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Deregulation of apoplastic polyamine oxidase affects development and salt response of tobacco plants

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ABSTRACT

Polyamine (PA) homeostasis is associated with plant development, growth and responses to biotic/abiotic stresses. Apoplastic PA oxidase (PAO) catalyzes the oxidation of PAs contributing to cellular homeostasis of reactive oxygen species (ROS) and PAs. In tobacco, PAs decrease with plant age, while apoplastic PAO activity increases. Our previous results with young transgenic tobacco plants with enhanced/reduced apoplastic PAO activity (S-*ZmPAO*/AS-*ZmPAO*, respectively) established the importance of apoplastic PAO in controlling tolerance to short-term salt stress. However, it remains unclear if the apoplastic PAO pathway is important for salt tolerance at later stages of plant development. In this work, we examined whether apoplastic PAO controls also plant development and tolerance of adult plants during long-term salt stress. The AS-*ZmPAO* plants contained higher Ca^{2+} during salt stress, showing also reduced chlorophyll content index (CCI), leaf area and biomass but taller phenotype compared to the wild-type plants during salt. On the contrary, the S-*ZmPAO* had more leaves with slightly greater size compared to the AS-*ZmPAO* and higher antioxidant genes/enzyme activities. Accumulation of proline in the roots was evident at prolonged stress and correlated negatively with *PAO* deregulation as did the transcripts of genes mediating ethylene biosynthesis. In contrast to the strong effect of apoplastic PAO to salt tolerance in young plants described previously, the effect it exerts at later stages of development is rather moderate. However, the different phenotypes observed in plants deregulating *PAO* reinforce the view that apoplastic PAO exerts multifaceted roles on plant growth and stress responses. Our data suggest that deregulation of the apoplastic PAO can be further examined as a potential approach to breed plants with enhanced/reduced tolerance to abiotic stress with minimal associated trade-offs.

1. Introduction

Salinity is a major abiotic threat to global agriculture due to increasing use of poor quality water for irrigation and soil salinizatictive aliphatic polycations named polyamines (PAs), the di-amine putrescine (Put), the tri-amine spermidine (Spd) and the tetra-amine spermine (Spm) affect developmental and metabolic processes, as well as a vast range of stress responses. PAs homeostasis is maintained by the fine orchestration of biosynthesis, conjugation, compartmentalization, transport and oxidation/back-conversion. The apoplastic flavoprotein PA oxidase (PAO) oxidizes Spd yielding Δ^1- pyrroline and Spm yielding 1,5-diazabicyclononane, along with 1,3-diaminopropane (1,3-Dap) and H_2O_2, affecting PA and H_2O_2 homeostasis, and the ratio of biosynthesis/catabolism (Mattoo et al., 2006; Moschou et al., 2008c, 2009).

Previously, we used young tobacco transgenic seedlings with increased (S-*ZmPAO;* overexpressing *ZmPAO*) and reduced apoplastic PAO activity (AS-*ZmPAO*; overexpressing the antisense cDNA from *ZmPAO* that blocks translation of tobacco PAO; Moschou et al.,

Abbreviations: PAs, polyamines; PAO, polyamine oxidase; WT, wild type tobacco; S-*ZmPAO*/AS-*ZmPAO*, sense/antisense-*ZmPAO* transgenic plants; A_{net}, net photosynthetic rate; CCI, chlorophyll content index; DAT, days after treatment; FW, fresh weight; DW, dry weight; FRAP, ferric reducing antioxidant power; LA, leaf area; QY, quantum yield of PSII; REL, root electrolyte leakage; ROS, reactive oxygen species.

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2008a,b) to study the effect of apoplastic PAO to salinity. Salinity or pathogens induce Spd exodus and oxidation in the apoplast which significantly contributes to regulation of H_2O_2 'signatures' defining the outcome of stress responses (Moschou et al., 2009; Moschou and Roubelakis-Angelakis, 2014). NADPH-oxidase and the apoplastic PAO constitute a feedforward ROS amplification loop, which impinges on oxidative state and culminates in the execution of programmed cell death (PCD). The data suggest that this loop is a central hub in the plethora of responses controlling salt stress tolerance, with potential functions extending beyond stress tolerance (Gémes et al., 2016). S-*ZmPAO* transgenic tobacco plants show increased inter-/intracellular levels of the reactive oxygen species (ROS) H_2O_2 and $O_2^{\bullet -}$ and expression of antioxidant genes, which are nevertheless insufficient to scavenge these ROS efficiently (Moschou et al., 2008a,b). The *AS-ZmPAO* tobacco young seedlings show tolerance to short-term salt stress, either due to increased PA content or decreased ROS levels from the impaired PA oxidation in the apoplast (Moschou et al., 2008a,b).

Given that the severity/mode(s) of response(s) to stress are cell/tissue/organ but also ontogenetic stage-specific processes, we assessed tolerance and examined growth characteristics of adult S-*ZmPAO* and AS-*ZmPAO* plants under control conditions or during long-term salt treatments. We also determined photosynthetic parameters, the intracellular monovalent and divalent cation contents, under prolonged/varying salt stress, and also the efficiency of the antioxidant enzymes and machinery. Our results highlight the developmental stage-specific role of apoplastic PAO in the regulation of development and plant responses to salt stress.

2. Materials and methods

2.1. Plant material, growth conditions and salt treatments

Partial cDNA cloning of the *PAO* gene (Yoda et al., 2006), vector construction, plant transformation and molecular analysis of the transgenics were described by Moschou et al. (2008a,b). Plants of WT (*Nicotiana tabacum* cv Xanthi) and the transgenic lines S2.2 and A2, over-/downexpressing the *ZmPAO* gene, respectively (Moschou et al., 2008a,b) were used. These lines from now-on will be referred as S-*ZmPAO* and AS-*ZmPAO*. The plants were grown as described earlier (Mellidou et al., 2016). In brief, seeds from the three genotypes were cold stratified (4 °C, 7 days), and immersed in a 1% potassium nitrate solution for 12 h. Then, the seeds were sown in 10-cm diameter pots filled with peat (Terraplant, Compo) and perlite (2:1; v/v) mixture, and placed under plastic cover for 3 d to preserve high relative humidity. At the four-true-leaf stage, 120 seedlings per genotype were transplanted in 0.25 L pots, filled with the aforementioned mixture and placed in a completely randomised design (split-split-plot arrangement). At the 4–5 pair true leaf stage (70 day-old plants), five salt treatments (0, 50, 100, 200, 300 mM NaCl) were applied, each on 24 plants per genotype. All plants were subjected to salt treatment through irrigation either with 100 mL aqueous solution of the respective NaCl dose or with 100 mL water (controls) at 0, 4, 7, 11, 14 and 18 days after the first treatment (DAT). At four sampling times (0, 7, 14, 21 DAT) six plants/genotype-treatment (each considered as a single replication) were harvested. Growth, developmental and physiological characteristics were evaluated on three plants and phytochemical parameters were determined on the other three.

In addition, seeds were surface sterilised with bleach for 10 min, washed four times with sterile water and plated on filter paper in plates with 1/2Murashige and Skoog medium [Duchefa Biochemie, Haarlem, Netherlands; supplemented with 0.05% MES/KOH, pH 5.7 (2-(*N*-morpholino) ethanesulfonic acid) (Sigma-Aldrich, St. Louis MO, USA), Gamborg B5 vitamins and micronutrient mixture (Duchefa), 2% (w/v) sucrose]. Seedlings were grown in a growth chamber for 13 days. Seedlings were transplanted in plates with the same medium containing 0 or 100 mM NaCl for 2 more days before being used. All plants were grown under long day conditions (16/8 h photoperiod) at 22/18 °C, and 110 μE m^{-2} s^{-1} PAR supplied by cool-white fluorescent tungsten tubes (Osram, Berlin, Germany).

2.2. RNA extraction and semi-quantitative RT-PCR and q-RT-PCR

Total RNA isolation and RT-PCR were performed as described earlier (Mellidou et al., 2016). Conditions of PCR amplification were as follows: 2 min at 94 °C, 1 min at 92 °C, 1 min at the appropriate annealing temperature, 35 s at 72 °C, and a final extension step of 5 min at 72 °C. All amplicons were separated by 1.4% (w/v) agarose gel electrophoresis, stained with EtBr and visualised under UV light. The total density of individual bands was measured with GelEval software package (v1.37, Frog Dance Software). In addition, to q-RT-PCR, isolation of total RNA was performed as previously described (Wi and Park, 2002). One μg of total RNA from leaves was reverse-transcribed for 30 min at 42 °C in a 20 μL reaction volume using a High Fidelity PrimeScript™ RT-PCR kit (Takara, Japan) according to the manufacturer's instructions. The q-RT-PCR reactions were carried in Chromo 4™ Continuous Fluorescence Detector (Bio-Rad, USA). Ct values were analyzed using MJ Opticon Monitor Software version 3.1 (Bio-Rad, USA) and then exported to Microsoft Excel for further analysis. The reference gene *β-ACTIN* was used. The primers used appear in Suppl. Table S1.

2.3. Protein extraction and in-gel enzyme assays

Proteins were extracted and treated as described in Papadakis and Roubelakis-Angelakis (2005). For native electrophoresis and enzyme activity stainings, proteins were electrophoretically resolved using native PAGE and then stained. For SOD activity staining the procedure was carried out according to Andronis et al. (2014). SOD enzyme activity was determined by incubating the gel in 50 mM potassium phosphate buffer (pH 7.4) containing 2 mg/mL NBT for 30 min in dark, and then in another 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mg/mL riboflavin and 0.25% TEMED for 20 min in dark. The gel was placed into distilled water in light until bands were detected.

2.4. Ion content

Ion content (K^+, Na^+, Ca^{2+}, and Mg^{2+}) was determined in shoots of AS-*ZmPAO*, S-*ZmPAO* transgenic and WT plants grown in the presence of 0, 50, 100, 200, and 300 mM NaCl at 0, 7, 14 and 21 DAT as already reported (Mellidou et al., 2016). The monovalent ions (K^+ and Na^+) were determined with a flame photometer (Jenway PFP 7, Gransmore Green, Felsted England), and the divalent ones (Ca^{2+} and Mg^{2+}) with an atomic absorption spectrophotometer (Shimadzu AA 6300, Japan).

2.5. Photosynthetic parameters: photosynthetic yield, chlorophyll content index and net photosynthesis

In an attempt to assess to what extent deregulation of *PAO* affects photosynthetic efficiency, photosynthetic yield [effective quantum yield of photochemical energy conversion in photosystem II or simply quantum yield (QY)], chlorophyll content index (CCI=% transmittance at 931 nm/% transmittance at 653 nm) and net photosynthetic rate (A_{net}, μmol $m^{-2}s^{-1}$) were determined in AS-*ZmPAO*, S-*ZmPAO* transgenics and WT plants, on the fifth fully expanded leaf (counting from the apex) of 70 day-old plants (time 0), subsequently of 77, and 84 day-old plants (7 and 14DAT) grown in the presence of 0, 50, 100, 200, and 300 mM NaCl, as described