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Short communication

Underexpression of apoplastic polyamine oxidase improves thermotolerance in *Nicotiana tabacum*

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ABSTRACT

Polyamines (PAs) and hydrogen peroxide (H_2O_2), the product of PA oxidation by polyamine oxidase (PAO), are potential players affecting plant growth, development and responses to abiotic/biotic stresses. Genetically modified *Nicotiana tabacum* plants with altered PA/H_2O_2 homeostasis due to over/underexpression of the *ZmPAO* gene (S-*ZmPAO*/AS-*ZmPAO*, respectively) were assessed under heat stress (HS). Underexpression of *ZmPAO* correlates with increased thermotolerance of the photosynthetic machinery and improved biomass accumulation, accompanied by enhanced levels of the enzymatic and non-enzymatic antioxidants, whereas *ZmPAO* overexpressors exhibit significant impairment of thermotolerance. These data provide important clues on PA catabolism/H_2O_2/thermotolerance, which merit further exploitation.

1. Introduction

Heat stress (HS) is a potential environmental threat resulting to severe crop losses. Depending on plant genotype/organ/tissue, the ontogenetic stage, and the HS magnitude/duration, HS can drastically reduce plant growth, development and productivity (Mittler and Blumwald, 2010). Plants have evolved molecular, biochemical, structural, and physiological strategies for HS tolerance/acclimation. Preservation of the photosynthetic apparatus and reactive oxygen species (ROS) homeostasis are among the key elements of the HS-protection/adaptations strategies (Gill and Tuteja, 2010). Understanding these mechanisms is therefore among the cutting-edge research topics in plant stress biology and of outmost importance for developing tolerant crops.

Polyamines (PAs), which include the triamine spermidine (Spd), the tetramines spermine (Spm) and thermospermine (TSpm), and their precursor, the diamine putrescine (Put), correlate with various developmental processes as well as with stress responses (Paschalidis and Roubelakis-Angelakis, 2005a,b; Moschou et al., 2008a,b; Minocha et al., 2014; Gémes et al., 2017 *inter alia*). The inter/intracellular PA homeostasis is controlled by the fine orchestration of biosynthesis, oxidation/back-conversion, acetylation, conjugation, transport, and compartmentalization (Minocha et al., 2014). Oxidation of Spd and Spm is catalyzed by the apoplastic polyamine oxidase (PAO; EC 1.5.3.3), or back-converted by the peroxisomal and cytoplasmic PAOs, with the simultaneous production of H_2O_2 (Fincato et al., 2011; Moschou et al., 2008c). Genetically engineered plants with deregulated PA homeostasis are useful tools to gain new insights on the potential mode(s) of action of PAs (Moschou et al., 2008a,b; Gill and Tuteja, 2010; Minocha et al., 2014; Liu et al., 2015; Mellidou et al., 2016; Gémes et al., 2016, 2017). Within this context, overexpression of *ZmPAO* in tobacco increased PAO-dependent H_2O_2 production, and although the enzymatic antioxidant machinery was highly induced, further increase in the intracellular H_2O_2 resulted in the activation of Programmed Cell Death (PCD; Moschou et al., 2008a,b). Under salinity, Spd is excreted into the apoplast where it is oxidized by the apoplastic PAO, generating H_2O_2. AS-*ZmPAO* plants accumulated less H_2O_2 and exhibited lower apoptosis-like cell death than the WT, in contrast to S-*ZmPAO*. Induction of either stress-responsive genes or of the PCD syndrome was dependent on the level of Spd-derived apoplastic H_2O_2. Recently, use of PAO-transgenics revealed an NADPH-oxidase/PAO feedback loop which controls oxidative burst under salinity (Gémes

Abbreviations: AAE, ascorbic acid equivalents; A_{net}, net photosynthetic rate; CAT, catalase; CCI, chlorophyll content index; FW, fresh weight; GAE, gallic acid equivalents; G_s, stomatal conductance; H_2O_2, hydrogen peroxide; HS, heat stress; PAs, polyamines; PAO, polyamine oxidase; PCD, programmed cell death; POX, peroxidase; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; Spm, spermine; S-*ZmPAO*/AS-*ZmPAO*, sense/antisense *Zea mays* polyamine oxidase transgenic plants

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et al., 2016).

The potential mode(s) of action of PAs under HS is an open question to a great extent. Polyamines protect against heat (Liu et al., 2015), possibly by maintaining thermostability of thylakoid membranes and affecting biosynthesis of HSPs (Königshofer and Lechner, 2002). In this work, the tobacco transgenics AS-*ZmPAO* with reduced PA catabolism and load of H_2O_2, and S-*ZmPAO* with higher PA catabolic activity and load of H_2O_2 (Moschou et al., 2008a,b) were assessed under HS. The results support that underexpression but not overexpression of *PAO* results to improved thermotolerance, more likely *via* the more tolerant photosynthetic apparatus and the induced antioxidant machinery.

2. Materials and methods

2.1. Plant material, growth conditions and HS treatments

Partial cDNA cloning of the *PAO* gene, vector construction, plant transformation and molecular analysis of the transgenics were described by Moschou et al. (2008a,b). Tobacco (*Nicotiana tabacum*) cv Xanthi plants (WT), and the transgenic lines A2 and S2.2, under/overexpressing the *ZmPAO* gene (AS-*ZmPAO* and S-*ZmPAO,* respectively) were used (Moschou et al., 2008a,b). The plants were grown as described earlier (Mellidou et al., 2016). At the four-pair true leaf stage (60-day old plants), 30 seedlings/genotype were transplanted in 0.25L pots filled with peat (Terraplant, Compo) and transferred to a growth chamber (Snijders Microclima 1750, Snijders Scientific BV, Netherlands) under non-stressed conditions at 16/8 h photoperiod and 25/22 °C. A week later, HS was applied for seven days as follows: 39 °C ± 1 °C (16 h light) with 60% RH and 30 °C ± 1 °C (8 h dark) with 65–70% RH. All plants were watered regularly every other day. Five plants were used per genotype/treatment.

2.2. Photosynthetic parameters and biomass allocation

Net photosynthetic rate (A_{net}, μmol m^{-2} s^{-1}), and chlorophyll content index were determined in AS-*ZmPAO*, S-*ZmPAO* transgenics and in WT plants, as described earlier (Mellidou et al., 2016), using a portable photosynthesis system (LI-6200, LI-COR Inc. Lincoln NE, USA), and an Opti-Sciences CCM-200 chlorophyll content meter (OptiSciences Inc., Tyngsboro MA). For CCI, 10 measurements were taken per genotype and treatment. For evaluating biomass allocation in the aboveground (aerial) plant parts, dry weight (DW) of shoots and leaves were recorded following drying at 70 °C, essentially as previously described (Mellidou et al., 2016).

2.3. Enzymatic and non-enzymatic antioxidants

The enzymatic activities of the antioxidant enzymes Catalase (CAT, EC 1.11.1.6) and Peroxidase (POX, EC 1.11.1.7) were assessed at the end of HS on the 4th and 6th top leaf of five plants. Proteins were extracted essentially as previously described (Mellidou et al., 2012), and passed through an ion-exclusion Sephadex G-25 column (PD 10, GE Healthcare) according to manufacturer' guidelines. The protein contents of the extracts were quantified according to Bradford's method (1976), using bovine serum albumin (BSA, Sigma) as standard. All measurements were carried out in duplicates. CAT and POX activities were determined as before (Mellidou et al., 2012). One CAT unit was defined as the amount of enzyme required to catalyse the decomposition of 1 μmol H_2O_2 s^{-1}. One POX unit was defined as the production of 1 mg of purpurogallin from pyrogallol after 15 s at 20 °C. Measurements were taken every 20 s for 4 min at 420 nm.

Total soluble phenolics and antioxidant capacity were determined as already described (Mellidou et al., 2016). The results were expressed as gallic acid equivalents (GAE) and as ascorbic acid equivalents (AAE) on a fresh weight (FW) basis, respectively. In order to compare short (3 h) *versus* long (7 days) HS, three week-old seedlings were subjected to 37 °C for three h. Quantification of total flavonoids was carried out by using the aluminium chloride colorimetric method (Chang et al., 2002).

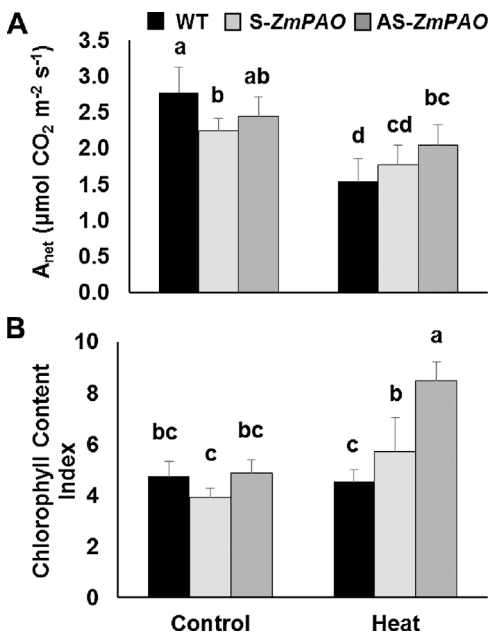

Fig. 1. Photosynthetic indices of WT, S-*ZmPAO* and AS-*ZmPAO* plants 7 d post-Heat Stress. (A) Net photosynthetic rate (A_{net}, μmol m^{-2} s^{-1}); (B) Chlorophyll Content Index (CCI). Data are means ± SD. Different letters indicate significant differences based on Duncan's multiple test ($P < 0.05$).

The standard curve was constructed by using sequential concentrations of rutin dissolved in methanol and expressed as mg RE gFW^{-1}. All samples were analyzed in triplicates.

2.4. Statistical analysis

Duncan's multiple test was performed by the statistical package SPSS (version 24) to determine statistical differences at a 5% significance level.

3. Results

3.1. Underexpression of the ZmPAO gene results to enhanced photosynthetic parameters and biomass allocation under HS

The photosynthetic machinery, as assessed by the rate of net CO_2 assimilated per leaf area (A_{net}), was severely impaired by HS in WT and S-*ZmPAO* compared to control plants (Fig. 1A), with a reduction of 44.6%, and 21.0%, respectively. Heat stress exerted no effect on A_{net} in AS-*ZmPAO* plants. Accordingly, plant biomass of the aerial plant parts, expressed as DW, was reduced by HS in WT and S-*ZmPAO* but not in the AS-*ZmPAO* in which DW was retained at the respective control levels (Fig. 2A). The greatest decrease in DW was observed in WT plants (34.9%). Furthermore, HS led to increased CCI in AS-*ZmPAO* by 73.6% and in S-*ZmPAO* by 51.2% compared to respective controls, with no particular effect in WT plants (Fig. 2B).

3.2. The AS-ZmPAO plants exhibit stronger antioxidant capacity by increased enzymatic and non-enzymatic antioxidants

A significant increase of CAT activity was recorded in heat-stressed AS-*ZmPAO* plants compared to the respective controls (37.4%; Fig. 3A). On the contrary, a remarkable decrease of CAT activity was observed in WT plants (58.0%), whereas S-*ZmPAO* plants exhibited a marginal, but no statistically significant reduction (16.7%). Heat stress exerted no

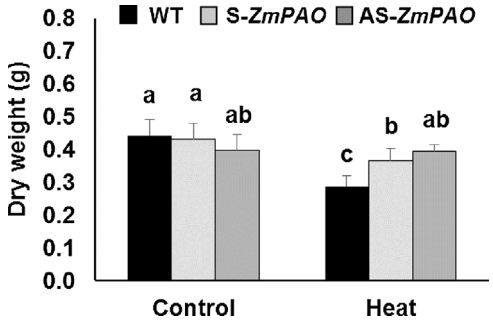

Fig. 2. Biomass allocation in the aerial parts of WT, S-*ZmPAO* and AS-*ZmPAO* plants 7 d post-Heat Stress. Data are means ± SD. Different letters indicate significant differences based on Duncan's multiple test ($P < 0.05$).

noticeable impact on POX activity in WT and S-*ZmPAO* plants, whereas it was significantly higher (153%) in the heat-stressed AS-*ZmPAO* plants compared to respective controls (Fig. 3B). Also, antioxidant activity was significantly higher in stressed AS-*ZmPAO* (37.9%), unaffected in S-*ZmPAO*, and lower in WT (34.3%) compared to respective controls (Fig. 3C).

In addition to enzymatic antioxidants, non-enzymatic compounds are significant for the antioxidant defence of cells. Within this context, a 29.6% increase in total soluble phenolics (yet not statistically significant), was recorded in the 7-d heat-stressed AS-*ZmPAO* compared to respective controls. On the contrary, a statistically significant decrease was evident in WT (20.9%), with no change in S-*ZmPAO* plants (Fig. 3C).

The qualitative analysis of total flavonoids in three week-old WT and transgenic plants following a short-term HS (3 h at 37 °C) showed no alteration in the zonation pattern between the WT and the transgenic plants, neither under control conditions nor under HS (Fig. 4A). However, at control conditions, the AS-*ZmPAO* plants contained significantly higher amounts (3-fold) of flavonoids, while the *S-ZmPAO* plants contained 48% less of these major phenolic compounds, compared to the WT (Fig. 4B). Upon short-term HS, WT plants responded considerably, exhibiting a prominent (2.8-fold) increase *versus* the respective controls after 3 h at 37 °C, whereas a moderate, yet significant, increase (25%) was recorded in AS-*ZmPAO* transgenic plants. On the contrary, S-*ZmPAO* plants did not respond to HS, as indicated by the similar quantity of total flavonoids before and after the treatment (Fig. 4).

4. Discussion

Polyamines and H_2O_2 act in concert as double edged swords in the responses of plants to stresses (Gupta et al., 2016). Polyamine oxidases participate in the regulation of the intra/intercellular homeostasis of PAs and H_2O_2. In addition, NADH-oxidase and PAO derived H_2O_2 signal/participate in downstream molecular, cellular and physiological processes affecting plant growth/development and response(s) to biotic and abiotic stresses (Moschou et al., 2008a, 2008b, 2008c; 2009; Wu et al., 2010; Tisi et al., 2011; Moschou et al., 2012; Gémes et al., 2016). We have previously shown that tobacco lines overexpressing *ZmPAO* exhibit significantly lower Spd and Spm compared to WT, whereas lines underexpressing *ZmPAO* contain significantly higher Put, Spd, and Spm titers (Moschou et al., 2008a, 2008b). In addition, the transgenic plants exhibit differences in the intra-/intercellular ROS load, which seem to signal different molecular pathways, depending on their "signatures" especially under stress (Moschou et al., 2008a,b; 2009; Gémes et al., 2016). Under salt stress the AS-*ZmPAO* genotype at 50–100 mM NaCl/7 DAT, showed a marked decrease in the A_{net}, suggesting an osmotic/ionic shock, which was overcome at 14 DAT and also taller phenotype, especially at the highest salt doses. Undoubtfully, drastic changes of temperature may affect the photosynthetic parameters at the physico-chemical/physiological/biochemical/molecular level, which have been proposed as indicators of thermotolerance (Wahid et al., 2007). Heat-stressed S-*ZmPAO* plants show a significant decrease in A_{net}, suggesting that these plants already suffer from heat injury, whereas the AS-*ZmPAO* plants maintain A_{net} at control levels, suggesting the existence/activation of a putative thermotolerance mechanism and/or the more efficient allocation of assimilates (Fig. 1A,B). This is also in line with the higher DW allocation and CCI in these transgenics under HS (Fig. 2).

Polyamine oxidation by PAO generates H_2O_2. Furthermore, under salinity, H_2O_2 and $O_2^{\cdot -}$ accumulate at the onset of stress and are dependent on NADPH-oxidase, which is upstream of apoplastic PAO, suggesting that NADPH-oxidase and the apoplastic PAO form a feed-forward ROS amplification loop, which impinges on oxidative state and culminates in the execution of PCD (Gémes et al., 2016). Whether this is

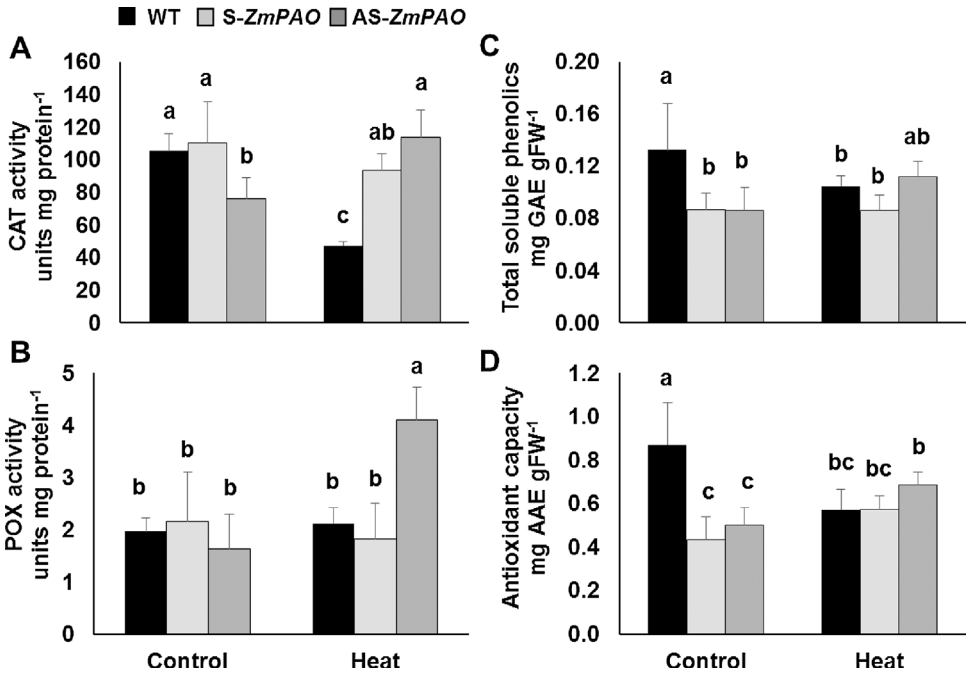

Fig. 3. Antioxidants in WT, S-*ZmPAO* and AS-*ZmPAO* plants 7 d post-Heat Stress. (A) CAT activity (units mg protein^{-1}); (B) POX activity (units mg protein^{-1}); (C) total soluble phenolics (mg GAE g FW^{-1}); and (D) antioxidant capacity (mg AAE g FW^{-1}). Data are means ± SD. Different letters indicate significant differences based on Duncan's multiple test ($P < 0.05$).

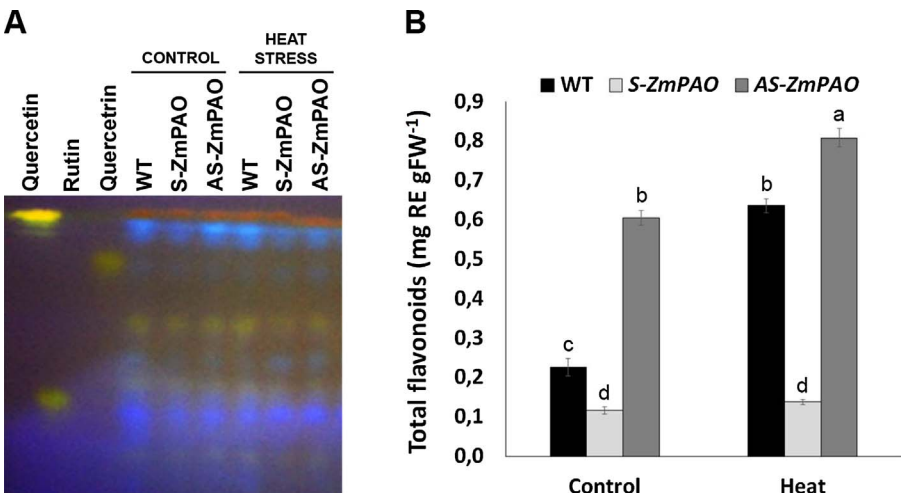

Fig. 4. Qualitative and quantitative profile of total flavonoids (mg RE g FW^{-1}) in WT, S-*ZmPAO* and AS-*ZmPAO* plants after 3 h at 37 °C. (A) TLC analysis of leaf extracts. Quercetin dehydrate, rutin and quercetin were used as standards; (B) Quantification of total flavonoids expressed as rutin equivalent (RE). Data are means ± SE, representing the mean of three independent experiments, which gave similar results. Different letters indicate significant differences based on Duncan's multiple test ($P < 0.05$).

the case under HS is yet unknown. At any case, the homeostasis of cellular enzymatic and non-enzymatic antioxidants is crucial for the protection against stresses (Gill and Tuteja, 2010; Baxter et al., 2014). In S-*ZmPAO* plants, HS alters neither the enzymatic nor the non-enzymatic antioxidants. In these transgenics, HS does not cause irreversible damage in growth potential (data not shown), suggesting that the generated ROS under moderate HS are successfully scavenged as previously reported under salt stress (Moschou et al., 2008a). On the contrary, the enhanced antioxidant machinery upon HS in the AS-*ZmPAO* plants, as illustrated by the increased antioxidant enzymatic activities, the total phenolics and flavonoids and the antioxidant capacity, both under short and long stress treatments (Figs. 3,4), can account for the improved thermotolerance of these trangenics. Another possible explanation of the superiority of AS-*ZmPAO* could be sought in the synergistic effect of low ROS titers and higher PA biosynthetic activity (Moschou et al., 2008b: Gémes et al., 2016). Accumulation of soluble phenolics, highest phenylalanine ammonia-lyase activity and lowest peroxidase and polyphenol oxidase activity in HS-treated tomato and watermelon was previously suggested as an acclimation mechanism (Rivero et al., 2001).

Taken together, the results reported herein reinforce the emerging importance of decreased PA catabolism in HS responses, and can be used as the basis for further elucidation of the relevant mechanisms of action and for developing strategies for increasing tolerance to HS omitting associated yield penalties.

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