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A study on the physiological roles of *Arabidopsis thaliana* polyamine oxidases

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ABBREVIATIONS

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

ABSTRACT

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

Polyamine oxidases (PAOs) are FAD-dependent enzymes which oxidatively de-aminate the polyamines Spm and Spd. The chemical identity of PAO reaction products depends on the enzyme source and reflects the mode of substrate oxidation. In particular, PAOs from the monocotyledonous plants so far characterized, such as the apoplastic maize PAO (ZmPAO), oxidise the carbon on the *endo*-side of the secondary amino group of Spm and Spd to produce 1,3-diaminopropane, H_2O_2 and an aminoaldehyde, and are thus considered to be involved in a terminal polyamine catabolic pathway. Instead, animal PAOs and spermine oxidases (SMOs) oxidise the carbon on the *exo*-side of the secondary amino groups of Spd and Spm (and/or their acetylated derivatives) to produce Put and Spd, respectively, in addition to an aminoaldehyde and H_2O_2, and are considered to be involved in a polyamine back-conversion pathway.

In *Arabidopsis thaliana,* five *PAO* genes (*AtPAO1-5*) are present with varying amino acid sequence homology to ZmPAO and subcellular localization. In particular, AtPAO1 and AtPAO5 have a predicted cytosolic localization, while for AtPAO2, AtPAO3 and AtPAO4 a peroxisomal localization was shown. Furthermore, *AtPAO2*, *AtPAO3* and *AtPAO4*, having a high sequence homology to each other and a very similar intron/exon organization, are considered to form a distinct *PAO* subfamily (*AtPAO2-4* subfamily). Heterologous expression and biochemical characterization of recombinant AtPAO1, AtPAO2, AtPAO3 and AtPAO4 showed that AtPAO1 oxidises only Spm, whereas AtPAO2, AtPAO3 and AtPAO4 oxidise both Spm and Spd. AtPAO5 catalytic properties have not been determined yet. Another important characteristic of the four *Arabidopsis* PAOs until now characterised is their involvement in a polyamine back-conversion pathway. Indeed, they produce Spd from Spm and Put from Spd similarly to the animal PAOs / SMOs and contrary to ZmPAO.

Accumulating data indicate that in plants polyamine catabolism is more than a biochemical process aiming to control polyamine homeostasis. Indeed, it has been demonstrated that extracellular PAOs constitute a nodal point during plant growth under physiological as well as abiotic and biotic stress conditions, giving rise to increased apoplastic H_2O_2, which signals primary and secondary developmental and defense responses. In contrast to the so far characterized apoplastic PAOs, little is known until now on the physiological roles of the intracellular PAOs.

In the present study, in an attempt to determine the physiological roles of the various AtPAOs, their tissue- and organ-specific expression pattern was analysed in detail through analysis of promoter activity in transgenic Arabidopsis plants expressing *AtPAO*::*β-glucuronidase*. Histochemical analysis of the transgenic plants revealed a distinct expression pattern for each one of the five *AtPAO*s, such as in the transition region between meristematic and elongation zones of roots and in anther tapetum for *AtPAO1*, in columella, stipules and pollen for *AtPAO2*, in hypocotyls, roots, stipules, columella, trichomes, guard cells and pollen for *AtPAO3*, in the meristematic/elongation transition zone of roots, guard cells, anther tapetum and pollen for *AtPAO4*, and in the vascular system of roots, hypocotyls and anther tapetum for *AtPAO5*. These studies also evidenced abscisic acid (ABA)-inducible expression of *AtPAO1* in the meristematic/elongation transition zone of the roots and of *AtPAO2* in guard cells, inducible expression of *AtPAO1* in roots under gravity stimulus, as well as cold- and Spm-inducible expression of *AtPAO5* in cotyledons. These data altogether indicate distinct physiological roles for the various *AtPAOs* during seedling growth and flower development and suggest functional diversity inside the *AtPAO* gene family.

In parallel, studies were performed to determine the physiological roles of the various *AtPAOs* using loss-of-function T-DNA insertional mutants. In particular, considering the expression of *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO4* in root apex, their involvement in gravitropism was analysed. The obtained data, however, evidenced no significant variation in root bending of single loss-of-function mutants comparing to the wild-type plants. Experiments are in progress with multiple loss-of-function mutants.

Since the plant hormone ABA plays a protective role in response to abiotic stresses, acting as a key regulator of stomatal apertures to restrict transpiration and reduce water loss, the ABA-inducible expression of *AtPAO2* in guard cells, together with the constitutive expression of *AtPAO3* and *AtPAO4* in the same cells, led us to hypothesize involvement of these enzymes in the control of stomatal opening and in plant defence responses to abiotic stresses. To test this hypothesis, the transpiration rate and the

stomatal opening of *atpao* loss-of-function mutants were analysed. Our data showed no difference in the transpiration rate between *atpao* mutants and wild-type plants, but evidenced a reduced ability to close stomata upon ABA supply for *atpao2*, *atpao3*, *atpao4* single mutants, as well as of *atpao2*/*atpao4* and *atpao3*/*atpao4* double mutants, comparing to the wild-type plants. Studies are in progress to make clear the underlying mechanisms in the contribution of the peroxisomal AtPAOs to the regulation of stomata movement. Studies are also in progress to determine AtPAO involvement in plant development and defence responses to various abiotic stresses.

In conclusion, the present study provides evidence for important differences in the spatial and temporal expression pattern of the various Arabidopsis PAOs, which, together with the distinct catalytic properties, suggest distinct physiological roles for each member of the *A. thaliana PAO* gene family. This study will contribute to a detailed analysis of the physiological roles of the polyamine catabolic pathways in plants.

Le poliammine putrescina (Put), spermidina (Spd) e spermina (Spm) sono piccole molecole cariche positivamente presenti in tutti gli organismi viventi. Sono coinvolte in numerosi processi cellulari e svolgono ruoli importanti nella morfogenesi, nella crescita, nel differenziamento e nella senescenza. Nelle piante, le poliammine sono anche coinvolte nelle risposte di difesa nei confronti di vari stress biotici e abiotici. L'omeostasi delle poliammine è attentamente regolata attraverso l'anabolismo e il catabolismo, ma anche attraverso la coniugazione, il trasporto e la compartimentalizzazione.

Le poliammino ossidasi (PAO) sono enzimi FAD-dipendenti che deaminano ossidativamente le poliammine Spm e Spd. L'identità chimica dei prodotti di reazione delle PAO dipende dalla provenienza dell'enzima e riflette la modalità di ossidazione del substrato. In particolare, le PAO provenienti dalle piante monocotiledoni caratterizzate finora, come la PAO apoplastica di mais (ZmPAO), ossidano l'atomo di carbonio interno adiacente al gruppo amminico secondario di Spm e Spd per produrre 1,3-diaminopropano, H_2O_2 e un'amminoaldeide, e sono quindi coinvolte in un catabolismo terminale delle poliammine. Le PAO animali e la spermina ossidasi (SMO) ossidano invece l'atomo di carbonio esterno adiacente al gruppo amminico secondario di Spm e Spd (e/o dei loro derivati acetilati) per produrre Spd e Put, rispettivamente, oltre a H_2O_2 e un'amminoaldeide, e sono quindi coinvolte in una via di interconversione delle poliammine.

In *Arabidopsis thaliana* sono presenti cinque geni *PAO* (*AtPAO1-5*) con un'omologia variabile con la sequenza amminoacidica della ZmPAO e con localizzazione subcellulare. In particolare, AtPAO1 e AtPAO5 hanno una presunta localizzazione citosolica, mentre per AtPAO2, AtPAO3 e AtPAO4 è stata dimostrata la localizzazione perossisomiale. Inoltre *AtPAO2*, *AtPAO3* e *AtPAO4*, avendo un'alta omologia di sequenza tra di loro e un'organizzazione genica molto simile, sono considerate una sottofamiglia *PAO* distinta (sottofamiglia *AtPAO2-4*). L'espressione eterologa e la caratterizzazione biochimica delle AtPAO1, AtPAO2, AtPAO3 e AtPAO4 ricombinanti hanno dimostrato che AtPAO1 ossida solo la Spm, mentre AtPAO2, AtPAO3 e AtPAO4 ossidano sia la Spm che la Spd. Le proprietà catalitiche di AtPAO5 non sono ancora state determinate. Un'altra importante caratteristica delle quattro PAO di Arabidopsis caratterizzate finora è il loro coinvolgimento in una via di interconversione delle poliammine, poiché infatti producono Spd da Spm e Put da Spd, in modo simile alle PAO/SMO animali e diversamente dalla ZmPAO.

Una gran quantità di dati indica che nelle piante il catabolismo delle poliammine è più di un processo biochimico atto al controllo dell'omeostasi delle poliammine. È stato dimostrato, infatti, che le PAO extracellulari svolgono un ruolo importante nella crescita delle piante sia in condizioni fisiologiche che durante gli stress biotici e abiotici, attraverso la produzione di perossido di idrogeno nell'apoplasto, che segnala risposte primarie e secondarie di sviluppo e di difesa. Al contrario delle PAO apoplastiche caratterizzate finora, le informazioni sul ruolo fisiologico delle PAO intracellulari sono ancora poche.

Nel presente studio, allo scopo di determinare i ruoli fisiologici delle varie AtPAO, è stato analizzato in dettaglio il loro profilo di espressione tessuto- e organo-specifico mediante l'analisi dell'attività dei promotori in piante transgeniche di Arabidopsis esprimenti *AtPAO::β-glucuronidasi*. L'analisi istochimica delle piante transgeniche ha rivelato un profilo di espressione diverso per ognuna delle cinque *AtPAO*, come ad esempio l'espressione nella zona di transizione tra il meristema e la regione di allungamento delle radici e nel tappeto delle antere per *AtPAO1*, nella columella, nelle stipule e nel polline per *AtPAO2*, nella columella, nelle radici, nell'ipocotile, nelle stipule, nei tricomi, nelle cellule di guardia e nel polline per *AtPAO3*, nella regione di transizione meristema/allungamento delle radici, nelle cellule di guardia, nel tappeto delle antere e nel polline per *AtPAO4*, e nel sistema vascolare delle radici, nell'ipocotile e nel tappeto delle antere per *AtPAO5*. Questi studi hanno anche evidenziato l'induzione da parte del l'ormone vegetale acido abscissico (ABA) dell'espressione di *AtPAO1* nella regione di transizione meristema/allungamento delle radici e

di *AtPAO2* nelle cellule di guardia, l'induzione dell'espressione di *AtPAO1* nelle radici durante lo stimolo gravitropico, e l'induzione dell'espressione di *AtPAO5* nei cotiledoni da parte del freddo e della Spm. Questi dati, nel loro insieme, indicano diversi ruoli fisiologici per le varie AtPAO durante la crescita delle piante e durante lo sviluppo del fiore e suggeriscono l'esistenza di una diversità funzionale nell'ambito della famiglia genica *AtPAO*.

In parallelo, sono stati condotti studi per determinare i ruoli fisiologici delle AtPAO mediante l'utilizzo di mutanti per inserzione di T-DNA. In particolare, considerando l'espressione di *AtPAO1*, *AtPAO2*, *AtPAO3* e *AtPAO4* nell'apice radicale, è stato analizzato il loro coinvolgimento nel gravitropismo. I dati ottenuti non hanno tuttavia evidenziato alcuna variazione statisticamente significativa nelle curvature delle radici dei mutanti inserzionali singoli *atpao1*, *atpao2*, *atpao3* e *atpao4* a paragone delle piante *wild-type*. Sono in corso esperimenti che prevedono l'utilizzo di mutanti inserzionali multipli.

Poiché l'ABA svolge un ruolo protettivo nei confronti degli stress abiotici, fungendo da regolatore delle aperture stomatiche per limitare la traspirazione e ridurre la perdita di acqua, l'espressione di *AtPAO2* indotta dall'ABA nelle cellule di guardia, insieme all'espressione costitutiva di *AtPAO3* e *AtPAO4* nelle stesse cellule, ci ha indotti a ipotizzare il coinvolgimento di questi enzimi nel controllo delle aperture stomatiche e nelle risposte di difesa dagli stress abiotici. Per verificare questa ipotesi, sono stati analizzati il tasso di traspirazione e l'apertura stomatica dei mutanti inserzionali *atpao*. I nostri dati non hanno mostrato differenze nel tasso di traspirazione tra i mutanti *atpao* e le piante *wild-type*, ma hanno evidenziato una ridotta capacità di chiudere gli stomi in seguito a trattamento con ABA da parte dei mutanti singoli *atpao2*, *atpao3* e *atpao4*, così come dei doppi mutanti *atpao2*/*atpao4* e *atpao3*/*atpao4*, rispetto alle piante *wild-type*. Al momento sono in corso studi volti a chiarire i meccanismi che stanno alla base del contributo delle AtPAO perossisomali alla regolazione dei movimenti stomatici. Sono anche in corso altri studi per determinare il coinvolgimento delle AtPAO nello sviluppo e nelle risposte di difesa da vari stress abiotici.

In conclusione, il presente lavoro fornisce evidenze di importanti differenze nel profilo di espressione spazio-temporale delle varie PAO di Arabidopsis, che, insieme alle loro differenti proprietà catalitiche, suggerisce ruoli fisiologici distinti per ogni membro della famiglia genica *AtPAO*. Questo studio può contribuire a un'analisi dettagliata dei ruoli fisiologici delle vie cataboliche delle poliammine nelle piante.

INTRODUCTION

Polyamines

General characteristics of polyamines

Polyamines are aliphatic polycations of low molecular weight found in both prokaryotic and eukaryotic cells. The most common polyamines in eukatyotes are the diamine putrescine (Put), the tri-amine spermidine (Spd), and the tetra-amine spermine (Spm) (Galston and Sawhney, 1990) (Fig. 1). In addition, other polyamines, such as 1,3-diaminopropane (Dap), cadaverine (Cad), thermospermine (Ther-Spm), norspermidine (Nor-Spd) and norspermine (Nor-Spm) (Fig. 1) are found in many organisms as minor components of the cellular polyamine pool and are referred to as uncommon polyamines (Cohen, 1998; Tavladoraki *et al.*, 2011).

Common polyamines

NH_2-$(CH_2)_4$-NH_2	**Putrescine**
NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2	**Spermidine**
NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2	**Spermine**

Uncommon polyamines

NH_2-$(CH_2)_3$-NH_2	**1,3-diaminopropane**
NH_2-$(CH_2)_5$-NH_2	**Cadaverine**
NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2	**Norspermidine**
NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2	**Norspermine**
NH_2-$(CH_2)_4$-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2	**Thermospermine**

Fig. 1. Structures of common and uncommon polyamines.

Polyamines occur in free soluble form, but they are also present in conjugated soluble or insoluble forms. Soluble conjugated polyamines are covalently bound to small molecules, such as phenolic compounds, while insoluble conjugated polyamines are covalently bound to macromolecules such as nucleic acids and proteins (Martin-Tanguy, 1997). Conjugation

alters the charge of polyamines and this probably changes their ability to interact with other molecules, and thereby their biological function. Acetylation of Spd and Spm is a signal to export these polyamines from animal cells; acylated Spd has an antifungal action (Walters *et al*., 2001), while hydroxycinnamic acid conjugates have been suggested to be implicated in plant protection against pathogens and in flower development (Martin-Tanguy, 1997; Grienenberger *et al*., 2009; Kaur *et al*., 2010).

Physiological roles of polyamines

The positive charge that polyamines have at physiological pH allows them to interact with negatively charged macromolecules, such as DNA, RNA, proteins and phospholipids. Therefore, they alter physical and chemical properties of numerous cellular components and, as a consequence, they are involved in the regulation of several fundamental cellular processes, including DNA replication, regulation of gene expression, RNA modification, translation, cell proliferation, ion-channel regulation, cell cycle regulation, modulation of cell signalling and membrane stabilization (Kusano *et al*., 2008; Tavladoraki *et al*., 2011). However, polyamines are associated with several cellular processes not only through their interaction with anionic macromolecules, but also through their metabolic products (Alcázar *et al*., 2010b).

In animals, polyamines have an important role in cell differentiation and proliferation, as well as in senescence and pathological conditions such as cancer cell growth (Pegg, 1988; Pegg and Feith, 2007; Eisenberg, 2009; Tavladoraki *et al*., 2011). In plants, polyamines have been suggested to play important roles in a wide array of developmental processes, including morphogenesis, growth, differentiation and senescence. Moreover, they are implicated in defence responses to various biotic and abiotic stresses (Bouchereau *et al*., 1999; Walters, 2003a; Kusano *et al*., 2007b; Cuevas *et al*., 2008).

Since polyamines are involved in such a wide variety of processes, their intracellular concentrations are strictly regulated through anabolic and catabolic processes, but also through conjugation, transport and compartimentalization (Alcázar *et al*., 2010b; Kusano *et al*., 2008; Moschou *et al*., 2008d; Tavladoraki *et al*., 2011).

Roles of polyamines during growth and differentiation

Polyamines are essential for cell growth and differentiation, as inhibition of polyamine biosynthesis blocks cell growth (Cohen, 1998; Igarashi and Kashiwagi, 2000; Thomas and Thomas, 2001; Hanfrey *et al*.,

2001). Indeed, inhibition of Spd synthesis through deletion of enzymes involved in polyamine biosynthesis is lethal at very early embryonic stages in mice (Wang *et al*., 2004) and leads to lethal defects in embryo development in *Arabidopsis thaliana* plants (Imai *et al*., 2004b; Urano *et al*., 2005). The primary importance of Spd for viability of eukaryotic cells may reside in the unusual Spd-derived amino acid hypusine, which is produced from the post-translational modification of the eukaryotic translational initiation factor 5A (eIF5A) and which is essential for eukaryotic cell growth and proliferation (Park *et al*., 2006). On the contrary, organisms lacking Spm are viable, but show different degrees of dysfunction. This indicates that Spm, though not essential, plays very important roles in growth and development (Imai *et al*., 2004a; Wang *et al*., 2004; Yamaguchi *et al*., 2007; Minguet *et al*., 2008; Pegg and Michael, 2010).

Polyamines are involved in programmed cell death, causing it when their cellular amounts are higher or lower with respect to physiological levels. On the other hand, polyamines can also protect cells against apoptosis; indeed, it has been demonstrated that Spm prevents an increase in caspase 3 activity, which generally precedes apoptosis. Other mechanisms that could be on the basis of the protective effect of Spm are inhibition of endonucleases, stabilization of DNA and protection of DNA against oxidative stresses (Schipper *et al*., 2000; Seiler and Raul, 2005).

In animals, polyamines are required for normal gut functions, maturation and repair of intestinal mucosa (Seiler and Raul, 2007). They are involved in the processes underlying brain ischemia (Li *et al*., 2007) and affect numerous processes in carcinogenesis. Indeed, in both tumor cell cultures and solid tumors, polyamines (particularly their acetylated conjugates N^1-acetyl-Spm and N^1-acetyl-Spd) are often present at increased concentrations, while polyamine depletion leads to inhibition of tumor growth (Tavladoraki *et al*., 2011).

In plants, polyamines have been suggested to play important roles in morphogenesis, growth, embryogenesis, organ development and leaf senescence (Kumar *et al*., 1997; Walden *et al*., 1997; Malmberg *et al*., 1998; Liu *et al*., 2000; Kusano *et al*., 2007a, Alcázar *et al*., 2006b). In general, cells undergoing division contain high levels of free polyamines, while cells undergoing expansion and elongation contain low levels of free polyamines (Galston and Sawhney, 1995). In plants, polyamines are also involved in vascular development. Indeed, a loss-of-function Arabidopsis mutant for an *S*-adenosylmethionine decarboxylase (SAMDC), an enzyme involved in polyamine biosynthesis and homeostasis, displays enlarged vascular systems in inflorescences, roots, and petioles (Ge *et al*., 2006). Furthermore, a loss-of-function mutant for ACAULIS5 (*acl5*), a

thermospermine synthase specifically expressed in the xylem, shows a dwarf phenotype, severely reduced secondary growth of the vascular tissue, dramatically altered morphology of the vessel element, and total lack of xylem fibers (Clay and Nelson, 2005; Kakehi *et al*., 2008). Polyamines are also involved in plant reproduction. Indeed, pollen germination and tube growth in lily are stimulated *in vitro* by exogenous polyamines and reduced by inhibitors of polyamine biosynthesis (Rajam, 1989). Moreover, γ-aminobutyric acid (GABA) and H_2O_2 derived from polyamine catabolism have been shown to regulate pollen tube attraction towards egg cells and pollen tube growth, respectively (Palanivelu *et al*., 2003; Higashiyama and Hamamura, 2008; Wu *et al*., 2010). Acylated Spd was found in pollen coat and was suggested to play a role in the defence against environmental stresses, but also against pathogens (Grienenberger *et al*., 2009).

Roles of polyamines during abiotic stresses

In plants, polyamine involvement in the response to abiotic stresses is largely documented. Indeed, they have been shown to play important roles in plant defences against drought, salinity, osmotic stress, low and high-temperature, heavy metals and oxidative stress (Bouchereau *et al*., 1999; Alcázar *et al*., 2006b, 2010a, b; Kusano *et al*., 2007b; Groppa and Benavides, 2008; Gill and Tuteja 2010). Indeed, several studies in different plant species showed that polyamine accumulation occurs in response to several adverse environmental conditions, and exogenous polyamine supply has been demonstrated to play a protective role against stress (Liu *et al*., 2007; Yamaguchi *et al*., 2007; Shi *et al*., 2010). Moreover, stress-inducible expression of biosynthetic enzymes has been shown for dehydration, salt and cold stress (Pérez-Amador *et al*., 2002; Urano *et al*., 2003; Alcázar *et al*., 2006b, 2011; Hummel *et al*., 2004; Vergnolle *et al*., 2005). Furthermore, inducible expression of plant enzymes involved in polyamine catabolism was observed under various stress conditions and following treatments with stress-related hormones (Møller *et al*., 1998; An *et al*., 2008; Xue *et al*., 2009; Hewezi *et al*., 2010; Quinet *et al*., 2010; Toumi *et al*., 2010; Tavladoraki *et al*., 2011), suggesting that polyamines may protect plants from environmental stresses also through their catabolic products. In Arabidopsis, K^+ deficiency induces an increase in Put accumulation, and several studies have established a specific role of Put in maintaining a cation-anion balance in plant tissues (Boucherau *et al*., 1999). In addition, different studies reported increase in Spd level in water-stressed and cold-tolerant tissues, indicating the stress-related role of this polyamine (He *et al*., 2002; Nayyar *et al*., 2005). Arabidopsis *acl5/spms* mutant plants, which are unable to produce Spm and Ther-Spm, are hypersensitive to salt and

drought stresses while exogenous Spm supply rescues the hypersensitivity. Arabidopsis *acl5/spms* mutant plants are also Ca^{2+} deficient and lose more water compared to control plants, due to a failure of stomatal closure during drought (Yamaguchi *et al*., 2006; Yamaguchi *et al*., 2007). These results suggest that the absence of Spm may cause deregulation of Ca^{2+} trafficking, resulting in a lack of proper adaptation to high sodium chloride or drought stresses (Kusano *et al*. 2007a; Kusano *et al*., 2007b). Moreover, polyamines were shown to block ion channels in a charge-dependent manner (Spm>Spd>Put) (Bruggemann *et al*., 1998), and to regulate stomatal responses through a direct effect on guard cell channel activity (Liu *et al*., 2000; Yamaguchi *et al*., 2007). On the other hand, it has been shown that intensifying polyamine biosynthesis via biotechnological approaches leads to an increased tolerance to salt, drought, cold and several other abiotic stresses (Kasabuke *et al*., 2004; Wi *et al*., 2006; Altabella *et al*., 2009; Alcázar *et al*., 2010a).

The underlying mechanisms by which polyamines exert their protective role against abiotic stresses are still unclear. It is plausible that one of the tasks of stress-induced polyamines is to modulate the activity of a certain set of ion channels to adapt ionic fluxes in response to environmental changes (Shabala *et al*., 2007; Zhao *et al*., 2007). Indeed, in mammals polyamines have been shown to have a direct effect on several ion channels and receptors, resulting in the regulation of Ca^{2+}, Na^{+} and K^{+} homeostasis (Kusano *et al*., 2008), while in plants Liu *et al*. (2000) demonstrated that cytoplasmic polyamines block the inward K^{+} currents across the plasma membrane of guard cells. However, some of polyamine functions in stress resistance have been attributed to their catabolic products, such as GABA or H_2O_2 (Angelini *et al*., 2010).

Roles of polyamines during biotic stresses

Polyamines are also involved in incompatible and compatible plant-pathogen interactions (Walters, 2003a). Accumulation of polyamines is widely described in plant tissues infected with various types of pathogens. For example, polyamine accumulation was observed in Arabidopsis inoculated with *P. syringae*, in rice inoculated with *Magnaporthe grisea*, in tobacco inoculated with *Pseudomonas cichorii*, in tobacco infected with the necrotrophic fungus *Sclerotinia sclerotiorum* and the biotrophic bacterium *Pseudomonas viridiflava* (Angelini *et al*., 2010). Infection of barley with powdery mildew fungus leads to increased accumulation of Put, Spd and Spm in infected leaves and these changes are accompanied by increased activities of polyamine biosynthesic enzymes (Walters *et al*., 1985, 1986). Similarly, infection of turnip with turnip yellow mosaic virus (TYMV) and

tobacco with tobacco mosaic virus (TMV) cause increased polyamine levels in the plants (Walters *et al*., 2003b; Xu *et al*., 2005). During the hypersensitive response (HR), which occurs as a consequence of an incompatible plant-pathogen interaction and which consists in rapid H_2O_2 production and cell death at the site of pathogen entry, an enhanced polyamine synthesis and an apoplastic accumulation of Spm were reported (Torrigiani *et al*., 1997; Marini *et al*., 2001). In the apoplast, Spm may directly interact with cation channel(s) affecting their functions and/or be catabolized to produce H_2O_2. The resulting changes in K^+/Ca^{2+} trafficking and the generation of H_2O_2 can lead to mitochondrial malfunction and cell death (Takahashi *et al*., 2003; Amirsadeghi *et al*., 2007; Kusano *et al*., 2007a). Polyamines are precursors for secondary metabolites that have been linked with plant–pathogen defence responses (Walters *et al*., 2003a; Walters *et al*., 2003b). Recently it was shown that Spm-signalling pathway plays a defensive role mediating cucumber mosaic virus (CMV)-induced HR for restricting the pathogen in Arabidopsis and tobacco (Mitsuya *et al*., 2009; Sagor *et al*., 2009). Exogenous application of Spm and Spd was shown to modulate resistance against TMV in tobacco and Arabidopsis (Mitsuya *et al*., 2009; Sagor *et al*., 2009).

Changes in polyamine metabolism were also reported during compatible plant-pathogen interactions. In particular, increased polyamine concentrations were found in green islands, which are formed on cereal or barley leaves infected by biotrophic fungal pathogens, like rust and powdery mildew (Walters, 2000). Green islands surround the infection sites and are thought to represent regions in which a juvenile condition is maintained to ensure absorption of nutrients by the pathogen (Walters, 1989). It was suggested that the increased polyamine concentration in infected cereal or barley leaves might be related to green island formation (Walters, 2003a).

In general, both in animals and plants, changes in polyamine metabolism occur in response to a variety of physiological and pathological conditions, and although the precise physiological function and mechanisms of action of polyamines remain unclear, polyamine metabolism is being used as a target for antiproliferative therapies in animals and stress tolerance strategies in plants (Tavladoraki *et al*., 2011).

Polyamine biosynthesis

The general mechanism of polyamine biosynthetic pathway is conserved from bacteria to animals and plants (Tabor and Tabor, 1984) and begins from the synthesis of the diamine Put, followed by successive

additions of aminopropyl groups to produce the triamine Spd and the tetramine Spm (Fig. 2).

In plants, the diamine Put can be synthesized through two alternative pathways (Bagni and Tassoni, 2001; Liu *et al*., 2007), starting either from arginine (Arg) or from ornithine (Orn), which are decarboxylated by arginine decarboxylase (ADC) or by ornithine decarboxylase (ODC), respectively. In the ADC pathway, Arg decarboxylation generates agmatine, which is subsequently converted to Put by the combined action of agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase. In the ODC pathway, Put is the direct product of ornithine decarboxylation. Put is then converted to Spd and Spd to Spm by the subsequent addition of an aminopropyl group onto Put and Spd, respectively, in reactions catalysed by the enzymes spermidine synthase (SPDS) and spermine synthase (SPMS), respectively. The donor of the aminopropyl group is *S*-adenosylmethionine (SAM), which is synthesized from methionine by methionine adenosyltransferase, and then decarboxylated by SAMDC (Fig. 2).

In bacteria, in addition to ADC and ODC, another enzyme is present involved in Put biosynthesis, agmatinase, which directly produces Put from agmatine. Furthermore, in bacteria Spm is not synthesized, since no *SPMS* gene is present (Wortham *et al*., 2007). In animals, Put is mainly synthesized through the ODC pathway, the ADC pathway being just a minor pathway in specific mammalian tissues (Gilad *et al*., 1996) (Fig. 2). In *A. thaliana*, Put is produced exclusively through the ADC pathway, since no ODC gene has been identified in the sequenced genome of this plant and the corresponding enzyme activity has not been detected (Hanfrey *et al*., 2001; Alcázar *et al*. 2010b), whereas all other polyamine biosynthetic genes are present. In detail, in *A. thaliana* genome there are two *ADC* genes (*ADC1* and *ADC2*) (Watson and Malmberg, 1996; Watson *et al*., 1997), two *SPDS* genes (*SPDS1* and *SPDS2*) (Hanzawa *et al*., 2002), one *SPMS* gene (Panicot *et al*., 2002; Clay and Nelson, 2005) and at least four *SAMDC* genes (*SAMDC1*, *SAMDC2*, *SAMDC3* and *SAMDC4*) (Urano *et al*., 2003; Ge *et al*., 2006). Among the genes involved in polyamine biosynthesis, *ACL5* was for a long time described as a *SPMS*, but it has been recently demonstrated to specifically synthesize Ther-Spm from Spd (Knott *et al*., 2007).

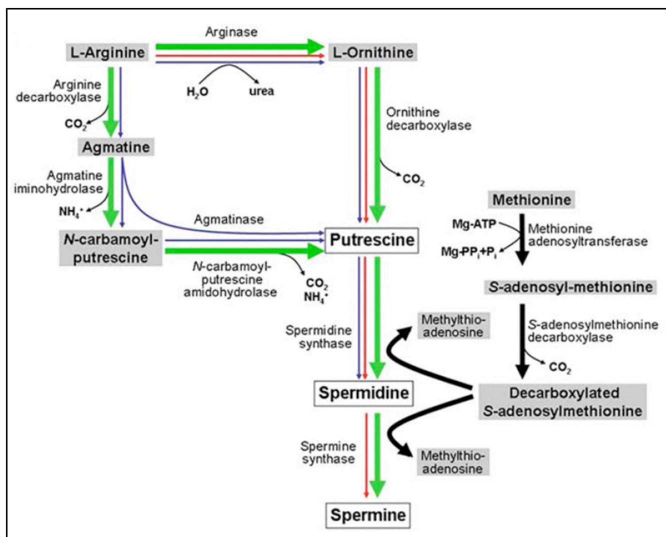

Fig. 2. Polyamine biosynthetic pathways. Plant pathway is indicated by green bold arrows. Blue and red arrows indicate bacterial and animal pathways, respectively. Figure taken from Kusano *et al*., 2008.

Polyamine catabolism

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

CuAOs catalyze the oxidation of Put at the primary amino group producing ammonia, H_2O_2 and 4-aminobutanal (Cohen, 1998) (Fig. 3). The latter spontaneously cyclizes to generate Δ^1-pyrroline and can be further converted to GABA by an aldehyde dehydrogenase. This is subsequently transaminated and oxidized to succinic acid, which is incorporated into the Kreb's cycle, ensuring the recycling of carbon and nitrogen from Put (Cona *et al*., 2006) (Fig. 3).

PAOs catalyze the oxidation of Spm, Spd and/or their acetylated derivatives at the secondary amino groups, generating different reaction products depending on the mode of substrate oxidation, which in turn is due to a different mode of substrate binding inside the catalytic site resulting in the oxidation of a different carbon atom. In particular, all the so-far characterized PAOs from monocotyledonous plants, such as the extracellular maize PAO (ZmPAO), oxidize the carbon on the *endo*-side of the N^4-nitrogen of Spd and Spm, producing 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, in addition to 1,3-

diaminopropane (Dap) and H_2O_2. Thus, these PAOs are considered involved in the terminal catabolism of polyamines (Angelini *et al*., 2010) (Fig. 3). On the contrary, animal PAOs oxidize the carbon on the *exo*-side of N^4-nitrogen of N^1-acetyl-Spm and N^1-acetyl-Spd to produce Spd and Put, respectively, in addition to 3-acetamidopropanal and H_2O_2 (Landry and Sternglanz, 2003; Vujcic *et al*., 2003; Wu *et al*., 2003) (Fig. 3). In this catabolic pathway, polyamine acetylation is catalyzed by the tightly regulated Spd/Spm N^1-acetyltransferase (SSAT), which is the rate-limiting factor (Wallace *et al*., 2003). The animal PAOs are considered involved in a polyamine back-conversion pathway since they produce a common polyamine by the oxidation of another common polyamine (Seiler, 2004). In animals another class of polyamine oxidases is present, the spermine oxidases (SMOs), which also oxidize the carbon on the *exo*-side of N^4-nitrogen of Spm to produce Spd, 3-aminopropanal, and H_2O_2, and thus also animal SMOs are considered involved in a polyamine back-conversion pathway (Wang *et al*., 2001; Vujcic *et al*., 2002; Cervelli *et al*., 2003; Landry and Sternglanz, 2003) (Fig. 3). Subsequently, 3-aminopropanal and 3-acetamidopropanal can be metabolized by an aldehyde dehydrogenase (ADH) to form β-alanine and *N*-acetyl-β-alanine, respectively. *N*-acetyl-β-alanine in turn can be converted to β-alanine by the action of a selective hydrolase (HDL) (Fig. 3).

For a long time it was thought that PAOs from animals and plants had distinct catabolic pathways. However, recently, biochemical characterization of four PAOs from *Arabidopsis thaliana* and three PAOs from *Oryza sativa* evidenced that also plant polyamines can catalyse a polyamine back-conversion pathway, since they produce a common polyamine by the oxidation of another common polyamine (Tavladoraki *et al*. 2006; Moschou *et al*. 2008c; Fincato *et al*. 2011a; Ono *et al*., 2011).

Interactions with other metabolic routes

Polyamine anabolic and catabolic pathways are deeply interconnected with other metabolic routes involved in the formation of various signalling molecules and metabolites that are relevant in plant stress responses (Fig. 4). For example, polyamines and ethylene have SAM as a common precursor, and antagonistic effects between these compounds occur during leaf and flower senescence and fruit ripening (Pandey *et al*., 2000; Wi and Park, 2002). Polyamine metabolism also influences nitric oxide (NO) formation (Yamasaki and Cohen, 2006). Indeed, Spd and Spm were shown to induce the production of NO, which may act as a link between polyamine-mediated stress responses and other stress mediators (Tun *et al*., 2006).

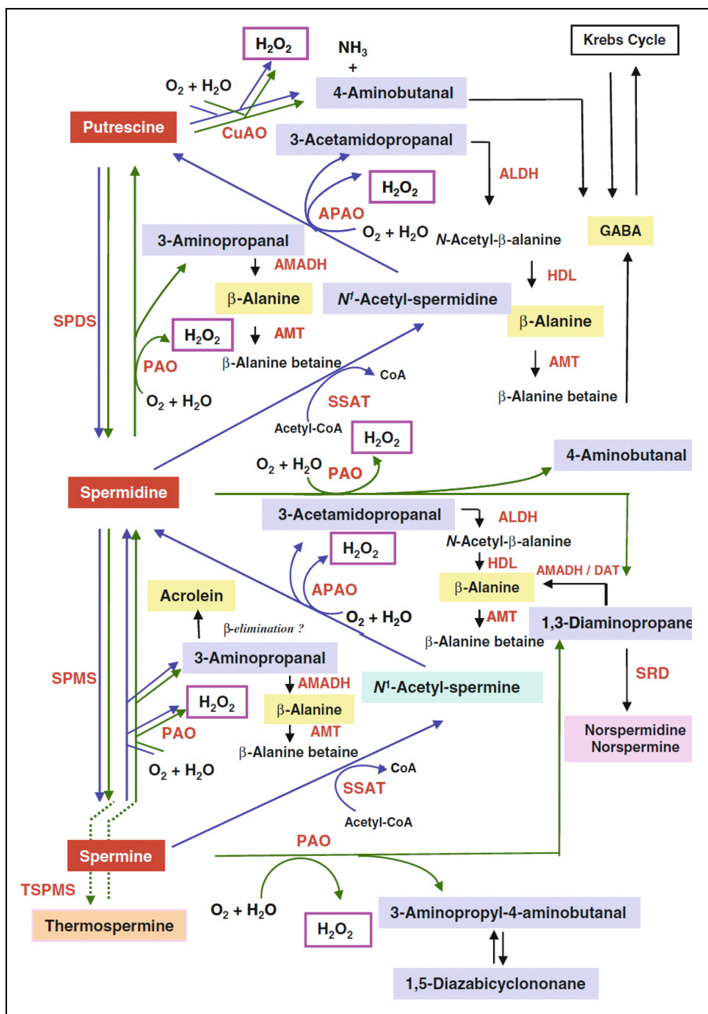

Fig. 3. Schematic representation of the polyamine catabolic pathways in animals and plants and related matabolites. Green arrows indicate polyamine metabolic pathways in plants, blue arrows indicate polyamine metabolic pathways in animals and black arrows indicate metabolic pathways related to polyamine catabolism. ALDH aldehyde dehydrogenase, ADC arginine decarboxylase, AMADH aminoaldehyde dehydrogenase, AMT β-alanine *N*-methyltransferase, DAT 1,3-diaminopropane-aminotransferase, GABA γ-aminobutyric acid, HDL *N*-acetyl-β-alanine amidohydrolase, ODC ornithine decarboxylase, PMT putrescine *N*-methyltransferase, SSAT spermidine-spermine N^1-acetyltransferase, SPDS spermidine synthase, SPMS spermine synthase, SRD Schiff-base reductase/decarboxylase, TSPMS thermospermine synthase. Figure modified from Tavladoraki *et al*., 2011.

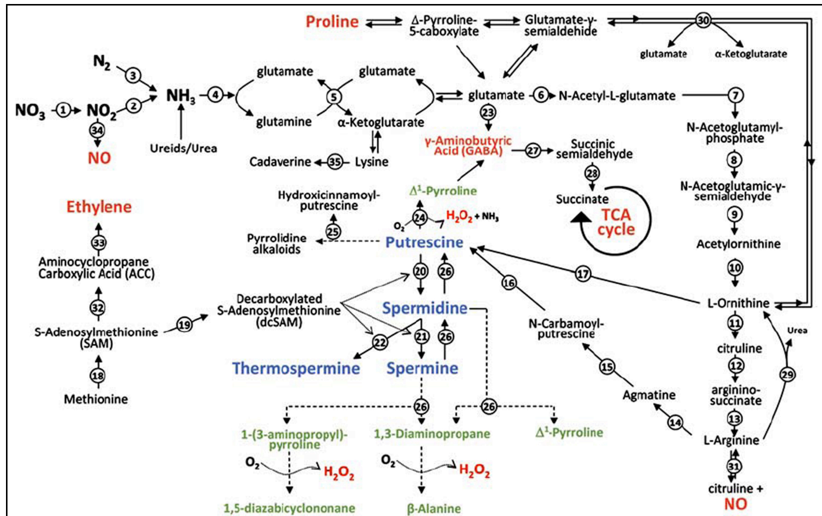

Fig. 4. Polyamine metabolism and interaction with other metabolic routes. Biosynthetic pathways for polyamines and related metabolites are indicated by continuous lines. Dashed lines show the formation of Put-derived alkaloids, polyamine conjugation and catabolic processes. Numbers refer to the following enzymes: *1* nitrate reductase, *2* nitrite reductase, *3* nitrogenase, *4* glutamine synthetase, *5* glutamate synthase, *6* glutamate *N*-acetyltransferase, *7* acetylglutamate kinase, *8* *N*-acetyl-γ-phosphate reductase, *9* acetylornithine transaminase, *10* acetylornithine deacetylase, *11* ornithine-carbamoyl transferase, *12* arginosuccinate synthase, *13* arginosuccinate lyase, *14* arginine decarboxylase, *15* agmatine iminohydrolase, *16* *N*-carbamoylputrescine amidohydrolase, *17* ornithine decarboxylase, *18* SAM synthetase, *19* SAM decarboxylase, *20* Spd synthase, *21* Spm synthase, *22* Ther-Spm synthase, *23* glutamate decarboxylase, *24* diamine oxidase, *25* Put hydroxycinnamoyl transferase, *26* polyamine oxidase, *27* γ-aminobutyrate aminotransferase, *28* succinic semialdehyde dehydrogenase, *29* arginase, *30* ornithine aminotransferase, *31* nitric oxide synthase, *32* ACC synthase, *33* ACC oxidase, *34* nitrate reductase, *35* lysine decarboxylase. Figure taken from Alcázar *et al*., 2010b.

H_2O_2 produced by the action of CuAOs and PAOs is involved in both biotic and abiotic stress signalling, as well as in developmental features and abscisic acid (ABA)-induced stomatal closure (Cona *et al*., 2006; An *et al*., 2008). As described above, another product of Put and Spd catabolism is GABA, (Cona *et al*., 2006) (Fig. 4) and since levels of Put, GABA, agmatine and some components of the TCA cycle increase under dehydration (Alcázar *et al*., 2006a; Urano *et al*., 2009), it has been hypothesized that a metabolic connection may exist between these routes in response to stress. In addition, Put and proline (Pro) share ornithine as a common precursor, and both Put and Pro levels increase in response to various abiotic stresses (Aziz *et al*., 1998; Sharma and Dietz 2006; Urano *et al*., 2009). In conclusion, polyamine metabolism is connected to several

important hormonal and metabolic pathways involved in development, stress responses, nitrogen assimilation and respiratory metabolism (Alcázar *et al*., 2010b).

Plant amine oxidases

Plant copper-containing amine oxidases

CuAOs are homodimers in which each subunit of about 70-90 kDa contains a copper ion coordinated to three His and a 2,4,5-trihydroxyphenylalanine quinone cofactor (TPQ), generated by a post-translational autocatalytic modification of a tyrosine residue in the active site (Medda *et al*., 1995). In plants, and in particular in *Pisum sativum*, *Lens culinaris* and *Cicer arietinum* seedlings, CuAOs represent the most abundant proteins of the extracellular fluids (Federico and Angelini, 1991) and they are loosely associated to cell walls of tissues often characterized by rapid extension growth (Cona *et al*., 2006).

In *A. thaliana*, 12 putative CuAO genes are present (*ATAOs*), some of which with a predicted apoplastic localization, other with an intracellular localization. In particular, for some of them, a peroxisomal localization was predicted by sequence analysis, thus representing the first CuAOs involved in a peroxisomal catabolic pathway of polyamines (Tavladoraki *et al*. 2011). ATAO1 is the only Arabidopsis CuAO until now biochemically characterized and was it shown to oxidize Put and, with a lower activity, Spd. Furthermore, the analysis of *ATAO1* expression pattern in *A. thaliana* revealed that the highest expression levels occurs in lateral root cap cells and in the differentiating vascular tissue of root (Møller and McPherson, 1998).

The expression of some plant CuAOs has been shown to be modulated during development, pathogen attack, wound healing and salt stress. Plant hormones, for example jasmonic acid (JA) and ABA, were also shown to regulate expression of plant CuAOs (Møller *et al*., 1998; Cona *et al*., 2006; An *et al*., 2008; Toumi *et al*., 2010; Quinet *et al*., 2010).

Plant polyamine oxidases

Polyamine oxidases are monomeric proteins of about 55 kDa that bear a non-covalently bound molecule of FAD as a cofactor. The first characterized plant PAO was the apoplastic ZmPAO (Federico and Angelini, 1991). The three existing *ZmPAO* genes (*ZmPAO1*, *ZmPAO2, ZmPAO3)* share a conserved gene organization and identical amino acid sequence (Tavladoraki *et al*., 1998; Cervelli *et al*., 2000).

ZmPAO shows a typical absorption spectrum of oxidised flavoproteins with absorption maxima at 280, 380 and 460 nm (Federico and Angelini, 1991). It catalyses the oxidation of Spm (*K*m = 1.6 μM; *k*cat = 32.9 s^{-1}) and Spd (*K*m = 2.1 μM; *k*cat = 50.2 s^{-1}) with an optimal pH of 6.5 (Polticelli *et al*., 2005). Another important biochemical characteristic of ZmPAO is the high value of *K*m for oxygen (0.2 mM), which suggests that the oxygen concentration may be a relevant rate-limiting factor *in vivo* (Bellelli *et al*., 1997). Furthermore, the aminoaldehydes produced during the reaction for Spd and Spm, respectively 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, are competitive inhibitors of the enzyme itself (with *K*i values being 400 μM and 100 μM, respectively) (Federico *et al*., 1990).

In barley (*Hordeum vulgare*), two PAO genes (*HvPAO1* and *HvPAO2*) were isolated which encode for two proteins that display a high overall sequence homology with each other (73%) and with ZmPAO (84% and 73%, respectively) (Cervelli *et al*., 2001). HvPAO1 enzyme was purified from immature caryopsis and was also *in vitro* synthesized. It oxidizes both Spm and Spd with *K*m values of 8.9 μM and of 5.0 μM respectively and a pH of optimum activity of 7.0 for both substrates, similarly to ZmPAO (Cervelli *et al*., 2006). The similar catalytic properties shown for HvPAO1 and ZmPAO could be ascribed to the close phylogenetic relationship existing between them (Fig. 6). On the contrary, HvPAO2 displays different enzymatic features from HvPAO1. Indeed, despite the elevated sequence homology between these two PAOs, HvPAO2 purified from barley seedlings has a pH of optimum activity (5.5 for Spm and 8.0 for Spd), which is different from that of HvPAO1. Furthermore, HvPAO2 has a higher affinity for Spm (*K*m = 4.8 μM) than for Spd (*K*m = 560 μM) (Cervelli *et al*., 2001). Recently, HvPAO2 symplastic localization was demonstrated. In particular, it has been shown that a *C*-terminal extension of eight amino acid residues present in the HvPAO2 sequence is a signal for protein targeting into the plant vacuoles (Cervelli *et al*., 2004). On the contrary, the presence in HvPAO1 of an *N*-terminal signal peptide specific for the secretory pathway and the lack of the *C*-terminal extension present in HvPAO2 could suggest an apoplastic localization for HvPAO1, similarly to ZmPAO. The differences between HvPAO1 and HvPAO2 in catalytic properties and localization suggest that, in barley, the two PAO genes evolved separately, after a duplication event, to encode for two distinct enzymes, and they are likely to play different physiological roles (Cervelli *et al*., 2006).

In *A. thaliana*, model organism of flowering plants, five PAO genes are present: *AtPAO1* (*At5g13700*), *AtPAO2* (*At2g43020*), *AtPAO3* (*At3g59050*), *AtPAO4* (*At1g65840*) and *AtPAO5* (*At4g29720*). AtPAO1, which has a predicted cytosolic localisation, shares with the extracellular ZmPAO a

45% identity at the amino acid level and a similar intron/exon organization (Tavladoraki *et al*., 2006); AtPAO2, AtPAO3, and AtPAO4 display low sequence identity (23% -24% identity) with ZmPAO, and the other AtPAOs, but a high sequence identity to each other (85% between AtPAO2 and AtPAO3, 58% between AtPAO2 and AtPAO4, 50% between AtPAO3 and AtPAO4) and a very similar intron/exon organization, all three genes containing eight introns with highly conserved positions (Fig. 5). This, together with the elevated sequence homology to each other, suggests that these three Arabidopsis genes are recent derivatives from a common ancestor, thus forming a distinct PAO subfamily (*AtPAO2–AtPAO4* subfamily). Interestingly, all the three members of this subfamily have a peroxisomal localization (Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008c). AtPAO5 has a higher amino acid sequence identity with mouse PAO and SMO (31%) than with ZmPAO (22% sequence identity) and with AtPAO2, AtPAO3 and AtPAO4 (23% identity). Furthermore, *AtPAO5* also presents a very different gene organization from that of *ZmPAO* and the other *AtPAOs* as it lacks introns (Fig. 5), and is the first *PAO* gene characterized so far with such an organization. Similarly to AtPAO1, also AtPAO5 has a predicted cytosolic localization (Fincato *et al*., 2011a).

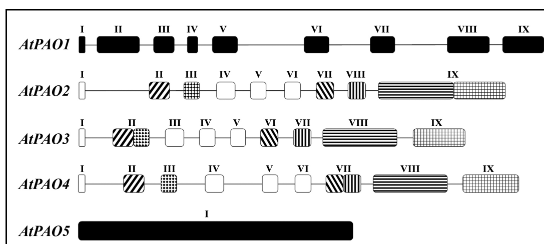

Fig. 5. Schematic representation of the exon/intron organization of *AtPAO* genes. Introns are represented by lines and exons by boxes. Exons are numbered in Roman numerals. Open and filled boxes indicate shared and unshared exons among the various *AtPAO* genes, respectively. Stripes and stipples show 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*., 2011a.

A phylogenetic analysis of AtPAOs and other known plant PAOs revealed that plant PAOs are divided into four clades (Ono *et al.*, 2011) (Fig. 6). AtPAO1 belongs to the same branch as NtPAO (*Nicotiana tabacum* PAO, Yoda *et al*., 2006) and apple MdPAO1 (*Malus domestica* PAO). Clade II comprises maize ZmPAO (Tavladoraki *et al*., 1998), barley HvPAO1, HvPAO2 (*Hordeum vulgare* PAOs, Cervelli *et al*., 2001), along with three rice PAOs, OsPAO2, OsPAO6, and OsPAO7 (Ono *et al*., 2011). Clade III includes AtPAO5, Brassica juncea BjPAO (Lim *et al*., 2006), and

OsPAO1 (Ono *et al.*, 2011). In clade IV, Arabidopsis peroxisome-localized members, AtPAO2, AtPAO3 and AtPAO4, are grouped together with three rice PAOs, OsPAO3, OsPAO4, and OsPAO5, whose peroxisomal localization was demonstrated as well (Ono *et al.*, 2011) (Fig. 6).

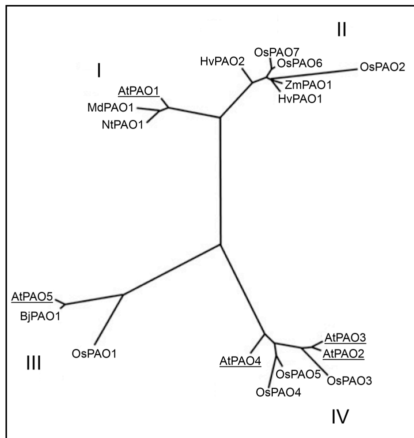

Fig. 6. Phylogenetic relationships of AtPAOs and other plant PAOs. Figure modified from Ono *et al*., 2011.

A. thaliana PAOs display different substrate specificities to each other (Table 1). AtPAO1 oxidizes only Spm and not Spd, differently from ZmPAO but similarly to the animal SMO; interestingly, AtPAO1 also shows high specificity towards the uncommon polyamines Ther-Spm and Nor-Spm (Tavladoraki *et al.* 2006). Recombinant AtPAO1 also oxidises N^1-acetyl-Spm but with a low efficiency (Tavladoraki *et al.,* 2006). AtPAO2, AtPAO3 and AtPAO4 oxidize both Spd and Spm with important differences to each other (Moschou *et al*., 2008c; Kamada-Nobusada *et al*., 2008; Fincato *et al*., 2011a; Takahashi *et al*., 2010) (Table 1). Indeed, while AtPAO2 is equally active with Spm and Spd, AtPAO3 is twofold more active with Spd than with Spm, and AtPAO4 is 40-fold more active with Spm than with Spd (Table 1). Recombinant AtPAO2, AtPAO3 and AtPAO4 are also able to oxidise Nor-Spm, Ther-Spm and N^1-acetyl-Spm, but less efficiently than Spm (Tavladoraki *et al.,* 2006; Fincato *et al*., 2011a) (Table 1). The much lower catalytic efficiency of the three peroxisomal AtPAOs towards N^1-acetyl-Spm than towards Spm and Spd suggests that acetylated polyamines may not be their physiological substrates, conversely to the mammalian peroxisomal PAOs, which are specifically implicated in the catabolism of the acetylated polyamines.

	AtPAO1	AtPAO2	AtPAO3	AtPAO4
	K_{cat} (s^{-1})			
Spm	2.5 ± 0.4	4.2 ± 1.2	1.7 ± 0.5	4.6 ± 1.0
Spd	0	4.6 ± 1.5	3.4 ± 1.4	0.1 ± 0.03
***N*[1]-acetyl-Spm**	0.2 ± 0.4	0.8 ± 0.2	0.02 ± 0.01	0.014 ± 0.004
Nor-Spm	6.9 ± 1.3	2.9 ± 0.8	1.1 ± 0.2	0.45 ± 0.1
Ther-Spm	5.7 ± 1.1	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.04

Table 1. Catalytic activity of recombinant AtPAOs and ZmPAO. The enzymatic activity of recombinant enzymes was determined in 100 mM Tris-HCl at the optimum pH (pH 8.0 for AtPAO1 and pH 7.5 for AtPAO2, AtPAO3, and AtPAO4). Data are the mean ± SE of at least three independent experiments (Fincato *et al*., 2011a).

In mammals, acetylated polyamines and their oxidation are considered to have a regulatory role. Indeed, it is believed that polyamines are acetylated in order to be excreted from the cells (Seiler, 1995; Wu *et al*., 2003) and PAOs may prevent this transport, resulting in increased intracellular Spd and Put levels (Wu *et al*., 2003). Furthermore, acetylation reduces the charge of polyamines and this probably alters their ability to interact with other molecules and thereby their biological function (Pegg, 2008). In this case, in animals peroxisomal PAOs may play an important role in the homeostasis of acetylated polyamines.

Moreover, AtPAO1, AtPAO2, AtPAO3, and AtPAO4 have different pH optima (7.0–8.0) than ZmPAO (pH optimum of 6.0; Polticelli *et al*., 2005). In particular, the optimum pH for AtPAO1 catalytic activity is 8 (Tavladoraki *et al*., 2006), while the optimum pH for AtPAO2 and AtPAO3 is 7.5 (Moschou *et al*., 2008c; Fincato *et al*., 2011a); as regards AtPAO4, its catalytic activity towards Spm displays a different pH dependence from that towards Spd. More specifically, the optimum pH for AtPAO4 catalytic activity towards Spd is 8.0, whereas that towards Spm is 7.0 (Fincato *et al*., 2011a). These differences could be related to different subcellular localization and/or physiological role(s).

As far as *AtPAO5* is concerned, all efforts to express this gene in a heterologous system were so far unsuccessful, probably due to protein degradation. Indeed, analysis of the AtPAO5 amino acid sequence indicated the presence of a putative PEST signal for protein degradation near the *N*-terminus of the protein, suggesting that AtPAO5 may be rapidly degraded. Therefore, no information is available yet about AtPAO5 catalytic properties.

Analysis of the AtPAO reaction products showed that all characterized *A. thaliana* PAOs are involved in a polyamine back-conversion pathway

(Tavladoraki *et al*., 2006; Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008c; Fincato *et al*., 2011a), similarly to the animal PAOs/SMOs and in contrast to the PAOs from monocotyledonous plants characterized thus far (such as, for example, ZmPAO), which are involved in a terminal polyamine catabolic pathway (Fig. 7). In this regard, the information so far available allows to propose the terminal catabolic pathway of polyamines as specifically active in the extracellular compartments, while the polyamine back-conversion pathway as mostly intracellular (Fincato *et al*., 2011a; Ono *et al*., 2011). Interestingly, a polyamine back-conversion pathway may also exist in maize, since putative PAOs with high sequence homology to AtPAO2–AtPAO4 (B6SYR8, B4F9F6, B6SW44) and to AtPAO5 (C0PE4) and with predicted intracellular localization were found by similarity searches in the maize genome, suggesting that both the terminal polyamine catabolic pathway and the polyamine back-conversion pathway co-exist in this plant.

More studies are necessary for a detailed understanding of the specific physiological roles of the polyamine back-conversion pathways in plants as opposed to the terminal catabolic pathways of polyamines, which have been shown to be involved in plant development and defence responses to biotic and abiotic stresses (Cona *et al*., 2006; Yoda *et al*., 2006, 2009; Kusano *et al*., 2008; Moschou *et al*., 2008b).

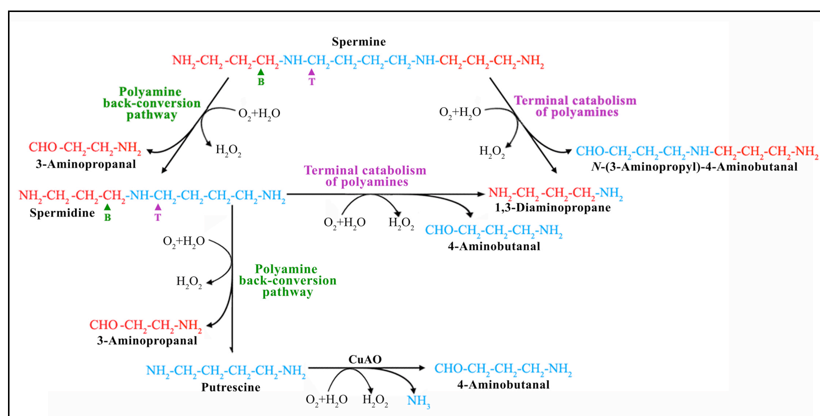

Fig. 7. Polyamine catabolic pathways. Polyamine back-conversion and terminal catabolic pathways are shown. B: back-conversion pathway. T: terminal catabolism. Figure taken from Angelini *et al*., 2010.

Physiological roles of CuAOs and PAOs in plants

CuAOs and PAOs were shown to contribute to important physiological processes through the regulation of cellular polyamine levels, but also through their reaction products: aminoaldehydes, Dap, and mainly H_2O_2 (Boucherau *et al*., 1999; Walters, 2003a; Cona *et al*., 2006; Angelini et al., 2010). In particular, as described before, 4-aminobutanal can be further metabolized to GABA (Figs. 3, 4), which is an important metabolite associated with various physiological processes (including the regulation of cytosolic pH, carbon fluxes into the citric acid cycle, deterrence from insects, wound healing, protection against oxidative stress and signalling) and is largely and rapidly produced in response to biotic and abiotic stresses (Bouché and Fromm, 2004; Xing *et al*., 2007). Dap is a precursor of β-alanine and uncommon polyamines (Terano and Suzuki, 1978; Koc *et al*., 1998) (Fig. 3), which in plants are associated with stress tolerance. H_2O_2 has a key role in several plant development processes, such as cell expansion, polar growth, gravitropism, stomatal aperture, flower development, but also in stress and defence responses. H_2O_2 is produced in the apoplast and in intracellular compartments by several enzymatic systems (Apel and Hirt, 2004; Mittler *et al*., 2004; Yesbergenova *et al*., 2005), and H_2O_2 produced via apoplastic degradation of polyamines was shown to drive peroxidase-mediated oxidative cross-linking of structural cell wall components contributing to cell-wall strengthening during development and under stress conditions, such as wound-healing and pathogen attack (Cona *et al*., 2006; Angelini *et al*., 2008, 2010; Moschou *et al*., 2009). In this regard, a positive spatial correlation between lignin, peroxidase and CuAO levels has been found in chick-pea and tobacco, supporting a functional correlation between the two enzymes (Angelini *et al*., 1990; Paschalidis and Roubelakis-Angelakis, 2005). In *A. thaliana*, H_2O_2 produced by Spd oxidation triggers the opening of hyperpolarization-activated Ca^{2+} permeable channels in pollen, thereby regulating pollen tube growth (Wu *et al*., 2010).

A role in programmed cell death (PCD) associated with developmental differentiation has been proposed for H_2O_2 produced by polyamine catabolism (Møller and McPherson, 1998; Møller *et al*., 1998). Indeed, the considerable presence of ZmPAO and ATAO1 in developing tracheary elements and root cap cells suggests the possibility of their specific involvement in the PCD which both cell types eventually undergo (Møller and McPherson, 1998; Cona *et al*., 2006). Very recently, Tisi *et al*. (2011) suggested that H_2O_2 derived from polyamine catabolism affects root development and xylem differentiation, by inducing differentiation of secondary wall and precocious cell death in xylem precursors of Spd-treated

maize primary root. Moreover, analyzing xylem tissue differentiation in transgenic tobacco plants overexpressing ZmPAO, they demonstrated that H_2O_2 generated by the PAO-mediated polyamine oxidation induces early differentiation of vascular tissues and increased cell death in root cap cells (Tisi *et al*., 2011).

H_2O_2 derived from polyamine catabolism has been also shown to contribute to HR cell death (Yoda *et al*., 2003). Indeed, it has been reported that in *Nicotiana tabacum* plants resistant to tobacco mosaic virus (TMV), PAO expression level increases in tissues exhibiting TMV-induced HR and that PAO inhibition by the specific inhibitor guazatine markedly reduces HR (Yoda *et al*., 2003). Further evidence regarding the participation of polyamine catabolism in defence mechanisms to biotic stresses came from the observation that CuAOs are part of the complex network leading to wound- or herbivore-induced systemic protection. Indeed, in chickpea tissue damage elicits a local and systemic increase in CuAO expression level. Jasmonic acid, which is a mobile wound signal, induces a further increase in CuAO expression level, whereas salicylic acid, a crucial component of systemic acquired resistance, exerts an opposite effect. Furthermore, inhibition *in vivo* of CuAOs by 2-bromoethylamine markedly reduces local and systemic wound-induced H_2O_2 accumulation (Rea *et al*., 2002).

In several cases, plant response to abiotic stress, such as drought, salinity, osmotic stress and heat stress, is associated with an enhancement of polyamine oxidation. In tomato leaf discs treated with sodium chloride, polyamine catabolism has been shown to be closely related to Pro accumulation, which is one of the most commonly induced metabolic responses to water stress or salinity in higher plants. Furthermore, treatment with aminoguanidine, an inhibitor of CuAOs, strongly inhibited the parallel accumulation of Pro (Aziz *et al*., 1998). In rape leaf discs, osmotic stress caused by polyethylene glycol has been associated with increases in Put, Cad and Dap levels as well as a decrease in Spd level. Inhibitor studies have indicated that the stress-induced reduction of Spd level is due to stimulation of Spd oxidation and not to a block in Spd biosynthesis (Aziz *et al*., 1997). These results support the idea that under osmotic stress, rape leaf cells are induced to produce Dap through activation of PAO. The specific effect of this end product of polyamine catabolism is not well understood, but it can involve biosynthesis of uncommon polyamines and/or β-alanine. Indeed, Dap can be converted to uncommon polyamines, such as Nor-Spm and Nor-Spd, which in plants are associated with stress tolerance, by the action of a Schiff-base reductase/decarboxylase (Cona *et al*., 2006) (Fig. 3). In addition, Dap can be also converted to β-alanine through the concerted action of both a Dap-aminotransferase, reported in bacteria but not yet

characterized in plants, and an aldehyde dehydrogenase. The role of β-alanine in stress responses may be due to its involvement, through the action of a β-alanine *N*-methyltransferase, in the production of β-alanine betaine (Fig. 3), an osmoprotectant found in species of *Plumbaginaceae* adapted to a wide range of adverse stress environments including saline and hypoxic conditions (Hanson *et al*., 1994; Raman and Rathinasabapathi, 2003).

Importantly, amine oxidases are also involved in the regulation of stomata opening. Indeed, H_2O_2 produced through polyamine catabolism has been proposed to act as a mediator in ABA signalling in stomatal closure in *Vicia faba* (An *et al*., 2008) and in *Vitis vinifera* (Paschalidis *et al*., 2010). Since ABA was shown to enhance both polyamine synthesis (Alcázar *et al*., 2006b) and catabolism (Moschou *et al*., 2008b), Toumi *et al*. (2010) proposed that this hormone may induce synchronized effects on the anabolic and catabolic polyamine pathways in an attempt to increase the cellular reactive oxygen species (ROS) load to signal downstream stress defence events (Toumi *et al*., 2010). Moreover, since Spd and Spm induce rapid biosynthesis of NO (Tun *et al*., 2006) and both H_2O_2 and NO are involved in ABA signalling, an interplay seems to exist between polyamines, H_2O_2 and NO signalling in ABA-mediated responses (Alcázar *et al*., 2010b). Polyamine catabolism in plants is also associated with heat stress. For example, Roy and Ghosh (1996) found that heat stress increased the amounts of PAO and ADC in rice callus. This increase was higher in calli raised from a heat-tolerant cultivar than in those from a heat-sensitive one and correlated well with the increased levels of free and conjugated polyamines. Interestingly, PAO and ADC levels were also well correlated with the accumulation of uncommon polyamines under non-stress and stress conditions (Roy and Ghosh, 1996).

The contribution of polyamine catabolism in plant defence responses is linked to polyamine transport in the apoplast, where only limiting amounts of polyamines are present under normal growth conditions (Kusano *et al*., 2008; Moschou *et al*., 2008b; Takahashi *et al*., 2010; Toumi *et al*., 2010). Indeed, it has been demonstrated that upon salt stress, Spd is secreted into the apoplast, where it is oxidised by PAOs generating H_2O_2 and leading to either tolerance responses or PCD, depending on the levels of intracellular polyamines: when polyamine anabolism predominates over polyamine catabolism, PCD fails to occur, whereas when the opposite occurs, PCD is induced (Moschou *et al*., 2008a). H_2O_2 produced by polyamine catabolism in the apoplast under stress conditions and/or the apoplastic polyamines themselves trigger a downstream signal cascade pathway leading to increased expression of specific genes for proteins, such as superoxide dismutase, ascorbate peroxidase, pathogenesis-related proteins, protein

kinases, transcriptional factors and other stress responsive proteins (Yamakawa *et al*., 1998; Mitsuya *et al*., 2007; Moschou *et al*., 2008a, 2009; Xue *et al*., 2009).

Recent studies using transgenic plants overexpressing or downregulating polyamine catabolic enzymes revealed the importance of polyamine catabolism in the induction of tolerance to a series of different biotic and abiotic (Yoda *et al*., 2006; Moschou *et al*., 2008a, b, c; 2009; Marina *et al*., 2008). These studies open the possibility to use biotechnological approaches to improve crop resistence to a broad spectrum of pathogens and environmental stresses in species of agronomic interest. A detailed study of the general polyamine metabolism in plants will also permit a combined strategy to obtain multi-resistant plant to a broad range of stesses manipulating in parallel both the polyamine biosynthetic and catabolic pathways, as well as polyamine transport.

AIM OF THE WORK

In plants, polyamine catabolism is more than a biochemical process aiming to control polyamine homeostasis. Indeed, it has been demonstrated that extracellular PAOs constitute a nodal point during plant growth under physiological and stress conditions, giving rise to increased apoplastic H_2O_2 which signals primary and secondary developmental and defense responses. However, in contrast to the so far characterized extracellular PAOs, little is known on the physiological role(s) of intracellular PAOs, as for example of the five *Arabidopsis thaliana* PAOs, which differ to each other in subcellular localization and catalytic properties. The main aim of the present work is to get detailed information on the tissue- and organ-specific expression pattern of all the members of the *AtPAO* gene family during seedling and flower growth and development under physiological and stress conditions through analysis of promoter activity in transgenic *Arabidopsis* plants expressing *AtPAO*::*GUS* constructs. The information obtained will be used as a guide to study the physiological roles of the various AtPAOs using *atpao* knock out mutants.

RESULTS

AtPAO space-temporal expression pattern

The existing studies on the various members of the *AtPAO* gene family evidenced important differences among them and in respect to the other plant PAOs characterized so far in substrate specificity, reaction products and subcellular localization, probably reflecting differences in the physiological roles. Since to determine the *AtPAO* physiological roles information on the tissue-specific and space-temporal expression pattern of each member of the *AtPAO* gene family could be valuable, in the present study *AtPAO* promoter activity was investigated in detail using *AtPAO::GUS* transgenic plants.

Preparation of AtPAO::GFP-GUS and AtPAO::GUS transgenic Arabidopsis plants

To study *AtPAO* promoter activity, 2,000–2,900 bp promoter regions, including the 5'-UTR, of *AtPAO1*, *AtPAO2*, *AtPAO5* (Dr L. Pomettini, graduation thesis) and *AtPAO4* (in collaboration with Dr A. Ahou) were amplified and cloned at the 5' end of the *green fluorescence protein* (*GFP*) gene in fusion to the *β-glucuronidase* (*GUS*) gene in pKGWFS7 binary vector (Karimi *et al*., 2002) using Gateway technology (Fig. 8). The *AtPAO3* promoter region was cloned into the pGWB3 binary vector (Nakagawa *et al*., 2007) to control expression of GUS gene alone (in collaboration with Dr P.N. Moschou; Fig. 8). The *AtPAO1* promoter region was also cloned into the pGWB3 binary vector to verify whether the two binary vectors provide identical GUS staining patterns. The resulting *AtPAO::GFP-GUS* and *AtPAO::GUS* constructs were used to transform *A. thaliana* wild-type plants by the *Agrobacterium tumefaciens*-mediated floral dip transformation method. Several transgenic lines were obtained for each construct through selection by kanamycin resistance and PCR analysis. At least five transgenic lines per construct were analysed by histochemical GUS staining at various developmental stages. Only highly reproducible results were taken into consideration.

The histochemical GUS assay

For our studies, GUS and GFP reporter systems were chosen for their numerous advantages. In particular, GFP allows a real time detection in

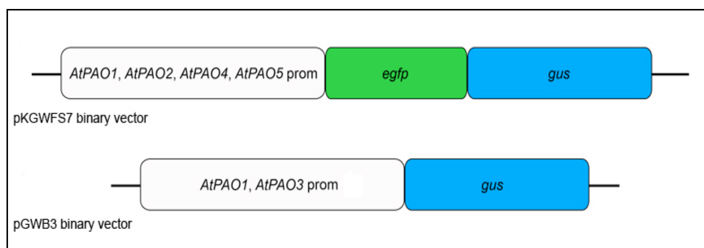

Fig. 8. Schematic representation of *AtPAO::GFP-GUS* and *AtPAO::GUS* constructs used to study *AtPAO* promoter activity in *A. thaliana* transgenic plants.

living tissues without the need of a substrate, though it can also give false positives in those cells which display an auto-fluorescence. GUS assay, which is practicable in plant tissues because they don't contain endogenous GUS activity, can provide a very detailed signal that endures in time, allowing the conservation of specimen and their subsequent embedding for sectioning. Moreover, GUS can use different glucuronides as substrates, allowing not only histochemical, but also spectrophotometrical and fluorimetrical detection. A major disadvantage of GUS assay is the irreversible tissue destruction, in addition to the requirement of a substrate and the time-consuming overall procedure.

In the present study, most of the results were obtained by histochemical GUS assay due to problems of low GFP-specific signal and tissue autofluorescence. The GUS histochemical assay was developed in 1987 by Jefferson (Jefferson, 1987) and exploits the reaction in which GUS cleaves its chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc), resulting in the production of an insoluble blue compound. Among the various protocols for GUS staining existing in literature, after several trials we chose the one that under our experimental conditions gave the best results in terms of specificity, reproducibility and low background. A careful optimization of staining protocol was anyway necessary. Acetone was always used as fixative in a pre-incubation step to increase the permeability of tissues and to minimize the diffusion of the enzyme. The presence of ferri- and ferrocyanide at an optimal concentration in the staining solution was essential, to ensure high enzyme activity, to protect the final reaction product (Fig. 9) from further oxidation to colourless or yellowish products, and to minimize diffusion of the primary reaction product, thus providing a specific signal. EDTA was also present in the staining solution to minimize background and to obtain reproducible results.

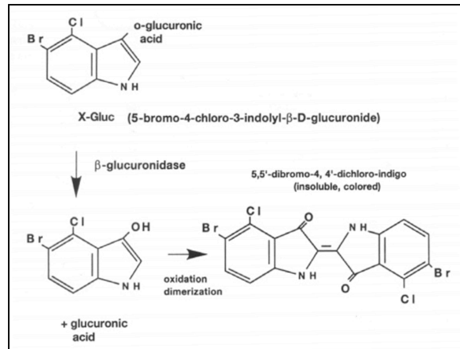

Fig. 9. GUS activity towards its chromogenic substrate X-gluc. X-gluc is cleaved to produce the colourless and soluble 5-bromo-4-chloro-3-indoxyl together with glucuronic acid. The former is then oxidized and dimerized to give 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble and intensely blue compound.

Incubation time was another important factor that was followed out, mainly when quantitative aspects were considered. Finally, to stop the reaction an ethanol-acetic acid extraction solution was generally preferred to ethanol followed by glycerol or chloralhydrate, as it ensures a rapid chlorophyll elimination, facilitates sample handling and increases image quality.

Analysis of AtPAO promoter activity during seedling growth

The tissue-specific and space-temporal analysis of *AtPAO* promoter activity during seedling growth was analysed at different developmental stages, ranging from 3 to 15 days after germination. From this analysis a distinct expression pattern for each *AtPAO* emerged.

AtPAO1-related GUS staining was detected in root tips, where it formed a characteristic blue spot in the transition zone between the meristematic and the elongation regions (Fig. 10), mainly involving cortical tissues and often with a unilateral distribution (Fig. 10b, d). This spot already appeared within 15-30 minutes of GUS staining and was present in all the analysed developmental stages. Interestingly, the *AtPAO1* promoter activity in this region was also evidenced through analysis of GFP specific fluorescence (Fig. 10a). In 3- to 4-day-old seedlings, the *AtPAO1*-related GUS staining in root was extended toward the maturation zone involving all tissues (Fig. 10e, f, g). At this developmental stage, staining was also detected in cotyledons (Fig. 10h). In 5- to 8-day-old seedlings, *AtPAO1*-specific staining appeared in shoot apex (Fig. 10i, j) and newly emerging leaves (Fig. 10i). Later in development, cotyledons and expanded leaves

showed GUS activity in tips and hydathodes, respectively (Fig. 10k, l). Staining of stipules was observed as well (Fig. 10j). No GUS activity was identified in hypocotyls in any developmental stage (Fig. 10e).

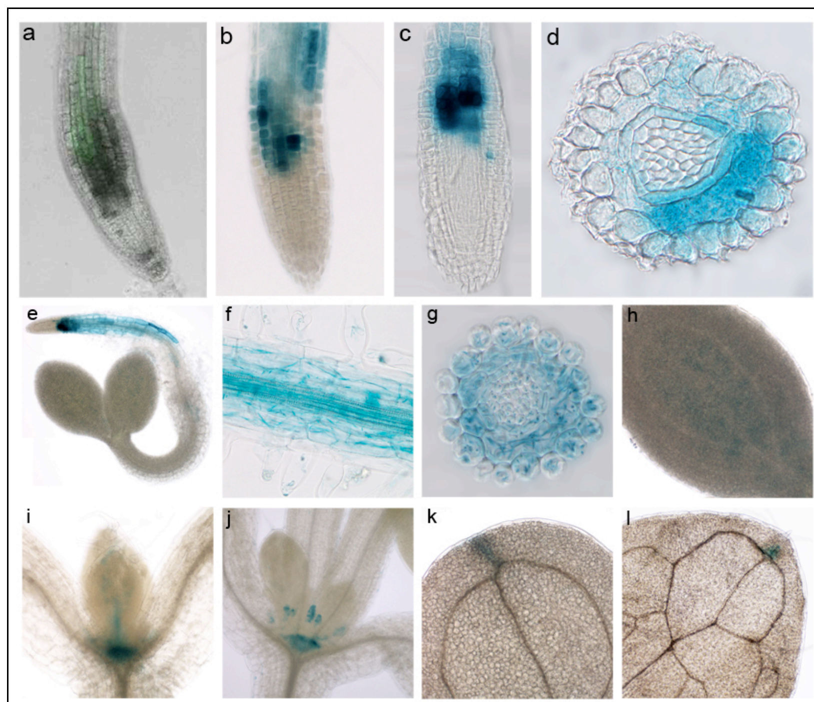

Fig. 10. *AtPAO1* promoter activity during seedling growth. GFP-specific fluorescence in root tip of *AtPAO1::GFP-GUS* transgenic plants (a) and histochemical GUS staining in root tip of *AtPAO1::GFP-GUS* and *AtPAO1::GUS* transgenic plants (b, c, d). Longitudinal (c) and transversal (d) sections obtained after GUS staining. Histochemical GUS staining of whole 3-day-old seedling (e); differentiation region of root (f); transversal section of root obtained after GUS staining (g); cotyledon of 3-day-old seedling (h); shoot apex and newly emerging leaves of 5-day-old seedling (i); shoot apex and stipules in 8-day-old seedling (j); cotyledon of 5-day-old seedling (k); leaf of 8-day-old seedling (l). Identical GUS-staining patterns were obtained from *AtPAO1::GFP-GUS* and *AtPAO1::GUS* transgenic plants.

AtPAO2-related GUS staining was detected in the root apex (Fig. 11a, b), in particular near the quiescent center and in columella initials (Fig. 11b). *AtPAO2*-specific GUS staining was also detected in the elongation and differentiation regions of roots up to the hypocotyl–root junction site involving all tissues (Fig. 11a, c, d). *AtPAO2* expression was observed in hypocotyls of very young seedlings (2- to 3-day-old, Fig. 11a) and disappeared right after. As cotyledons are concerned, the only *AtPAO2*-

related signal in these organs appeared later in development, from 5 days on, and only in the cotyledonary tips (Fig. 11e). In 5- to 8-day old seedlings, GUS activity was observed in stipules and newly expanding leaves (Fig. 11f, g). In the latter case, staining was mainly localized around the vascular tissues and in the petioles, and gradually diminished during leaf development in parallel with an increasing signal in leaf hydathodes (Fig. 11g). *AtPAO2* expression was also detected in few guard cells (Fig. 11h).

AtPAO3-related GUS signal was present in lateral root cap and columella (Fig. 12b). In addition, GUS activity was present in the elongation and differentiation zones of the roots up to the hypocotyl–root junction site (Fig. 12a, c) involving epidermis, cortex, pericycle and the vascular system but not endodermis, as cross sections showed (Fig. 12d). Hypocotyls appeared stained in the region adjacent to the hypocotyl–root junction site (Figs. 12a). From 5 to 8 days on, *AtPAO3*-related GUS staining was also present in stipules (Fig. 12e, f), trichomes (Fig. 12f, g) and guard cells (Fig. 12h).

As far as *AtPAO4* is concerned, a strong GUS staining was detected in roots, from the meristem/elongation transition region up to the hypocotyl–root junction site (Fig. 13a). A part of the meristematic region, but no part of the root cap, was also stained. (Fig. 13b). This *AtPAO4*-related GUS staining in the root was present in all the analysed developmental stages and already appeared after 15-30 minutes of GUS staining. Lateral roots display the same expression pattern (Fig. 13d). In 5- to 8-day-old seedlings, *AtPAO4*-related GUS staining appeared in shoot apex (Fig. 13f, g), cotyledonary and leaf tips (Fig. 13e, f, g), as well as in stipules (Fig. 13f, g). No GUS activity was identified in hypocotyls at any developmental stage (Fig. 13a). *AtPAO4*-related GUS staining was also observed in guard cells (Fig. 13h).

AtPAO5-related GUS staining was present in roots, hypocotyls and cotyledons (Fig. 14a). In roots, the signal was extended from the transition region to the hypocotyl–root junction region and was absent from the root cap (Fig. 14b). *AtPAO5* expression in roots only involved the vascular tissue (Figs. 14b, d). This *AtPAO5*-related GUS staining was also observed through analysis of GFP specific fluorescence (Fig. 14c). In hypocotyls, GUS activity was detected from the shoot apex to the hypocotyl–root junction, involving the stele (Figs. 14a, e). In cotyledons, a rather diffused signal was observed (Fig. 14a). In 5- to 8-day-old seedlings, AtPAO5-related GUS activity also appeared at the distal ends of newly emerging leaves (Fig. 14f), which was extended to the whole leaf area as leaf development proceeded (Fig. 14g). Moreover, staining was found at the base of the trichomes (Fig. 14h). Later during development, staining in hypocotyls, cotyledons and leaves appeared reduced (data not shown).

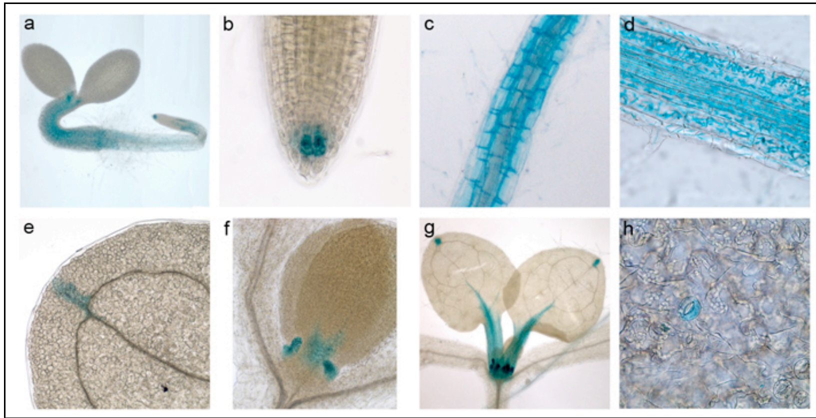

Fig. 11. ***AtPAO2* promoter activity during seedling growth.** Histochemical GUS staining of *AtPAO2::GFP-GUS* transgenic plants. Whole 3-day-old seedling (a); root tip of 5-day-old seedling (b); differentiation region of root (c); longitudinal section of root obtained after GUS staining (d); cotyledon of 5-day-old seedling (e); shoot apex and newly emerging leaves of 5-day-old seedling (f); leaves and stipules in 8-day-old seedling (g); cotyledon of 8-day-old seedling (h).

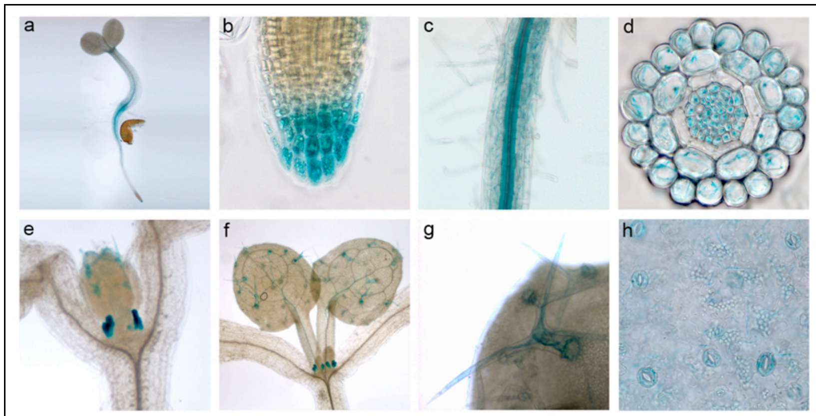

Fig. 12. ***AtPAO3* promoter activity during seedling growth.** Histochemical GUS staining of *AtPAO3::GUS* transgenic plants. Whole 3-day-old seedling (a); root tip of 5-day-old seedling (b); differentiation region of root (c); transversal section of root obtained after GUS staining (d); shoot apex and newly emerging leaves of 5-day-old seedling (e); leaves and stipules in 8-day-old seedling (f); leaf trichomes in 8-day-old seedling (g); stomata on cotyledon of 8-day-old seedling (h).

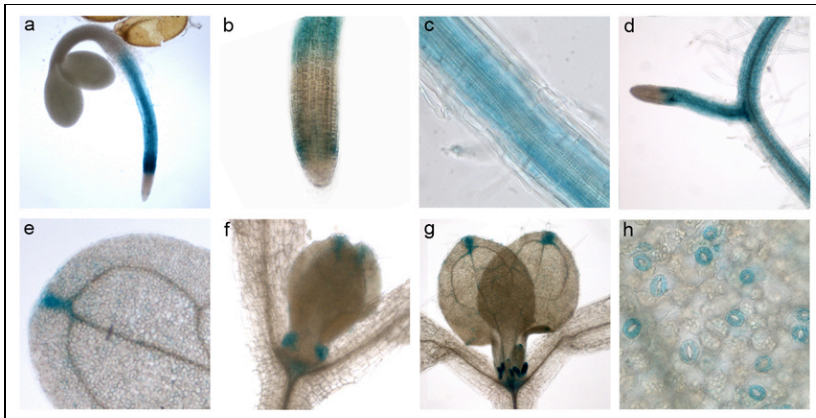

Fig. 13. ***AtPAO4*** **promoter activity during seedling growth.** Histochemical GUS staining of *AtPAO4::GFP-GUS* transgenic plants. Whole 3-day-old seedling (a); root tip of 5-day-old seedling (b); differentiation region of root (c); emerging lateral root (d); cotyledon of 5-day-old seedling (e); shoot apex and newly emerging leaves of 5-day-old seedling (f); leaves and stipules in 8-day-old seedling (g); guard cells on cotyledon of 8-day-old seedling (h).

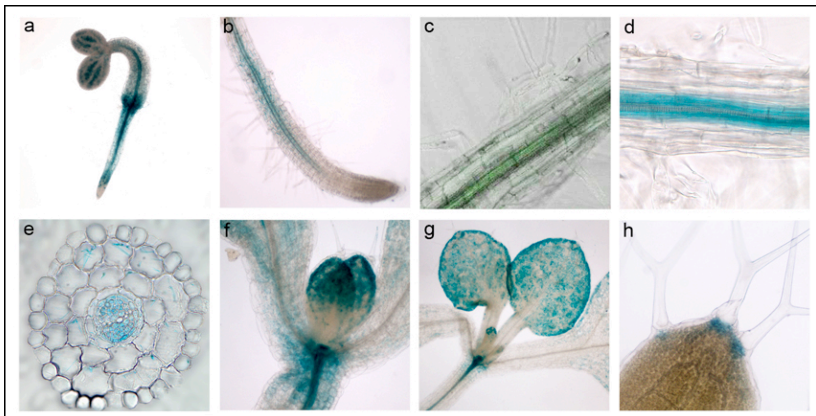

Fig. 14. ***AtPAO5*** **promoter activity during seedling growth.** Histochemical GUS staining (a, b, d, e, f, g, h) and GFP-specific fluorescence of *AtPAO5::GFP-GUS* transgenic plants (c). Whole 3-day-old seedling (a); root tip of 5-day-old seedling (b); differentiation region of roots (c, d); transversal section of hypocotyl obtained after GUS staining (e); shoot apex and newly emerging leaves of 5-day-old seedling (f); leaves in 8-day-old seedling (g); leaf trichomes in 8-day-old seedling (h).

Analysis of AtPAO promoter activity in flowers

For the analysis of *AtPAO* promoter activity in flowers, whole inflorescences were examined, in order to obtain data from different developmental stages, from completely closed flower buds to siliques. Also in this case a specific expression pattern for each *AtPAO* emerged.

When *A. thaliana* inflorescences were screened for *AtPAO1* expression, a strong GUS signal was observed in very young, completely closed flower buds (Fig. 15a, b, c). A close-up revealed that *AtPAO1* expression was localized in the anthers (Fig. 15c, e) and in particular in the microspores and the tapetum (Figs. 15d, f). Microspore staining was gradually decreased during flower development, being very high in pollen mother cells (Fig. 15d) and not at all evident in mature pollen grains (Fig. 15g, h). Anther staining was finally restricted to the anther–filament junction (Fig. 15g, h). *AtPAO1*-related GUS activity appeared also in receptacle and stem, involving mainly vascular tissues (Fig. 15a, b); this signal gradually diminished during flower development. No activity was identified in pistils, petals, sepals, siliques or seeds (data not shown).

AtPAO2 was expressed in very young buds (Fig. 16b). As flower development proceeded and before pollen maturation, a strong signal appeared in pistils (stigma and ovary wall; Figs. 16c, d), which gradually decreased and finally disappeared (Fig. 16h). Mature pollen grains were stained as well (Fig. 16e). Interestingly, this staining persisted during pollination and pollen tube growth (Fig. 16f, g, h). No *AtPAO2*-related GUS staining was detected in petals, sepals, receptacles, stems, siliques or seeds (data not shown).

Also *AtPAO3*-related GUS activity was present in very young flower buds. Later during flower development, pistils and anthers were stained. In particular, in young flowers *AtPAO3*-related GUS signal was found in pistil walls and septum (Figs. 17a, b, c), while in mature flowers *AtPAO3* expression was identified in anther filaments and pollen grains (Fig. 17e). Similarly to *AtPAO2*, pollen staining persisted during pollination and pollen tube growth (Fig. 17f, g, h). Nectars (Fig. 17h) and guard cells of petals and sepals (Fig. 17d) were also positive to GUS activity.

In *AtPAO4::GUS* transgenic plants, a strong GUS signal was observed at the base of very young, completely closed flower buds, and in the guard cells of petals (Fig 18a). As buds developed, a staining appeared in anthers, probably in the tapetum (fig. 18b, c); this GUS activity rapidly diminished during development until it completely disappeared, while a signal appeared in pistil walls and stigma (Fig. 18d). In mature flowers, a strong *AtPAO4*-related GUS signal in pollen grains was detected (Fig. 18e, f, g, h). As in the case of *AtPAO2* and *AtPAO3*, pollen staining persisted during

pollination and pollen tube growth (Fig. 18f, g, h). Guard cells of stems appeared also stained (data not shown).

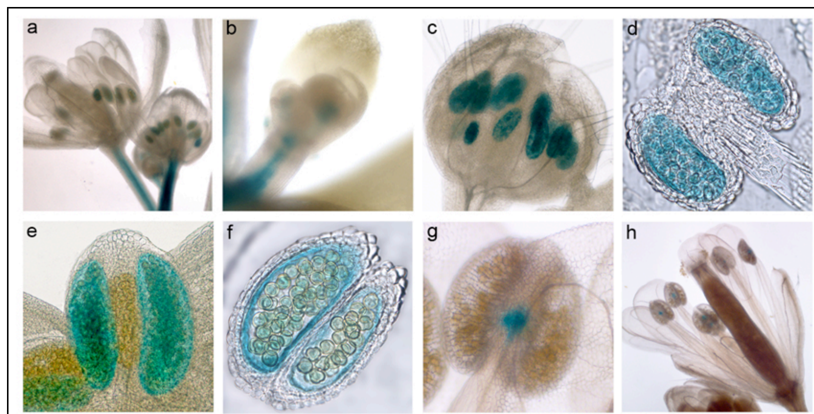

Fig. 15. *AtPAO1* promoter activity in inflorescences. Histochemical GUS staining of *AtPAO1::GFP-GUS* and *AtPAO1::GUS* transgenic plants. Whole flower (a); very young flower bud (b); young flower bud (c); longitudinal section of young flower bud obtained after GUS staining (d); young anther (e); longitudinal section of young anther obtained after GUS staining (f); mature anther (g); mature flower during pollination (h). Identical GUS-staining patterns were obtained from *AtPAO1::GFP-GUS* and *AtPAO1::GUS* transgenic plants.

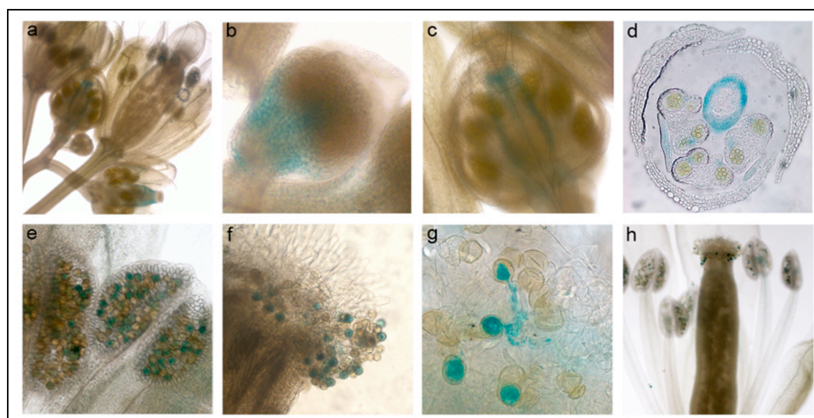

Fig. 16. *AtPAO2* promoter activity in inflorescences. Histochemical GUS staining of *AtPAO2::GFP-GUS* transgenic plants. Whole flower (a); very young flower bud (b); young flower bud (c); transversal section of young flower bud obtained after GUS staining (d); mature anthers (e); pollen grains on pistil during pollination (f); pollen grains during pollination and pollen tube growth (g); mature flower during pollination (h).

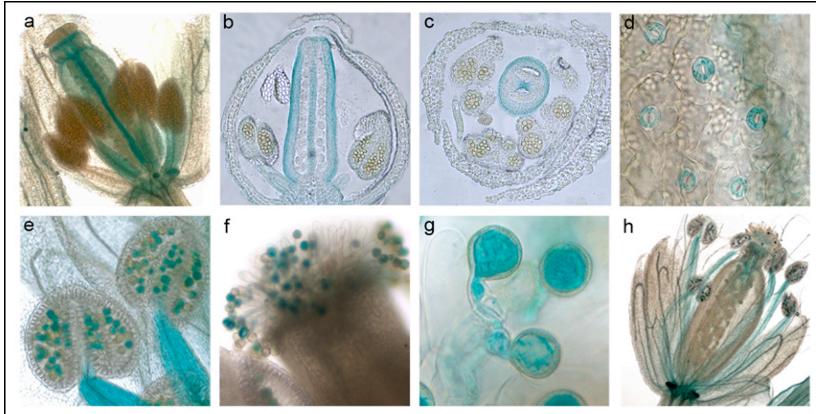

Fig. 17. *AtPAO3* promoter activity in inflorescences. Histochemical GUS staining of *AtPAO3::GUS* transgenic plants. Young flower bud (a); longitudinal (b) and transversal (c) sections of young flower buds obtained after GUS staining; guard cells on petal (d); mature anthers (e); pollen grains on pistil during pollination (f); pollen grains during pollination and pollen tube growth (g); mature flower during pollination (h).

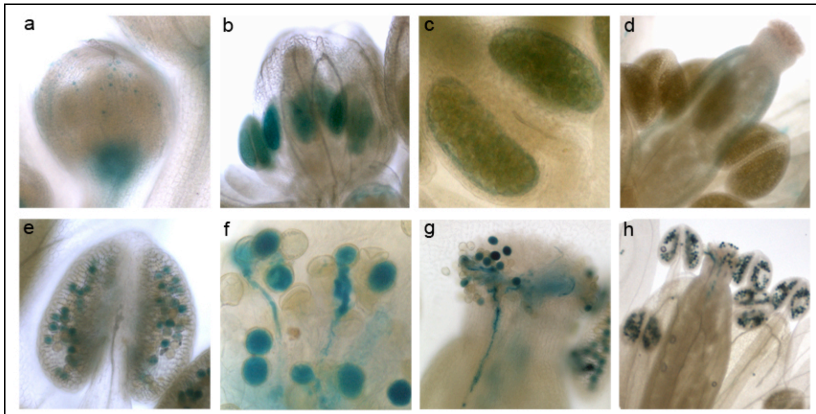

Fig. 18. *AtPAO4* promoter activity in inflorescences. Histochemical GUS staining of *AtPAO4::GFP-GUS* transgenic plants. Very young flower bud (a); young flower bud (b); young anthers (c); guard cells on stem (d); mature anther (e); pollen grains on pistil during pollination (f); pollen grains during pollination and pollen tube growth (g); mature flower during pollination (h).

AtPAO5-related GUS activity was observed already in very young, completely closed flower buds (Fig. 19a). A close-up showed staining localization in the anthers (Fig. 19b) and in particular in anther tapetal cells (Fig. 19c) as in the case of *AtPAO1* and *AtPAO4*. This coloration was gradually reduced during flower development and was finally localized in the anther–filament junction site, similarly to *AtPAO1*, and in the upper part of the filament (Fig. 19e). *AtPAO5* expression was also observed in sepals, petals and pistils (Fig. 19a), but not in pollen grains (Fig. 19f). Pistils were stained in the stigma and in the septum before pollination (Fig. 19d), whereas after pollination pistil staining drastically decreased (Fig. 19f). GUS signal was strong in receptacles and stems, particularly in the cortex and in vascular tissues (Fig. 19h), from the early stages of flower development to silique formation (Fig. 19a, g), whereas siliques and seeds were not stained.

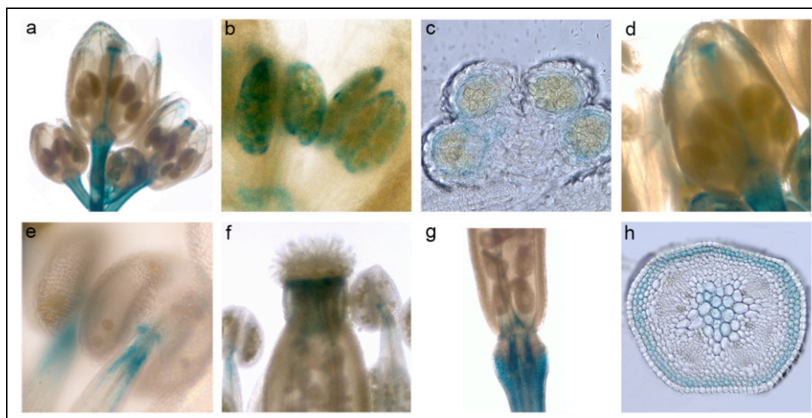

Fig. 19. *AtPAO5* promoter activity in inflorescences. Histochemical GUS staining of *AtPAO5::GFP-GUS* transgenic plants. Whole flower (a); young anthers (b); transversal section of young anther obtained after GUS staining (c); young flower bud (d); mature anthers (e); mature pistil during pollination (f); silique (g); transversal section of stem obtained after GUS staining (h).

AtPAO promoter expression following hormone and stress treatments

Polyamines are widely reported to be involved in several biotic and abiotic stress responses, and the expression of their biosynthetic and catabolic enzymes is regulated by various stimuli (Alcázar *et al*., 2011; Tavladoraki *et al*., 2011). In the present study we used the *AtPAO::GFP-*

GUS and *AtPAO::GUS* Arabidopsis transgenic plants to determine whether various hormone treatments and stress conditions alter promoter activity of the various *AtPAOs*.

AtPAO promoter activity upon hormone treatment

The *AtPAO::GFP-GUS* and *AtPAO::GUS* Arabidopsis transgenic plants for *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO5* were treated with three different plant hormones: Abscisic acid (ABA), involved in stomata closing and stress response; Indole-acetic acid (IAA), implicated in cell division and elongation and thus in plant growth and development; Jasmonic acid (JA), involved in the regulation of plant responses to abiotic and biotic stresses and in plant growth and development. The analysis of *AtPAO4*-related GUS staining following hormone treatments is still in progress since the corresponding transgenic plants were obtained only very recently.

Under our experimental conditions, treatments of the *AtPAO::GFP-GUS* and *AtPAO::GUS* Arabidopsis transgenic plants with IAA and JA determined no significant variation in the GUS-staining pattern of all the analysed *AtPAOs*. Only following ABA treatment, an increase in the intensity of *AtPAO1*-related GUS signal in the meristematic/elongation transition region of the roots was observed (Fig. 20). It should be noticed that to evidence this increase, the GUS reaction was allowed to proceed for a limiting time period. ABA treatment also induced a conspicuous increase in the number of GUS-stained guard cells in *AtPAO2::GFP-GUS* Arabidopsis transgenic plants as compared the untreated plants (Fig. 20).

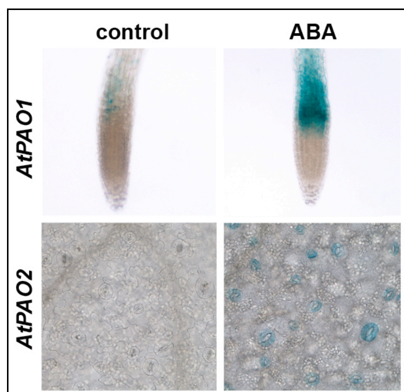

Fig. 20. *AtPAO* promoter activity in ABA-treated seedlings. Histochemical GUS-staining of *AtPAO1::GFP-GUS* and *AtPAO2::GFP-GUS* Arabidopsis transgenic plants treated with 10 μM ABA for 6 hours. For *AtPAO1*, GUS-staining reaction was allowed to proceed for 15-30 minutes, until a visible staining was produced, to permit a comparative analysis before colour saturation was reached.

On the contrary, no increase was evident either in the number or in the intensity of the GUS-stained guard cells in the *AtPAO3::GUS* transgenic plants following ABA treatment, despite the fact that *AtPAO2* and *AtPAO3* are considered recent derivatives from a common ancestor (Fincato *et al.*, 2011a).

To confirm the changes in *AtPAO1* and *AtPAO2* promoter activity following ABA treatment in the roots and the guard cells of the leaves, respectively, *AtPAO* expression levels upon ABA treatment of wild-type Arabidopsis seedlings were analysed through semi-quantitative RT-PCR using gene-specific primers. This analysis showed an increase in *AtPAO1* transcript accumulation levels compared to the untreated controls not only in roots, but also in leaves (Fig. 21).

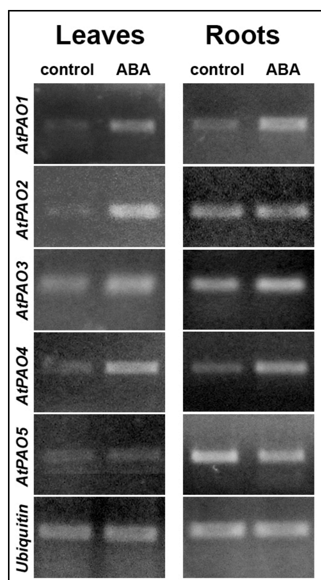

Fig. 21. Analysis of *AtPAO* expression levels following ABA treatment. The expression levels of the five *AtPAOs* were analyzed in leaves and roots of wild-type Arabidopsis plants by semi-quantitative RT-PCR using gene-specific primers. Expression of *UBQ5* was used as loading control. Results at the exponential phase (at 35 cycles for *AtPAO1* and at 30 cycles for *AtPAO2*, *AtPAO3***,** *AtPAO4*, *AtPAO5* and *UBQ5*) are shown.

The RT-PCR analysis also evidenced an ABA-inducible increase in *AtPAO2* expression levels in leaves but not in roots, in agreement with the GUS-staining results. In parallel, an increase in *AtPAO3* expression levels was also observed in leaves of ABA-treated seedlings as compared to those of the untreated seedlings, which, however, was not evident by the GUS-staining experiments (data not shown). After ABA treatment, *AtPAO4* expression levels resulted to be enhanced in both leaves and roots, while *AtPAO5* transcript levels didn’t seem to vary significantly (Fig. 21).

Discrepancies between results obtained by GUS assay and RT-PCR analysis could be due to the very small differences in *AtPAO* expression, not easily detectable by GUS assay.

AtPAO promoter activity under stress conditions

The *AtPAO::GFP-GUS* and *AtPAO::GUS* Arabidopsis transgenic seedlings for *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO5* were analyzed for GUS activity after treatment with 50 mM NaCl (salt stress), 10% (w/v) polyethylene glycol (osmotic stress and dehydration), 10 µM H_2O_2 (cell death inductor), after wounding, after cold (4°C), and heat (37°C).

Among the above mentioned treatments, only growth of the seedlings at 4°C for 18 hours determined an increase in *AtPAO5*-related GUS staining in cotyledonary surface as compared the untreated control seedlings (Fig. 22). This was confirmed by semi-quantitative RT-PCR of cold treated wild-type plants, which also revealed an increase in *AtPAO3* expression levels following the same treatment (Fig. 23).

AtPAO expression upon exogenous polyamine supply

When exogenous Spd or Spm were added to *AtPAO::GFP-GUS* seedlings, *AtPAO5*-related GUS signal increased in cotyledonary surface as compared the untreated control seedlings (Fig. 22). This result is of particular importance, since *AtPAO5* catalytic properties haven't been determined yet and no data is available about its catalytic activity towards polyamines. As regards the other analysed *AtPAOs*, their promoter activity didn't result altered following Spm or Spd supply comparing to the untreated controls. The effect of Spm and Spd on *AtPAO5* expression levels was further confirmed by semi-quantitative RT-PCR analysis of wild-type plants treated or not with these two polyamines (Fig. 23). This analysis also evidenced a Spm- and Spd-induced increase in *AtPAO2* and *AtPAO3* expression levels (Fig. 23), variations which however were not detectable by the GUS-staining experiments with the *AtPAO::GFP-GUS* seedlings (data not shown). The RT-PCR analysis also showed no Spm- and Spd-inducible change in *AtPAO1* and *AtPAO4* transcript levels. However, in the case of *AtPAO1* after Spm and Spd treatment one more band appeared at 570bp, which matches to the corresponding unspliced fragment (Fig. 23). The presence of this band suggests Spm- and Spd-inducible alternative splicing, since genomic DNA was completely absent from the DNAse-treated RNA used for the reverse transcriptase reaction, as controlled by PCR using *UBQ*- and *AtPAO1*-specific primers. More experiments are necessary to make clear the regulatory role of the alternative splicing in *AtPAO1* expression.

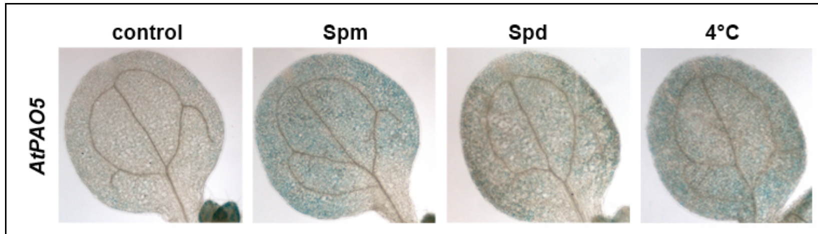

Fig. 22. *AtPAO5* promoter activity in substrate- and cold-treated seedlings. Histochemical GUS-staining of *AtPAO5::GFP-GUS* Arabidopsis transgenic plants kept at 4°C for 18 hours or treated with 500 µM Spm or Spd. GUS-staining reaction was allowed to proceed for 2 hours, until a visible staining was produced, to permit a comparative analysis before colour saturation was reached. Experiments were repeated 3 times with similar results.

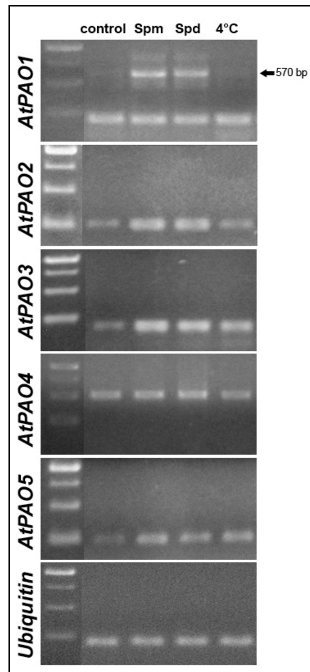

Fig. 23. Expression levels of *AtPAOs* following exogenous polyamine supply and cold treatment. The expression levels of the five *AtPAO* genes in whole wild-type seedlings kept at 4°C or treated with 500 µM Spm or Spd for 18 hours were analyzed by semi-quantitative RT-PCR using gene-specific primers. Expression of *UBQ5* was used as loading control. Results at the exponential phase (at 35 cycles for *AtPAO1* and at 30 cycles for *AtPAO2*, *AtPAO3*, *AtPAO4*, *AtPAO5* and *UBQ5*) are shown.

Analysis of *AtPAO* promoter activity during gravity response

AtPAO1, *AtPAO2*, *AtPAO3* and *AtPAO4* expression pattern in root apex may suggest their involvement in gravity response. Plants have the ability to sense the direction of gravity and to modify their growth pattern when reoriented, so that stems always head upwards and root tips always head downwards. The events leading to the curvature response (gravity perception, transmission of information and differential growth) take place in different sites. The initial event in gravity perception is the sedimentation of starch-filled amyloplasts within the columella cells (in which *AtPAO2* and *AtPAO3* are expressed as described above; Figs. 11, 12), followed by an asymmetrical redistribution of auxin in the lateral root cap, which is transmitted to the transition and the elongation regions. The asymmetrical distribution of auxin determines a differential growth of the transition and elongation regions (in which *AtPAO1* and *AtPAO4* are expressed as described above; Figs. 10, 13) so that cells in the upper part of these regions elongate faster than cells in the lower part, resulting in gravitropic curvature. To verify the involvement of AtPAOs in gravity response, *AtPAO1::GFP-GUS*, *AtPAO2::GFP-GUS* and *AtPAO3::GUS* seedlings were analysed for GUS activity after reorientation.

AtPAO1-related GUS staining in the transition region increased following reorientation by 90° (Fig. 24), supporting the hypothesis of AtPAO1 involvement in gravitropism. On the contrary, when *AtPAO2::GFP-GUS* and *AtPAO3::GUS* seedlings were reoriented by 90°, no change in GUS staining of root tips was observed (data not shown).

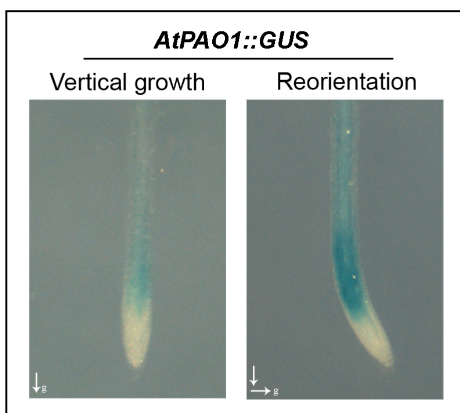

Fig. 24. *AtPAO1* expression in root tip after reorientation. Plates were reoriented by 90° after 6 days of vertical growth and histochemical GUS assay was performed 3 hours later. GUS reaction proceeded for 15-30 minutes, until a visible staining was produced, to allow a comparative analysis before colour saturation was reached. Experiments were repeated 5 times with similar results (n=10). Representative seedlings are shown. White arrows represent the direction of gravity vector (g).

Physiological roles of AtPAOs

The expression pattern of *AtPAOs* determined by histochemical GUS assay suggests a role for these enzymes in several physiological processes, as for example gravity response, control of stomata opening, pollen development and pollination. With the aim to analyse the involvementof the various *AtPAOs* in these processes, Arabidopsis insertional knockout mutants for each one of the five *AtPAOs* were obtained from the collections of Arabidopsis seeds SALK (Salk Institute Genome Analysis Laboratory collection) and SAIL (Syngenta Arabidopsis Insertion Library) (Fig. 25) and homozygous plants for the T-DNA insertion were selected (Dr L. Pomettini, graduation thesis). Furthermore, *atpao2/atpao4* and *atpao3/atpao4* double mutant plants were obtained by sexual crossing of the corresponding single mutant plants (in collaboration with Dr A. Ahou). The preparation of triple *atpao2/atpao3/atpao4* knockout mutant is currently in progress.

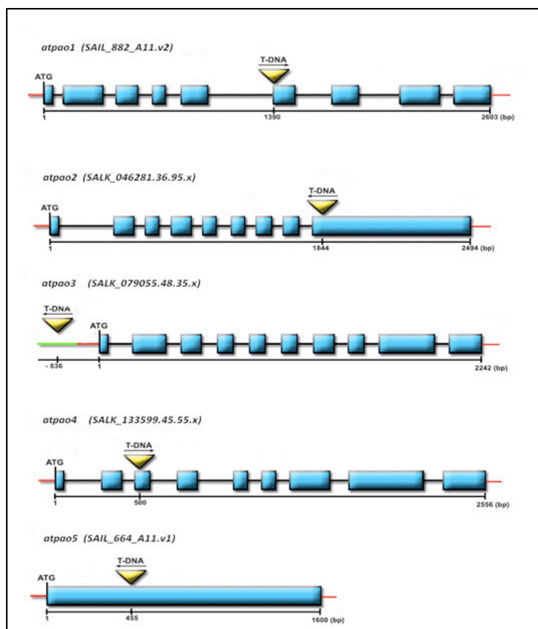

Fig. 25. Schematic representation of the T-DNA insertion site in the *atpao1*, *atpao2*, *atpao3*, *atpao4* and *atpao5* mutants. Yellow triangles indicate the T-DNA insertion. Exons of *AtPAO* genes are represented as blue boxes, whereas introns are represented as black lines. Red lines represent the 5' untranslated regions. Figure taken from Dr L. Pomettini, graduation thesis.

Gravity response in atpao *knockout mutant plants*

In order to verify whether AtPAOs take part in gravity response, single *atpao* mutants were used to measure the angle of root tip curvature after reorientation by 90°. No statically significant difference in rotation angles comparing to wild-type control plants was observed (Fig. 26). Since the lack of alterations in the gravity response of the single *atpao* insertional mutants may be due to gene redundancy within the *AtPAO* gene family, experiments to determine the gravity response of multiple *atpao insertional* mutants are in progress.

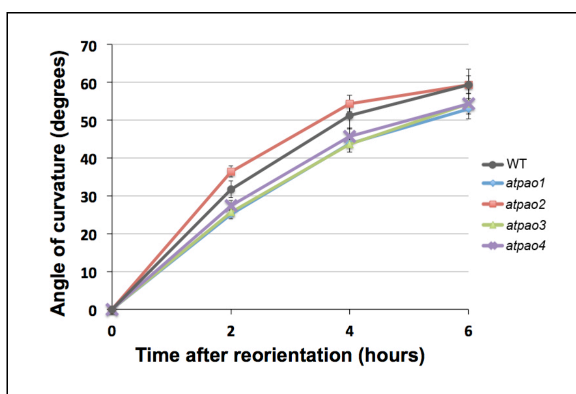

Fig. 26. Gravitropic response of *atpao* mutants. After 7 days of vertical growth of *atpao1*, *atpao2*, *atpao3* and *atpao4* knockout mutants and wild-type (WT) plants plates were reoriented by 90°. Pictures were taken at specific time points after reorientation and the angle of curvature was calculated. Experiments were repeated 5 times with similar results. Bars indicate standard deviation.

Stomatal response in atpao *knockout mutant plants*

Since ABA plays a central role in the control of stomata movements, the ABA-inducible expression of *AtPAO2* in guard cells, together with the constitutive expression of *AtPAO3* and *AtPAO4* in the same cells, lead to hypothesize involvement of the *AtPAO2-4* subfamily in the control of stomata opening. To verify this hypothesis, experiments were conducted aiming to determine stomatal closure of *atpao* knockout mutants following treatment with ABA.

Our results showed that, after ABA treatment, *atpao2*, *atpao3* and *atpao4* single knockout mutants display a reduced stomatal closure as compared to wild-type plants. Interestingly, this reduced closure was even

more pronounced for the *atpao2/atpao4* and *atpao3/atpao4* double mutants (Fig. 27). These data further suggest the involvement of the peroxisomal *AtPAOs* in the control of stomatal movements.

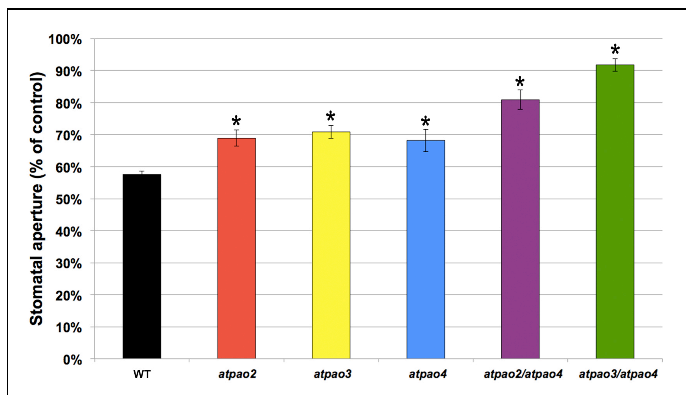

Fig. 27. Stomatal aperture in *atpao* knockout single and double mutants. Epidermal strips from *atpao2*, *atpao3* and *atpao4* single knockout mutants as well as from *atpao2/atpao4* and *atpao3/atpao4* double mutants and wild-type (WT) plants were treated with 50 µM ABA for 1 hour. Stomatal apertures (width/length) were measured and percentages of opening relative to the corresponding untreated controls are shown. Experiments were repeated 4 times with similar results and a representative experiment is shown. Asterisks indicate values statistically different from wild-type plants by one-way ANOVA test ($p<0.001$).

Transpiration in atpao knockout mutant plants

Considering the reduced stomatal closure shown by *atpao2*, *atpao3* and *atpao4* single and double mutants and since water evaporation depends on stomata opening, experiments were performed to determine the transpiration rate of *atpao* mutants and wild-type plants. Water loss was measured in terms of fresh weight reduction during drought stress. As can be seen in Fig. 28, no statistically significant difference between *atpao* mutants and wild-type plants emerged. However, to assess more precisely the role of AtPAOs in transpiration, the triple *atpao2/atpao3/atpao4* mutant plants have still to be analysed. Furthermore, experiments aiming to assess the susceptibility *of atpao2-4* mutants to dehydration are in progress.

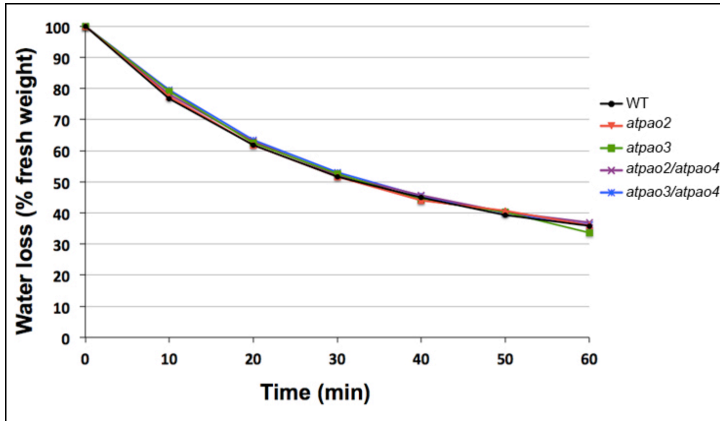

Fig. 28. Transpiration rate of *atpao* mutant plants. Experiments were repeated 5 times with similar results and a representative experiment is shown. Error bars are not shown for reason of clarity.

Part of this study was published in:
Fincato P., Moschou P. N., Ahou A., Angelini R., Roubelakis-Angelakis K. A., Federico R., Tavladoraki P. (2011b) The members of *Arabidopsis thaliana PAO* gene family exhibit distinct tissue- and organ-specific expression pattern during seedling growth and flower development. *Amino Acids*. DOI: 10.1007/s00726-011-0999-7.

Discussion

Accumulating data suggest that polyamine catabolism is more than a biochemical process aiming to control polyamine homeostasis in plants. Indeed, the H_2O_2 produced by polyamine catabolism participates in the signaling network; depending on its levels, it signals important events in different developmental processes, such as PCD and pollen tube elongation, as well as in defence responses to biotic and abiotic stresses (Yoda *et al.*, 2003, 2006; Marina *et al.*, 2008; Moschou *et al.*, 2008a, b, c, 2009; Angelini *et al.*, 2010; Wu *et al.*, 2010). In particular, H_2O_2 production by extracellular CuAO and PAO has been shown to be involved in peroxidase-mediated synthesis of suberin and lignin taking place during cell wall maturation in the course of plant development, as well as during wound healing and cell wall strengthening following pathogen attack (Angelini *et al.*, 2010). Furthermore, Yoda et al. (2003, 2006) reported that Spd and Put accumulation and PAO expression increase in the extracellular space of *Nicotiana tabacum* cells exhibiting TMV-induced hypersensitive response. Interestingly, they further showed that the cell death caused by TMV infection was partially mediated by H_2O_2 generated through polyamine catabolism in the extracellular space. In addition, Takahashi *et al.* (2003) provided evidence correlating Spm oxidation in the apoplast to induction of HR-associated defense-related genes. Polyamine catabolism in the extracellular space was also shown to be involved in defence responses against various necrotrophic and biotrophic pathogens (Marina *et al.* 2008; Moschou *et al.* 2009; Angelini *et al.* 2010). On the other hand, it has been reported that abiotic stress induces Spd exodous into the apoplast, where it is oxidized by apoplastic PAO generating H_2O_2 (Moschou *et al.*, 2008b). These data taken together reveal that in plants, the extracellular PAOs constitute a nodal point under specific abiotic and biotic stress conditions giving rise to increased apoplastic H_2O_2, which signals primary and secondary defence responses.

In contrast to the so far characterized apoplastic PAOs, which are involved in a terminal catabolic pathway of polyamines, little is known until now about the physiological role(s) of the intracellular PAOs, as for example of the cytosolic AtPAO1 and AtPAO5 and the peroxisomal AtPAO2, AtPAO3 and AtPAO4, which are involved in a polyamine back-conversion pathway. In an attempt to elucidate these roles, we analysed the expression pattern of the various AtPAOs during seedling and flower growth and development through analysis of promoter activity in *AtPAO::GUS* and *AtPAO::GFP-GUS* transgenic plants.

Our data evidenced a distinct expression pattern for each one of the five members of the *AtPAO* gene family. In particular, *AtPAO1* is highly expressed in the meristematic/elongation transition zone of the root (Fig. 18a-e), while *AtPAO2* and *AtPAO3* are expressed in the root cap (Figs. 11b; 12b). Interestingly, in the root cap differences exist also between *AtPAO2* and *AtPAO3,* although they belong to the same *AtPAO* subfamily (Fincato *et al*., 2011a). Indeed, while *AtPAO2* is expressed only near the quiescent center and columella initials, *AtPAO3* is expressed in lateral root cap and the whole columella (Figs. 11b; 12b). As far as *AtPAO4* is concerned, this gene does not seem to be expressed in the root cap, differently from the other two members of the subfamily it belongs, but it is expressed in the meristematic/elongation transition zone of the root, similarly to *AtPAO1* (Fig. 13a). *AtPAO4*-related GUS staining is also present in a small region of the meristematic zone immediately proximal to the quiescent center (Fig. 13b), whose significance is not clear yet. Furthermore, while all five genes are expressed in the differentiation zone of the roots, *AtPAO5* is specifically expressed in the vascular system of this zone (Fig. 14b-e), the other four being present also in cortical tissues (Figs. 10g; 11d; 12d).

All five *AtPAOs* exhibit distinct expression patterns also in the aerial part of the plant: *AtPAO1* is specifically expressed in the shoot apical meristem, the stipules and the leaf hydathodes (Figs. 10i, j, l), *AtPAO2* in the stipules and the leaf hydathodes (Fig. 11f, g), *AtPAO3* in the stipules, the trichomes and the guard cells (Fig. 12e-h), *AtPAO4* in the shoot apical meristem, the stipules, the leaf hydathodes and the guard cells (Fig. 13f-h), and *AtPAO5* in the vascular system of the hypocotyl and the base of the trichomes (Fig. 14a, h). In inflorescences, *AtPAO1* and *AtPAO5* are specifically expressed in the microspores and the tapetum (Figs. 15c-f; 20b, c), while *AtPAO2* and *AtPAO3* are specifically expressed in young pistils (Figs. 16c, d; 17a-c; 18d) and pollen grains (Figs. 16e-h; 17e-h; 18e-h). Converesely, *AtPAO4* seems to have an overlapping expression pattern with that of the other *AtPAOs*, being expressed in the microspores and the tapetum (Fig. 18b, c), similarly to *AtPAO1* and *AtPAO5*, as well as in pistil walls and pollen grains (Fig. 18d-h), similarly to *AtPAO2* and *AtPAO3*. Also *AtPAO5* exhibits a overlapping expression pattern with that of the other *AtPAOs*, though with some differences. In particular, although it is initially expressed in anther tapetal cells (Fig. 19b, c) and then in the anther–filament junction site (Fig. 19e) similarly to *AtPAO1*, *AtPAO5* is also expressed in the upper part of the filament (Fig. 19e). In addition, *AtPAO5* is expressed in the stigma (similarly to *AtPAO2*, *AtPAO3* and *AtPAO4*) and in the septum (similarly to *AtPAO2* and *AtPAO3*), but not in the ovary wall (Fig. 19a, d) as do instead *AtPAO2*, *AtPAO3* and *AtPAO4* (Figs. 16c, d; 17a-c; 19d). Furthermore, *AtPAO5* is not expressed in pollen

grains, in contrast to *AtPAO2*, *AtPAO3* and *AtPAO4*. All these data together indicate important differences in the tissue- and organ-specific expression patern among the various AtPAOs, which probably indicate distinct physiological role(s) for each member of the *AtPAO* gene family.

Some features of this analysis of *AtPAO* expression pattern during seedling and flower development are in agreement with the information available on TAIR website (Arabidopsis eFP Browser), as for example *AtPAO2*, *AtPAO3* and *AtPAO4* expression in mature pollen grains; on the contrary, some important and highly repeatable GUS-staining patterns are missing from TAIR website, as for example *AtPAO3* expression in columella, *AtPAO1*, *AtPAO4* and *AtPAO5* expression in the tapetum and *AtPAO5* expression in vascular tissues.

The expression in root tip of *AtPAO1*, *AtPAO2*, *AtPAO3* and *AtPAO4* could suggest their involvement in gravity perception and/or response. Indeed, a change in plant orientation within the gravity field is initially sensed through sedimentation of starch-filled plastids in the columella; this information is then transmitted to the transition and elongation regions inducing cells in the upper part of these regions to elongate faster than cells in the lower part thus allowing root bending towards the gravity vector (Blancaflor and Masson, 2003). This differential elongation is promoted by a gravity-induced auxin gradient, whose asymmetrical distribution is initiated in the lateral root cap and reaches the elongation region. Nevertheless, when exogenous IAA was supplied to *AtPAO1::GFP-GUS*, *AtPAO2::GFP-GUS* and *AtPAO3::GUS* seedlings, no change in *AtPAO* expression levels was observed. Also following seedling reorientation, no change in the expression pattern of *AtPAO2* and *AtPAO3* was observed. Only for *AtPAO1*, reorientation induced an increase in GUS-staining intensity in the meristematic/elongation transition zone of the root, supporting the hypothesis that AtPAO1 is involved in the differential growth of the cells during root bending toward the gravity vector (Fig. 24). However, when the angles of root curvature following reorientation were measured for *atpao1*, *atpao2, atpao3* and *atpao4* insertional mutants, no statistically significant difference was observed as compared the wild-type plants (Fig. 26). Although multiple mutants still have to be analysed, the existing data seem to exclude a role of AtPAO1, AtPAO2, AtPAO3 and AtPAO4 in the gravitotropic response.

ABA plays a pivotal role in the response of plants to abiotic stresses, such as drought, salinity and cold, acting as a key regulator of stomatal apertures to restrict transpiration and reduce water loss. Several components are involved in the ABA signalling network, among which ROS are important second messengers. In *A. thaliana*, two plasma membrane-associated NADPH oxidases (AtrbohD and AtrbohF) were shown to be

implicated in ABA-induced production of ROS in guard cells (Kwak *et al*., 2003). Furthermore, in *Vicia faba* a CuAO is an essential enzymatic source for H_2O_2 production in ABA-induced stomatal closure via Put oxidation (An *et al*., 2008). The ABA-inducible *AtPAO2* expression in guard cells, together with the constitutive expression of *AtPAO3* and *AtPAO4* in guard cells, suggests a role of these enzymes in the control of stomata closure/aperture through H_2O_2 production. Indeed, single and double loss-of-function mutants for these three peroxisomal AtPAOs displayed a reduced stomatal closure when ABA was supplied (Fig. 27). However, a role of the peroxisomal *AtPAOs* in the ABA-inducible modulation of stomatal apertures through regulation of polyamine homeostasis is also possible. Indeed, polyamines have been shown to modulate stomatal apertures through a direct effect on the voltage-dependent inward K^+ channel in the plasma membrane of the guard cells (Liu *et al*., 2000). Studies will be performed to determine the underlying mechanisms in the contribution of the peroxisomal AtPAOs to the regulation of stomata movements.

The ABA-inducible expression of *AtPAO1* in the root transition zone and of *AtPAO2* in guard cells also implies a role of these enzymes in plant responses to abiotic stresses. An ABA-dependent environmental stress-inducible expression was also observed for other genes involved in polyamine metabolism, as for example an arginine decarboxylase gene (*ADC2*), a spermidine-synthase gene (*SPDS1*) and a spermine-synthase gene (*SPMS*) (Alcázar *et al*., 2010b). Furthermore, Toumi *et al*. (2010) reported that ABA signals reorientation of polyamine metabolism to regulate the generation of H_2O_2, which further signals stress responses. Further work will explore the physiological roles of AtPAOs in stress responses.

Interestingly, *AtPAOs* are expressed in meristematic tissues. In particular, *AtPAO1* is present in the shoot apical meristem, *AtPAO2* in the quiescent center and columella initials, *AtPAO4* in the shoot apical meristem and in a part of root meristem. Moreover, all five *AtPAO* genes are expressed in pericycle, from which lateral roots originate. In these tissues, AtPAOs could participate in cell cycle progression through regulation of polyamine levels. Indeed, polyamine concentration has been shown to vary during cell cycle. In particular, Put level increases during S and G2 phases, that of Spd during the entire cycle and that of Spm mainly during G1 and S phases (Thomas and Thomas, 2001). It has been suggested that Put is essential for the cell to enter S-phase, possibly driving the cell through the G1 restriction point prior to DNA synthesis and by the regulation of cyclin D1 (Wallace *et al*., 2003).

The expression of *AtPAO2*, *AtPAO3* and *AtPAO4* in mature pollen grains and in pollen tubes (Figs. 16e-h; 17e-h; 18e-h), may indicate their involvement in pollen tube growth and in fertilization. Indeed, recent results showed that in *A. thaliana*, H_2O_2 produced by PAO-mediated Spd oxidation triggers the opening of hyperpolarization-activated Ca^{2+}-permeable channels in pollen, thereby regulating pollen tube growth (Wu *et al*., 2010). Furthermore, in pollen from *atpao3* loss-of-function mutants, the activation of Ca^{2+} currents by Spd was significantly disrupted resulting in reduced pollen tube growth and seed number (Wu *et al*., 2010). The role in pollen tube growth of the peroxisomal metabolic pathways in general and of the peroxisomal polyamine catabolic pathways in particular is still unknown. Functional peroxisomes have been shown to be necessary for Arabidopsis fertilization (Boisson-Dernier *et al*., 2008), probably for their production of a large range of signalling molecules, among which ROS and NO (Nyathi and Baker, 2006). Importantly, NO has been shown to be produced in peroxisomes of pollen tubes, and to reorient pollen-tube growth *in vitro* (Prado *et al*., 2004).

AtPAO1, *AtPAO4* and *AtPAO5* expression in the tapetum (Figs. 15c-f; 18b, c; 19b, c) is of great interest, since during stamen maturation this tissue is characterized by the production and secretion of large quantities of proteins and a complex array of small metabolites essential for maturation and differentiation of pollen grains. Following maturation of pollen grains, the tapetum is lysed and undergoes PCD releasing the materials necessary for the formation of pollen cell wall, which displays a high degree of mechanical resistance and is structurally the most complex type of plant cell wall. The exact role of AtPAO1, AtPAO4 and AtPAO5 in these steps of flower development is not clear yet. They may have a role in stamen maturation through their reaction products (as for example H_2O_2, which, as described above, is a well-known inducer of PCD) and/or regulation of polyamine levels. Indeed, both free and conjugated polyamines have been shown to be involved in flower development (Rastogi and Sawhney, 1990). Interestingly, genes involved in the production of conjugated Spd have been recently shown to be specifically expressed in anther tapetum cells, while RNAi plants and knockout mutants presented alterations in pollen grains and impaired silique development (Fellenberg *et al*., 2008; Grienenberger *et al*., 2009).

The semi-quantitative RT-PCR analysis of ABA, Spm, Spd and cold treatments confirmed the results obtained through histochemical GUS assay, but also showed some variations in *AtPAO* expression levels that weren't detectable through GUS assay. These discrepancies could be ascribed to the high stability of GUS signal, which makes it difficult to evidence small quantitative differences and to assess dynamic changes in

gene expression. Interestingly, semi-quantitative RT-PCR showed a putative Spm- and Spd-inducible alternative splicing for *AtPAO1* (Fig. 23), whose physiological significance is not clear yet and which is currently under investigation. This alternative splicing may have a regulatory role, as already assessed for other PAOs. Indeed, in mouse different gene splicing variants of SMO were found, which display similar biochemical characteristics but different subcellular localization (Cervelli *et al*., 2004).

In conclusion, the present study provides evidence for important differences in the spatial and temporal expression pattern of the various Arabidopsis PAOs, which, together with the distinct catalytic properties, suggest distinct physiological roles for each member of *A. thaliana PAO* gene family. In particular, our results suggest that Arabidopsis PAOs may be involved in root growth, in pollen maturation and thus in plant reproduction, in the control of stomatal opening and in vascular system development and function. This study will contribute to a detailed analysis of the physiological roles of the polyamine catabolic pathways in plants.

References

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

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

Alcázar R., Planas J., Saxena T., Zarza X., Bortolotti C., Cuevas J.C., Bitrián M., Tiburcio A.F., Altabella T. (2010a) Putrescine accumulation confers drought tolerance in transgenic Arabidopsis plants over-expressing the homologous Arginine decarboxylase 2 gene. *Plant Physiol. Biochem.* **48**:547–52.

Alcázar R., Altabella T., Marco F., Bortolotti C., Reymond M., Koncz C., Carrasco P., Tiburcio A.F. (2010b) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* **231**:1237–1249.

Alcázar R., Bitrián M., Bartels D., Koncz C., Altabella T., Tiburcio A.F. (2011) Polyamine metabolic canalization in response to drought stress in Arabidopsis and the resurrection plant *Craterostigma plantagineum. Plant Signal. Behav.* **6**:243-250.

Allan A.C., Fluhr R. (1997) Two distinct sources of elicited reactive oxygen. *Plant Cell*. **9**:1559-1572.

Altabella T., Tiburcio A.F., Ferrando A. (2009) Plant with resistance to low temperature and method of production thereof. Spanish patent application WO2010/004070.

Amirsadeghi S., Robson C.A., Vanlerberghe G.C. (2007) The role of the mitochondrion in plant responses to biotic stress. *Physiol. Plant.* **129**: 253-266.

An Z.F., Jing W., Liu Y.L., Zhang W.H. (2008) Hydrogen peroxide generated by copper amine oxidase is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *J. Exp. Bot.* **59**:815–825.

Angelini R., Manes F., Federico R. (1990) Spatial and functional correlation between diamine-oxidase and peroxidase activities and their dependence upon de-etiolation and wounding in chick-pea stems. *Planta* **182**:89-96.

Angelini R., Tisi A., Rea G., Chen M.M., Botta M., Federico R., Cona A. (2008) Involvement of polyamine oxidase in wound healing. *Plant Physiol*. **146**:162–177.

Angelini R., Cona A., Federico R., Fincato P., Tavladoraki P., Tisi A. (2010) Plant amine oxidases on the “move”: an update. Plant *Physiol. Biochem*. **48**:560–564.

Apel K., Hirt H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**:373-399.

Aziz A., Martin-Tanguy J., Larher F. (1997) Plasticity of polyamine metabolism associated with high osmotic stress in rape leaf discs and with ethylene treatment. *Plant Growth Regul.* **21**:153-163.

Aziz A., Martin-Tanguy J., Larher F. (1998) Stress-induced changes in polyamine and tyramine levels can regulate proline accumulation in tomato leaf discs treated with sodium chloride. *Physiol. Plant*. **104**:195–202.

Bagni N., Tassoni A. (2001) Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. *Amino Acids* **20**:301–317.

Baluska F, Mancuso S, Volkmann D, Barlow PW. (2010) Root apex transition zone: a signalling-response nexus in the root. *Trends Plant Sci*. Jul;15(7):402-8.

Bellelli A., Angelini R., Laurenzi M., Federico R. (1997) Transient kinetics of polyamine oxidase from *Zea mays* L. *Arch. Biochem. Biophys.* **343**:146-148.

Blancaflor E.B., Masson P.H. (2003) Plant gravitropism. Unraveling the ups and downs of a complex process. *Plant Physiol*. **133**4:1677-90.

Boisson-Dernier A., Frietsch S., Kim T.H., Dizon M.B., Schroeder J.I. (2008) The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition. *Curr Biol*. **18**:63-8.

Bouché N., Fromm H. (2004) GABA in plants: just a metabolite? *Trends Plant Sci.* **9**:110-115.

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

Bruggemann L.I., Pottosin I.I., Schonknecht G. (1998) Cytoplasmic polyamines block the fast-activating vacuolar cation channel. *Plant J*. **16**:101–105.

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

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

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

Cervelli M., Bellini A., Bianchi M., Marcocci L., Nocera S., Polticelli F., Federico R., Amendola R., Mariottini P. (2004) Mouse spermine oxidase gene splice variants: nuclear subcellular localization of a novel active isoform. *Eur. J. Biochem*. **271**:760–770.

Cervelli M., Bianchi M., Cona A., Crosatti C., Stanca M., Angelini R., Federico R., Mariottini P. (2006) Barley polyamine oxidase isoforms 1 and 2, a peculiar case of gene duplication. *FEBS J.* **273**: 3990-4002.

Clay N., Nelson T. (2005) Arabidopsis thickvein mutation affects vein thickness and organ vascularization, and resides in a provascular cellspecific spermine synthase involved in vein definition and in polar auxin transport. *Plant Physiol*. **138**:767–777.

Cohen S.S. (1998) A guide to polyamines. Oxford University Press, New York.

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

Cuevas J.C., Lopez-Cobollo R., Alcázar R., Zarza X., Koncz C., Altabella T., Salinas J., Tiburcio A.F., Ferrando A. (2008) Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. *Plant Physiol*. **148**:1094–1105.

Eisenberg T., Knauer H., Schauer A., Büttner S., Ruckenstuhl C., Carmona-Gutierrez D., Ring J., Schroeder S., Magnes C., Antonacci L., Fussi H., Deszcz L., Hartl R., Schraml E., Criollo A., Megalou E., Weiskopf D., Laun P., Heeren G., Breitenbach M., Grubeck-Loebenstein B., Herker E., Fahrenkrog B., Fröhlich K.U., Sinner F., Tavernarakis N., Minois N., Kroemer G., Madeo F. (2009) Induction of autophagy by spermidine promotes longevity. *Nat. Cell. Biol*. **11**:1305–1314.

Federico R., Cona A., Angelini R., Schininà M.E., Giartosio A. (1990) Characterization of maize polyamine oxidase. *Phytochemistry* **29**:2411-2414.

Federico R., Angelini R. (1991) Polyamine catabolism. In: Slocum R. D., Flores H.E. (Eds.), Biochemistry and physiology of polyamines in plants. *CRC Press*, Boca Raton, FL. 41-56.

Fellenberg C., Milkowski C., Hause B., Lange P.R., Böttcher C., Schmidt J., Vogt T. (2008) Tapetum-specific location of a cation-dependent O-methyltransferase in Arabidopsis thaliana. *Plant J*. **56**:132-45.

Fincato P., Moschou P.N., Spedaletti V., Tavazza R., Angelini R., Federico R., Roubelakis-Angelakis K.A., Tavladoraki P. (2011a) Functional diversity inside the Arabidopsis polyamine oxidase gene family. *J. Exp. Bot*. **62**:1155–1168.

Fincato P., Moschou P. N., Ahou A., Angelini R., Roubelakis-Angelakis K. A., Federico R., Tavladoraki P. (2011b) The members of *Arabidopsis thaliana PAO* gene family exhibit distinct tissue- and organ-specific expression pattern during seedling growth and flower development. *Amino Acids*. DOI: 10.1007/s00726-011-0999-7.

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

Galston A.W. and Sawhney R.K. (1995) Polyamines as endogenous growth regulators. In *Plant hormones: Physiology, biochemistry and molecular biology (2nd edn)*. Davies PJ (Ed). Kluwer Academic Publishers, Dordrecht, The Netherlands. 158-178.

Ge C., Cui X., Wang Y., Hu Y., Fu Z., Zhang D., Cheng Z., Li J. (2006) *BUD2*, encoding an S-adenosylmethionine decarboxylase, is required for *Arabidopsis* growth and development. *Cell Res.* **16**:446-456.

Gilad G.M., Gilad V.H., Rabey J.M. (1996) Arginine and ornithine decarboxylation in rodent brain: coincidental changes during development and after ischemia. *Neurosci. Lett.* **216**:33-36.

Gill S., Tuteja N. (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signal. Behav*. **5**:26-33.

Grienenberger E., Besseau S., GeoVroy P., Debayle D., Heintz D., Lapierre C., Pollet B., Heitz T., Legrand M. (2009) A BAHD acyltransferase is expressed in the tapetum of Arabidopsis anthers and is involved in the synthesis of hydroxycinnamoyl spermidines. *Plant J.* **58**:246–259.

Groppa M.D., Benavides M.P. (2008) Polyamines and abiotic stress: recent advances. *Amino Acids* **34**:35–45.

Han W, Rong H, Zhang H, Wang MH. (2009) Abscisic acid is a negative regulator of root gravitropism in *Arabidopsis thaliana*. *Biochem Biophys Res Commun*. Jan 23;378(4):695-700 nel testo c’è scritto 2008

Hanfrey C, Sommer S, Mayer MJ, Burtin D, Michael AJ (2001) Arabidopsis polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. *Plant J.* **27**:551–560.

Hanson A.D., Rathinasabapathi B., Rivoal J., Burnet M., Dillon M.O., Gage D.A. (1994) Osmoprotective compounds in the *Plumbaginaceae*: a natural experiment in metabolic engineering of stress tolerance. *Proc. Natl. Acad. Sci. USA* **91**:306-310.

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

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

He L., Nada K., Kasukabe Y., Tachibana S. (2002) Enhanced susceptibility of photosynthesis to low-temperature photoinhibition due to interruption of chill-induced increase of S-adenosylmethionine decarboxylase activity in leaves of spinach (*Spinacia oleracea* L.). *Plant Cell Physiol.* **43**:196-206.

Hewezi T., Howe P.J., Maier T.R., Hessey R.S., Mitchum M.G., Davis E.L., Baum T.J. (2010) Arabidopsis spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiol*. **152**:968–984.

Higashiyama T., Hamamura Y. (2008) Gametophytic pollen tube guidance *Sex. Plant Reprod*. **21**:17–26.

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

Hummel I., Bourdais G., Gouesbet G., Couée I., Malmberg R.L., El Amrani A. (2004) Differential expression of arginine decarboxylase *ADC1* and *ADC2* in *Arabidopsis thaliana*: characterization of transcriptional regulation during
seed germination and seedling development. *New Phyt.* **163**:519-531.

Igarashi K., Kashiwagi K. (2000) Polyamines: mysterious modulators of cellular functions. *Biochem. Biophys. Res. Commun*. **271**:559–564.

Imai A., Akiyama T., Kato T., Sato S., Tabata S., Yamamoto K.T., Takahashi T. (2004a) Spermine is not essential for survival of Arabidopsis. *FEBS Lett*. **556**:148–152.

Imai A., Matsuyama T., Hanzawa Y., Akiyama T., Tamaoki M., Saji H., Shirano Y., Kato T., Hayashi H., Shibata D., Tabata S., Komeda Y., Takahashi T. (2004b) Spermidine synthase genes are essential for survival of Arabidopsis. *Plant Physiol*. **135**:1565–1573.

Jefferson R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep*. **5**:387–405.

Kakehi J.I., Kuwashiro Y., Niitsu M., Takahashi T. (2008) Thermospermine is required for stem elongation in *Arabidopsis thaliana*. *Plant Cell Physiol*. **49**:1342–1349.

Kamada-Nobusada T., Hayashi M., Fukazawa M., Sakakibara H., Nishimura M. (2008) A putative peroxisomal polyamine oxidase, AtPAO4, is involved in polyamine catabolism in *Arabidopsis thaliana*. *Plant Cell Physiol*. **49**:1272–1282.

Karimi M., Inzé D., Depicker A. (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* **7**:193-195.

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

Kaur H., Heinzel N., Schöttner M., Baldwin I.T., Gális I. (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoidpolyamine conjugates, which are essential for local and systemic defense against insect herbivores in Nicotiana attenuata. *Plant Physiol*. **152**:1731–1747.

Knott J.M., Romer P., Sumper M. (2007) Putative spermine synthases from *Thalassiosira pseudonana* and *Arabidopsis thaliana* synthesize thermospermine rather than spermine. *FEBS Lett*. **581**:3081–3086.

Koc E.C., Bagga S., Songstad D.D., Betz S.R., Kuehn G.D., Phillips G.C. (1998) Occurrence of uncommon polyamines in cultured tissues of maize. *In vitro Cell. Dev. Biol. Plant* **34**:252-255.

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

Kusano T., Yamaguchi K., Berberich T., Takahashi Y. (2007a) Advances in polyamine research in 2007. *J. Plant Res.* **120**:345-350.

Kusano T., Yamaguchi K., Berberich T., Takahashi Y. (2007b) The polyamine spermine rescues *Arabidopsis* from salinity and drought stresses. *Plant Signal Behav*. **2**:250-251.

Kusano T., Berberich T., Tateda C., Takahashi Y. (2008) Polyamines: essential factors for growth and survival. *Planta* **228**:367-381.

Kwak J.M., Mori I.C., Pei Z.M., Leonhardt N., Torres M.A., Dangl J.L., Bloom R.E., Bodde S., Jones J.D., Schroeder J.I. (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J*. **22**:2623-33.

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

Li J., Doyle K.M., Tatlisumak T. (2007) Polyamines in the brain: distribution, biological interactions, and their potential therapeutic role in brain ischaemia. *Curr. Med. Chem.* **14**:1804–1813.

Lim T.S., Chitra T.R., Han P., Pua E.C., Yu H. (2006) Cloning and characterization of *Arabidopsis* and *Brassica juncea* flavincontaining amine oxidases. *J. Exp. Bot*. **57**:4155–4169.

Liu .K, Fu H., Bei Q., Luan S. (2000) Inward potassium channel in guard cells as a target for polyamine regulation of stomatal movements. *Plant Physiol*. **124**:1315–1326.

Liu J.H., Kitashiba H., Wang J., Ban Y., Moriguchi T. (2007) Polyamines and their ability to provide environmental stress tolerance to plants. *Plant Biotechnol.* **24**:117-126.

Malmberg R.L., Watson M.B., Galloway G.L., Yu W. (1998) Molecular genetic analyses of plant polyamines. *Crit. Rev. Plant Sci*. **17**:199–224.

Marina M., Maiale S.J., Rossi F.R., Romero M.F., Rivas E.I., Gárriz A., Ruiz O.A., Pieckenstain F.L. (2008) Apoplastic polyamine oxidation plays different roles in local responses of tobacco to infection by the necrotrophic fungus *Sclerotinia sclerotiorum* and the biotrophic bacterium *Pseudomonas viridiflava*. *Plant Physiol.* **147**:2164–2178.

Marini F., Betti L., Scramagli S., Biondi S., Torrigiani P. (2001) Polyamine metabolism is upregulated in response to tabacco mosaic virus in hypersensitive, but not in susceptible, tobacco. *New Phytol.* **149**:301-309.

Martin-Tanguy J. (1997) Conjugated polyamines and reproductive development: Biochemical, molecular and physiological approaches. *Physiol Plant* **100**:675–688.

Medda R., Padiglia A., Pedersen J.Z., Rotilio G., Finazzi Agrò A., Floris G. (1995) The reaction mechanism of copper amine oxidase: detection of intermediates by the use of substrates and inhibitors. *Biochemistry* **34**:16375-16381.

Minguet E.G., Vera-Sirera F., Marina A., Carbonell J., Blazquez M.A. (2008) Evolutionary diversiWcation in polyamine biosynthesis. *Mol. Biol. Evol*. **25**:2119–2128.

Mitsuya Y., Takahashi Y., Uehara Y., Berberich T., Miyazaki A., Takahashi H., Kusano T. (2007) Identification of a novel Cys2/ His2-type zinc finger protein as a component of a spermine signaling pathway in tobacco. *J. Plant Physiol*. **164**:785–793.

Mitsuya Y., Takahashi Y., Berberich T., Miyazaki A., Matsmura H., Takahashi H., Terauchi R., Kusano T. (2009) Spermine signaling plays a significant role in the defence response of *Arabidopsis thaliana* to cucumber mosaic virus, *J. Plant Physiol*. **166**:626–643.

Mittler R., Vanderauwera S., Gollery M., Van Breusegem F. (2004) Reactive oxygen gene networks of plants. *Trends Plant Sci.* **9**:490-498.

Møller S.G., McPherson M.J. (1998) Developmental expression and biochemical analysis of the Arabidopsis *ATAO1* gene encoding an H_2O_2-generating diamine oxidase. *Plant J*. **13**:781–791.

Møller S.G., Urwin P.E., Atkinson H.J., McPherson M.J. (1998) Nematode-induced expression of atao1, a gene encoding an extracellular diamine oxidase associated with developing vascular tissue. *Physiol Mol Plant Pathol* **53**:73–79.

Moschou P.N., Sanmartin M., Andriopoulou A.H., Rojo E., Sanchez-Serrano J.J., Roubelakis-Angelakis K.A. (2008a) Bridging the gap between plant and mammalian polyamine catabolism: a novel peroxisomal

polyamine oxidase responsible for a full back-conversion pathway in Arabidopsis. *Plant Physiol*. **147**:1845–1857.

Moschou P.N., Delis I.D., Paschalidis K.A., Roubelakis-Angelakis K.A. (2008b) Transgenic tobacco plants overexpressing polyamine oxidase are not able to cope with oxidative burst generated by abiotic factors. *Physiol. Plant*. **133**:140–156.

Moschou P.N., Paschalidis K.A., Delis I.D., Andriopoulou A.H., Lagiotis G.D., Yakoumakis D.I., Roubelakis-Angelakis K.A. (2008c) Spermidine exodus and oxidation in the apoplast induced by abiotic stress is responsible for H_2O_2 signatures that direct tolerance responses in tobacco. *Plant Cell* **20**:1708–1724.

Moschou P.N., Paschalidis K.A., Roubelakis-Angelakis K.A. (2008d) Plant polyamine catabolism: the state of the art. *Plant Signal. Behav*. **3**:1061–1066.

Moschou P.N., Sarris P.F., Skandalis N., Andriopoulou A.H., Paschalidis K.A., Panopoulos N.J., Roubelakis-Angelakis K.A. (2009) Engineered polyamine catabolism preinduces tolerance of tobacco to bacteria and oomycetes. *Plant Physiol*. **149**:1970–1981.

Nakagawa T., Suzuki T., Murata S., Nakamura S., Hino T., Maeo K., Tabata R., Kawai T., Tanaka K., Niwa Y., Watanabe Y., Nakamura K., Kimura T., Ishiguro S. (2007) Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci Biotechnol Biochem.* **71**:2095-100.

Nayyar H., Kaur S., Singh S., Kumar S., Singh K.J., Dhir K.K. (2005) Involvement of polyamines in the contrasting sensitivity of chickpea (*Cicer arietinum* L.) and soybean (*Glycine max* (L.) Merrill.) to water deficit stress. *Bot. Bull. Acad. Sin.* **46**:333-338.

Nyathi, Y., and Baker, A. (2006). Plant peroxisomes as a source of signalling molecules. *Biochim. Biophys. Acta* **1763**:1478–1495.

Ono Y., Kim D.W., Watanabe K., Sasaki A., Niitsu M., Berberich T., Kusano T., Takahashi Y. (2011) Constitutively and highly expressed *Oryza sativa* polyamine oxidases localize in peroxisomes and catalyze polyamine back conversion. *Amino Acids* DOI 10.1007/s00726-011-1002-3.

Palanivelu R., Brass L., Edlund A.F., Preuss D. (2003) Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. *Cell* **114**:47–59.

Pandey S., Ranade S.A., Nagar P.K., Kumar N. (2000) Role of polyamines and ethylene as modulators of plant senescence. *J. Biosci*. **25**:291–299.

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

Park J.H., Aravind L., Wolff E.C., Kaevel J., Kim Y.S., Park M.H. (2006) Molecular cloning, expression, and structural prediction ofdeoxyhypusine hydroxylase: a HEAT-repeat-containing metalloenzyme. *Proc. Natl. Acad. Sci. USA* **103**:51–56.

Paschalidis K.A., Roubelakis-Angelakis K.A. (2005) Sites and regulation of polyamine catabolism in the tobacco plant. Correlations with cell division/expansion, cell cycle progression, and vascular development. *Plant Physiol.* **138**:2174-2184.

Paschalidis K.A., Toumi I., Moschou P.N., Roubelakis-Angelakis K.A. (2010) ABA-dependent amine oxidases-derived H_2O_2 affects stomata conductance. *Plant Signal. Behav*. **5**:1153-1156.

Pegg A.E. (1988) Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer Res*. **48**:759–774.

Pegg A.E. (2008) Spermidine/spermine-N^1-acetyltransferase: a key metabolic regulator. *Am. J. Physiol. Endocrinol. Metab*. **294**:E995-1010.

Pegg A.E., Feith D.J. (2007) Polyamines and neoplastic growth. *Biochem. Soc. Trans*. **35**:295–299.

Pegg A.E., Michael A. (2010) Spermine synthase. *Cell Mol. Life Sci*. **67**:113–121.

Pérez-Amador M.A., Leon J., Green P.J., Carbonell J. (2002) Induction of the arginine decarboxylase *ADC2* gene provides evidence for the

involvement of polyamines in the wound response in *Arabidopsis*. *Plant Physiol.* **130**:1454-1463.

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

Prado, A.M., Porterfield, D.M., Feijo, J.A. (2004) Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development* **131**:2707–2714.

Quinet M., Ndayiragije A., Lefèvre I., Lambillotte B., Dupont-Gillain C.C., Lutts S. (2010) Putrescine differently influences the effect of salt stress on polyamine metabolism and ethylene synthesis in rice cultivars differing in salt resistance. *J. Exp. Bot*. **61**:2719–2733.

Rajam M.V. (1989) Restriction of pollen germination and tube growth in lily pollen by inhibitors of polyamine metabolism. *Plant Sci.* **59**:53-56.

Raman S.B., Rathinasabapathi B. (2003) β-alanine *N*-methyltransferase of *Limonium latifolium* cDNA cloning and functional expression of a novel *N*-methyltransferase implicated in the synthesis of the osmoprotectant β-alanine betaine. *Plant Physiol.* **132**:1642-1651.

Rastogi R., Sawhney V.K. (1990) Polyamines and flower development in the male sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum* Mill.). I. Level of polyamines and their biosynthesis in normal and mutant flowers. *Plant Physiol*. **93**:439–445.

Rea G., Metoui O., Infantino A., Federico R., Angelini R. (2002) Copper amine oxidase in defense response to wounding and *Ascochyta rabiei* invasion. *Plant Physiol.* **128**:865-875.

Roy M., Ghosh B. (1996) Polyamines, both common and uncommon, under heat stress in rice (*Oryza sativa*) callus. *Physiol. Plant.* **98**:196-200.

Sagor G.H.M., Cong R.Z., Berberich T., Takahashi H., Takahashi Y., Kusano T. (2009) Spermine signaling in defense reaction against avirulent viral pathogen in *Arabidopsis thaliana*. *Plant Signal. Behav*. **4**:316–318.

Schipper R.G., Penning L.C., Verhofstad A.A. (2000) Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin. Cancer Biol.* **10**:55-68.

Seiler N. (1995) Polyamine oxidase, properties and functions. *Prog. Brain Res.* **106**:333-344.

Seiler N. (2004) Catabolism of polyamines. *Amino Acids* **26**:217-233.

Seiler N., Raul F. (2005) Polyamines and apoptosis. *J. Cell Mol. Med.* **9**:623–642.

Seiler N., Raul F (2007) Polyamines and the intestinal tract. *Crit. Rev. Clin. Lab. Sci*. **44**:365–411.

Shabala S., Cuin T.A., Pottosin I. (2007) Polyamines prevent NaCl-induced K^+ efflux from pea mesophyll by blocking non-selective cation channels. *FEBS Lett*. **581**:1993-1999.

Sharma S.S., Dietz K.J. (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J. Exp. Bot*. **57**:711–726.

Shi J., Fu X.Z., Peng t., Huang X.S., Fan Q.J., Liu J.H. (2010) Spermine pretreatment confers dehydration tolerance of citrus in vitro plants via modulation of antioxidative capacity and stomatal response. *Tree Physiol*. **30**:914-922.

Tabor C.W., Tabor H. (1984) Polyamines. *Annu. Rev. Biochem*. **53**:749–790.

Takahashi Y., Berberich T., Miyazaki A., Seo S., Ohashi Y., Kusano T. (2003) Spermine signalling in tobacco: activation of mitogen-activated protein kinases by spermine is mediated through mitochondrial dysfunction. *Plant J.* **36**:820-829.

Takahashi Y., Cong R., Sagor G.H., Niitsu M., Berberich T., Kusano T. (2010) Characterization of five polyamine oxidase isoforms in *Arabidopsis thaliana*. *Plant Cell Rep*. **29**:955–965.

Tavladoraki P., Schininà M.E., Cecconi F., Di Agostino S., Manera F., Rea G., Mariottini P., Federico R., Angelini R. (1998) Maize polyamine

oxidase: primary structure from protein and cDNA sequencing. *FEBS Lett.* **426**:62-66.

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

Tavladoraki P., Cona A., Federico R., Tempera G., Viceconte N., Saccoccio S., Battaglia V., Toninello A., Agostinelli E. (2011) Polyamine catabolism: target for antiproliferative therapies in animals and stress tolerance strategies in plants. *Amino Acids* DOI: 10.1007/s00726-011-1012-1.

Terano S., Suzuki Y. (1978) Formation of β-alanine from spermine and spermidine in maize shoots. *Phytochemistry* **7**:148-149.

Thomas T., Thomas T.J. (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell. Mol. Life Sci.* **58**:244-258.

Tisi A., Federico R., Moreno S., Lucretti S., Moschou P.N., Roubelakis-Angelakis K.A., Angelini R., Cona A. (2011) Perturbation of polyamine catabolism can strongly affect root development and xylem differentiation. *Plant Physiol*. **157**:200–215.

Torrigiani P., Rabiti A.L., Bortolotti G., Betti L., Marani F., Canova A., Bagni N. (1997) Polyamine synthesis and accumulation in the hypersensitive response to TMV in *Nicotiana tabacum*. *New Phytol*. **135**: 467-473.

Toumi I., Moschou P.N., Paschalidis K.A., Daldoul S., Bouamama B., Chenennaoui S., Ghorbel A., Mliki A., Roubelakis-Angelakis K.A. (2010) Abscisic acid signals reorientation of polyamine metabolism to orchestrate stress responses via the polyamine exodus pathway in grapevine. *J. Plant Physiol.* **167**:519–525.

Tun N.N., Santa-Catarina C., Begum T., Silveira V., Handro W., Floh I.S., Scherer G.F.E. (2006) Polyamines induce rapid biosynthesis of nitric oxide (NO) *in Arabidopsis thaliana* seedlings, *Plant Cell Physiol*. **47**:346–354.

Urano K., Yoshiba Y., Nanjo T., Igarashi Y., Seki M., Sekiguchi F., Yamaguchi-Shinozaki K., Shinozaki K. (2003) Characterization of Arabidopsis genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. *Plant Cell Environ*. **26**:1917–1926.

Urano K., Hobo T., Shinozaki K. (2005) Arabidopsis ADC genes involved in polyamine biosynthesis are essential for seed development. *FEBS Lett*. **579**:1557–1564.

Urano K., Maruyama K., Ogata Y., Morishita Y., Takeda M., Sakurai N., Suzuki H., Saito K., Shibata D., Kobayashi M., Yamaguchi- Shinozaki K., Shinozaki K. (2009) Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. *Plant J*. **57**:1065–1078.

Vergnolle C., Vaultier M.N., Taconnat L., Renou J.P., Kader J.C., Zachowski A., Ruelland E. (2005) The cold-induced early activation of phospholipase C and D pathways determines the response of two distinct clusters of genes in Arabidopsis cell suspensions. *Plant Physiol*. **139**:1217-1233.

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

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

Walden R., Cordeiro A., Tiburcio A.F. (1997) Polyamines: Small Molecules Triggering Pathways in Plant Growth and Development. *Plant Physiol.* **3**:1009-1013.

Wallace H.M., Fraser A.V., Hughes A. (2003) A perspective of polyamine metabolism. *Biochem. J.* **376**:1-14.

Walters D.R. (1989) Polyamines and plant disease. *Plants Today* January-February. 22-26.

Walters D.R. (2000) Polyamines in plant-microbe interactions. *Physiol. Mol. Plant Pathol.* **57**:137-146.

Walters D.R. (2003a) Polyamines and plant disease. *Phytochemistry* **64**:97-107.

Walters D.R. (2003b) Resistance to plant pathogens: possible roles for free polyamines and polyamine catabolism, *New Phytol*. **159**:109–115.

Walters D.R., Wilson P.W.F., Shuttleton M.A., (1985) Relative changes in levels of polyamines and activities of biosynthetic enzymes in barley infected with the powdery mildew fungus, *Erysiphe graminis* DC. Ex Merat f.sp. *hordei* Marchal. *New Phytol*. **101**:695–705.

Walters D.R., Wylie M.A. (1986) Polyamines in discrete regions of barley leaves infected with the posdery mildew fungus *Erysiphe graminis. Physiol. Plant*. **67**:630–633.

Walters D.R, Meurer-Grimes B., Rovira I. (2001) Antifungal activity of three spermidine conjugates. FEMS *Microbiol. Lett.* **201**:255-258.

Wang Y., Devereux W., Woster P.M., Stewart T.M., Hacker A., Casero R.A. Jr (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res*. **61**:5370–5373.

Wang X., Ikeguchi Y., McCloskey D.E., Nelson P., Pegg A.E. (2004) Spermine synthesis is required for normal viability, growth, and fertility in the mouse. *J. Biol. Chem.* **279**:51370-51375.

Watson M.B., Malmberg R.L. (1996) Regulation of *Arabidopsis thaliana* (L.) Heynh Arginine decarboxylase by potassium deficiency stress. *Plant Physiol.* **111**:1077-1083.

Watson M.W., Yu W., Galloway G.L., Malmberg R.L. (1997) Isolation and characterization of a second arginine decarboxylase cDNA from *Arabidopsis* (PGR97–114). *Plant Physiol*. **114**:1569.

Wi S.J., Park K.Y. (2002) Antisense expression of carnation cDNA encoding ACC synthase or ACC oxidase enhances polyamine content and abiotic stress tolerance in transgenic tobacco plants. *Mol. Cells* **13**:209–220.

Wi S.J., Kim W.T., Park K.Y. (2006) Overexpression of carnation *S-adenosylmethionine decarboxylase* gene generates a broad-spectrum

tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep*. **25**:1111–1121.

Wortham B.W., Patel C.N., Oliveira M.A. (2007) Polyamines in bacteria: pleiotropic effects yet specific mechanisms. *Adv. Exp. Med. Biol.* **603**:106-115.

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

Wu J,, Qu H., Shang Z., Jiang X., Moschou P.N., Roubelakis-Angelakis K.A., Zhang S. (2010) Spermidine oxidase derived H_2O_2 regulates pollen plasma membrane hyperpolarization-activated Ca^{2+}-permeable channels and pollen tube growth. *Plant J*. **63**:1042–1053.

Xing S.G., Jun Y.B., Hau Z.W., Liang L.Y. (2007) Higher accumulation of γ-aminobutyric acid induced by salt stress through stimulating the activity of diamine oxidases in *Glycine max* (L.) Merr. roots. *Plant Physiol. Biochem*. **45**:560–566.

Xu M.J., Dong J.F., Zhu M.Y., (2005) Nitric oxide mediates the fungal elicitor-induced hypericin production *of Hypericum perforatum* cell suspension cultures through a jasmonic acid-dependent signal pathway, *Plant Physiol*. **139**:991–998.

Xue B., Zhang A., Jiang M. (2009) Involvement of polyamine oxidase in abscisic acid-induced cytosolic antioxidant defense in leaves of maize. *J. Integr. Plant Biol*. **51**:225–234.

Yamaguchi K., Takahashi Y., Berberich T., Imai A., Miyazaki A., Takahashi T., Michael A., Kusano T. (2006) The polyamine spermine protects against high salt stress in *Arabidopsis thaliana*. *FEBS Lett*. **580**:6783-6788.

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

Yamakawa H., Kamada H., Satoh M., Ohashi Y. (1998) Spermine is a salicylate-independent endogenous inducer for both tobacco acidic

pathogenesis-related proteins and resistance against Tobacco mosaic virus infection. *Plant Physiol*. **118**:1213–1222.

Yamasaki H., Cohen M.F. (2006) NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants? *Trends Plant Sci*. **11**:522–524.

Yesbergenova Z., Yang G., Oron E., Soffer D., Fluhr R., Sagi M. (2005) The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. *Plant J.* **42**:862-876.

Yoda H., Yamaguchi Y., Sano H. (2003) Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. *Plant Physiol*. **132**:1973–1981.

Yoda H., Hiroi Y., Sano H. (2006) Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. *Plant Physiol*. **142**:193–206.

Zhao F., Song C.P., He J., Zhu H. (2007) Polyamines improve K^+/Na^+ homeostasis in barley seedlings by regulating root ion channel activities. *Plant Physiol.* **145**:1061-1072.