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Antioxidative response in variegated *Pelargonium zonale* leaves and generation of extracellular H_2O_2 in (peri)vascular tissue induced by sunlight and paraquat

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ABSTRACT

In this study we exposed variegated leaves of *Pelargonium zonale* to strong sunlight (>1100 μmol m^{-2} s^{-1} of photosynthetically active radiation) with and without paraquat (Pq), with the aim to elucidate the mechanisms of H_2O_2 regulation in green and white tissues with respect to the photosynthetically-dependent generation of reactive oxygen species (ROS). Sunlight induced marked accumulation of H_2O_2 in the apoplast of vascular and (peri)vascular tissues only in green sectors. This effect was enhanced by the addition of Pq. In the presence of diphenyl iodide, an NADPH oxidase inhibitor, H_2O_2 accumulation was abolished. Distinct light-induced responses were observed: in photosynthetic cells, sunlight rapidly provoked ascorbate (Asc) biosynthesis and an increase of glutathione reductase (GR) and catalase activities, while in non-photosynthetic cells, early up-regulation of soluble ascorbate peroxidase, dehydroascorbate reductase (DHAR) and GR activities was observed. Paraquat addition stimulated DHAR and GR activities in green sectors, while in white sectors activities of monodehydroascorbate reductase, DHAR and class III peroxidases, as well as Asc content rapidly increased. Differential antioxidative responses in the two tissues in the frame of their contrasting metabolisms, and the possible role of (peri)vascular H_2O_2 in signaling were discussed.

1. Introduction

In green leaves, the photosynthetic electron transport chain and photorespiration are the main sources of reactive oxygen species (ROS), even under optimal growth conditions (Foyer and Shigeoka, 2011). In addition to photosynthetic tissue, green-white variegated leaves (such as in variegated *Pelargonium zonale*) have non-photosynthetic tissue lacking functional chloroplasts and peroxisomes (Vidović et al., 2015). Therefore, in the cells of white sectors, the most important sources of ROS are the mitochondrial electron transport chain and apoplast (Møller, 2001; Sierla et al., 2013). Nevertheless, the respiration rate in non-photosynthetic leaf tissue of variegated *P. zonale* was significantly lower compared to the photosynthetic tissue (Toshoji et al., 2011).

In our previous studies, two different antioxidative systems in photosynthetic and non-photosynthetic leaf tissues of *P. zonale* under optimal light conditions have been characterized (Vidović et al., 2016, 2015). Non-photosynthetic tissue had constitutively higher activities of enzymes involved in ascorbate-glutathione (Asc-GSH) cycle and Cu/Zn superoxide dismutase (SOD) compared to the photosynthetic tissue. Moreover, higher cytosolic ascorbate and total cellular glutathione contents in non-photosynthetic cells than in the photosynthetic ones were observed. However, higher total ascorbate content, as well as higher catalase (CAT) and thylakoid ascorbate peroxidase (APX) activities were found in photosynthetic tissue compared to non-photosynthetic one.

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; Asc, reduced ascorbate; CAT, catalase; DAB, 3, 3′-diaminobenzidine; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; MDAR, monodehydroascorbate reductase; $O_2^{\bullet}$, superoxide anion radical; PAR, photosynthetically active radiation (400–700 nm); PODs, class III peroxidases; PS, photosystem; ROS, reactive oxygen species; RsA, redox state of ascorbate; RsG, redox state of glutathione; Pq, paraquat (methyl viologen); SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; SOD, superoxide dismutase.

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Under optimal growth conditions, photosynthetic tissue contained higher level of carbonylated proteins than non-photosynthetic tissue, which was even more pronounced under high light, confirming more intensive pro-oxidative conditions in this tissue type (Vidović et al., 2015).

High light intensity accelerates superoxide anion radical ($O_2^{\bullet-}$) generation at photosystem I (PS I) through reduction of molecular oxygen in the Mehler reaction (Asada, 2006). In addition to high light, the herbicide paraquat (Pq, known as methyl viologen) is commonly used to induce oxidative stress in chloroplasts through predominant acceleration of the Mehler reaction (Neuhaus and Stitt, 1989; Kangasjärvi et al., 2008; Scarpeci et al., 2008; Stonebloom et al., 2012). In addition to its toxicity to chloroplasts, Pq^{2+} is also toxic to mitochondria (but to a lesser extent), since it can be reduced by electrons "leaking" from complex I, and therefore impair the redox homeostasis in these organelles (Asada, 2000; Vicente et al., 2001; Cochemé and Murphy, 2008; Lascano et al., 2012). Intensive Pq treatment (prolonged application or higher concentrations) can result in severe membrane damage and cell death (Li et al., 2013).

In this study, we investigated the dynamics of Asc-GSH cycle components, CAT and class III peroxidases (POD) in variegated *P. zonale* leaves during a 9-h period under increased cellular H_2O_2 availability. Considering that photosynthetic and non-photosynthetic leaf cells have different constitutive H_2O_2 regulating systems under optimal growth conditions, we expected that the two tissues would have distinct responses to oxidative stress induced by light excess and the pro-oxidative agent, Pq. We hypothesized that accumulation of H_2O_2 would be higher in photosynthetic tissue due to increased ROS generation dependent from photosynthesis. Furthermore, we examined whether photosynthetically derived H_2O_2 accumulation might be involved in intercellular signaling between photosynthetic and non-photosynthetic leaf tissue.

2. Material and methods

2.1. Plant material and experimental conditions

The variegated *Pelargonium zonale* cv. "Ben Franklin" plants were purchased from Fir Trees Pelargonium (Stokesley, North Yorkshire, UK) nursery. Plants were grown and propagated under 250 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR, 400–700 nm) under 14/10 h day/night photoperiod, 26/18 °C day/night temperature and relative humidity of 60–70%. This cultivar is a periclinal chimera with white leaf margins, caused by the lack of functional chloroplasts in L2 and L3 cell layers (hypodermis and mesophyll), while chloroplasts were observed in the guard cells in L1 layer (epidermis) (Vidović et al., 2016).

For controls, white and green sectors of dark adapted leaves (four fully developed leaves per plant, four plants in total) were separated, immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Further, three dark adapted leaves per plant from ten plants in total were excised at the base of stems with a razor blade, and one half was placed in 1.5 mL tubes covered with Alu foil filled with water, and the other half in 100 μM Pq solution. All leaves were exposed to direct solar radiation over 9 h on a clear, sunny day, when maximal PAR was almost 2000 μmol m^{-2} s^{-1} at noon (Fig. A1), which exceeds the intensity required for achieving maximal net CO_2 assimilation rate (400 μmol m^{-2} s^{-1}, light curves are not shown).

The dynamics of the responses of antioxidants in *P. zonale* leaves treated with Pq were monitored in relation to only sunlight exposed leaves and dark adapted leaves. The amount of Pq taken up by the whole leaf was calculated by measuring the volume of remained solution per leaf. The average amounts of Pq absorbed by whole leaf were: 16.2 ± 1.6; 120.4 ± 7.0; 262.9 ± 20.9 nmol g^{-1}_{FW}, after the 1st, the 4th and the 9th h of exposure, respectively. The experiments were repeated three times in the period from June to September.

2.2. Ascorbate and glutathione redox status analysis

Measurements of reduced ascorbate (Asc), dehydroascorbate (DHA), reduced and oxidized glutathione (GSH and GSSH) contents in the photosynthetic and non-photosynthetic leaf tissue were performed by high-performance liquid chromatography (HPLC, Shimadzu LC-20AB Prominence liquid chromatograph, Shimadzu, Kyoto, Japan) as described by Vidović et al. (2016). The redox state of ascorbate (RsA) was calculated as a percentage of Asc in total ascorbate (Asc + DHA) content, while the redox state of glutathione (RsG) was calculated as: 100% × [GSH/(GSH + 2GSSG)].

2.3. Enzyme extractions and assays

Extraction of soluble ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), glutathione reductase (GR, EC 1.8.1.7), class III peroxidases (PODs, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) was performed as reported previously (Vidović et al., 2016).

The activities of antioxidative enzymes were determined spectrophotometrically, in triplicates, using a temperature-controlled spectrophotometer (Shimadzu, UV-160, Kyoto, Japan), as described in our previous study (Vidović et al., 2016).

2.4. SDS-PAGE and SOD gel blot analysis

For SOD gel blot analysis, 7 μg of total soluble proteins of green and white leaf extracts were separated by SDS-PAGE (15% gel) and electrotransferred to a polyvinylidene difluoride membrane according to Vidović et al. (2016). For Cu/Zn SOD detection primary antiserum: rabbit anti-Arabidopsis chloroplastic Cu/ZnSOD (CSD2) antiserum (AS06 170, Agrisera) (diluted 1:1000) was used. Equal loading was confirmed by silver staining of replicate gels.

2.5. Detection of accumulated H_2O_2

Detection of H_2O_2 was based on the 3,3′-diaminobenzidine (DAB) 'up-take' method according to Fryer et al. (2003). After exposing leaves to high light (1100 ± 100 μmol m^{-2} s^{-1}) and 100 μM Pq for 1 h (at least twenty variegated leaves, from ten plants in total per treatment), they were placed in tubes filled with solution of 1 mg mL^{-1} DAB in 100 mM sodium acetate buffer (pH 3.8) and returned to the same light irradiance. After 1 h, leaves were cleared in boiling 70% (v/v) ethanol (for 10 min) with 10% glycerol. The same results were obtained when leaves were incubated in DAB (with and without Pq) immediately upon light exposure. In addition, different leaf morphotypes, sectorial chimeras, totally green and totally white leaves were used. As a negative control, detached leaves were placed in the buffer solution containing no DAB, and after chlorophyll removal they showed no brown precipitate. Only leaves that absorbed equal amounts of DAB (350 ± 25 μg g^{-1}_{FW} on average) were analyzed. Each leaf was photographed before and after chlorophyll removal, and high resolution images were made using an Olympus BX41light microscope equipped with Olympus C7070 camera (Olympus Optical Co., Hamburg, Germany).

Densitometric analysis of H_2O_2 accumulation was performed using ImageJ software 1.45 version (http://imagej.nih.gov/ij/) similar to the method described by Morina et al. (2016). Accumulation of H_2O_2, measured as staining intensity is presented in arbitrary units.