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Abscisic acid signals reorientation of polyamine metabolism to orchestrate stress responses via the polyamine exodus pathway in grapevine

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ABSTRACT

Polyamines (PAs) have been suggested to be implicated in plant responses to abiotic and biotic stress. Grapevine is a model perennial plant species whose cultivars respond differently to osmotic stress. In this study, we used two cultivars, one sensitive (S) and one tolerant (T) to drought. In adult vines subjected to drought under greenhouse conditions, total PAs were significantly lower in the control T- and higher in the control S-genotype and significantly increased or decreased, respectively, post-treatment. Soluble Put and Spd exhibited the greatest increase on d 8 post-treatment in the T- but not in the S-genotype, which accumulated soluble Spm. Abscisic acid (ABA) was differentially accumulated in T- and S-genotypes under drought conditions, and activated the PA biosynthetic pathway, which in turn was correlated with the differential increases in PA titers. In parallel, polyamine oxidases (PAOs) increased primarily in the S-genotype. ABA at least partially induced PA accumulation and exodus into the apoplast, where they were oxidized by the apoplastic amine oxidases (AOs), producing H_2O_2, which signaled secondary stress responses. The results here show that the ABA signaling pathway integrates PAs and AOs to regulate the generation of H_2O_2, which signals further stress responses or the PCD syndrome.

Introduction

Drought stress increasingly affects global agriculture, and tolerant plants employ a wide array of molecular and physiological strategies to cope with drought. In addition to morphological and anatomical adaptive alterations, tolerant plants express an array of specific transcription factors and genes. These genes encode, among other things, for enzymes regulating homeostasis of various cellular components, such as reactive oxygen species (ROS), abscisic acid (ABA), low molecular-weight osmolytes such as proline (Kishor et al., 1995; Zhu and Liu, 1998), polyamines (PAs), and others (Flores, 1991). Thus, conclusions drawn by the correlation and interplay of different pathways are important for further understanding the so-called 'tolerance traits'. Recently, using metabolomics, Urano et al. (2009) characterized the metabolic phenotypes of *Arabidopsis* wild type and a knockout mutant of the *NCED3* gene (*nc3-2*) under dehydration stress. *NCED3* plays a role in the dehydration-inducible biosynthesis of ABA, a phytohormone that is important in the dehydration stress response in higher plants. Metabolite profiling has revealed that accumulation of amino acids and PAs was dependent on ABA production, suggesting integration of ABA signaling to accumulation of protective molecules.

PAs, mainly the diamine putrescine (Put), the triamine spermidine (Spd), and the tetraamine spermine (Spm), are polycationic compounds of low molecular weight that are present in all living organisms. In plants, Put is derived from either Arg or Orn, *via* the Arg decarboxylase (ADC; EC4.1.1.19) or the Orn decarboxylase (ODC; EC4.1.1.17) pathways, respectively. Spd and Spm biosyntheses require the concerted action of spermidine synthase (SPDS; EC 2.5.1.16)/*S*-adenosyl-L-Met decarboxylase (SAMDC; EC 4.1.4.50) and spermine synthase (SPMS; EC 2.5.1.22)/SAMDC, respectively (Paschalidis et al., 2009).

To date, the best-known enzymes that catabolize higher PAs, generating hydrogen peroxide (H_2O_2) and reducing intracellular PA titers, are two amine oxidases (AOs), the diamine oxidase (DAO; EC 1.4.3.6), and polyamine oxidases (PAOs; EC 1.5.3.3). DAOs and PAOs are localized in peroxisomes and the apoplast (Moschou et al., 2008a; Rea et al., 2004; Reumann et al., 2009). Recently, we showed that during abiotic stress, apoplastic PAO is responsible for ROS generation (Moschou et al., 2008b). On abiotic stress, Spd is secreted into the apoplast, where it is oxidized by

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PAO, producing H_2O_2. Depending on its levels ('signatures') and the intracellular PA homeostasis, the generated H_2O_2 signals tolerance-effector genes to abiotic stress or induces execution of the programmed cell death (PCD) syndrome.

Extensive literature has presented the alterations in PA homeostasis under environmental stresses (Alcazar et al., 2006; Groppa and Benavides 2008; Liu and Moriguchi, 2007, and references therein. Interestingly, endogenous levels of individual and total PAs in roots of seedlings subjected to water stress increased to a significantly greater extent in the tolerant chickpea than in the sensitive soybean plants, and stress injury was more evident as PA levels declined in both plant species (Nayyar et al., 2005). The reduced levels of PAs in soybean, especially Put and Spd, when compared with chickpea, correlated to higher stress injury and decreased water content. Exogenous Put and Spd mitigated the stress-induced effects, particularly in soybean. Barley seedlings treated with Spd prior to a water deficit period reverted the increase in catalase and guaicol peroxidase activities produced by this stress, suggesting that PAs are able to affect the activity of H_2O_2-scavenging enzymes, moderating this signal at molecular level (Kubis, 2003).

Significant information has been accumulated on the protective role of PAs by the use of transgenic plants. Rice plants overexpressing ADC showed increased endogenous Put levels as well as drought tolerance, but the higher titers of Put were not enough to trigger the conversion of Put into Spd and Spm (Capell et al., 2004). Also, rice plants overexpressing SAMDC, showing increased Spd and Spm but not Put titers, exhibited enhanced recovery from water stress, but not increased tolerance during stress, correlating Put with direct tolerance and higher PAs with recovery efficiency (Peremarti et al., 2009). In summary, transgenic plants overexpressing PA biosynthetic genes were significantly more tolerant to drought (Kasukabe et al., 2004; Wen et al., 2008). On the other hand, a loss-of-function mutation in *Arabidopsis* of one of the two ADC encoding genes, the stress inducible one *ADC2*, resulted in plants that were more sensitive to salinity (Urano et al., 2004). Furthermore, the *Arabidopsis acl5/spms* mutant, which is unable to produce Spm, was hypersensitive to drought (Yamaguchi et al., 2007). These stress-sensitive phenotypes were reversed by the addition of exogenous Spm, and Spm also rescued *Arabidopsis* from drought stress (Kusano et al., 2008). On the other hand, transgenic tobacco plants overexpressing the *PAO* gene were not able to cope with excessive oxidative burst induced by abiotic factors, in contrast with tobacco plants with down-regulated *PAO* (Moschou et al., 2008a, b).

PAs have been shown to up-regulate stress-protective genes, additionally possessing a direct signaling effect. Transgenic pine plants overexpressing the gene *CaPF1*, which encodes for the ethylene responsive factor ERF/AP2-type transcription factor, exhibited increased tolerance to drought, freezing, and salt stress (Tang and Newton, 2005). Also, recent results have provided evidence for the direct involvement of PA oxidation in plant adaptation to stresses, both abiotic and biotic (Moschou et al., 2008a, b, 2009; Yoda et al., 2003, 2006). Hydrogen peroxide, derived from PA catabolism, could also exert signaling effects (Moschou et al., 2008b) such as expression of the gene encoding for the α-subunit of glutamate dehydrogenase under salinity, leading to increased Pro synthesis (Skopelitis et al., 2006). Further, molecules produced through PAO action can exert signaling effects. In particular, 4-aminobutanal can be further metabolized to GABA through the action of an aldehyde dehydrogenase. DAP, on the other hand, is involved in tolerance, as it is a precursor of β-alanine and uncommon PAs.

In order to further explore the potential mechanism(s) linking PA homeostasis to stress tolerance, we compared drought-tolerant (T-) and sensitive (S-) genotypes of a perennial model plant, the grapevine, subjected to osmotic stress using *in planta* as well as *in vitro* models, such as leaf discs and cells. The results demonstrate that the intrinsic ABA signal up-regulates PA metabolism, which in turn increases endogenous H_2O_2 load through the apoplastic PA exodus/catabolism pathway, affecting among others secondary stress responses. Moreover, increased rates of PA synthesis in the tolerant genotypes *versus* the sensitive ones are linked to abiotic stress tolerance.

Materials and methods

Plant material and culture conditions

Two *Vitis vinifera* cultivars contrasting in drought tolerance, Kahli kerkennah and Guelb sardouk (Toumi et al., 2008), were obtained from dormant cuttings planted in sand in a controlled culture room (28 °C temperature, 90% humidity, and 16/8 h photoperiod) before being transferred to a soil substrate and cultivated in a greenhouse (16 h light period at a photosynthetic active radiation of at least 300 $\mu E\ m^{-2}\ s^{-1}$, temperature between 25 and 28 °C, and relative humidity ranging from 60% to 70%). Six-month old plants were subjected to water shortage for 8 d.

ABA extraction and quantification

Leaves (100 mg) were homogenized in cold methanol (80%) at room temperature for 20 min. Samples were centrifuged for 15 min at 10,000 rpm and 4 °C. This extraction was repeated twice and the supernatant was extracted and filtered through Whatman No. 5 filter paper. The supernatants were re-homogenized with 1 volume of chloroform, with the same solution mixture and the extracts were combined. Samples were centrifuged for 15 min at 10,000 rpm and 4 °C. The organic phase was separated in a new tube and evaporated under vacuum at 40 °C. The dry pellet was dissolved in acetonitrile. Samples were separated using a silica gel G TLC plate and developed with benzene/acetone/acetic acid (70:30:1), giving an ABA zone in the region of R_f 0.40. After drying, the chromatograph was sprayed with 10% aqueous sulfuric acid and heated at 130 °C for 8 min. ABA treated with sulfuric acid in this way fluoresces under UV light. The fluorescing bands were scrapped off and quantified using spectrophotometric analysis against ABA standards. The spectrum of the scrapped zone was also verified against the standard.

ABA treatments

Leaf discs were obtained from mature leaves of the drought-tolerant Kahli kerkennah and the drought-sensitive Guelb sardouk and floated in MES medium (50 mM KCl and 10 mM MES [2-(Nmorpholino) ethanesulfonic acid], pH 5.7) before to be treated with 10 μM ABA or 200 mM mannitol for a maximal period of 24 h.

Cell suspension preparation

Cell suspensions were initiated from shoot segment-generated callus in Roubelakis basal medium (Roubelakis-Angelakis and Zivanovitc, 1991) supplemented with BAP (0.47 $mg\ L^{-1}$) and NAA (0.93 mgL^{-1}) and adjusted to pH 6.4. Cell cultures were maintained in a rotary shaker (150 rpm) at 24 °C. After 6 weeks, cell suspensions were divided into 100 mL aliquots and supplemented with mannitol (200 mM) or ABA (10 μM) for 48 h. At different