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Research Paper

Chilling stress and hydrogen peroxide accumulation in *Chrysanthemum morifolium* and *Spathiphyllum lanceifolium*. Involvement of chlororespiration

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ABSTRACT

Plants of *Chrysanthemum morifolium* (sun species) and *Spathiphyllum lanceifolium* (shade species) were used to study the effects of chilling stems under high illumination. The stress conditions resulted in a greater accumulation of H_2O_2 in *C. morifolium* than in *S. lanceifolium*, and in the down–regulation of photosynthetic linear electron transport in both species. However, only a slight decrease in the maximal quantum yield of PSII was observed under unfavorable conditions in both species, suggesting that mechanisms exist in the chloroplasts that dissipate excess excitation energy and prevent damage to the photosynthetic apparatus. Additionally, changes were observed in the PGR5 polypeptide involved in cyclic electron flow around PSI and in chlororespiratory enzymes (plastidial NDH complex and PTOX). The level of PGR5 increased significantly only in chilled plants of *C. morifolium*, whereas the levels of the PTOX and NDH-H polypeptide of the plastidial NDH complex and the NDH activity increased significantly only in chilled plants of *S. lanceifolium*. These findings suggest that the cyclic electron flow involving PGR5 is more active in *C. morifolium*, while in *S. lanceifolium*, other mechanisms involving chlororespiratory enzymes are stimulated in response to chilling and high light, resulting in less H_2O_2 being accumulated in leaves.

1. Introduction

A novel type of chilling injury induced by high root temperature has been described in tropical and subtropical species. Temperatures of about 10 °C produced damage only when stems, but not roots, were chilled, but no damage when both stems and roots were chilled to the same low (but not freezing) temperature simultaneously (Suzuki et al., 2008, 2011, 2013; Paredes and Quiles, 2015; Segura and Quiles, 2015). Although the biochemical basis of such chilling stress is not clearly understood, Suzuki et al. (2011) suggested that chilling in stems while the root temperature remains high blocks electron transport between Q_A and Q_B in PSII, so that PSII is overreduced in the light.

Chilling stress under light results in an imbalance between the capacity to harvest light energy and the capacity to dissipate this energy through metabolic activity, which results in excess PSII excitation pressure (Öquist and Huner, 2003). The imbalance between the reducing equivalents produced in excess and the consumption capacity of photosynthesis can potentially result in the generation of reactive oxygen species (ROS), which may cause photooxidative damage and the photoinhibition of photosystems, photosystem II (PSII) being the most sensitive in this respect. Among the various ROS, hydrogen peroxide in particular has received considerable interest in the last decade. Hydrogen peroxide possesses the highest half-life (1 ms) of the ROS. This comparatively long life span and the small size of H_2O_2 molecules permit them to traverse cellular membranes to reach different cellular compartments, facilitating signaling functions.

Several studies have demonstrated that hydrogen peroxide can enhance abiotic stress tolerance by regulating gene expression and multiple stress-responsive pathways (Hossain et al., 2015). Tolerance to stress results from events that occur at all levels, from morphological to molecular. At the biochemical level,

Abbreviations: F_0, minimal fluorescence yield in the dark adapted state; F_m, maximal fluorescence yield in the dark adapted state; F'_m, maximal fluorescence yield in the light adapted state; F_v, variable fluorescence; NADH-PQR, NADH-plastoquinone oxidoreductase; NDH, NADH dehydrogenase; NPQ, non-photochemical quenching of excitation energy; PAM, pulse amplitude modulation; PPFD, photosynthetic photon flux density; PS, photosystem; PTOX, plastid terminal oxidase; ROS, reactive oxygen species; Y(II), the effective PS II quantum yield of illuminated samples.

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the metabolism of plants changes to withstand environmental stress, and alternative pathways to photosynthetic linear electron transport, including cyclic electron flow around PSI and chlororespiration, may come to play a role. Chlororespiration is a respiratory electron transport chain in the thylakoids of chloroplasts that interacts with photosynthetic electron transport, in a way that involves both non-photochemical reduction and oxidation of plastoquinones, with the corresponding consumption of oxygen (Bennoun, 1982, 1994). Two chlororespiratory enzymes – the plastid-encoded NADH dehydrogenase (NDH) complex (Quiles, 2005; Rumeau et al., 2005) and the nucleus-encoded plastid-localized terminal oxidase (PTOX) (Kuntz, 2004) – have been characterized molecularly. The NDH complex is an entry point for electrons into the photosynthetic electron-transport chain, involving the non-photochemical reduction of plastoquinones, and PTOX is a point of electron transfer from plastoquinol to molecular oxygen, leading to the formation of water in the stroma and a reduction in ROS formation (Peltier and Cournac, 2002). The NDH complex is also involved in cyclic electron flow around PSI, where two parallel cyclic pathways exist (Joët et al., 2001), one involving the above mentioned NDH complex and another which is sensitive to antimycin A, and where one of the essential components is the thylakoid membrane protein encoded by *pgr5* gene (PGR5) (Munekage et al., 2002, 2004). However, it is difficult to establish the exact physiological significance of the chloroplast electron pathways operating around PSI, although, while their role seems to be unimportant in non-stress conditions (Bendall and Manasse, 1995; Joët et al., 2002), they seem to contribute to the flexibility of the electron transfer reactions that is needed to balance ATP and NADPH requirements in changing environmental conditions (Rumeau et al., 2007; Joliot and Johnson, 2011). It has been suggested that the chlororespiration and cyclic electron pathways may play a part in the protective or adaptive mechanisms of plants in the face of stresses that increase ROS formation and cause oxidative stress (Casano et al., 2001; Rizhsky et al., 2002; Quiles and López, 2004; Streb et al., 2005; Quiles, 2006; Díaz et al., 2007; Tallón and Quiles, 2007). However, little is known about the relationship of those pathways that may improve plant tolerance to stress and hydrogen peroxide production. It has been suggested that the high production of hydrogen peroxide from the plastoquinone pool by thylakoids of the halophytic *Thellungiella* might be an important element in the preparedness of this highly stress-resistant species to combat salinity stress, a phenomenon associated with the activity of PTOX (Wiciarz et al., 2015). However, the possible relation of hydrogen peroxide production with the cyclic electron pathways and chlororespiration in conditions of chilling stress is unknown.

This work examines the effects of chilling the stems under high light on photosynthesis, the accumulation of hydrogen peroxide, chlororespiratory enzymes and PGR5 in the sun species *Chrysanthemum morifolium,* which is cold-tolerant, and in the shade species of tropical origin *Spathiphyllum lanceifolium,* which is cold-sensitive.

2. Materials and methods

2.1. Plant material, treatments and isolation of stroma and thylakoid membranes

Chrysanthemum morifolium (sun plant) and *Spathiphyllum lanceifolium* (shade plant) were grown until the adult state in soil in 500 mL pots at 22–25 °C in a greenhouse with a natural photoperiod, with daytime irradiation maxima of around 800 and 200 μmol m^{-2} s^{-1} PPFD (sun and shade plants, respectively) and controlled watering to avoid drought stress (control conditions). Treatments at different temperatures under photoperiods of high light intensity were performed in adult plants as described in detail previously (Paredes and Quiles, 2015), using temperatures (°C stem/°C root) of 24 °C/24 °C (not chilling) and 10 °C/24 °C (chilling). In each treatment, the photoperiod (18 h) and night period (6 h) temperatures were the same, and the measurements were performed after the third night period in mature leaves.

Chloroplasts were isolated from leaves as described in detail previously (Paredes and Quiles, 2015) and fractionated into stroma and thylakoids as described previously (Paredes and Quiles, 2013). The thylakoid membrane pellet was resuspended in buffer (pH 7.5) containing 200 mM sorbitol, 130 mM KCl and 5 mM potassium phosphate at a chlorophyll concentration of 0.4 mg mL^{-1}.

2.2. Parameter measurements

Chlorophyll fluorescence was analyzed using a PAM-210 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as described previously (Paredes and Quiles, 2015).

Both the PSI activity and NADH-plastoquinone oxidoreductase (NADH-PQR) activity were measured spectrophotometrically, as described in Soto et al. (2014) and Paredes and Quiles (2015), respectively.

Hydrogen peroxide was detected using 3,3-diaminobenzidine (DAB), and the concentration was determined by the FOX-1 method, as described in Ibañez et al. (2010).

2.3. Other determinations

Gel electrophoresis and immunoblot analysis were performed as previously described (Ibañez et al., 2010). An ACTIB 1D digital image analyzer (Microptic, Barcelona, Spain) was used to make densitometric analyses and estimate the polypeptide molecular masses.

Chlorophyll was measured following the method described by Lichtenthaler and Wellburn (1983) using 80% (v/v) acetone as solvent. Protein was determined using the method described by Lowry et al. (1951). The control and treatments at different temperatures under high light intensity were compared by ANOVA using Statistix 9 (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1. Photosynthetic parameters

Fluorescence imaging was used to test photosynthesis in intact leaves from *C. morifolium* and *S. lanceifolium* plants at the start the experiment (control) and after exposure to three photoperiods with high illumination and different temperatures (°C stem/°C root): 24 °C/24 °C (not chilling) and 10 °C/24 °C (chilling). The maximal quantum yield of PS II (F_v/F_m), effective PS II quantum yield (Y(II)) and non-photochemical quenching (NPQ) are depicted in Fig. 1. As can be seen, F_v/F_m remained high for all treatments in both *C. morifolium* and *S. lanceifolium*, the chloroplasts seemingly protected by mechanisms that dissipate excess excitation energy. However, there was a significant decrease in the effective PSII quantum yield in the plants of both species when exposed to chilling treatments, particularly in *S. lanceifolium*. This decrease was associated with a slight increase in the NPQ, the higher values being seen in *S. lanceifolium* plants. NPQ, an indicator of excessive energy dissipated safely as heat at PSII, was always lower in *C. morifolium* than that in *S. lanceifolium*, suggesting that the electron transport at PSII is controlled more efficiently in *C. morifolium*, considering together with the smaller responses in both Y(II) and NPQ to chilling.

The PSI activity in thylakoid membranes isolated from the leaf chloroplasts of *C. morifolium* and *S. lanceifolium* plants are shown in Fig. 2, where it can be seen that the activity was higher in *C.*