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Research article

Effect of mitochondrial ascorbic acid synthesis on photosynthesis

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ABSTRACT

Ascorbic acid (AA) is synthesized in plant mitochondria through the oxidation of L-galactono-1,4-lactone (L-GalL) and then distributed to different cell compartments. AA-deficient *Arabidopsis thaliana* mutants (*vtc2*) and exogenous applications of L-GalL were used to generate plants with different AA content in their leaves. This experimental approach allows determining specific AA-dependent effects on carbon metabolism. No differences in O_2 uptake, malic and citric acid and NADH content suggest that AA synthesis or accumulation did not affect mitochondrial activity; however, L-GalL treatment increased CO_2 assimilation and photosynthetic electron transport rate in *vtc2* (but not wt) leaves demonstrating a stimulation of photosynthesis after L-GalL treatment. Increased CO_2 assimilation correlated with increased leaf stomatal conductance observed in L-GalL-treated *vtc2* plants.

1. Introduction

Ascorbic acid (AA) is a metabolite present in large quantities in plant tissues (Foyer and Noctor, 2011). It is well recognized its crucial participation in many physiological processes being lethal its absence for the plant (Dowdle et al., 2007). The main function originally proposed for AA was the role as antioxidant with a particular importance in the detoxification of reactive oxygen species (ROS) generated by photosynthesis (Foyer and Halliwell, 1976). Other several functions were attributed to this compound like the participations in photoprotection and growth and development of plants. As its function as a cofactor, AA participates in the synthesis of some hormones and in the conversion of violaxanthin to zeaxanthin plus antheraxanthin that are involved in the thermal dissipation of energy in chloroplasts protecting photosynthesis from excess irradiance (Gilmore, 1997). In addition, zeaxanthin was proposed as a blue-light receptor in guard cells implicated in the stomatal aperture (Frechilla et al., 1999). AA was indicated as a main player in the water–water cycle proposed by Asada (1999) important for both photochemistry and ROS detoxification. AA participates in the growth of the plants since it is needed for the normal elongation and division of the cells (Smirnoff, 1996). The study of AA metabolism and accumulation in plants is also important since it was recognized as a vitamin (Vitamin C) for human beings (Buettner and Jurkiewicz, 1996).

AA is synthesized in plant mitochondria and then distributed throughout different cell compartments but with particularly high abundance in chloroplasts (Foyer and Shigeoka, 2011). The last biosynthetic step that is shared by different pathways proposed in plants consists in the oxidation of L-galactono-1,4-lactone (L-GalL) forming AA. This reaction is catalyzed by L-GalL dehydrogenase (L-GalLDH) located in the inner mitochondrial membrane facing the intermembrane space (Bartoli et al., 2000). Several Arabidopsis mutants with low AA were obtained (Conklin et al., 2000). Among them *vtc2* mutant presents the lowest AA content compared with wt plants. These plants lack the protein VTC2 (GDP-L-galactosephosphorylase) that contributes with most of the activity (Linster and Clarke, 2008). The small remnant activity corresponds to the enzyme encoded by the homologue gene *VTC5* getting the low AA accumulation observed in *vtc2* plants (Dowdle et al., 2007). These mutant plants with decreased AA synthesis and content present phenotype alterations: reduced growth, altered flowering response to photoperiod, roots with lost response to gravity, and others (Barth et al., 2006; Olmos et al., 2006). However, these modifications might not all be related with AA deficiency. A tomato mutant with both low GDP-D-mannose-3,5-epimerase activity and AA content, also presents decreased levels for D-galactose affecting plant cell formation and would be responsible of growth reduction

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(Voxeur et al., 2011). The utilization of AA mutants and the possibility to increase the AA content through an external supplement can be used to unravel the specific modifications in the phenotype observed as a consequence of changes in AA accumulation.

The hypothesis of this work is that the AA synthesised in plant mitochondria affects photosynthetic activity. Considering the above comments, *vtc2* mutants and external L-GalL supplementation were used here to get plants with different levels of AA for studying the participation of this metabolite in the regulation of photosynthesis.

2. Material and methods

2.1. Plant material

Experiments were performed with *Arabidopsis thaliana* L. Heynh plants with low AA, *vtc2* and its wild background, Col-0. Seeds were kindly provided by Christine Foyer (University of Leeds, UK) and plants described in Kerchev et al. (2011). Plants were grown at 23 ± 1 °C, 160 μmol m^{-2} s^{-1} (PPFD) and 10/14 h light/dark periods, respectively.

2.2. L-GalL treatments

L-GalL was supplemented to increase the accumulation of AA in leaves of both genotypes using plants before flowering. Three L-GalL treatments were applied to the rosette leaves starting at the beginning of the 8th week after germination. Leaves were sprayed with 4 mL/plant of a solution containing 10 mM L-GalL and 0.01% tween 20. Control plants were sprayed with the solution without L-GalL. L-GalL was supplemented three times: 4 and 2 days before measurements and the last at the sampling day. The treatment was done after lights switched on and the measurements performed 5 h thereafter. Some determinations were made in samples freezed in liquid nitrogen and stored at −80 °C, taken at the previous indicated time. All measurements were done in complete expanded leaves.

2.3. Metabolite analysis

AA and GSH were determined as previously reported (Bartoli et al., 2006 and Griffith, 1980, respectively). AA was measured by HPLC system using a C-18 column (MicroSpher C18 S 100 × 4.6 mm, Agilent Technologies).

Malic and citric acids were measured in leaves ground in 6% phosphoric acid and centrifuged at 13000g for 10 min. The supernatants were used for the measurements following the same protocol used for AA but HPLC detector was set at 214 nm for organic acid detection (Ergönül and Nergiz, 2010).

Chlorophyll was extracted with dimethylformamide and quantified as reported by Inskeep and Bloom (1985).

NAD(P)/NAD(P)H levels were basically determined as Queval and Noctor (2007). Briefly, leaves were ground in 0.2 N HCl or 0.2 M NaOH for oxidized or reduced forms, respectively. Then samples were centrifuged and supernatants boiled for the differential degradation of the reduced or oxidized forms in HCl or NaOH, respectively. Samples were neutralize and the nucleotide pyridine contents were measured spectrophotometrically using specific enzymatic assays, alcohol dehydrogenase or glucose-6-phosphate dehydrogenase for NAD(H) or NADP(H), respectively.

2.4. Leaf gas exchange

Photosynthesis was measured by both CO_2 fixation and electron transport rate (ETR) with an infra red gas analyzer (PLC 6, Ciras-2 PPSystems) and a chlorophyll fluorescence modulated system (FMS-2, Hansatech Instruments Ltd), as previously described (Bartoli et al., 2005a, b). ETR was calculated as follows: (Φ_{PSII} x PFDa x 0.5), where Φ_{PSII} is the quantum yield of PSII and PFDa is the absorbed light (Genty et al., 1989). Measurements were made inside the plant growth chamber at the growing conditions indicated above.

Transpiration was determined as leaf conductance simultaneously with CO_2 fixation. In addition, transpiration of each leaf side was determined with a steady state diffusion leaf poromoter (SC-1, Decagon Devices).

About 1 g of detached leaves was placed in a gas-tight chamber equipped with a Clark type O_2 electrode (Hansatech Instruments Ltd) for respiration measurements. Plants were previously adapted at darkness for 20 min and then O_2 consumption was followed for 10 min (Bartoli et al., 2005b).

2.5. Stomatal density

The stomatal density (the number of stomata per leaf area unit) was quantified as described by Tambussi et al. (2005). Briefly, both sides of leaves were coated with nail polish and left to dry for some minutes. Then the polishes were peeled off and observed with a microscope (Olympus BX51) with a magnification of 200x for stomata counting. Measurements were made in 4 different experiments and at least five microscope fields per leaf were observed for each determination.

2.6. Rubisco content

Leaf samples were homogenized in a buffer 50 mM Tris pH 8 containing 2 mM EDTA and 20 mM PMSF. Then samples were analyzed in SDS denaturating gels and the large rubisco subunit was detected by western blot as previously described by Martínez et al. (2008) and quantified with ImageJ software.

2.7. Statistical analysis

Data were obtained from at least 4 independent experiments for each physiological parameter and the analysis of media performed through the Fisher test (ANOVA, $P \leq 0.05$).

3. Results

3.1. Accumulation of AA in leaves with different L-GalL supplementation

Leaves of *vtc2* plants presented around 10% AA content compared with those of the wt (Fig. 1). The treatments with L-GalL increased leaf AA content in both wt and *vtc2* plants. AA content in L-GalL treated mutant plants was similar to that observed in non-treated wt plants. Redox state was similar for all treatments. These data show that L-GalLDH capacity is not altered in *vtc2* plants.

3.2. Effect of L-GalL supplementation on respiration

The rate of O_2 uptake was similar in leaves of both wt and *vtc2* plants and L-GalL supplementation does not produce any change (Fig. 2A). Malic and citric acids levels were measured since they would indicate modifications in mitochondria metabolism. Neither of the acids show any change for any genotype or treatment (Fig. 2B and C, respectively).