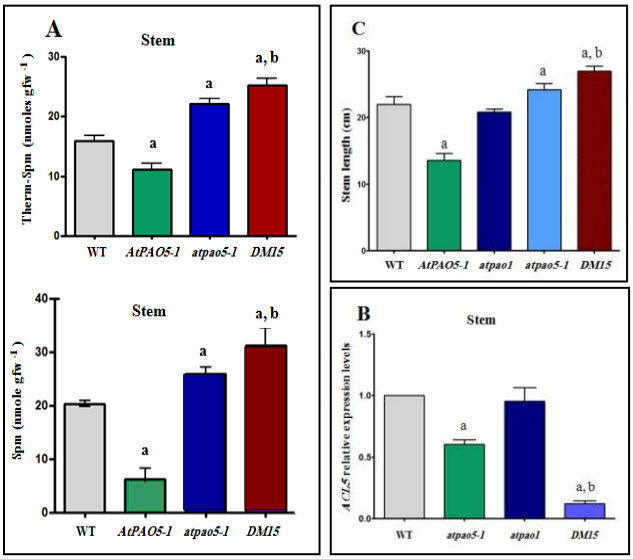

Fig. 21. Analysis of PA levels, stem length and *ACL5* expression levels in *DM15* plants. (A) Spm and Therm-Spm levels in *AtPAO5-1*, *atpao5-1*, *DM15* and wild-type (WT) plants. (B) *ACL5* expression levels in *atpao5-1*, *atpao1*, *DM15* and WT plants. (C) Stem length of 5-weeks-old *AtPAO5-1*, *atpao5-1*, *atpao1*, *DM15* and WT plants. Numbers are mean values ± SE of three independent repetitions of the experiment. Statistical analysis was performed by one-way ANOVA test. Symbols (a) and (b) indicate statistically significant differences compared with WT plants and *atpao5-1* single mutant, respectively.

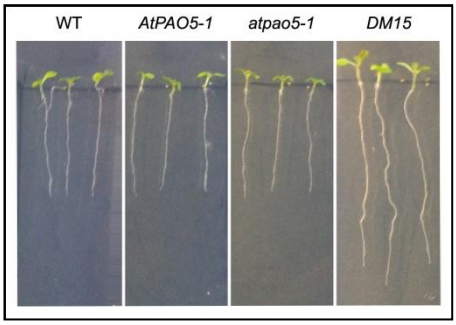

Fig. 22. Roots growth of 7-days old *DM15*, *atpao5-1*, *AtPAO5-1* and wild-type (WT) plants.

To investigate whether the *AtPAO5-1* transgenic plants, the *atpao5* single mutants and the *DM15* double mutant with altered levels of Spm and Therm-Spm respond differently from the wild-type plants to abiotic stresses, the growth of these plants was examined under conditions of drought and salt stress. Drought stress was applied in 30-day-old plants grown either in soil pots (Fig. 23) or in a hydroponics system (Fig. 24). In the former case, water

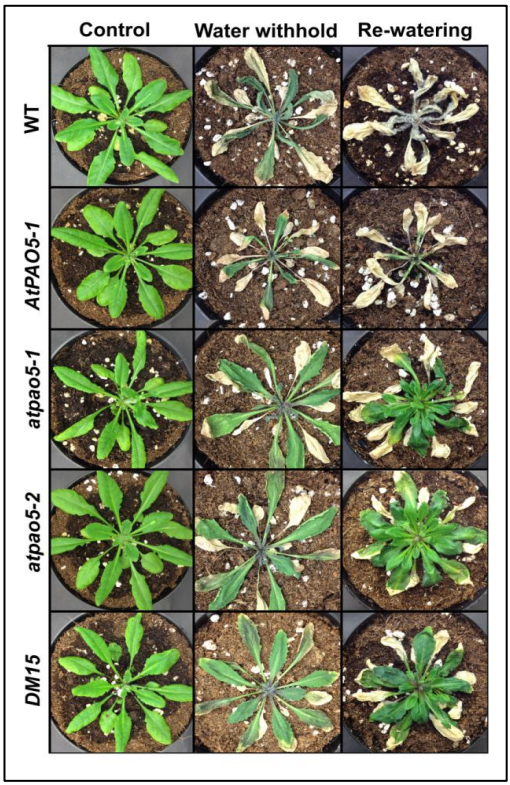

Fig. 23. Response to drought of plants with altered *AtPAO1* and *AtPAO5* expression levels. Drought treatment of soil pot-grown plants. In 30-day-old plants grown soil pot under well-watered conditions, water was withhold for 20 days. Following re-watering, plants were left to recover for 3 days. Plants were photographed before re-watering or after the 3 days of recovery. Control plants continuously grown under well-watered conditions were also photographed. The experiment has been repeated twice.

was withhold for 20 days, while in the latter for 30 or 40 h. Successively, plants were re-watered and left to recover for 3 days. Under these conditions, the wild-type and the *AtPAO5-1* plants were not able to survive, whereas the *atpao5* and *DM15* plants managed to recover (Fig. 23; Fig. 24).

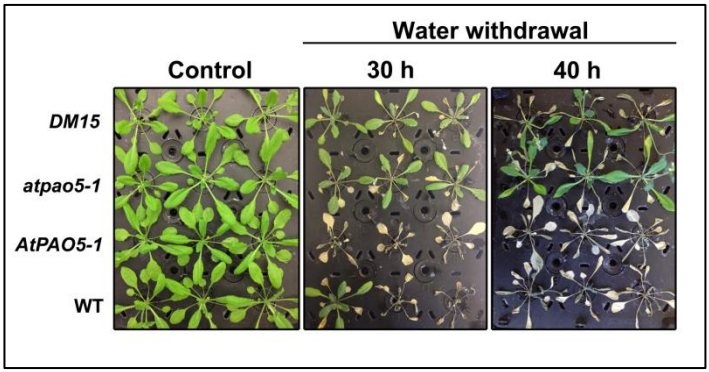

Fig. 24. Drought treatment of plants with altered *AtPAO1* and *AtPAO5* expression levels. Water was withdrawn for 30 or 40h in 30-day-old plants grown in a hydroponic system under well-watered conditions. Following re-watering, plants were left to recover for 3 days. Plants were photographed before re-watering and/or after the 3 days of recovery. Control plants continuously grown under well-watered conditions were also photographed. The experiment has been repeated three times.

The *AtPAO5-1*, *atpao5-1* and *DM15* plants were also tested under salt stress conditions. In particular, plants grown in soil under well-watered conditions for 25 days, were watered with gradually increasing concentrations of NaCl (50 mM- 200 mM; see Materials and Methods) to avoid plasmolysis. After 24 days of NaCl treatment, all the leaves of the *AtPAO5-1* and wild-type plants became yellow. Conversely, the *atpao5-1* and *atpao5-2* single mutants had some green leaves in the center of the rosette (Fig. 25), whereas all *DM15* rosette leaves remained slightly green (Fig. 25).

Similar results were obtained with plants grown in the hydroponic system (Fig. 26). After 25 days of growth under physiological conditions, the plants were treated with different concentrations of NaCl (50 mM, 75 mM and 100 mM) for 10 days. Under these conditions, the *AtPAO5-1* and wild-

type plants presented high sensitivity to the salt stress, the *AtPAO5-1* plants showing a higher number of chlorotic leaves and more delayed growth than the wild-type plants. Conversely, the *atpao5-1* and *DM15* plants appeared much less affected by the salt stress than the wild-plants, the *atpao5-1* plants presenting only a low number of chlorotic leaves and the *DM15* ones not at all (Fig. 26).

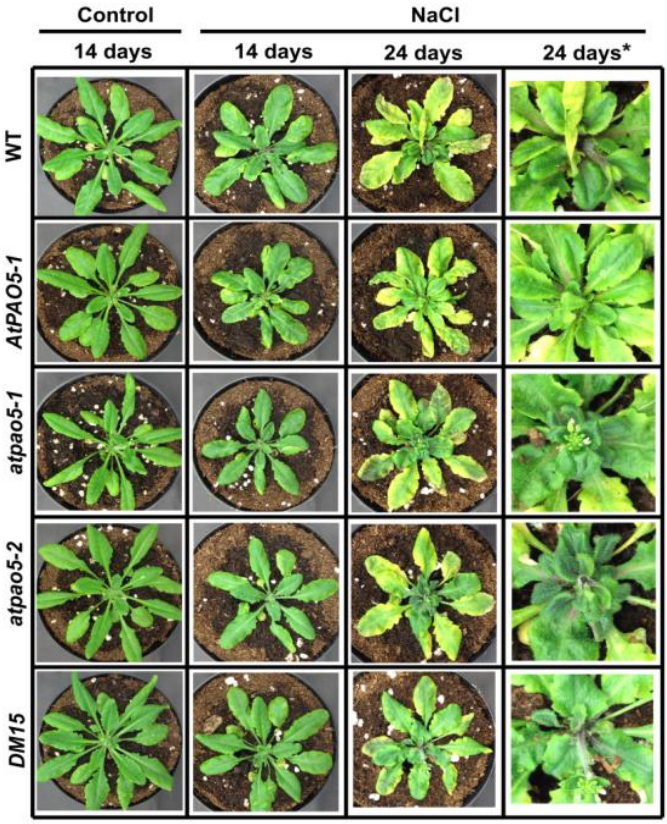

Fig. 25. Salt stress response of plants with altered *AtPAO1* and *AtPAO5* expression levels. Plants, grown on soil for 25 days under standard conditions, were watered with gradually increasing concentrations of NaCl (50 mM - 200 mM). The plants were photographed after 14 and 24 days of NaCl treatment. Untreated plants grown in parallel (Control) were also photographed. The experiment has been repeated two times.

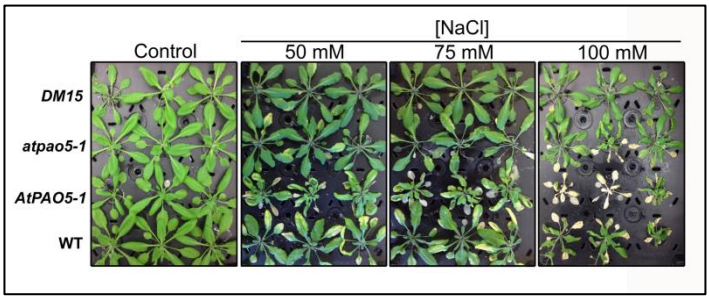

Fig. 26. Salt stress of plants with altered *AtPAO1* and *AtPAO5* expression levels. Twenty five-day-old plants, grown in a hydroponic system under standard conditions, were treated with different concentrations of NaCl (50 mM, 75 mM and 100 mM). The plants were observed daily and photographed after 10 days of NaCl treatment. Untreated plants grown in parallel (Control) were also photographed. The experiment has been repeated three times.

Taken together, the stress studies evidenced that the *atpao5* and *DM15* mutants are more tolerant to drought and salt stress than the *AtPAO5-1* and wild-type plants, the *DM15* presenting a slightly increased tolerant phenotype comparing with the *atpao5* single mutants under salt stress. These data suggest that *AtPAO1* and *AtPAO5* contribute to plant defense responses to environmental stresses. Further studies are necessary to determine the underlying mechanisms.

DISCUSSION

The results presented in this study provide evidence that AtPAO5 is involved in the homeostasis of Spm, N^1-acetyl-Spm and Therm-Spm levels and contributes in a dose-dependent way to the feedback mechanism controlling *ACL5* and *SAMDC4*. Indeed, *ACL5* and *SAMDC4* are up-regulated in *AtPAO5-1* transgenic plants with lower levels of Spm, N^{1-}acetyl-Spm and Therm-Spm than wild-type plants and down-regulated in *atpao5-1* and *atpao5-2* mutants with higher Spm, N^{1-}acetyl-Spm and Therm-Spm levels than wild-type plants. Instead, no difference in *ACL5* and *SAMDC4* expression levels was observed in *AtPAO5-2* transgenic plants from those in wild-type plants. This feedback mechanism appears to be active throughout the entire plant, since altered *ACL5* and *SAMDC4* expression levels were

observed both in whole seedlings and in specific organs, such as roots and inflorescence stems. It should be noticed, however, that the altered *ACL5* and *SAMDC4* expression levels in the *AtPAO5-1* and *atpao5* plants comparing with those in the wild-type plants seem not to be sufficient to restore PA levels. Differently from *ACL5* and *SAMDC4* expression levels and despite the altered Spm levels comparing with the wild-type plants, *SPMS* expression levels remained unaltered in the *AtPAO5-1* and *atpao5* plants which suggests that Spm levels are not controlled by a similar feedback mechanism to that controlling Therm-Spm levels. This further suggests that the different PAs undergo different regulatory mechanisms and participate in different physiological processes.

The altered phenotype of *AtPAO5-1* transgenic plants regarding stem length and vascularization are similar to those of *acl5* and *bud2/samdc4* mutants which also present a dwarf phenotype and excessive xylem differentiation (Hanzawa *et al*., 2000; Clay and Nelson, 2005; Ge *et al*., 2006; Kakehi *et al*., 2008; Muñiz *et al*., 2008; Cui *et al*., 2010). These data, together with the participation of *ACL5*, *SAMDC4* and *AtPAO5* in feedback mechanisms controlling Therm-Spm levels, indicate that the dwarf phenotypes of both *APAO5-1* transgenic plants and *bud2/samdc4* mutants are mainly due to reduced Therm-Spm levels. In agreement, in the present study it was observed that the *atpao5-1* and *atpao5-2* mutants with increased levels of Therm-Spm present long stem phenotype. Instead, it is difficult to explain the different data published during the course of the present study on the same *atpao5* mutants (Kim *et al*., 2014). A reasonable explanation may be the different growth conditions which may have influenced the dynamic equilibrium of various metabolic pathways, thus establishing a different homeostasis in Therm-Spm levels and hormone metabolism.

The *AtPAO5-1* and *atpao5* plants present altered expression levels of genes implicated in auxin signaling and up-regulated by auxin, such as *ATHB8*, *PHB*, *CAN*, *PIN1* and *PIN6*. In particular, the expression level of these genes are increased in the *AtPAO5-1* plants and decreased in the *atpao5* plants as compared with the wild-type plants, thus suggesting enhanced auxin signaling in the *AtPAO5-1* plants with reduced Therm-Spm levels and diminished auxin signaling in the *atpao5* mutants with increased Therm-Spm levels. Altered auxin signaling has been also observed in Arabidopsis and Populus plants with modulated *ACL5* expression levels (Kakehi *et al*., 2010; Vera-Sirera *et al*., 2010; Takano *et al*., 2012; Yoshimoto *et al*., 2012a; Milhinhos *et al*., 2013; Baima *et al*., 2014; Tong *et al*., 2014), as well as in Arabidopsis plants treated with Therm-Spm (Tong *et al*., 2014). The

AtPAO5-1 and *atpao5-1* plants seem to additionally present altered cytokinin signaling. Indeed, the expression levels of the negative regulator of cytokinin signaling *AHP6* which is up-regulated by auxin and down-regulated by cytokinins (Bishopp *et al*., 2011; Besnard *et al*., 2014) is increased in the *AtPAO5-1* transgenic plants and decreased in the *atpao5* mutants as compared with the wild-type plants. *ARR5*, *ARR7* and *ARR16* are also differently regulated in *AtPAO5-1* and *atpao5* plants comparing with wild-type plants, but only in the presence of BAP. Indeed, these genes are less induced by BAP in the *AtPAO5-1* plants and more induced in *atpao5* mutant plants when compared with wild-type plants. Remarkably, the *AHP6* was shown to be down-regulated by Therm-Spm, while the *ARRs* genes to be up-regulated by Therm-Spm. These data suggest that Therm-Spm functions not only in the repression of the auxin signaling pathway (Takano *et al*., 2012; Yoshimoto *et al*., 2012a,b; Milhinhos *et al*., 2013), but also in the stimulation of the cytokinin signaling pathway. These data also suggest that *AtPAO5* is involved in the auxin/cytokinin antagonistic interactions.

Auxin and cytokinin form a mutually-inhibitory feedback loop, in which cytokinin modulates the bisymmetric distribution of the auxin efflux proteins PINs, and auxin, in turn, promotes *AHP6* expression, which inhibits the cytokinin signaling. This reciprocal inhibition between auxin and cytokinin plays an important role in specifying vascular pattern in the root meristem (Bishopp *et al*., 2011; Kondo *et al*., 2014). Coherently with the alterations in the auxin- and cytokinin-related pathways, modulation of *AtPAO5* expression causes modifications in stem and root growth as well as in vasculature development. At early developmental stages, ectopic expression of *AtPAO5* on the one hand and *AtPAO5* knock-out on the other hand interfere specifically with BAP-induced inhibition of protoxylem differention in roots, but not in the BAP-mediated control of root elongation and meristem size. In particular, comparing with the wild-type plants, the *AtPAO5* transgenic plants are less responsive to BAP as considered inhibition of protoxylem differentiation, while on the contrary the *atpao5* mutants are more responsive. The specific effect of *AtPAO5* on protoxylem differentiation may be mediated by *AHP6* which functions to facilitate protoxylem specification by down-regulating the cytokinin signaling in a spatially specific manner, being specifically expressed with a bisymmetric pattern at the protoxylem position and the adjacent pericycle cells (Mähönen *et al*., 2006; Bishopp *et al*., 2011). This is consistent with the altered *AHP6* expression levels in the *AtPAO5-1* and the *atpao5* plants.

At later developmental stages, differences were observed among *AtPAO5-1*, *atpao5* and wild-type plants regarding primary xylem development in the mature region of the roots and in the inflorescence stems, the *AtPAO5-1* transgenic plants presenting an increased number of metaxylem vessel elements and the *atpao5* mutants a decreased one comparing with the wild-type plants. In agreement with these changes, a number of genes involved in the regulation of xylem differentiation (*ATHB8*, *PHB*, *CAN*, *VND6*, *VND7*) and cell-wall formation (*XTH3*) are up-regulated in the *AtPAO5-1* transgenic plants and down-regulated in the *atpao5-1* mutant plants both in stems and roots. In contrast to what takes place at the level of the primary xylem, the *AtPAO5-1* plants exhibit reduced production of secondary xylem in stems, while on the opposite the two *atpao5* mutants exhibit increased production of secondary xylem comparing to the wild-type plants. This difference between primary and secondary xylem differentiation in the *AtPAO5*-1 and *atpao5* plants may be due to differences in the timing and/or extent of cell death of procambial/cambial cells into xylem elements, an early cell death probably exerting a stimulatory effect on primary xylem differentiation but an inhibitory one on secondary xylem differentiation. In agreement, *XCP1*, a marker of xylem differentiation associated with the programmed cell-death (Funk *et al*., 2002; Avci *et al*., 2008; Lucas *et al*., 2013), is upregulated in the *AtPAO5-1* plants and down-regulated in the *atpao5-1* plants comparing with the wild-type plants. These data suggest misregulation of xylem maturation in the *AtPAO5-1* and *atpao5-1* plants, thus not allowing proper organ extension. Reduced secondary xylem growth was also observed in *acl5* mutants (Muñiz *et al*., 2008) which similarly to *AtPAO5-1* plants present higher *XCP1* expression levels than the wild-type plants (Baima *et al*., 2014). However, in contrast to large metaxylem cells present in *AtPAO5-1* stems and roots, the *acl5* plants produce a large amount of xylem cells of a reduced size, a difference which may be attributed to a still earlier xylem differentiation in the *acl5* mutants than in the *AtPAO5-1* plants. This difference between the *AtPAO5-1* and *acl5* plants may be due to differences in Therm-Spm, auxin and cytokinin homeostasis in these plants, considering that in the former plants reduction of Therm-Spm levels is based on AtPAO5 catalytic reaction, while in the latter ones on impairment of Therm-Spm biosynthesis. Differences between *AtPAO5* and *ACL5* in the temporal, hormone-dependent and tissue-specific expression patterns may also explain the differences between *AtPAO5-1* and *acl5* plants in xylem differentiation.

The *AtPAO5-1* and *atpao5* plants present altered xylem differentiation also in the aerial organs, and in particular in the veins of cotyledons, leaves and hypocotyls, thus suggesting that *AtPAO5* interferes with this process throughout the whole plant, consistently with the altered expression levels of the auxin- and xylem-related genes both in whole seedlings and in specific organs (i.e., roots and stems). It should, however, be noticed that no difference was observed among *AtPAO5-1*, *atpao5* and wild-type plants as concerned meristem size, hypocotyl length, leaf size and flower development which suggests the *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 has been attributed to an increase of 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* transgenic plants, the *bud2/samdc4* mutant plants have been reported to be hypersensitive to cytokinins concerning root elongation and callus growth and to have increased levels of cytokinins (Cui *et al*., 2010). These differences among *AtPAO5-1*, *acl5* and *bud2/samdc4* plants can be explained with tissue-specific differences in Therm-Spm and/or auxin and/or cytokinin homeostasis, as described above.

Treatment of *Arabidopsis* plants with Therm-Spm bears protoxylem defects in the roots which are more evident in the *atpao5* mutants and less evident in the *AtPAO5* over-expressing transgenic plants. These observations are in agreement with previous results showing that Therm-Spm and Nor-Spm, but not Spm, reduce development of lignified elements in *acl5-1spms-1* double mutant seedlings and block trans-differentiation of mesophyll cells into tracheary elements in an *in vitro Zinnia elegans* xylogenic culture system (Kakehi *et al*., 2010). These observations are also consistent with the modifications in xylem differentiation in the roots and the stems of the *AtPAO5-1* and *atpao5* plants. The mechanism through which Therm-Spm affects xylem differentiation is still not clear. The effect may be indirect through interaction with hormone-dependent pathways controlling expression of genes involved in xylem differentiation, as for example the auxin- and cytokinin-dependent pathways. In the presence of increased levels of Therm-Spm, the decreased levels of the auxin signaling, which stimulates xylem differentiation, and the increased levels of the cytokinin signaling, which together with the *AHP6* down-regulation inhibits protoxylem differentiation, may contribute to the appearance of the xylem defects through reduced cell-

wall thickenings and thus diminished resistance to the stretching forces that appear during organ development. However, a direct effect of Therm-Spm on xylem differentiation as well as involvement of other hormonal pathways, as for example that of ethylene which shares with PAs a common precursor (Pommerrenig *et al*., 2011; Waduwara-Jayabahu *et al*., 2012; Sauter *et al*., 2013) and contributes to xylem differentiation (Milhinhos and Miguel, 2013) cannot be excluded. Indeed, several hormonal signals have been shown to be involved in the control of xylem vessel formation (Milhinhos and Miguel, 2013) which is central to the efficiency of water and nutrient transport from roots to leaves through the stem and may strongly influence the growth in height of the plant.

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 PAs 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 between Therm-Spm, auxin and cytokinins necessary for proper xylem differentiation and plant growth. *AtPAO5* contributes to this regulatory network participating in the feedback mechanisms which control

Therm-Spm levels (Fig. 27) and which specifically operate at the xylem level as a safeguard mechanism against the damaging and/or inhibitory effects on the xylem of increased levels of Therm-Spm and cytokinins and an excessive xylem proliferation by decreased levels of Therm-Spm and increased ones of auxin which would negatively affect organ expansion and plant growth (Yoshimoto *et al*., 2012). 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). The molecular mechanisms of Therm-Spm action in the control of gene expression, auxin/cytokinin interplay, xylem differentiation and plant growth has still to be studied in detail.

AtPAO5 presents some similarities (as for example subcellular localization, substrate specificity and tissue-specific expression pattern), but also important differences (regarding catalytic activity towards N^1-acetyl-Spm, catalytic mechanism and expression in the vascular system) to *AtPAO1*. In the present study, it is shown that *AtPAO1* participates, together with *AtPAO5*, to Spm and Therm-Spm homeostasis, as well as to the control of stem and root development, with *AtPAO5* playing however a major role. More studies are necessary to determine the mechanism underlying the contribution of *AtPAO1* to plant growth and development, as well as to understand the *AtPAO1*/*AtPAO5* interplay in these processes.

AtPAO1 and *AtPAO5* are also involved in the plant defense responses to abiotic stresses. Indeed, the *AtPAO5-1* transgenic plants resulted less tolerant to drought and salt stress than the wild-type plants, whereas the *atpao5* mutants more tolerant. Furthermore, the *DM15* double mutant plants present a slightly increased tolerance comparing with the *atpao5* single mutants. The existing data do not permit to understand whether *AtPAO1* and *AtPAO5* are involved in plant defense responses contributing to PA homeostasis and/or controlling xylem differentiation.

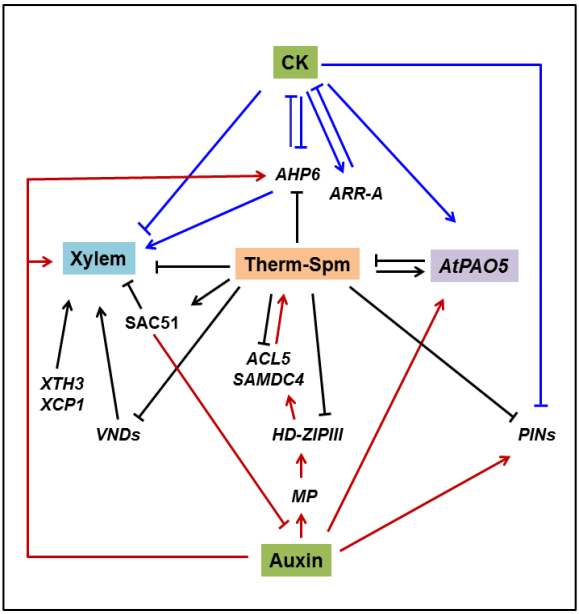

Fig. 27. Schematic representation of the cytokinin/auxin/Therm-Spm/AtPAO5 interplay involved in xylem differentiation. *AtPAO5*, *ACL5*, and *SAMD4* participate in positive and negative feedback loops which finely regulate Therm-Spm homeostasis and which specifically operate at the xylem level as safeguard mechanisms against the damaging and/or inhibitory effects on the xylem of increased levels of Therm-Spm and cytokinins and an excessive xylem proliferation by increased levels of auxin.

MATERIAL AND METHODS

1. *Plant material and growth conditions*

All experiments were performed with *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia. Two T-DNA insertion alleles of *AtPAO5* (At4g29720) were used, one (*atpao5-1*; Ahou *et al*., 2014) obtained from Syngenta *Arabidopsis* Insertion Library (allele SAIL_664_A11.v1; Sessions *et al*., 2002) and the other (*atpao5-2*) from SALK collection (SALK_053093; Alonso *et al*., 2003). A T-DNA insertion allele of *AtPAO1* (At4g29720) was also used, (SALK_142477.31.30x). Transgenic *35S::AtPAO5-6His* (*AtPAO5*) and *AtPAO5::GFP-GUS* plants obtained as described previously (Fincato *et al*., 2012; Ahou *et al*., 2014) were additionally analyzed. 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 75% RH. For *in vitro* growth, seeds were first sterilized and stratified for three days at 4°C and then put on agar plates containing half-strength Murashige and Skoog basal medium with Gamborg's vitamins and 0.5% (w/v) sucrose (½MS). For qRT-PCR analysis following hormone or polyamine treatment, 5-day-old seedlings grown on ½MS agar plates were transferred in ½MS liquid medium and were grown 5 more days. After addition of fresh medium, seedlings were treated with BAP, IAA or Therm-Spm. Samples were removed at various time intervals, frozen with liquid nitrogen and conserved at -80°C until further use. For analysis of *AtPAO5* promoter activity through histochemical GUS staining of *AtPAO5::GFP-GUS Arabidopsis* transgenic plants following hormone treatments, 7-days-old seedlings grown onto ½MS agar plates were transferred vertically in ½MS liquid containing the treatments for the indicated time intervals. For stem and root physiological studies, plants were grown either on soil or in a hydroponic growing system (Araponics).

2. *Phenotypical analysis*

For height growth measurements, plants were grown on soil or in hydroponic growing systems for 5 weeks and the distance from the base to the apex of the main bolt was measured in individual plants. For studies on root elongation and vascular differentiation following hormone or PA treatment, 5-day-old seedlings grown on vertically oriented 1/2MS agar plates were transferred on new agar plates containing or not BAP, IAA or Therm-Spm

and were left to grow vertically for 5 more days. Root lengths were marked on the plates every two days and measured using ImageJ software. To determine root vascular differentiation, the distances from the QC of the first protoxylem vessels with spiral/annular secondary cell-wall thickenings and of the first metaxylem vessels were measured in seedlings stained with fuchsin using confocal microscope. The root apical meristematic activity was determined measuring the distance from the QC to the first cells displaying elongation as well as the width of the meristematic zone at the TZ.

3. *Application of Drought stress in plants*

3.1. *Drought stress in soil-grown plants*

Plants were grown in soil pots under well-watered conditions for 30 days. Progressive drought treatments were applied by withholding watering up to 20 days. Plants were re-watered and left to recover for 3 days. Control plants were continuously grown under well-watered conditions.

3.2. *Drought stress in plants grown in hydroponic system*

Plants were grown in hydroponics system for 30 days. Changing the nutrient solution every 5 days. Then, the nutrient solution was removed from the hydroponic pots for up to 40 h. The plants were re-watered and left to recover for 3 days.

4. *Application of Salt Stress in plants*

4.1. *Salt stress in soil-grown plants*

Plants were grown in soil pots for 25 days under well-watered conditions. Then the plants were watered every 3 or 4 days with NaCl solution of gradually increasing concentration starting from 50 mM up to 200 mM for 24 days.

4.2. *Salt stress in plants grown in hydroponic system*

Plants were grown in hydroponics system for 25 days were treated with different concentrations of NaCl (50 mM, 75 mM and 100 mM). In the case

of high salt concentration (75 and 100 mM), treatment initiated with 50mM and salt concentration was gradually (every 5 days) increased up to the final concentration. Plants were left to grow until appearance of chlorotic leaves.

5. *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

In order to avoid amplification from 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 (0,5 μg of total RNA). The mixture was incubated at 70°C for 5 min and was then placed on ice-water 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 MgCl2, 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 iCycler™ 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.

6. *Agarose gel electrophoresis of DNA*

Samples containing DNA were mixed with gel-loading buffer (6X buffer; 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and loaded on 1-2% agarose gel prepared in Tris-acetate-EDTA buffer (40 mM Trisacetate, 1 mM EDTA, pH 8.0) and containing ethidium bromide (0.5 μg/mL). The electrophoresis run was performed at 100V in Tris-acetate-EDTA buffer and the Gene Ruler ™ 1 kb DNA Ladder (Fermentas) was used as molecular weight marker to estimate the size of the separated and ethidium bromide-stained DNA fragments, which were visualized as fluorescent bands under UV light.

7. *Quantitative RT-PCR analysis*

Total RNA was isolated from whole *Arabidopsis* seedlings using the RNeasy Plant Mini kit (QIAGEN). To degrade genomic DNA, DNase digestion was performed during RNA purification using the RNase-Free DNase Set (QIAGEN). 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 ($2^{-\Delta\Delta Ct}$) or as difference ($-\Delta\Delta Ct$) from the controls. The gene for ubiquitin-conjugating enzyme 21 (*UBC21*; At5g25760) was chosen as a reference gene. All the gene-specific oligonucleotides used are listed in Supplementary Table S1.

8. *Analysis of polyamine levels in plants*

8.1. *Extraction of total free polyamines from plants*

Total free polyamine levels were determined in whole Arabidopsis seedlings as well as from Arabidopsis roots, rosette leaves and flowers. For polyamine extraction fresh plant material leaf was 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 were clarified by centrifugation. The supernatant was used to analyze total free polyamines by HPLC following derivatization with dansyl chloride.

8.2. *Derivatization of polyamines with dansyl chloride*

For each aliquot of 100 µL of polyamine solution in PCA, an equal volume of saturated sodium carbonate was added which allows bringing the pH of the sample to neutrality. Subsequently, 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 2 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. Subsequently, the organic phase was collected and dried using a vacuum speed.

8.3. *Analysis of dansyl polyamines by HPLC*

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.

8.4. *Polyamine determination by gas chromatography–mass spectrometry*

Polyamine analysis by gas chromatography–mass spectrometry was performed as described by Rambla *et al*., (2010). In detail, 100 mg fresh weight of 12-day-old Arabidopsis seedlings was frozen in liquid nitrogen and homogenized with mortar and pestle. To this frozen powder, 4.8 vol of0.2 M PCA and 0.2 vol of a 100 µg/mL 1,6-diaminohexane solution in 0.2M PCA as internal standard were added, and the slurry mixed in a vortex.After centrifugation at 13,000 g for 15 min at room temperature, polyamines in the supernatant were further purified by ion-pair extraction with bis-(2-ethylhexyl) phosphate in chloroform, and 1 ml of the clean extract was then derivatized with heptafluorobutyric anhydride. The derivatized extract was evaporated to dryness under a steam of nitrogen, and finally solved in 100 µl ethyl acetate. Two microliters of the heptafluorobutyrated derivatives was injected in a splitless mode in a 6890 N gas chromatograph (Agilent Technologies) coupled to a Pegasus 4D TOF mass spectrometer (LECO). For

polyamine separation, the following chromatographic conditions were used: BPX35 (30 m x 0.32 mm x 0.25µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as carrier gas, constant flow 2 ml/min. The liner was set at 230°C. Oven program was 85°C for 2 min, 15°C/min ramp until 280°C, and 280°C for 4min, for a total run time of 19 min. Mass spectra were collected at 6.25spectra s-1 in the m/z range 35–900 and ionization energy of 70 eV.

9. *Histochemical GUS analysis of AtPAO5::GFP-GUS Arabidopsis transgenic plants*

Staining for β-glucuronidase (GUS) activity in *Arabidopsis* plants was performed as previously described (Fincato *et al*., 2012; Ahou *et al*., 2014). The reaction was allowed to proceed at 37°C for 30 min to 1 h to observe staining of the roots and overnight to observe staining of the rest of the plant. Images were acquired by a Leica DFC310FX digital camera applied to a Zeiss Axioplan 2 microscope.

10. *Confocal microscopy analysis and imaging*

Fuchsin staining of vasculature tissues was carried out as described previously (Mähönen *et al*., 2000). In detail, the seedlings were cleared by treatment with acidified methanol [10 mL of methanol, 2 mL of HCl (37%), 38 mL of H_2O] at 56°C for 15 min and then were transferred into a basic solution of ethanol (7% NaOH in 60% ethanol). Following incubation for 15 min at room temperature, the seedlings were rehydrated by successive washings with decreasing concentrations of ethanol (40%, 20% and 10% ethanol). The seedlings were then stained by 0.01% basic fuchsin solution for 5 min, de-stained with 70% ethanol and rehydrated, as described above to 10% ethanol solution. After addition of an equal volume of 50% glycerol and incubation for 30 min the seedlings were examined with a Leica TCS-SP5 confocal (488nm excitation with an argon ion laser and 568nm detection) using the software *Advanced Fluorescence* (LAS AF; Leica).

11. *Histology*

To obtain transverse and longitudinal sections of roots and stems, the specific tissues were first embedded in Technovit 7100 resin (Kulzer). Sections (20 µm) were made using a Microm HM330 microtome. For histochemical localization of GUS activity in stained *AtPAO5::GFP-GUS* transgenic plants sections were observed under light microscope using a Zeiss Axioplan 2 microscope connected to a digital camera (Leica DFC310FX) for image acquisition. For histochemical localization of lignin, sections from *AtPAO5* transgenic plants, *atpao5* mutants and wild-type plants were first stained in a 0.05% (w/v) toluidine blue solution and then observed and photographed under light microscope. 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. Sections were also observed under UV light for autofluorescence of cell-wall phenolics. For observation of vein patterning and thickness, young seedlings or leaves of 2-week-old plants were fixed in ethanol/acetic acid mixture (6:1) overnight, incubated twice in 100% ethanol for 30 min at room temperature, once in 70% ethanol for 30 min, and cleared with a chloral hydrate:glycerol:water mixture (8:1:2,w:v:v) overnight. Samples were examined under dark-field illumination or UV light to observe autofluorescence of the cell walls.

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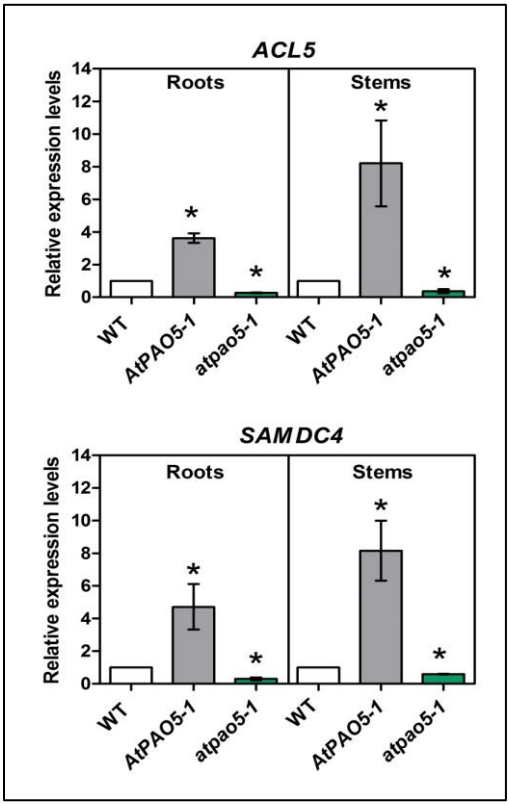
SUPPLEMENTARY DATA

Supplemental Table 1. Sequences of primers used for genotyping.

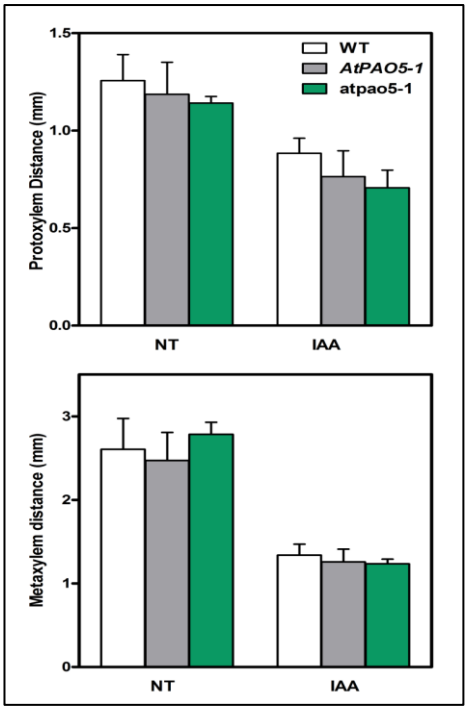
Mutant	Name	Sequence (5'-->3')
atpao5-1	*FP1*	GTGGAAGCCCTGTTTATAGAATCG
	RP1	GCACACCTAAAGAGACAGTAACAA
	T-DNA specific primer	GAATTTCATAACCAATCTCGATAC
atpao5-2	*FP2*	GAGAGTGAGTATCAGATGTTTCCAG
	RP2	GTAGGCACCATGAGTTGTGGGAG
	T-DNA specific primer	GATGGTTCACGTAGTGGGCCATCGC

Supplemental Table 2. Sequences of primers used for gene expression analysis by qRT-PCR.

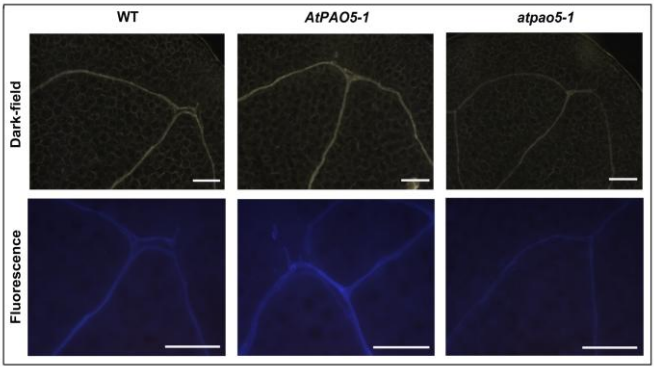
Gene name	AGI Code	Forward (5'-->3')	Reverse (5'-->3')
AtPAO5	At4G29720	GAGAGTGAGTATCAGATGTTTCCAG	AGCACACCTAAAGAGACAGTAACAA
ACL5	At5G19530	GGACCACGATTTGACGTTG	TCTAGCGCGAGAGAGATGGTT
SAMDC4	At5G18930	GGTGACCGTTACTCAACTATCCA	CGAAGCTCGCGTAGCTAAA
SPMS	At5G53120	GATGCTAAGTGTCACTCCACTGTTG	GTGAGTGTGCTTCTCCTGGC
PIN1	At1G73590	GGAGACTTAAGTAGGAGCTCAGCA	CCAAAAGAGGAAACACGAATG
PIN6	At1G77110	AATCTCCTTCTCTGGCACTGA	CCGAAGATGATGTGGGAGTC
ATHB8	At4G32880	GGGCACTACCAAGAAGACATGA	TGCAAATATAAGCTCTGCGCAT
PHB	At2G34710	CCTCAAAACAAACGCTTCACC	AGGGCTACAAGAGTCGTTTCCA
CNA	At1G52150	CTCTTGATTCCGCAAAGCAGGAAGT	ACCAATTTCCAGTGCCGAAG
VND6	At5G62380	CTTCCACATAACTCTTGGATGTCC	ATGGAAAGTCTCGCACACAT
VND7	At1G71930	CTTGTCTCTACCCGTGGCTT	ATGGGATATACAAGCGAGGTGT
XCP1	At4G35350	CGGACTTGCCTAAATCCGTA	AAAATGCCCAACAGCTACCA
XTH3	At3G25050	ATCTTCAGGTGGTGGGTTCG	TGACGATGCCGCCAGTATTT
PXY	At5G61480	GCTTGTACACAACAACGCAA	CGGTTACATTGCACCAGAATAT
ARR5	At3G48100	CATCAGCTTTCAGAGAAATACCAG	CAGCTCCTTCTTCAAGACATCTATC
ARR7	At1G19050	GGCAAAGGGGCTTCTAATCTT	TCCTGAAAGTCCTGGCATTG
ARR15	At1G74890	CAGCACTCAGAGAAATCCCAGT	TCATACATTGTTCTATACGAGGTTG
ARR16	At2G40670	CCTGTAACGTTATGAAGGTGAGTC	GACTCCTGCTTCACTTTCTTGAGTAGC
AHP6	At1G80100	CGCCCAGGGTGCTTGA	TCTTGAGGTAATGATACTCATGCTCTACT
SAC51	At5G64340	AATTGCCAGGCTGAGTACTT	GACCGACCTACTATATCCTT
SCR	At3G54220	CTTCACTCTCTTCGCCGTAGC	CACTCTCTGGTTACTCCAAAGATTAGC
UBQ21	At5G25760	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC



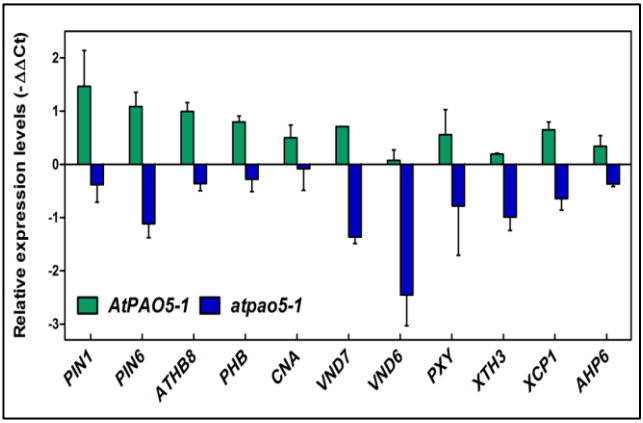
Supplemental Fig. S1. *ACL5* and *SAMDC4* expression levels in stems and roots of *AtPAO5-1* and *atpao5-1* plants. Expression levels were determined by qRT-PCR analysis. The analysis was repeated at least three times. Numbers are mean values ±SE. Statistical analysis was performed by one-way ANOVA test. Asterisks indicate statistically significant differences in respect to the wild-type (WT) plants.



Supplemental Fig. S2. Xylem differentiation in the presence of auxin. (A) Distance of the first protoxylem cells with secondary cell-wall thickening from the quiescent center. (B) Distance of the first metaxylem elements from the quiescent center. Protoxylem and metaxylem distances were measured after 7 days of treatment or not with 0.1 μM IAA of 7 day-old plants initially grown under physiological conditions. For protoxylem and metaxylem visualization seedlings were stained with fuchsin and observed under confocal microscope. NT: not treated control.



Supplemental Fig. S3. *AtPAO5* contribution to vein formation. Veins of cotyledons in *AtPAO5-1*, *atpao5-1* and wild-type (WT) plants as observed by dark-field and fluorescence microscopy. Bars indicate 100 μm.



Supplemental Fig. S4. Relative expression levels of auxin and cytokinin-related genes in whole seedlings as determined by qRT-PCR. Mean values ± SE of at least three independent experiments are shown.

PUBLICATIONS

Alabdallah O, Ahou A, Macone A, Mancuso N, Stano P, Cona A, Angelini R, Tavladoraki P (2016). The *Arabidopsis* polyamine oxidase/dehydrogenase 5 contributes to cytokinin / auxin interplay controlling xylem differentiation. Manuscript in preparation.

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