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3. Polyamine metabolism and signaling in plant abiotic stress protection

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Abstract. Polyamines (PAs) are small polycationic compounds present in all living organisms. Compelling evidences indicate a role for PAs in plant protection against stress. During the recent years, genetic, molecular and 'omic' approaches have been undertaken to unravel the role of PAs in stress signaling. Overall, results point to intricate relationships between PAs, stress hormone pathways and ROS signaling. Such cross-regulations condition stress signaling through the modulation of abscisic acid (ABA) and ROS amplification-loops. In this chapter we compile our recent findings which elucidate molecular mechanisms and signaling pathways by which PAs contribute to stress protection in plants.

1. Introduction

1.1. The importance of crop protection against abiotic stress

Abiotic stresses such as cold/freezing, salinity, heat and drought represent serious threats to agriculture. About 70% of yield losses among crops are attributed to abiotic stresses. Climatic change is predicted to increase global temperature, alter precipitation patterns, and intensify drought, increasing the need to grow crops in saline soil [1,2]. Within the

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European Union, the area affected by drought has doubled in 1991-2006, causing an estimated loss of € 8.7 billion in 2003, and up to 25% of yield losses in 2006. The specific threat of drought was acknowledged by an EU impact assessment calling for a multi-faceted policy including the 'use of more drought resistant crops'. Drought damages cannot be however viewed in isolation as they are often accompanied in the field by other stresses, such as heat, high light and increasing ozone concentrations. According to environmental predictions, during the 21st century global effects of desertification, salinization and atmospheric pollutants will produce severe limitations in arable lands with dramatic consequences in crop productivity.

In response to abiotic stress, plants initiate a plethora of biochemical and physiological changes. Importantly, a remarkable natural diversity exists in the ability of plants to cope with various stresses, ranging from highly sensitive plants to more tolerant ones. Thus, there is a genetic potential for plants to adapt to these stresses, preserving growth and/or high yield, but this potential has not been the main selective criteria in the domestication process, which yielded many crop cultivars that poorly cope with stress conditions. The past decade of international research characterized about 40 to 50% of gene functions conserved in the model plants Arabidopsis and rice, and sorted them into specific pathways. These efforts have created network models of stress and hormone regulatory pathways, as well as the definition of frameworks of co-regulated target genes of abiotic stress response pathways e.g. [3]. Except for few regulatory genes, transgenic approaches with individual stress-regulated candidate genes made so far little impact in breeding [4]. By contrast, regulation of the metabolism of compatible osmolytes, proline and polyamines (PAs) in particular, has emerged as more promising approach to practical applications. Elevated levels of PAs are one of the most remarkable changes that occur in plants in response to abiotic stress conditions [5]. The PA pathway interacts with metabolic routes of several signaling molecules (i.e. ethylene, NO, hydrogen peroxide) involved in abiotic stress responses [5]. Transcriptomic studies revealed differential regulation of genes in PA metabolism in response to different types of abiotic stress indicating that PAs are key regulatory molecules in abiotic stress signaling [6]. Here we focus on recent advances about the role of PAs in drought stress performed in the model species *Arabidopsis thaliana*, and discuss future perspectives and potential applications in crop protection.

1.2. Polyamine biosynthesis, catabolism and conjugation

Polyamines (PAs) are small polycationic compounds of low molecular weight which are present in all living organisms [5]. Most abundant

polyamines are the diamine putrescine (Put), the triamine spermidine (Spd) and the tetramine spermine (Spm) (Fig. 1). Presence of amine groups in their chemical structure provide positive charges under physiological pH, which allows the binding of PAs to negatively charged macromolecules such as DNA, proteins and phospholids [5,7]. In all living organisms, the first PA synthesized is Put by decarboxylation of ornithine through an enzymatic reaction catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17; Fig. 1). Plants and bacteria contain an alternative route to Put production by decarboxylation of arginine by arginine decarboxylase (ADC, EC. 4.1.1.19; Fig. 1). The product of ADC activity is agmatine, which is converted into Put in two enzymatic steps catalyzed by agmatine iminohydrolase (AIH, EC 3.4.3.12) and N-carbamoyl putrescine amidohydrolase (CPA, EC 3.5.1.53) (Fig. 1). Higher molecular weight PAs are produced by sequential addition of aminopropyl moieties to the Put skeleton through enzymatic reactions catalyzed by spermidine and spermine synthases (SPDS, EC 2.5.1.16 and SPMS, EC 2.5.1.22; Fig. 1). Donor of aminopropyl groups is decarboxylated S-adenosyl methionine (dcSAM), which is synthesized from decarboxylation of S-adenosyl methionine (SAM) by SAM decarboxylases (SAMDC, EC 4.1.1.50; Fig. 1).

The levels of free PAs depend on their biosynthesis, but also catabolism, transport and conjugation [5]. Polyamines are catabolized through diamine oxidases (DAO, EC 1.4.3.6) and polyamine oxidases (PAO; EC 1.5.3.3). DAOs catalyze the oxidation of Put producing 4-aminobutanal, H_2O_2 and ammonia. DAOs are present in monocots and dicots, but genes encoding these enzymes have been documented in few species [8]. PAOs bear a non-covalently bound molecule of flavin adenine dinucleotide (FAD) and are present at high levels in monocots [9]. PAOs are involved either in catabolism or back-conversion of PAs [5]. From the first group of PAOs, the maize PAO (ZmPAO) is the best characterized. ZmPAO is involved in the terminal catabolism of Spd and Spm producing 4-aminobutanal or (3-aminopropyl)-4-aminobutanal, along with 1,3-diaminopropane (Dap) and H_2O_2 [8], Fig. 1. The second group of plant PAOs resembles mammalian Spm oxidases (SMO, EC 1.5.3.3) that catalyze the back-conversion of Spm to Spd with concomitant production of 3-aminopropanal and H_2O_2 [10].

As anticipated, regulation of free PA contents is also achieved through their conjugation to hydroxycinnamic acids. So far, caffeoylputrescine, coumaroylputrescine, feruloylputrescine, coumaroylagmatine, dicoumaroyls-permidine, diferuloylspermidine, diferuloylspermine and feruloyltyramine have been identified in different plant species [11]. The ratios between free and conjugated PAs vary between plant species, being the conjugated forms especially abundant in Solanaceae [5].

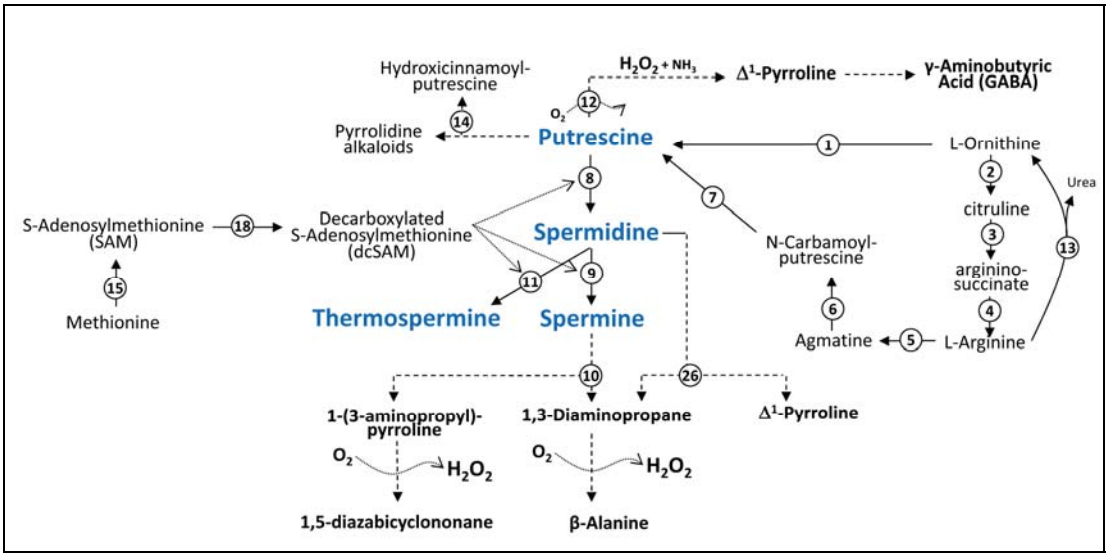

Figure 1. Polyamine metabolic pathway. Numbers refer to the following enzymes: 1, ornithine decarboxylase; 2, ornithine-carbamoyl transferase; 3, argininosuccinate synthase; 4, argininosuccinate lyase; 5, arginine decarboxylase; 6, agmatine iminohydrolase; 7, N-carbamoylputrescine amidohydrolase; 8, spermidine synthase; 9, spermine synthase; 10, polyamine oxidase (also involved in back-conversion to Spd and Spm); thermospermine synthase ACL5; 12, diamine oxidase; 13, arginase; 14, putrescine hydroxycinnamoyl transferase; 15, SAM synthetase; 18, SAM decarboxylase; 26, polyamine oxidase.

1.3. *Arabidopsis thaliana*: A model for polyamine research

Arabidopsis thaliana is a wild species distributed all over the world, mainly in the northern hemisphere. Its reduced size, easy transformation, high natural genetic variation and large number of molecular and genetic tools have made this species a model for molecular and genetic analyses. Also, knowledge derived from *A. thaliana* has successfully been applied to crop species.

The sequencing of the *A. thaliana* genome revealed the absence of the ODC pathway in this plant species [12]. Remarkably, ODC encoding genes are also absent in the genome of *A. lyrata*, ancestor of *A. thaliana*, thus suggesting that loss of *ODC* pathway occurred before the split between *A. thaliana* and *A. lyrata* lineages. *AIH* and *CPA* are found as single copy genes in *A. thaliana* [13,14]. Mutations in either *AIH* or *CPA* that disrupt their enzymatic activities lead to embryo lethality, thus evidencing the importance of keeping a minimum pool of PAs for plant survival (Alcázar *et al.*, unpublished results). Whereas *AIH* and *CPA* are found as single copy genes, *ADC* and *SPDS* encoding genes are found as duplicates (*ADC1*, *ADC2*, *SPDS1* and *SPDS2*) [7]. The finding of gene duplicates in *A. thaliana*

is frequent, due to the occurrence of large genome duplications in this species. However, the different gene paralogs may have evolved different *cis* elements in their promoters that provide differential transcriptional responses under stress [7]. This is the case for *ADC1* and *ADC2*. Whereas the expression of *ADC1* is highly up-regulated in response to cold [15], *ADC2* is responsive to drought, oxidative stress, salinity and biotic stress [7]. In addition, *ADC2* mRNA levels under non-stressed conditions are much lower than *ADC1*, which shows a more constitutive expression [16]. SPDS are also encoded by two genes namely *SPDS1* and *SPDS2*, whereas only one gene is found to encode SPMS [7]. Before its functional characterization, the *ACL5* gene was annotated as a spermine synthase [17]. This gene was identified in screens for mutants showing reduced stem size [17]. However *acl5* mutants do not show obvious reductions in Spm content (Alcázar *et al.*, unpublished observations). In the recent years it has been demonstrated that *ACL5* does not code for a spermine synthase, but for thermospermine synthase [18,19], thus evidencing that Spm biosynthesis also depends on a single gene (*SPMS*). Whereas depletion of Put and Spd levels in *A. thaliana* lead to loss of viability [20,21], the double *acl5/spms* mutant is still viable, thus evidencing that Spm and tSpm are not required for cell survival [22]. Nonetheless, *acl5/spms* mutants are more sensitive to stress conditions [23], although it remains to be clarified if this is due to pleiotropic effects of *acl5* mutation (Alcázar *et al.*, unpublished observations).

For an efficient metabolic canalization, some enzymatic pathways are assembled in macromolecular complexes called metabolomes. In the recent years our group has reported the first metabolon in plants involving aminopropyl transferases SPDS and SPMS [24]. Through a yeast two hybrid screen using one SPDS as bait, our group identified SPDS and SPMS interacting proteins [24]. Remarkably, ACL5 did not interact with SPDS or SPMS [24]. Through gel fractionation experiments from plant cell protein extracts, SPDS-SPMS protein assemblies were found associated to higher molecular weight complexes for which the molecular partners have not yet been identified [24]. The association between SPDS and SPMS in *A. thaliana* would provide an efficient canalization of the PA Put to Spm, something that has recently been observed in different species [25]. It remains to be studied at the proteomic level if the other components of the PA biosynthetic pathway (e.g. AIH, CPA, ADC and SAMDC) belong to the same macromolecular complex. Efforts are currently underway.

Arabidopsis thaliana contains five genes encoding putative PAOs [7]. PAO1 and PAO4 catalyze the same reaction as SMO [26,27], while PAO3 acts in the back-conversion pathway, converting Spm to Spd and Spd to Put [10]. 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 [28]. LSD1 acts as a histone demethylase, representing an important regulator of chromatin structure and gene expression [29]. *Arabidopsis* has four LSD1-related genes, some of which participate in the repression of *FLC*, a negative regulator of flowering time [30,31].

In the recent years, increasing interest has been shown to characterize the function of PAs in abiotic stress tolerance. In the following sections, we summarize where indications are found that PAs are key molecules in abiotic stress signaling and protection. Due to space limitations, we have focused on the role of PAs in drought stress, although our research laboratory is also interested in cold, salinity and we plan to get insight into biotic stress as well.

2. Polyamines in drought stress

The perception of water stress is rapidly sensed by plants and translated into a molecular signal which involves activation of mitogen-activated protein (MAP) kinase cascades, protein phosphatases, phospholipid signaling and multiple posttranslational modifications [32]. These signals induce transcriptional reprogramming of drought responsive genes, which are required to survive dehydration [32]. Eventually, stress signaling pathway activation leads to the accumulation of different osmolytes to cope with dehydration conditions. PAs accumulate to high levels in response to drought, consistent with their role on drought protection. In the recent years, molecular and genetic studies have revealed specific mechanisms of PAs in drought protection and signaling pathways involved. We describe in the following sections recent findings performed by our group evidencing PA cross-regulations with stress hormone pathways and metabolic canalizations of PAs in response to dehydration, in line with the PA-metabolon previously described [24].

2.1. ABA regulates PA-responsiveness to drought

The phytohormone abscisic acid (ABA) plays a key role in drought signaling and protection. Many drought-inducible genes are ABA-responsive, but also ABA-independent pathways are activated in response to drought conditions [33]. In order to determine the involvement of ABA in the transcriptional regulation of the PA biosynthetic pathway, Alcázar *et al.* [34] analyzed the expression of PA biosynthetic genes *ADC1, ADC2, AIH, CPA, SPDS1, SPDS2, SPMS, ACL5, SAMDC1* and *SAMDC2* in *A. thaliana* wild type plants and mutants impaired in ABA biosynthesis (*aba2-3*) or signaling (*abi1-1*). The ABA-deficient *aba2* mutants are blocked in the conversion of

xanthoxin to ABA-aldehyde and contain reduced levels of ABA in seeds and leaves [35]. These mutants also show reduced accumulation of ABA in response to drought conditions [35]. The *abi1* mutation affects ABA sensitivity in vegetative tissues and several ABA-mediated stress responses [36].

Wild type plants, *aba2-3* and *abi1-1* mutants where exposed to severe dehydration conditions during 24 h, and the expression of PA biosynthetic genes analyzed by quantitative RT-PCR after 0, 1, 2, 4, 8 and 24 h of treatment. *ADC2*, *SPDS1* and *SPMS* genes were among the most responsive to drought treatment under the imposed drought conditions (Fig. 2). Indeed, *ADC2* and *SPDS1* expression increased up to 32- and 25-fold respectively, whereas *SPMS* expression increased 75-fold after 24 h of treatment [34]. These observations suggested a key role of *ADC2, SPDS1* and *SPMS* conferring drought tolerance. Interestingly, whereas *ADC2* and *SPDS1* expression increased several fold after drought treatment, the expression of their gene paralogs *ADC1* and *SPDS2* did not change substantially [34]. These observations are consistent with the acquisition of certain stress-specificity probably due to divergent evolution of *cis* regulatory elements in their promoters. Indeed, different *cis* regulatory elements are found in the promoters of PA biosynthetic genes (Fig. 3). ABA-responsive elements (ABRE) or ABRE-related motifs are also found in the promoters of *ADC2, SPDS1* and *SPMS* [7], highly up-regulated in response to drought stress (Fig. 3).

The analysis in *aba2-3* and *abi1-1* mutants exposed to drought conditions showed much more moderate increases in *ADC2*, *SPDS1* and *SPMS* expression (Fig. 2) [34]. Hence, *ADC2* increased to a maximum of 7.5-fold after 24 h of treatment, which represented a reduction of 78% in fold induction compared to the stressed wild type [34]. *SDPS1* only increased up to 3.2-fold after 8 h (87% less increase than wild type) and *SPMS* increased 3.2-fold after 24 h (96% less than wild type). These results evidence that transcriptional up-regulation of *ADC2*, *SDPS1* and *SPMS* imposed by drought stress is mediated by ABA. Hence, ABA is an upstream regulator of PA biosynthesis in response to drought (Fig. 4).

To determine the effect of the transcriptional regulation of PA biosynthetic genes on PA levels, we analyzed the content of Put, Spd and Spm levels in response to drought. Wild type plants showed a progressive accumulation of Put in response to drought conditions, whereas this accumulation was absent in *aba2-3* and *abi1* mutants (Fig. 5) [34]. Hence, the ABA-dependent up-regulation in *ADC2* expression observed under drought conditions leads to an effective Put accumulation. An interesting finding from these results was the progressive reduction is Spm levels observed during dehydration, which was identified later on as a Put to Spm metabolic canalization coupled to back-conversion which serves as reactive oxygen species (ROS) amplification signal [25].

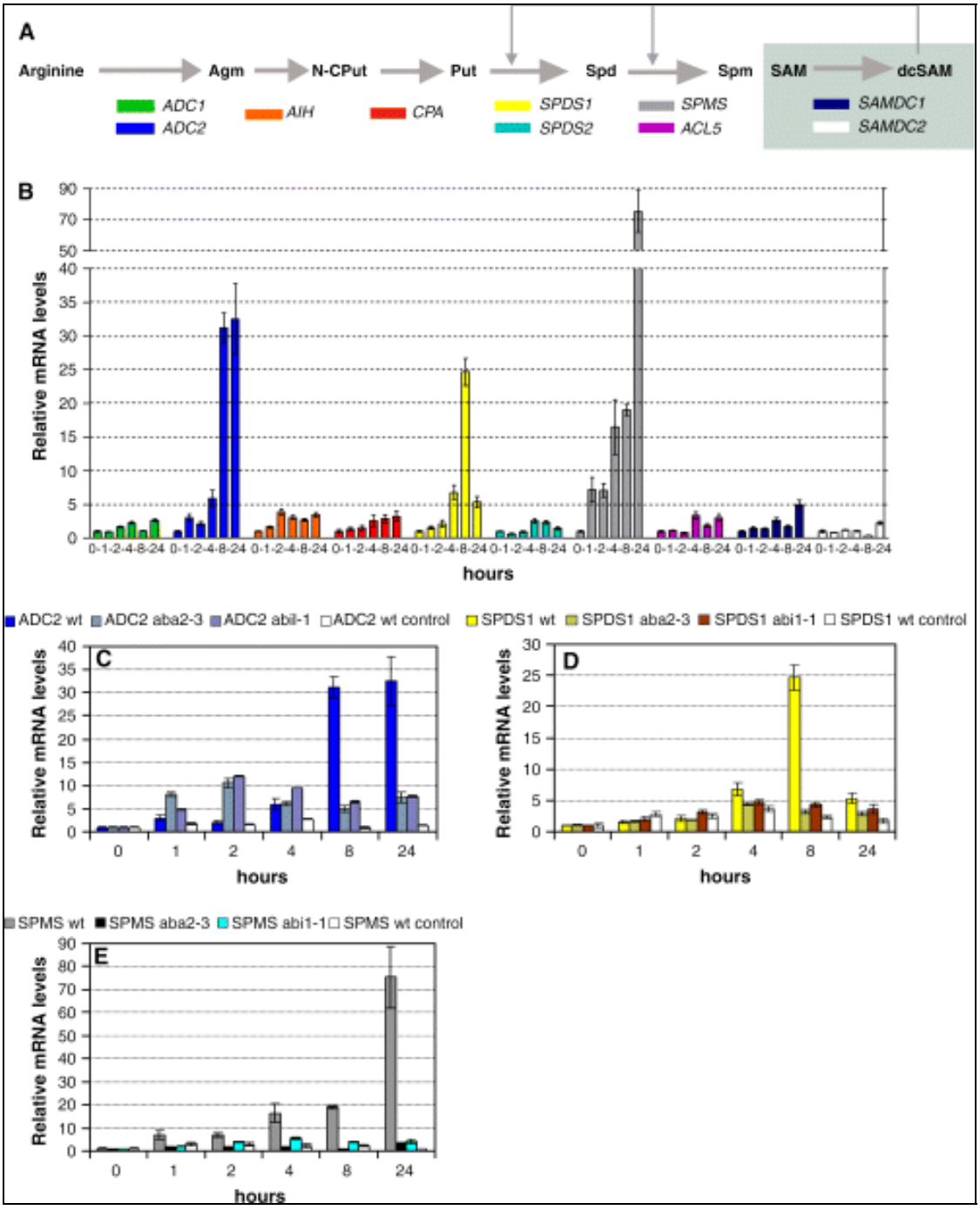

Figure 2. (A) Polyamine (PA)-biosynthetic pathway in *Arabidopsis thaliana*. **(B)** Relative transcript levels of PA-biosynthetic genes encoding arginine decarboxylase (*ADC1*, *ADC2*), agmatine iminohydrolase (*AIH*), N-carbamoylputrescine amidohydrolase (*CPA*), spermidine synthase (*SPDS1, SPDS2*), spermine synthase (*SPMS, ACL5*), S-adenosylmethionine decarboxylase (*SAMDC1*, *SAMDC2*) in wild-type (wt) plants exposed to water stress after 0, 1, 2, 4, 8 and 24 h of treatment. **(C)** Relative transcript levels of *ADC2*, **(D)** *SPDS1* and **(E)** *SPMS* in wt, *aba2-3* and *abi1-1* plants subjected to water stress, and non-stressed wt. These results were published by Alcázar *et al*. [34].

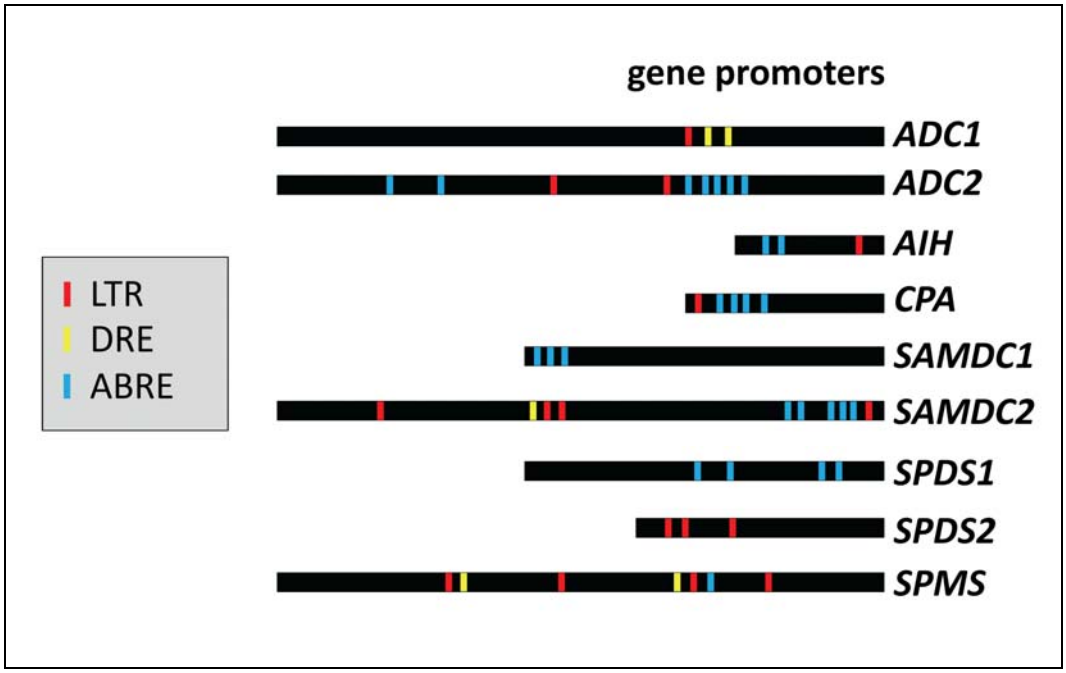

Figure 3. Cis-regulatory elements found in the promoters of PA biosynthetic genes. LTR, low temperature response element; DRE, dehydration responsive element; ABRE, ABA-responsive element. Picture adapted from Alcázar *et al.* [7].

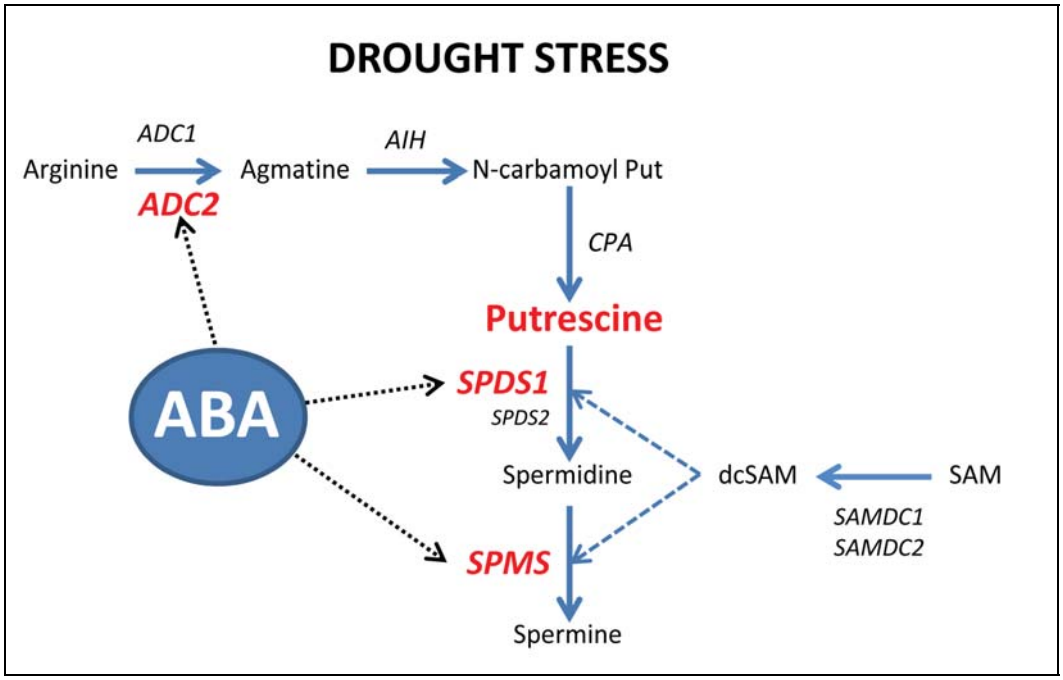

Figure 4. Scheme of the transcriptional regulation of PA biosynthesis by ABA. Drought stress leads to an increase in ABA levels which enhances the expression of ABA-responsive *ADC2*, *SPDS1* and *SPMS* genes. The increase in *ADC2* expression leads to Put accumulation, whereas increases in the expression of *SPDS1* and *SPMS* do not lead to accumulation of Spd or Spm.

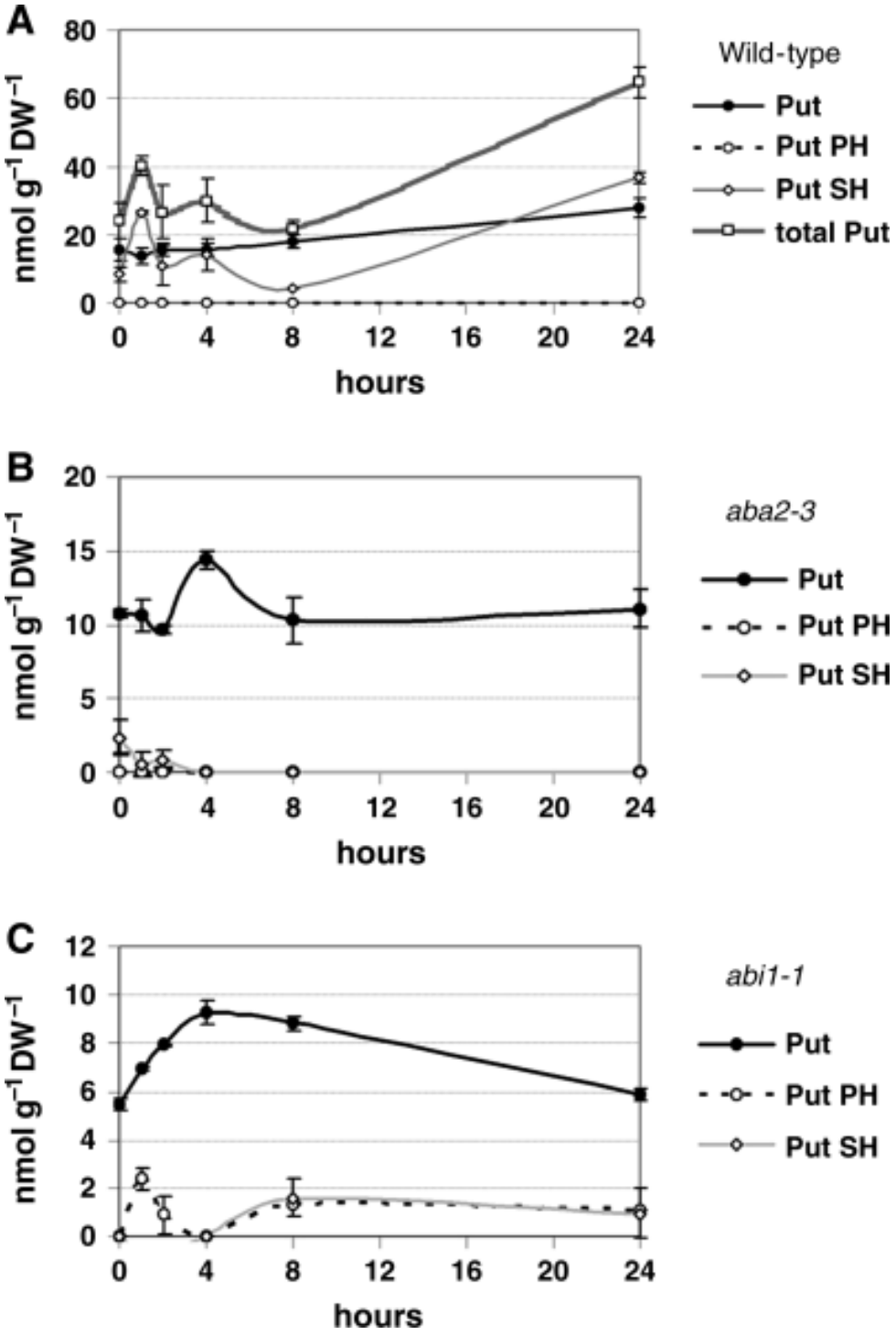

Figure 5. (A) Levels of putrescine (Put) from different polyamine fractions in wild-type, (B) *aba2-3* and (C) *abi1-1* plants exposed to water stress. Leaves from at least five plants per point of analysis were sampled after 0, 1, 2, 4, 8 and 24 h of stress treatment. Free, insoluble conjugated and soluble conjugated Put levels are referred as nmol g−1 dried weight (DW). Total levels of Put are also shown for wild-type plants. Values are mean ± standard error of three replicates in each one from three independent experiments. Published by Alcázar *et al.* [34].

2.2. Putrescine to spermine canalization in response to drought in *A. thaliana* and resurrection plants

A number of drought tolerant species have been used to study the molecular basis of desiccation tolerance. Among them, the most well characterized example is the South African resurrection plant *Craterostigma plantagineum*.

In a recent article by Alcázar *et al.* [25] we have compared the PA profiles and transcriptional responses of *A. thaliana* wild type plants and PA levels of *adc1-3*, *adc2-3*, *spds1-2*, *spds2-3* and *spms-2* mutants under a gradual drought acclimation response to the PA profiles of the resurrection plant *C. plantagineum* exposed to desiccation treatment. This is the first report on PA levels in a resurrection plant that provided clue on the differential regulation of the PA biosynthetic pathway between drought tolerant (*C. plantagineum*) and drought sensitive (*A. thaliana*) species.

In this work, we exposed plants to a progressive drought acclimation response by withholding water for 16 days and analyzed PA levels after 0, 2, 4, 6, 8, 10, 12, 14 and 16 days of treatment. The levels of Put accumulated to 1.8-fold after 2 days of treatment in wild type plants, which was in agreement with previous observations [34]. Interestingly, higher Put accumulation was observed in *spds1-2* mutant compared to wild type after 2 and 6 days (Fig. 6) [25]. As described in the introduction, *SPDS1* encodes one of the two *SPDS* gene paralogs that catalyze the conversion of Put to Spd. The accumulation of the precursor (Put) in *spds1-2* mutant under drought stress indicates that SPDS1 enzyme is involved in the Put to Spd conversion in response to dehydration [25]. Remarkably, peaks for Put accumulation correlate with higher *ADC2* expression [25]. On the other hand, mutations in *SPDS2* in the *spds2-3* mutant did not lead to evident increases in Put content in response to drought compared to wild type plants (Fig. 6) [25]. These observations evidence that *SPDS1* and not *SPDS2* is involved in the conversion from Put to Spd under drought stress [25,34].

An interesting finding in this work [25] was the absence of Spd accumulation (Fig. 6) even though a Put to Spd conversion was detected and mediated by *SPDS1*, thus suggesting that conversion to higher molecular weight polyamines (Spm) or degradation of Spd by PAO activity may contribute to Spd homeostasis. Indeed, the expression of deoxyhypusine synthase (*DHS*) followed similar kinetics to *SPDS1* and other ABA-inducible genes (e.g. *RD29A* and *RD22*) [25].

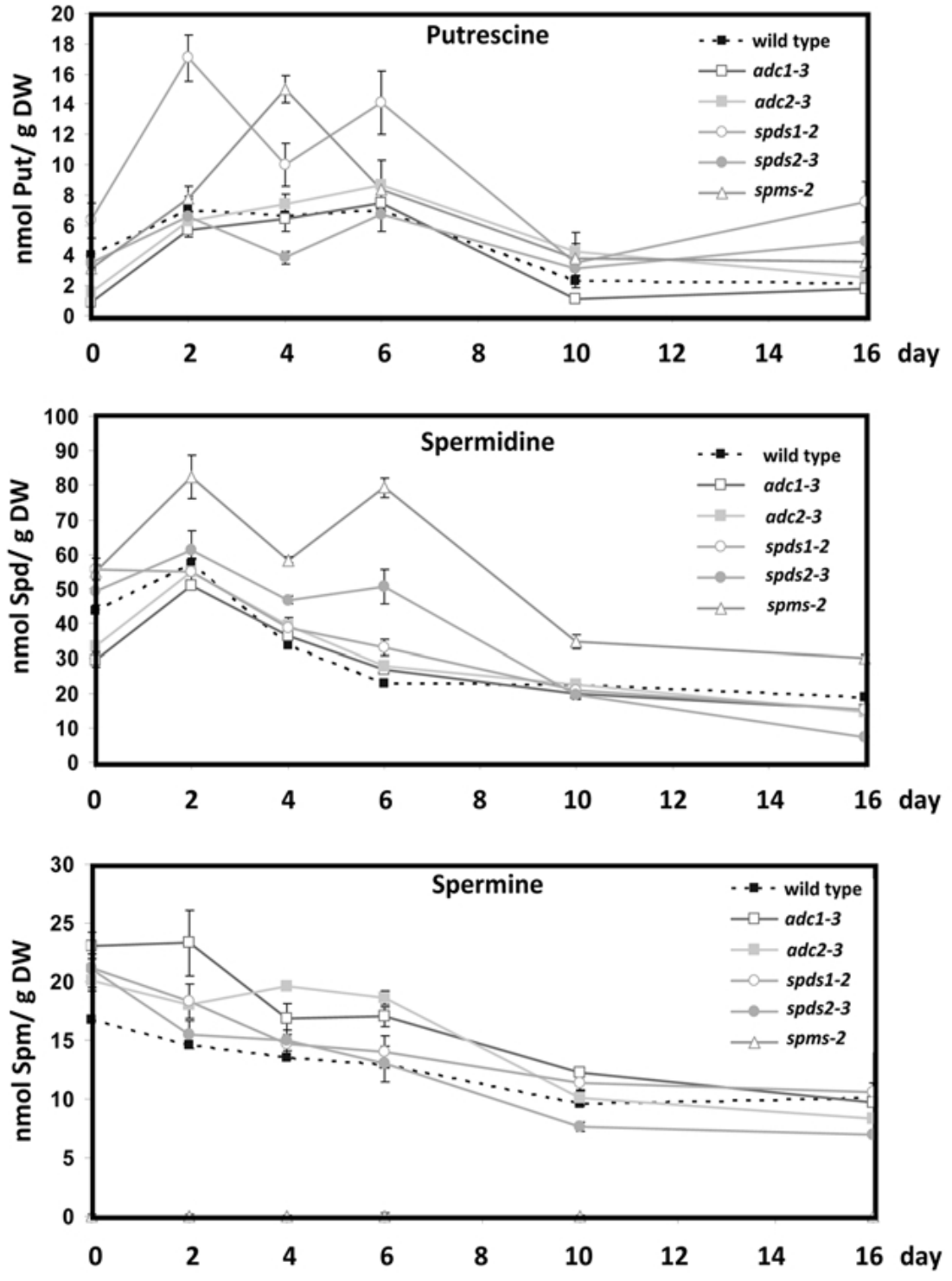

Figure 6. Polyamine (PA) profiles under drought stress in PA-biosynthetic mutants and wild-type *Arabidopsis thaliana* plants. Free putrescine, spermidine and spermine levels were analyzed in wild-type, *adc1–3*, *adc2–3*, *spds1–2*, *spds2–3* and *spms-2* mutants after 0, 2, 4, 6, 10 and 16 days of drought treatment. Values are the mean from three biological replicates ±standard deviation (SD). DW, dry weight. Results published by Alcázar *et al.* [25].

The deoxyhypusine synthase enzyme catalyzes the NAD-dependent formation of deoxyhypusine in the eukaryotic translation initiation factor 5A (eIF-5A), which requires Spd as substrate [37]. However, whether DHS activity significantly contributes to Spd homeostasis requires further analysis.

The conversion of Spd to Spm under drought stress was not observable in wild type plants (Fig. 6), which instead of accumulating Spm showed a progressive reduction is Spm levels [25]. However, loss of function of *SPMS*, involved in the enzymatic conversion of Spd and Spm, lead to evident increases in Spd and Put precursor in response to drought [25]. These observations were consistent with a Put to Spm canalization in response to drought that did not lead to the accumulation of the higher molecular weight polyamine Spm. To further determine a possible role of PAO in the oxidative deamination of Spm and depletion of Spm pools in response to drought, we measured Spm oxidase activity by detection of radiolabelled 1,5-diazabicyclononane in protein extracts supplemented with [^{14}C] Spm (Figure 7) [25]. SMO activity was detectable in wild type protein extracts, but did not increase in response to the imposed drought conditions [25].

These observations indicated that depletion of Spm pools was not due to Spm degradation. The reason of why Spm did not accumulate regardless of a strong Put to Spm canalization and absence of Spm degradation could involve the back-conversion pathway (Fig. 8). In the recent years, PAO involved in the back-conversion of Spm to Spd and Put have been characterized [5]. In *A. thaliana*, PAOs *AtPAO2* and *AtPAO3* are involved in the back-conversion of Spm to Put via Spd [10]. Interestingly, the expression of these two *PAO* is induced by ABA [10], which suggests a possible role in

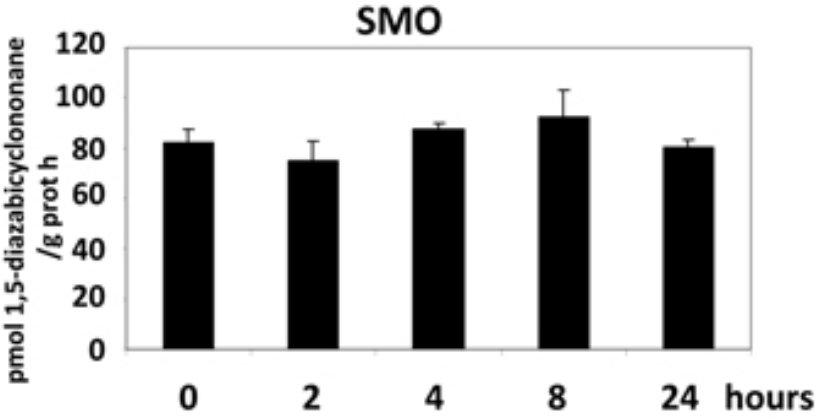

Figure 7. SMO enzymatic activities under drought. Wild type Arabidopsis plants exposed to drought stress were used for the analysis of spermine oxidase (SMO) activity at different time points of 24 h drought treatment. Values are the mean from three biological replicates ±SD. Results published by Alcázar *et al.* [25].

drought stress. Indeed, an increased expression of *AtPAO2* in response to drought was observed in wild type plants and followed similar expression kinetics as ABA-inducible genes *RD29A* and *RD22* [25]. Therefore, our results pointed to an active participation of the back-conversion pathway in the depletion of Spd and Spm pools during drought stress (Fig. 8).

A possible scenario for the contribution of PA back-conversion pathway under drought stress is the occurrence of a PA recycling loop that would serve as ROS signaling amplification by recurrent generation of hydrogen peroxide. Indeed, ROS signaling mediates many abiotic and biotic stress responses and is involved in activation of mitogen activated protein (MAP) kinase cascades [38].

In this work [25], we also analyzed the PA levels in response to drought in the resurrection plant *C. plantagineum*. During the course of the dehydration treatment, the levels of Spd and Spm in *C. plantagineum* progressively increase up to 3-fold and 8-fold, respectively, during 96 h of treatment (Fig. 9) [25]. Accumulation of Spd and Spm and consumption of the Put precursor correlated with enhanced drought tolerance. Hence, it is likely that Put to Spm canalization is an evolutionary conserved response between species, whereas the capability to accumulate high Spd and Spm levels discerns between drought tolerant or intolerant plants. These observations open a gate to manipulate PA levels for the development of plants with enhanced drought resistance. The modification of PA levels in plants can be achieved through transgenic manipulation or by exploitation of the natural variation in PA levels already present in nature.

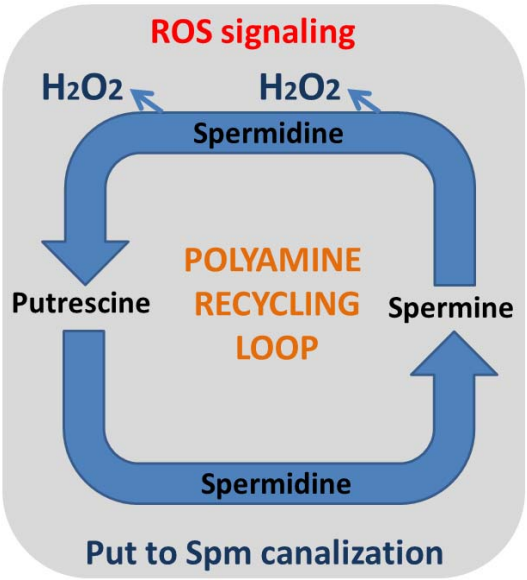

Figure 8. Polyamine recycling loop. Drought treatment leads to a Put to Spm canalization which is coupled with a Spm to Put back-conversion, releasing hydrogen peroxide that would serve as amplification of ROS signaling.

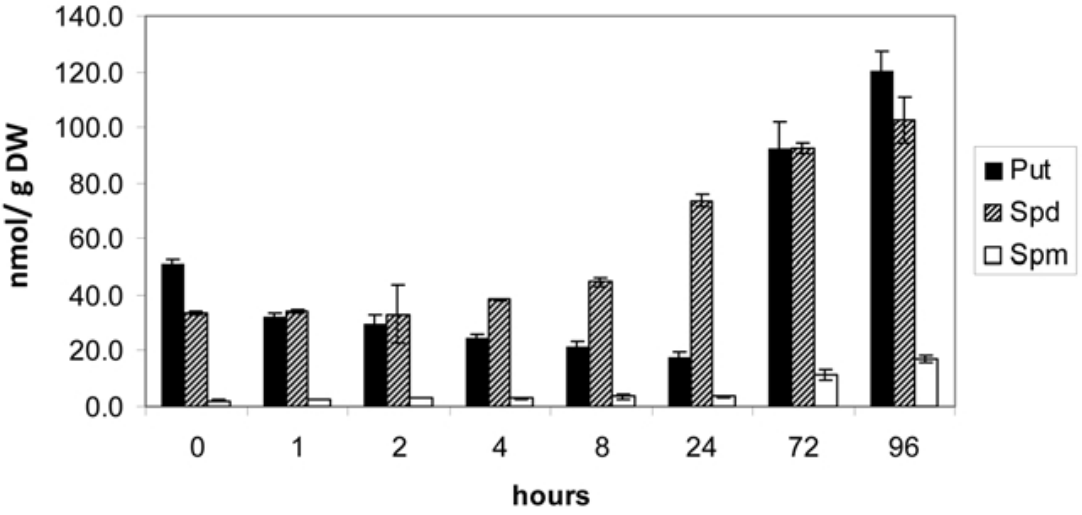

Figure 9. Free putrescine (Put), spermidine (Spd) and spermine (Spm) levels in *Craterostigma plantagineum* plants exposed to drought stress conditions for 0, 1, 2, 4, 8, 24, 72 and 96 hours. Values are the mean from three biological replicates ±SD. DW, dried weight. Results were published by Alcázar *et al.* [25].

2.3. Drought tolerance by genetic engineering of PA levels

The expression of *ADC2* is highly up-regulated in response to drought, and translated in an accumulation of Put. To determine a potential role of *ADC2* in conferring drought tolerance, we transformed *A. thaliana* plants with the homologous *ADC2* gene under the constitutive CaMV 35s promoter. The different lines analyzed (2.1, 3.6, 7.2) showed contrasting degrees of *ADC2* expression and Put accumulation [16]. Total Put content was between 12- and 2-fold higher than wild type depending on the transgenic line (Put content line 2.1 > 3.6 > 7.2 > wild type) [16,39]. These lines and wild type were exposed to drought stress conditions by withholding watering for 14 days, and their survival rates determined (Fig. 10) [39]. The extent of tolerance was scored by counting the number of plants that resumed growth after 7 days of recovery after re-watering. Interestingly, plants which accumulated higher levels of Put were more resistant to drought stress (Fig. 10) [39]. Hence, the line 2.1 which accumulated 12-fold more Put showed a survival rate of 75% compared to the wild type (12%) (Fig. 9) [39]. The enhanced drought tolerance correlated with a reduced stomata aperture and transpiration rate [39]. These observations are consistent with a role of PAs in the regulation of stomata aperture through modulation of ROS and NO signaling [5]. Our observations indicate that enhanced drought tolerance in plants can be achieved by manipulation of the PA pathway.

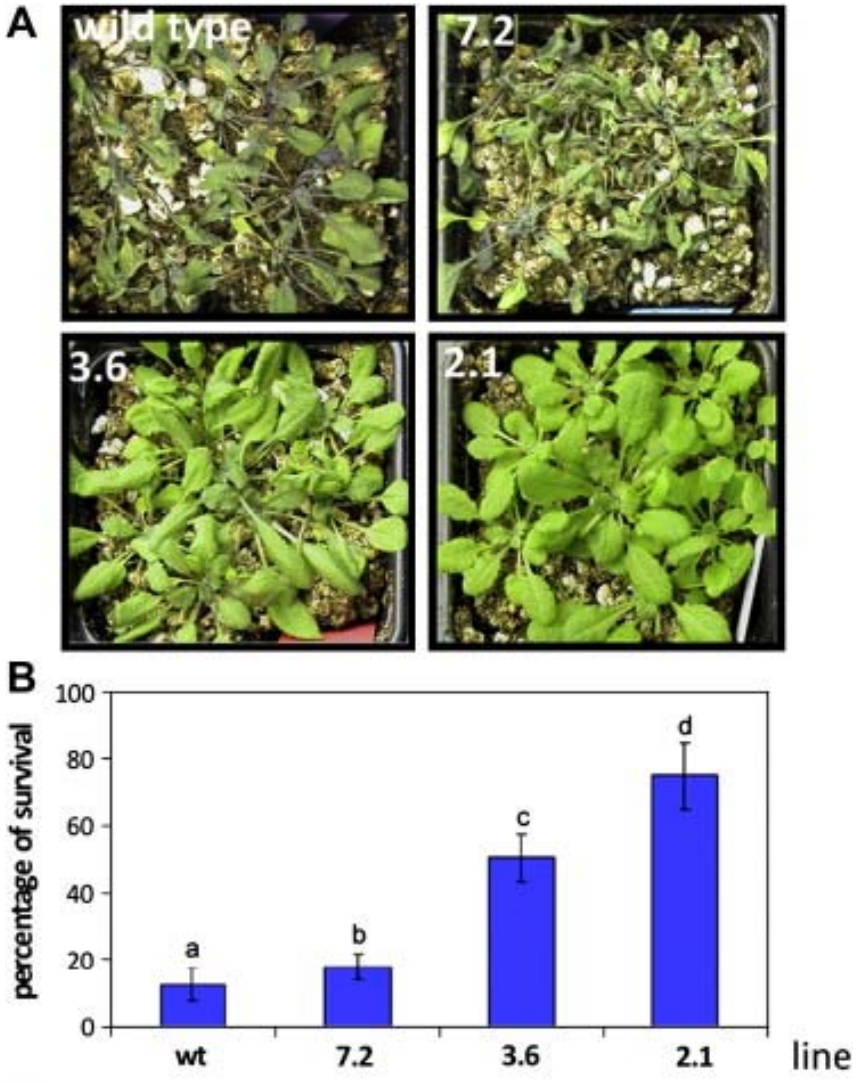

Figure 10. Drought resistance phenotypes of 35s *ADC2* lines 2.1, 3.6, 7.2 compared to wild-type. **(A)** Phenotype of 4-weeks-old plants dehydrated during 14 days. Wild-type, 35s *ADC2* lines 7.2, 3.6 and 2.1 have increased Put content which correlates with enhanced drought tolerance. **(B)** Survival percentages from 35s *ADC2* and wild-type plants after rehydration. Results were published by Alcázar *et al.* [39].

3. Genetics of natural variation for polyamine content

3.1. QTL analyses for PA levels using recombinant inbred line populations (RILs)

In addition to genetic engineering for PA content manipulation, the study of natural variation for PA content provides an alternative to the achievement of plants with enhanced PA levels and associated stress tolerance. In this regard, the study of natural variation for PA content in the model species *A. thaliana* is a good starting point. In the recent months, we have undertaken

some experiments to determine the feasibility of cloning QTLs for PA content in *A. thaliana*.

A preliminary analysis for PA content at 16ºC was performed using a recombinant inbred line (RIL) population of 164 lines derived from the cross between the European accession Landsberg *erecta* (L*er*) and the central Asian accession Kashmir-2 (Kas-2) [40]. These lines show a high transgression in different phenotypes including growth, flowering time and pathogen resistance [40]. We measured the Put, Spd and Spm levels after 5 weeks of growth at 16ºC and analyzed QTLs for Put, Spd and Spm contents. QTL detection was performed using the software MapQTL5. We could detect QTLs for Put, Spd and Spm which genetically explained significant variation of PA levels (Alcázar *et al.* unpublished results). These analyses evidenced that (i) QTLs for PA content are detectable in *A. thaliana*, (ii) part of the phenotypic variation for PA levels can be explained genetically and (iii) the mapping of QTLs for Put, Spd and Spm levels should identify novel genes contributing to PA homeostasis.

The identification of genes and natural alleles contributing to the modulation for PA contents may reveal geographical patterns of adaptation thus pointing to a close relationship between PA levels and local environments. These studies open a new gate to implement PA content regulation to breeding programs dedicated to pursue enhanced drought stress tolerance.

4. Concluding remarks and future perspectives

The genetic manipulation of PA levels enhances drought tolerance in different plant species. Nowadays, the mechanisms of action by which PAs confer enhanced drought resistance are beginning to be unraveled. Evidences point to the involvement of ROS signaling, possibly through PA-recycling loops involving PA back-conversion, as well as cross-talks with key stress hormone ABA. We anticipate that gaining insight into PA functions and exploiting natural variation for PA content regulation will provide new perspectives for crop protection against environmental change.

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