

Polyamines: molecules with regulatory functions in plant abiotic stress tolerance

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Abstract Early studies on plant polyamine research pointed to their involvement in responses to different environmental stresses. During the last few years, genetic, transcriptomic and metabolomic approaches have unravelled key functions of different polyamines in the regulation of abiotic stress tolerance. Nevertheless, the precise molecular mechanism(s) by which polyamines control plant responses to stress stimuli are largely unknown. Recent studies indicate that polyamine signalling is involved in direct interactions with different metabolic routes and intricate hormonal cross-talks. Here we discuss the integration of polyamines with other metabolic pathways by focusing on molecular

mechanisms of their action in abiotic stress tolerance. Recent advances in the cross talk between polyamines and abscisic acid are discussed and integrated with processes of reactive oxygen species (ROS) signalling, generation of nitric oxide, modulation of ion channel activities and Ca^{2+} homeostasis, amongst others.

Keywords Polyamine metabolism · Abiotic stress · Plant tolerance · Abscisic acid · Signalling

Abbreviations

ABA	Abscisic acid
ACC	Amino cyclopropane carboxylic acid
ACL5	Acaulis5
ADC	Arginine decarboxylase
AIH	Agmatine iminohydrolase
CPA	<i>N</i> -Carbamoyl putrescine amidohydrolase
DAO	Diamine oxidase
Dap	1,3-Diaminopropane
dcSAM	Decarboxylated SAM
FAD	Flavin adenine dinucleotide
GABA	γ -Aminobutyric acid
LSD	Lysine-specific demethylase
NO	Nitric oxide
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
Pro	Proline
Put	Putrescine
SAM	<i>S</i> -Adenosyl methionine
SAMDC	<i>S</i> -Adenosyl methionine decarboxylase
ROS	Reactive oxygen species
SMO	Spermine oxidase
Spd	Spermidine
SDPS	Spermidine synthase
Spm	Spermine

This review is dedicated to the memory of Prof. Arthur W. Galston (Yale University, New Haven, CT, USA) for his pioneering work and important contribution to the plant polyamine field.

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SPMS	Spermine synthase
TCA	Tricarboxylic acid
tSpm	Thermospermine

Introduction

Polyamines can be considered as one of the oldest group of substances known in biochemistry (Galston 1991). Indeed, the tetramine spermine (Spm) was discovered more than 300 years ago in ageing human spermatozoa (van Leeuwenhoek 1678), whilst the diamine putrescine (Put) and cadaverine (Cad) were identified in putrefying cadavers more than 100 years ago (Brieger 1885). The structure and chemistry of the most abundant polyamines Put, Spm and the triamine spermidine (Spd) were further elucidated in the 1920s, and it was revealed that they are nitrogen-containing compounds of low molecular weight (Dudley et al. 1926, 1927). Nowadays, Put and Spd are believed to be ubiquitous in all living cells. Earlier contentions that Spm does not occur in prokaryotes are incorrect, since this tetramine is also present in various bacterial cells (Pegg and Michael 2009). It has been suggested that plants had acquired a part of the polyamine biosynthetic pathway from an ancestral cyanobacterial precursor of the chloroplast (Illingworth et al. 2003). Therefore, it can be assumed that this is an ancient metabolic route in plants, which is also present in all organisms (Minguet et al. 2008). Many results support the contention that polyamines are essential for life. Thus, chemically or genetically induced depletion of Put and/or Spd levels is lethal in yeast, protists and plants (Hamasaki-Katagiri et al. 1998; Roberts et al. 2001; Imai et al. 2004b; Urano et al. 2005). Organisms deficient in Spm are viable, but show different degrees of dysfunction. This indicates that Spm, albeit not essential, must also play very important roles in growth and development (Imai et al. 2004a; Wang et al. 2004; Yamaguchi et al. 2007; Minguet et al. 2008).

Since polyamines are protonated at normal cellular pH, their biological function was initially associated with the capability of binding different anionic macromolecules (DNA, RNA, chromatin and proteins), thus confining them as substances with a structural role. However, it was later confirmed that in addition to stabilizing macromolecular structures, polyamines act as regulatory molecules in many fundamental cellular processes (Igarashi and Kashiwagi 2000; Seiler and Raul 2005; Alcázar et al. 2006b; Kusano et al. 2008). These include cell division, differentiation and proliferation, cell death, DNA and protein synthesis and gene expression (Igarashi and Kashiwagi 2000; Childs et al. 2003; Seiler and Raul 2005). In plants, polyamines have been implicated in many physiological processes, such as organogenesis, embryogenesis, floral initiation and development, leaf senescence, fruit development and

ripening, and abiotic and biotic plant stress responses (Galston and Kaur-Sawhney 1990; Kumar et al. 1997; Walden et al. 1997; Malmberg et al. 1998; Bouchereau et al. 1999; Bagni and Tassoni 2001; Alcázar et al. 2006b; Kusano et al. 2008).

Changes in plant polyamine metabolism occur in response to a variety of abiotic stresses (Bouchereau et al. 1999; Alcázar et al. 2006b; Groppa and Benavides 2008). The importance of this process is illustrated by the fact that in stressed plants, the levels of Put may account for 1.2% of the dry matter, representing at least 20% of the nitrogen (Galston 1991). However, the physiological significance of increased polyamine levels in abiotic stress responses is still unclear (Alcázar et al. 2006b; Kusano et al. 2008; Gill and Tuteja 2010). Complete sequencing of the Arabidopsis genome has facilitated the use of global 'omic' approaches in the identification of target genes in polyamine biosynthesis and signalling pathways. Loss and gain of function mutations affecting polyamine metabolism provide useful tools to gain new insights into molecular mechanisms underlying polyamine functions. Recent studies indicate that polyamines may act as cellular signals in intricate cross talk with hormonal pathways, including abscisic acid (ABA) regulation of abiotic stress responses. A progress in unravelling the molecular functions of polyamines has also facilitated the generation of Arabidopsis transgenic plants resistant to various stresses. However, the transfer of the latter technology to valuable crops have some current constraints in the agricultural industry despite the fact that breeding stress-resistant varieties by making use of naturally occurring compounds is a basic prerequisite of sustainable agriculture. We envisage that further exploitation of natural variability can open new alternatives for both fundamental and applied plant polyamine research.

Polyamine biosynthesis

Metabolic studies indicate that the intracellular levels of polyamines in plants are mostly regulated by anabolic and catabolic processes, as well as by their conjugation to hydroxycinnamic acids. Schematic representation of these processes and their interactions with other metabolic pathways are depicted in Fig. 1. The biosynthesis of polyamines is initiated with the formation of diamine Put. In mammals and fungi, Put is formed from ornithine in a reaction catalysed by ornithine decarboxylase (ODC, EC 4.1.1.17). In plants and bacteria, however, there is an alternative pathway for the formation of Put, which is synthesized from arginine in a reaction catalysed by arginine decarboxylase (ADC, EC 4.1.1.19) followed by two successive steps catalysed by agmatine iminohydrolase (AIH, EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA, EC

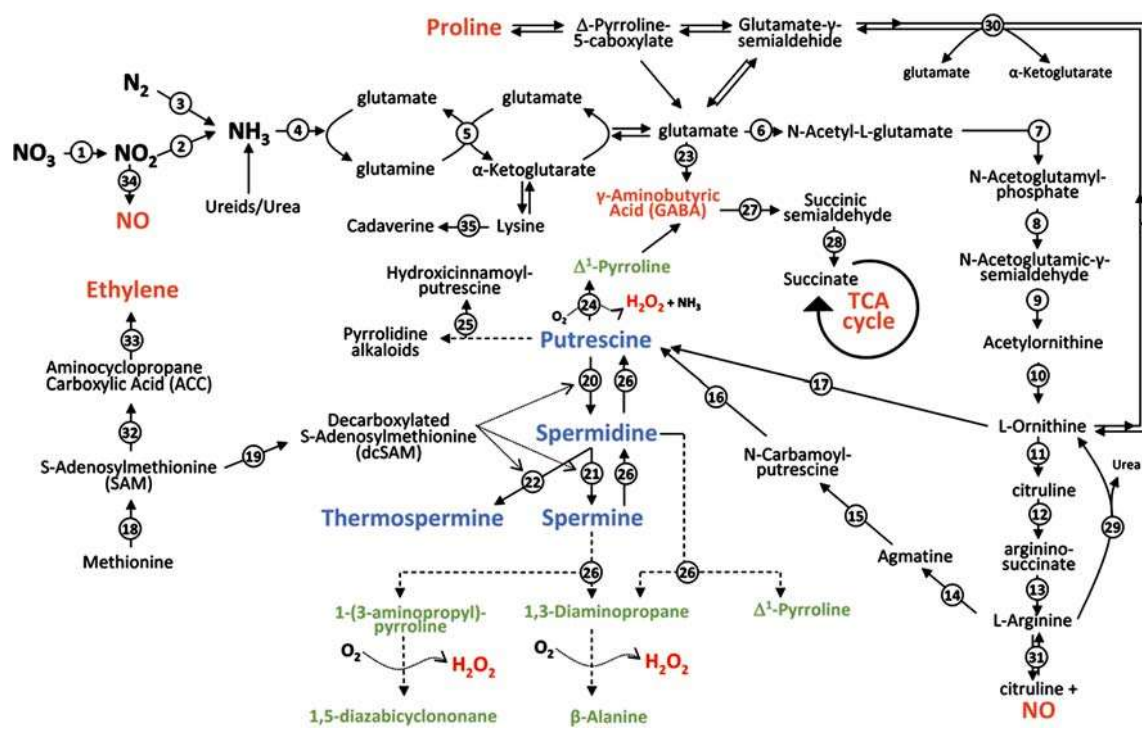


Fig. 1 Polyamine metabolism and interaction with other metabolic routes. Biosynthetic pathways for polyamines and related metabolites are indicated by continuous lines. Dashed lines show the formation of putrescine-derived alkaloids, polyamine conjugation and catabolic processes. Numbers refer to the 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 spermidine synthase, 21 spermine synthase, 22 thermospermine synthase, 23 glutamate decarboxylase, 24 diamine oxidase, 25 putrescine hydroxycinnamoyl transferase, 26 polyamine oxidase, 27 γ-aminobutyrate aminotransferase, 28 succinyl semialdehyde dehydrogenase, 29 arginase, 30 ornithine aminotransferase, 31 nitric oxide synthase, 32 ACC synthase, 33 ACC oxidase, 34 nitrate reductase, 35 lysine decarboxylase. It has been shown recently that polyamine oxidase (26) is not only involved in the terminal catabolism of polyamines, but also in the back-conversion of spermine to spermidine and spermidine to putrescine. Other by-products from the back-conversion are 3-aminopropanal and H₂O₂ (not shown)

3.5.1.53) (Slocum et al. 1984). The overexpression of homologous ADC2 in Arabidopsis is sufficient to promote Put accumulation, which suggests that ADC is the limiting step for Put biosynthesis in plants (Alcázar et al. 2005). The oat ADC is localized in chloroplasts associated with the thylakoid membranes (Borrell et al. 1995), whereas a nuclear or chloroplastic localization is observed in different tobacco tissues (Bortolotti et al. 2004). These observations indicate that subcellular compartmentalization of the ADC pathway occurs in plants, which might lead to gradient concentrations of Put within the cell. In yeast and *E.coli*, genes encoding polyamine transport systems have been described (Igarashi and Kashiwagi 2000). However, transport or shuttle mechanisms for polyamines in plants have not yet been reported. Recently, it has been suggested that ADC/ODC alternative pathways reflect their different evolutionary origins. Thus, ADC, AIH and CPA in plants could originate from a cyanobacterial ancestor of chloroplast, whereas ODC could derive from bacterial genes present in a common

ancestor of plants and animals that acquired the cyanobacterial endosymbiont (Illingworth et al. 2003). Once Put is formed via ODC and/or ADC, the diamine serves as a precursor of triamine Spd and tetramine Spm, which are formed by successive additions of aminopropyl groups in reactions catalysed, respectively, by Spd synthase (SPDS, EC 2.5.1.16) and Spm synthases (SPMS, EC 2.5.1.16). Decarboxylated S-adenosylmethionine (dcSAM), the donor of aminopropyl groups, is formed by decarboxylation of SAM in a reaction catalysed by SAM decarboxylase (SAMDC, EC 4.1.1.50) (Slocum et al. 1984) (Fig. 1).

The ODC pathway has been traditionally considered universal in all living organisms. ODC activity has been detected in most analysed plants species and genes coding for ODC have also been characterized (Michael et al. 1996; Imanishi et al. 1998). Surprisingly, Arabidopsis does not contain a gene coding for ODC and the corresponding enzyme activity cannot be detected in this plant (Hanfrey et al. 2001). Hence, in Arabidopsis Put is produced solely

through the ADC pathway. Arabidopsis carries duplicated ADC genes (*ADC1* and *ADC2*), but only one single gene coding for AIH and CPA (Janowitz et al. 2003; Piotrowski et al. 2003). In addition, the Arabidopsis genome carries four SAM decarboxylase (*SAMDC 1-4*; Urano et al. 2003) and two spermidine synthase (*SPDS1* and *SPDS2*) genes, whereas *SPMS* is encoded by a single gene (see also Fig. 2). The presence of a metabolon constituted by the interaction of aminopropyltransferases, *SPDS1*, *SPDS2* and *SPMS*, was discovered in Arabidopsis by Panicot et al. (2002), who suggested that Spd formed by the action of *SPDS* is effectively channelled to *SPMS* controlling the formation of the end product Spm (Panicot et al. 2002). Due to difficulties in separation of Spm and thermospermine (tSpm) by routine analytical methods (Rambla et al. 2010), *ACAULIS5* (*ACL5*) was originally reported to code for a putative *SPMS* in Arabidopsis (Hanzawa et al. 2000). However, recent findings indicate that *ACL5* catalyses the conversion of Spd to thermospermine, and not to Spm. Thus, *ACL5* functions as a thermospermine synthase (tSPMS) (Knott et al. 2007; Kakehi et al. 2008).

The diamine Put is also a precursor of several alkaloid families (Tiburcio et al. 1990). In some plant species, Put is methylated by *N*-methyltransferases (PMT) using SAM as a methyl donor (Ghosh 2000). The Arabidopsis PMT shares a high degree of sequence similarity with *SPDS*, *SPMS* and tSPMS, but nevertheless displays different substrate specificity (Teuber et al. 2007). Evolution of different

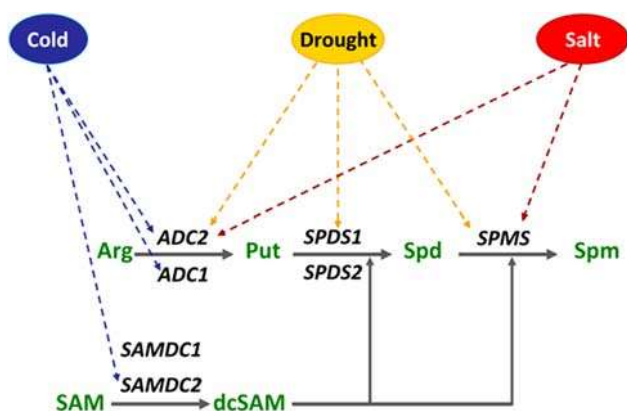


Fig. 2 Effects of drought, salt and cold treatments on polyamine biosynthesis in Arabidopsis. Drought induces the expression of *ADC2*, *SPDS1* and *SPMS* genes. The levels of Put increase, whilst Spd and Spm contents do not increase above basal levels (Alcázar et al. 2006a). Salt treatment induces the expression of *ADC2* and *SPMS* genes and results in increased Put and Spm levels (Urano et al. 2003). Cold induces the expression of *ADC1*, *ADC2* and *SAMDC2* genes. The levels of Put increase on cold treatment and this correlates with the induction of *ADC* genes. The content of Spd and Spm remain constant or even decrease in response to low temperature. The absence of correlation between enhanced *SAMDC2* expression and the decrease of Spm levels has been suggested to be due to an increase in Spm catabolism (Cuevas et al. 2008)

substrate specificities of these enzymes is suggested to reflect an unusually high rate of diversification of pre-existing functions (Minguet et al. 2008). All *SPDS*s derive from a common ancestor, but they have been the origin of a variety of new activities. It is possible that diversification and duplication of ancient *SPMS* sequences have led to the evolution of functionally distinct *SPDS* genes, whereas the appearance of tSPMS in some organisms was accompanied by a loss of *SPDS* gene function. The synthesis of nicotine and tropane alkaloids in Solanaceae implies that the appearance of PMT enzyme likely originated from duplication and diversification of *SPDS* sequences (Minguet et al. 2008).

Polyamine conjugation

In addition to their free forms, polyamines occur in plants as hydroxycinnamic acid conjugates that are referred to as hydroxycinnamic acid amides (HCCAs; Fig. 1). Caffeoyl-putrescine (paucine) was first discovered in 1893 as a component of some leguminous seeds (reviewed by Tiburcio et al. 1990). Coumaroylputrescine, feruloylputrescine, coumaroylagmatine, dicoumaroylspermidine, diferuloylspermidine, diferuloylspermine and feruloyltyramine were further identified in a wide range of plant species (Martin-Tanguy 1997). Recent studies have revealed the presence of a series of novel hydroxycinnamic acid conjugates of Spd in flower buds of Arabidopsis (Fellenberg et al. 2009). A gene encoding an Spd hydroxycinnamoyl transferase (SHT) has been characterized and suggested to participate in the formation of tricoumaroyl-, tricaffeoyl- and triferuloyl-Spd in the tapetum of Arabidopsis anthers (Grienenberger et al. 2009). Furthermore, two novel acyltransferase genes regulating the accumulation of disinapoyl-Spd and sinapoyl-(glucose)-Spd have been functionally characterized in Arabidopsis seeds (Luo et al. 2009). However, genes encoding *N*-hydroxycinnamoyl transferases, which acylate other polyamines, remain to be identified in Arabidopsis.

Polyamine catabolism

Polyamines are catabolized through the activity of one or more diamine oxidases (DAO, EC 1.4.3.6) and polyamine oxidases (PAO; EC 1.5.3.3). DAOs are copper-containing enzymes that catalyse the oxidation of diamines Put and Cad at the primary amino groups. The reaction products from Put are 4-aminobutanal (which spontaneously cyclizes to Δ^1 pyrroline), H_2O_2 and ammonia (Fig. 1). It is known that DAOs occur at high levels in dicots, but genes encoding these enzymes have been identified so far only in

few species (Cona et al. 2006). *Arabidopsis* contains 12 DAO-like genes (Alcázar et al. 2006b), but only one of them (*ATAOI*) has been characterized (Moller and McPherson 1998). In contrast to DAOs, PAOs are enzymes that bear a non-covalently bound molecule of FAD and occur at high levels in monocots (Sebela et al. 2001). They are classified into families, which are involved either in terminal catabolism or back-conversion of polyamines. Members of a third related protein family also carry similar PAO domains, but do not deaminate polyamines (Moschou et al. 2008). Maize PAO (ZmPAO), the best characterized enzyme of the first class, catalyses terminal catabolism of Spd and Spm producing 4-aminobutanal or (3-aminopropyl)-4-aminobutanal, along with 1,3-diaminopropane (Dap) and H₂O₂ (Cona et al. 2006; Fig. 1). The second group of plant PAOs resemble the mammalian Spm oxidase (SMO, EC 1.5.3.3) that catalyses the back-conversion of Spm to Spd with concomitant production of 3-aminopropanal and H₂O₂ (Moschou et al. 2008; Fig. 1). The *Arabidopsis* genome contains five genes encoding putative PAOs (Alcázar et al. 2006b). PAO1 and PAO4 catalyse the same reaction as SMO (Tavladoraki et al. 2006; Kamada-Nobusada et al. 2008), whilst PAO3 acts in the back-conversion pathway, converting Spm to Spd and Spd to Put (Moschou et al. 2008). The third class of plant PAO-domain proteins are relatives of the human lysine-specific demethylase 1 (LSD1) that possesses an amine oxidase domain similar to that of FAD-dependent PAOs (Shi et al. 2004). LSD1 acts as a histone demethylase, representing an important regulator of chromatin structure and gene expression (Huang et al. 2007). *Arabidopsis* has four *LSD1*-related genes, some of which participate in the repression of *FLC*, a negative regulator of flowering time (Jiang et al. 2007; Krichevsky et al. 2007).

Interactions with other metabolic routes

The polyamine metabolic pathway is also interconnected with other metabolic routes involved in the formation of various signalling molecules and metabolites that are relevant in plant stress responses (Fig. 1). Thus, polyamine and ethylene biosynthesis are connected through SAM that acts as a common precursor. 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). Polyamines induce the production of NO that may act as a link between polyamine-mediated stress responses and other stress mediators (Tun et al. 2006). H₂O₂ generated by the action of DAOs and/or PAOs is involved in both biotic and abiotic stress signalling, as well as in ABA-induced stomatal closure (Cona et al. 2006; An et al. 2008). Another product of Put

and Spd catabolism is γ -aminobutyric (GABA) that is formed via pyrroline (Cona et al. 2006; Fig. 1). The levels of GABA, agmatine (a precursor of Put) and some components of the TCA cycle increase under dehydration (Urano et al. 2009) along with an increase in Put content (Alcázar et al. 2006a), which suggests a metabolic connection between these routes in response to stress. In addition, proline (Pro) levels increase in response to various abiotic stresses (Sharma and Dietz 2006; Urano et al. 2009) and polyamine catabolism is closely related to Pro accumulation in response to salt stress (Aziz et al. 1998). Interactions between stress-induced Pro and polyamine accumulations may reflect the fact that they share ornithine as a common precursor (Mohapatra et al. 2009; Fig. 1). In conclusion, the polyamine metabolism is connected to several important hormonal and metabolic pathways involved in development, stress responses, nitrogen assimilation and respiratory metabolism.

Polyamines and abiotic stress

In the early stages of polyamine research, Richards and Coleman (1952) observed the presence of a predominant unknown ninhydrin positive spot that accumulated in barley plants exposed to potassium starvation. After isolation and crystallization, this compound was identified as Put. Later on it was shown that K⁺-deficient shoots fed with L-¹⁴C-arginine produced labelled Put in a more rapid way compared to feeding with labelled ornithine. These results suggested that decarboxylation of arginine was the main way of accumulation of Put under K⁺-deficiency (Smith and Richards 1964). The relevance of the ADC pathway in plant responses to abiotic stress was later on established by Galston et al. at Yale University (Flores and Galston 1982). Further work in different plant species has shown that polyamine accumulation occurs in response to several adverse environmental conditions, including salinity, drought, chilling, heat, hypoxia, ozone, UV-B and UV-C, heavy metal toxicity, mechanical wounding and herbicide treatment (for review see Bouchereau et al. 1999; Alcázar et al. 2006b; Groppa and Benavides 2008). However, the physiological significance of these responses remained unclear, and it had to be evaluated whether elevated polyamine levels were a result of stress-induced injury or a protective response to abiotic stress.

Classical approaches, using exogenous polyamine application and/or inhibitors of enzymes involved in polyamine biosynthesis, pointed to a possible role of these compounds in plant adaptation/defence to several environmental stresses (for a review, Bouchereau et al. 1999; Alcázar et al. 2006b; Groppa and Benavides 2008). More recent studies using either transgenic overexpression or loss-of-function

mutants support this protective role of PAs in plant response to abiotic stress (Alcázar et al. 2006b; Kusano et al. 2008; Gill and Tuteja 2010). Indeed, heterologous overexpression of *ODC*, *ADC*, *SAMDC* and *SPDS* from different animal and plant sources in rice, tobacco and tomato has shown tolerance traits against a broad spectrum of stress conditions (see Table 1 and references therein). Enhanced tolerance always correlated with elevated levels of Put and/or Spd and Spm. Antisense silencing of ethylene biosynthesis genes ACC synthase and ACC oxidase in tobacco has also shown to favour the flux of SAM to polyamines (Wi and Park 2002), which leads to an increased tolerance to salt, drought and a broad spectrum of other abiotic stresses (Kasukabe et al. 2004; Wi et al. 2006) (Table 1). Similar results have been obtained by homologous overexpression of polyamine biosynthetic genes in Arabidopsis (Table 1). Thus, overexpression of *SAMDC1* in Arabidopsis leads to elevated Spm levels and enhanced tolerance to various abiotic stress conditions. The *SAMDC1* overexpressing plants show elevated levels of ABA due to the induction of *NCED3*, a key enzyme involved in ABA biosynthesis (reviewed by Alcázar et al. 2006b). High Put levels induced by homologous overexpression of *ADC1* enhances freezing tolerance in Arabidopsis (Altabella et al. 2009). Similarly, elevated levels of Put by overexpressing *ADC2* produces drought tolerance in Arabidopsis, which may be related to reduction of water loss by the induction of stomata closure (Alcázar et al. 2010) (Table 1).

The results obtained from loss-of-function mutations in polyamine biosynthetic genes further support the protective role of polyamines in plant response to abiotic stress. For example, EMS mutants of *Arabidopsis thaliana spe1-*

1 and *spe2-1* (which map to *ADC2*) displaying reduced ADC activity are deficient in polyamine accumulation after acclimation to high NaCl concentrations and exhibit more sensitivity to salt stress (Kasinathan and Wingler 2004). Insertion mutants affecting the *Arabidopsis ADC2* gene present also altered responses to abiotic stress. Thus, *ADC2* induction by osmotic stress is impaired in the loss-of-function mutant *en9*, obtained by PCR screening of an En-1 mutagenized *Arabidopsis* population for insertions at the *ADC2* locus (Soyka and Heyer 1999). In the same way, a Ds insertion mutant (*adc2-1*), the Put content of which is diminished up to 75% of the control, is more sensitive to salt stress, whereas salt-induced injury was partly reverted by the addition of exogenous Put (Urano et al. 2004). Other *ADC1* (*adc1-2*, *adc1-3*) and *ADC2* (*adc2-3*, *adc2-4*) mutant alleles are more sensitive to freezing, and this phenotype is partially rescued by adding exogenous Put (Cuevas et al. 2008). Moreover, *acl5/spms* Arabidopsis double mutants that do not produce Spm are hypersensitive to salt and drought stresses, and the phenotype is mitigated by application of exogenous Spm (Kusano et al. 2007).

All these examples illustrate that genetic modification of the polyamine biosynthetic pathway has been useful to discern the function of polyamines in plant responses to abiotic stress in both crops and model plants. Collectively, the available results indicate that elevated polyamine levels represent a stress-induced protective response. A further challenge is to elucidate the mechanism of action by which polyamines protect plants from abiotic stress. This is discussed in the following sections.

Table 1 Abiotic stress tolerance in transgenic plants overproducing polyamines

Gene name	Gene source	Species	Overexpression	Overproduction	Tolerance	Reference
<i>ADC</i>	Oat	Rice	Inducible	Put	Salt	Roy and Wu (2001)
<i>ODC</i>	Mouse	Tobacco	Constitutive	Put	Salt	Kumria and Rajam (2002)
<i>SAMDC</i>	Tritordeum	Rice	Inducible	Spd and Spm	Salt	Roy and Wu (2002)
<i>ACC synthase</i>	Carnation	Tobacco	Antisense	Put and Spd	Broad spectrum	Wi and Park (2002)
<i>ACC oxidase</i>	Carnation	Tobacco	Antisense	Put and Spd	Broad spectrum	Wi and Park (2002)
<i>SAMDC</i>	Human	Tobacco	Constitutive	Put and Spd	Salt, osmotic	Waie and Rajam (2003)
<i>ADC</i>	<i>Datura stramonium</i>	Rice	Inducible	Spd and Spm	Drought	Capell et al. (2004)
<i>SPDS</i>	<i>Cucurbita ficifolia</i>	Arabidopsis	Constitutive	Spd	Broad spectrum	Kasukabe et al. (2004)
<i>SAMDC</i>	Carnation	Tobacco	Constitutive	Put, Spd and Spm	Broad spectrum	Wi et al. (2006)
<i>SAMDC1</i>	Arabidopsis	Arabidopsis	Constitutive	Spm	Broad spectrum	Alcázar et al. (2006b)
<i>ADC1</i>	Arabidopsis	Arabidopsis	Constitutive	Put	Freezing	Altabella et al. 2009
<i>SAMDC</i>	Yeast	Tomato	Constitutive	Spd and Spm	Heat	Cheng et al. (2009)
<i>SPDS</i>	Apple	Pear	Constitutive	Spd	Broad spectrum	Wen et al. (2009)
<i>ADC2</i>	Arabidopsis	Arabidopsis	Constitutive	Put	Drought	Alcázar et al. (2010)

Polyamines and ABA in drought, salt and cold stresses

The use of *Arabidopsis* has opened new perspectives in functional dissection of the polyamine metabolic pathway and its role in the control of abiotic stress responses (Ferrando et al. 2004; Alcázar et al. 2006b; Kusano et al. 2008; Takahashi and Kakehi 2009; Gill and Tuteja 2010). Annotation of the *Arabidopsis* genome (<http://www.arabidopsis.org>) has allowed a full compilation of the polyamine biosynthetic pathway (<http://www.plantcyc.org>). Figure 2 summarizes the transcription responses of the polyamine biosynthetic pathway in *Arabidopsis* subjected to drought, salt and cold treatments. Transcript profiling by using Q-RT-PCR has revealed that water stress induces the expression of *ADC2*, *SPDS1* and *SPMS* genes (Alcázar et al. 2006a) (Fig. 2). The expression of some of these genes is also induced by ABA treatment (Perez-Amador et al. 2002; Urano et al. 2003). To get a further insight into ABA regulation of polyamine pathway, the expression of *ADC2*, *SPDS1* and *SPMS* was analysed in the ABA-deficient (*aba2-3*) and ABA-insensitive (*abi1-1*) mutants subjected to water stress (Alcázar et al. 2006a). These three genes display reduced transcriptional induction in the stressed *aba2-3* and *abi1-1* mutants compared to the wild type, indicating that ABA modulates polyamine metabolism at the transcription level by up-regulating the expression of *ADC2*, *SPDS1* and *SPMS* genes under water stress conditions (Alcázar et al. 2006a). In addition, Put accumulation in response to drought is also impaired in the *aba2-3* and *abi1-1* mutants compared to wild-type plants. This result is further supported by metabolomic studies showing that polyamine responses to dehydration are also impaired in *nced3* mutants (Urano et al. 2009). All these observations support the conclusion that up-regulation of PA-biosynthetic genes and accumulation of Put under water stress are mainly ABA-dependent responses.

Under salt stress conditions, there is a rapid increase in the expression of *ADC2* and *SPMS* (Fig. 2), which is maintained during the 24-h treatment and results in increased Put and Spm levels (Urano et al. 2003). Spm-deficient mutants are sensitive to salt, whilst the addition of Spm suppresses the salt sensitivity, suggesting a protective role of this polyamine to high salinity (Yamaguchi et al. 2006). It is likely that polyamine responses to salt stress are also ABA-dependent, since both *ADC2* and *SPMS* are induced by ABA (see above). In fact, stress-responsive, drought-responsive (DRE), low temperature-responsive (LTR) and ABA-responsive elements (ABRE and/or ABRE-related motifs) are present in the promoters of the polyamine biosynthetic genes (Alcázar et al. 2006b). This reinforces the view that in response to drought and salt treatments, the expression of some of the genes involved in polyamine biosynthesis are regulated by ABA.

Transcript profiling has also revealed that cold enhances the expression of *ADC1*, *ADC2* and *SAMDC2* genes (Urano et al. 2003; Cuevas et al. 2008; Cuevas et al. 2009) (Fig. 2). Free Put levels are increased on cold treatment and this correlates with the induction of *ADC* genes. Surprisingly, the levels of free Spd and Spm remain constant or even decrease in response to cold treatment. The absence of correlation between enhanced *SAMDC2* expression and the decrease of Spm levels may be a result of increased Spm catabolism (Cuevas et al. 2008). Since double mutants completely devoid of ADC activity are not viable in *Arabidopsis* (Urano et al. 2005), two independent mutant alleles for both *ADC1* and *ADC2* were used to study their response to freezing. As indicated in “Polyamines and abiotic stress”, the *adc1* and *adc2* mutations caused higher sensitivity to freezing conditions, in both acclimated and non-acclimated plants, whilst addition of Put complemented this stress sensitivity (Cuevas et al. 2008, 2009). Reduced expression of *NCED3* and several ABA-regulated genes was detected in the *adc1* mutants at low temperature. Complementation analyses of *adc1* mutants with ABA and reciprocal complementation of *aba2-3* mutant with Put supported the conclusion that this diamine controls the levels of ABA in response to cold by modulating ABA biosynthesis at the transcriptional level (Cuevas et al. 2008, 2009). All these results suggest that Put and ABA are integrated in a positive feedback loop, in which ABA and Put reciprocally promote each other’s biosynthesis in response to abiotic stress (see Fig. 3). This highlights a novel mode of action of polyamines as regulators of ABA biosynthesis.

Interplay between ABA, polyamines, ROS (H₂O₂) and NO in stomata regulation

Abscisic acid is an endogenous anti-transpirant that reduces water loss through stomatal pores on the leaf surface. Enhanced biosynthesis of ABA occurs in response to water deficit, resulting in the redistribution and accumulation of ABA in guard cells. This results in the release of water, efflux and influx of ions, and loss of turgor of guard cells, causing a closure of stomata (Bray 1997). The ABA signalling pathway in stomata regulation involves many different components such as ABA receptors, G-proteins, protein kinases and phosphatases, transcription factors and secondary messengers, including Ca²⁺, reactive oxygen species (ROS) and NO (Kuppusamy et al. 2009). It has been reported that Put, Spd and Spm also regulate stomatal responses by reducing their aperture and inducing closure (Liu et al. 2000; An et al. 2008). In addition, Put modulates ABA biosynthesis in response to abiotic stress, as discussed in “Polyamines and ABA in drought, salt and cold stresses”. It is therefore likely that polyamines participate in

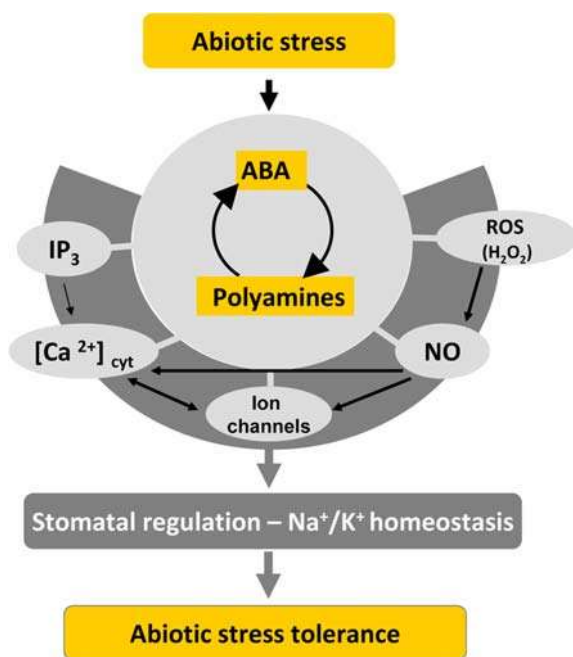


Fig. 3 Simplified model for the integration of polyamines with ABA, ROS (H₂O₂), NO, Ca²⁺ homeostasis and ion channel signalling in the abiotic stress response

ABA-mediated stress responses involved in stomatal closure. In this regard, evidences point to an interplay between polyamines with ROS generation and NO signalling in ABA-mediated stress responses (Yamasaki and Cohen 2006) (see Fig. 3). The generation of ROS is tightly linked to polyamine catabolic processes, since amino oxidases generate H₂O₂, which is a ROS associated with plant defence and abiotic stress responses (Cona et al. 2006). Furthermore, polyamines are reported to promote the production of NO in Arabidopsis (Tun et al. 2006). Both H₂O₂ and NO are involved in the regulation of stomatal movements in response to ABA, in such a way that NO generation depends on H₂O₂ production (Neill et al. 2008). In Arabidopsis guard cells, the production of H₂O₂ induced by ABA arises from superoxide generated by isoforms of NAD(P)H oxidases encoded by the *AtrbohD* and *AtrbohF* genes that are involved in ROS-dependent activation of Ca²⁺ channels and cytosolic Ca²⁺ increase (Kwak et al. 2003; Desikan et al. 2004; She et al. 2004). Besides NADPH oxidases, apoplasmic amino oxidases are also sources of ROS production (Cona et al. 2006; Fig. 3). Indeed, ABA has been reported to activate Put catabolism and H₂O₂ production through DAO activities during the induction of stomatal closure in *Vicia faba* guard cells (An et al. 2008). ABA and Put promote an enhancement of Ca²⁺ concentration in guard cells, and this increase is impaired by DAO inhibitors. This suggests that the effect of H₂O₂ from DAO-catalysed Put oxidation in guard cells is mediated by Ca²⁺ ions (Fig. 3). In contrast to the effects of Put,

Spd and Spm did not contribute to ABA-promoted H₂O₂ generation in *V. faba* guard cells (An et al. 2008) despite the fact that the three polyamines induce stomatal closure (Liu et al. 2000). It was previously hypothesized that NO production in plants was mediated through the action of either nitric oxide synthase (NOS)-like or nitrate reductase (NR) activities (see Fig. 1). However, recent data argue against the involvement of NOS-like activity in H₂O₂-induced NO synthesis in guard cells (Bright et al. 2006; Neill et al. 2008). Tun et al. (2006) demonstrated that Spd and Spm induce rapid biosynthesis of NO, but Put application had little or no effect. The promotion by Spd and Spm of the 14-3-3-dependent inhibition of phospho-NR (Athwal and Huber 2002), which down-regulates nitrate assimilation and NO production from nitrite, suggests the involvement of other sources for Spd and Spm-induced NO production (Yamasaki and Cohen 2006). The occurrence of a still unknown enzyme responsible for direct conversion of polyamines to NO thus cannot be ruled out. In any case, polyamines appear to regulate stomatal closure by activating the biosynthesis of signalling molecules (H₂O₂ and NO) through different routes (Yamasaki and Cohen 2006). Altogether, the available data indicate that polyamines, ROS (H₂O₂) and NO act synergistically in promoting ABA responses in guard cells (Fig. 3).

Polyamines and ion channels

The role of polyamines in plant stress responses implies additional layers of complexity, since polyamines have also been reported to block different ion channels. Some of these act downstream of H₂O₂ production in the ABA signalling pathway of guard cells (Fig. 3). At physiological pH, polyamines are positively charged compounds, which can interact electrostatically with negatively charged proteins, including ion channels. Indeed, polyamines at their physiological concentration block the fast-activating vacuolar cation channel in a charge-dependent manner (Spm, 4⁺ > Spd 3⁺ >> Put 2⁺), at both whole-cell and single-channel level, thus indicating a direct blockage of the channel by polyamines (Bruggemann et al. 1998). These authors reported that under optimal conditions, leaf cells of young barley plants contain 50–100 μM of Put and Spd each, and 10–30 μM Spm. Considering the K_d values of these PAs for blocking the fast vacuolar (FV) channels (Bruggemann et al. 1998), Put has no effect on FV channel activity, whilst a substantial portion of these channels is blocked by Spd and Spm. Thus, any change in Spd and Spm concentration affects FV channel activity. As mentioned above, in response to different abiotic stresses, such as potassium deficiency, Put levels are increased drastically (reaching millimolar concentrations), whereas the levels of Spd and

Spm are not significantly affected and this increase of Put may significantly reduce FV channel activity. At high salinity all PA levels increase, and the enhanced Spm concentration probably blocks FV channel activity (Bruggemann et al. 1998). Inhibition of the inward K^+ and especially Na^+ currents by extracellular polyamines (1 mM) has also been reported in barley root epidermal and cortical cells. Polyamine-induced repression of Na^+ influx into roots and prevention of K^+ loss from shoots improved K^+/Na^+ homeostasis in barley seedlings and tolerance to high salinity (Zhao et al. 2007). As in animal and bacterial cells (Delavega and Delcour 1995; Johnson 1996), polyamines in plants may thus modulate ion channel activities through direct binding to the channel proteins and/or their associated membrane components. However, not all effects on modulation of ion channels by polyamines require a direct binding. Indeed, inhibition of I_{Kin} (inward K^+ channels) in guard cells of *V. faba* (Liu et al. 2000) and NSCC (non-selective K^+ and Na^+ permeable cation channels) in pea mesophyll protoplasts (Shabala et al. 2007) appears to be mediated by a cytoplasmic pathway, which is different from the direct blockage reported in other systems. Moreover, in these cases the polyamine effect is not a mere charge event, but rather a signalling component in the modulation of channel activities (Liu et al. 2000; Shabala et al. 2007). Regulation of ion channels by polyamines is confined within the multiple and versatile mechanisms through which polyamines participate in the stress response. For instance, micromolar concentrations of polyamines block both inward and outward currents through the NSCC channels in pea mesophyll protoplasts, thus assisting the adaptation to salinity by reducing the uptake of Na^+ and leakage of K^+ from mesophyll cells (Shabala et al. 2007). In *V. faba*, polyamines at 1 mM concentration target inward K^+ channels in guard cells and modulate stomatal movements, providing a link between stress conditions, polyamine levels, ion channels and stomata regulation (Fig. 3).

Under stress, polyamine levels may increase from a range of 10–100 micromolar to submillimolar and millimolar concentrations. The results reported above suggest that polyamines are active compounds that modulate a number of ion channels and mediate stomata closure through different signalling pathways at concentrations that can be reached under stress conditions. This supports the idea that PAs serve as stress “messengers” for plants to respond to the encountered stress (Liu et al. 2000). From a practical point of view, polyamines are ideally suited as physiological channel blockers, since they are the only organic polycations present in sufficient quantities to perform the role of channel blockage, without compromising cell metabolism. Inorganic polycations (e.g. Al^{3+} , Gd^{3+} , La^{3+}) are also efficient channel blockers, but most of them are highly toxic and, hence, cannot be accumulated in the cytosol at the

required concentrations for a “safe control” of cellular homeostasis.

Regarding the molecular mode of action of polyamines in ion channels, evidences point to specific polyamine-binding proteins in cytoplasmic (Apelbaum et al. 1988; Mehta et al. 1991) and plasma membrane fractions (Tassoni et al. 1998, 2002) that might mediate regulatory effects of polyamines on ion channel activities. Phosphorylation and dephosphorylation of ion channel proteins are closely related to their activities (Bethke and Jones 1997; Michard et al. 2005). Thus, polyamines could also affect protein kinase and/or phosphatase activities to regulate ion channel functions. Indeed, polyamines regulate the activity of certain protein kinases and a Tyr phosphatase in both animal and plant cells (Kuehn et al. 1979; Datta et al. 1987; Gupta et al. 1998). Identification of ion channel structural elements and/or receptor molecules regulated by polyamines would be of extraordinary relevance for elucidating the molecular mechanisms underlying polyamine action (Zhao et al. 2007).

Polyamines and Ca^{2+} homeostasis

As mentioned in previous sections, stress responses involve the generation of second messengers such as Ca^{2+} . The increase in cytosolic Ca^{2+} modulates the stress signalling pathways controlling stress tolerance. This increase of cytosolic Ca^{2+} levels may result from extracellular source (apoplastic space) and also from activation of PLC (phospholipase C), leading to hydrolysis of PIP_2 to IP_3 and subsequent release of Ca^{2+} from intracellular stores (Mahajan and Tuteja 2005). In guard cells, the increase in cytosolic Ca^{2+} may activate different ion channels and induce stomatal closure (Blatt et al. 1990; Gilroy et al. 1990). In animal systems, polyamines increase the IP_3 pool by stimulating biosynthesis (Singh et al. 1995) and decreasing catabolic activities of IP_3 5-phosphatase (Seyfred et al. 1984). Recently, Wilson et al. (2009) reported that mutations affecting the Arabidopsis SAL1 enzyme, which dephosphorylates both dinucleotide phosphates and inositol phosphates, result in enhanced drought tolerance. The *SAL1* mutant *alx8* shows very high Put levels (15-fold higher than wild type) that correlate with an increase in *ADC2* expression. The authors suggested that these high Put levels may be responsible for the improved drought tolerance phenotype and proposed that the high levels of Put might alter the phosphoinositol pools (see also Fig. 3). As discussed above, overexpression of *ADC2* in Arabidopsis also results in elevated levels of Put, which correlates with higher degree of water stress tolerance and a reduction of stomatal aperture (Alcázar et al. 2010). Yamaguchi et al. (2006, 2007) proposed that the protective role of Spm against high salt and drought stress is a consequence of altered control

of Ca^{2+} allocation through regulating Ca^{2+} -permeable channels, including CAXs. The increase in cytoplasmic Ca^{2+} results in prevention of Na^+/K^+ entry into the cytoplasm, enhancement of Na^+/K^+ influx to the vacuole or suppression of Na^+/K^+ release from the vacuole, which in turn increases salt tolerance (Yamaguchi et al. 2006; Kusano et al. 2007). Moreover, as mentioned above, changes of free Ca^{2+} in the cytoplasm of guard cells are involved in stomatal movement that may explain drought tolerance induced by Spm. All these data point to a possible link between polyamines, Ca^{2+} homeostasis and stress responses, which should be further explored.

Future perspectives in polyamine research

Many known “stress tolerance” genes act only in a narrow range of stress conditions that are often not relevant in the field. Therefore, genetic variations at these loci induce a limiting phenotypic variation in elite breeding or domesticated materials. As shown in this review, metabolic regulation of polyamines has now emerged as a promising approach to practical applications. Natural variation arises as an alternative approach to the modulation of polyamine content by genetic engineering. A remarkable natural diversity exists in polyamine content between different cultivars/accessions, which broadly correlates with stress tolerance traits (Bouchereau et al. 1999). Thus, there is a genetic potential for plants to modulate their polyamine levels to cope with stress conditions. The identification of genes underlying the differential regulation of polyamine levels can be achieved by traditional quantitative trait locus (QTL) mapping and cloning (Alonso-Blanco et al. 2009) or by genome-wide association mapping (Nordborg and Weigel 2008). The exploitation of the information revealed using plant models and the transfer of knowledge to a wide range of crop species for breeding purposes is a current challenge for the improvement of plant tolerance by modulation of polyamine content.

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