



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 O₂ uptake, malic and citric acid and NADH content suggest that AA synthesis or accumulation did not affect mitochondrial activity; however, L-Gall treatment increased CO₂ assimilation and photosynthetic electron transport rate in *vtc2* (but not wt) leaves demonstrating a stimulation of photosynthesis after L-Gall treatment. Increased CO₂ assimilation correlated with increased leaf stomatal conductance observed in L-Gall-treated *vtc2* plants.

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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 (Smirnov, 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, $160 \mu\text{mol m}^{-2} \text{s}^{-1}$ (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 frozen 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 \times 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: $(\Phi_{\text{PSII}} \times \text{PFDA} \times 0.5)$, 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 porometer (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 denaturing 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).

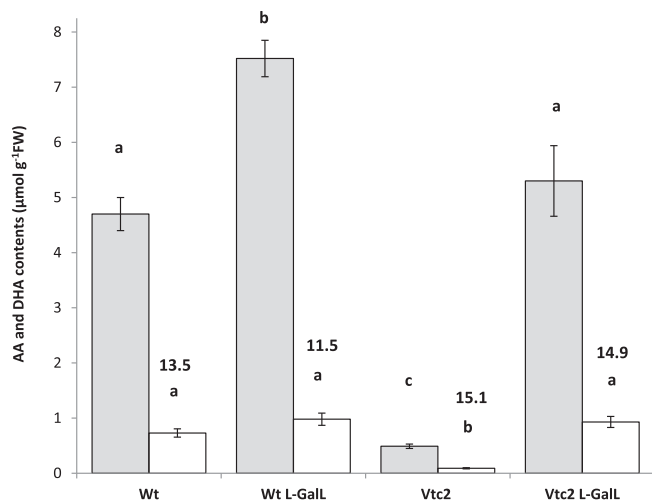


Fig. 1. AA and DHA contents in leaves of wt and *vtc2* Arabidopsis plants treated or not with L-Gall. The numbers above bars indicate AA redox state. Results with the same letters indicate statistical differences (ANOVA, $P < 0.05$).

3.3. Effect of L-Gall supplementation on photosynthesis

CO_2 assimilation was not statistically different between wt and *vtc2* leaves; however, photosynthesis of mutant plants (but not wt) increased around 25% after L-Gall supplementation (Fig. 3A).

Photosynthetic ETR was lower in wt leaves but L-Gall treatment raised it to wt levels (Fig. 3B).

3.4. Effect of L-Gall supplementation on leaf parameters

Since the photosynthetic activity of *vtc2* leaves was increased by AA recovery other leaf characteristics were analyzed.

Stomatal conductance was similar in wt and *vtc2* leaves, but it increased two times only in *vtc2* plants after the L-Gall treatment (Fig. 4A). Furthermore, Fig 4B shows that the higher transpiration observed in *vtc2* treated with L-Gall was due to increments in both sides of the leaves. In spite of stomatal aperture, this modification on leaf gas exchange may be the consequence of differences in the abundance of stomata. The stomatal density was significantly higher in both sides of *vtc2* leaves than in those of wt (Fig 5) but it was not altered in neither of the genotypes by L-Gall supplement.

The amount of rubisco in *vtc2* leaves was increased compared with those of the wt; however, the L-Gall addition does not produce modifications in neither of the genotypes (Fig 6). The chlorophyll content in *vtc2* leaves was similar to that measured in wt leaves (treated or not with L-Gall), but L-Gall treatment induced an increase in *vtc2* leaves (Supplemental Fig. 1).

3.5. Effect of L-Gall supplementation on pyridine nucleotides and GSH contents

These metabolites were measured since they are tightly connected with AA participating together in the optimization of the carbon metabolism of plant cells (Foyer and Noctor, 2011). NADH contents were not affected by the genotype or treatment, however, NAD presented higher accumulation in *vtc2* leaves when AA was increased (Table 1). Both NADPH and NADP presented similar levels in wt and *vtc2* leaves but increased when L-Gall was exogenous supplied (Table 1). In contrast, GSH content was higher in *vtc2* than in wt leaves and the increment of AA does not modify GSH levels in wt or *vtc2* plants (Table 1).

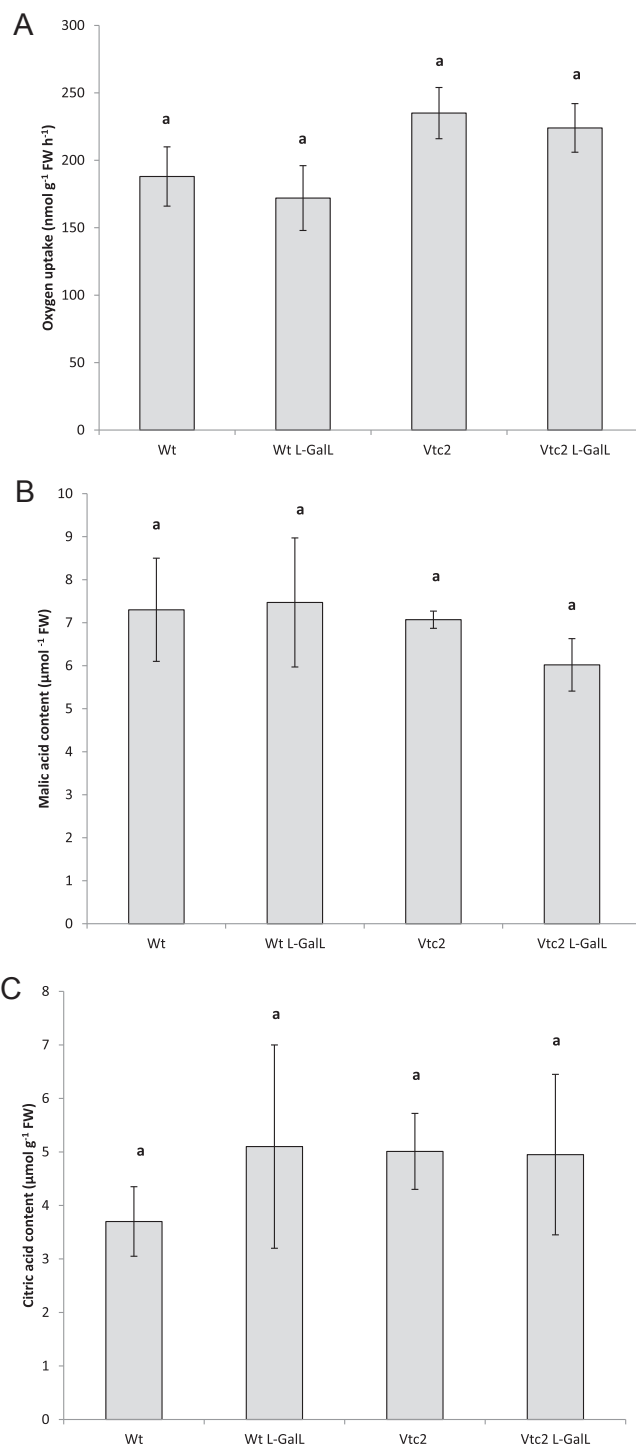


Fig. 2. Effects of different AA content/L-Gall treatments on leaf mitochondria metabolism of wt and *vtc2* Arabidopsis plants: A, O_2 uptake; B, malic acid content, and C, citric acid content. Results with the same letters indicate statistical differences (ANOVA, $P < 0.05$).

4. Discussion

Mutant Arabidopsis plants with very low AA and external application of L-Gall were used here to study the impact of different concentrations of AA on carbon metabolism. External supply of AA may not be effective in order to get an increased content in its reduced form inside the cell since it may be accumulated in the apoplast, and additionally, converted in its oxidized

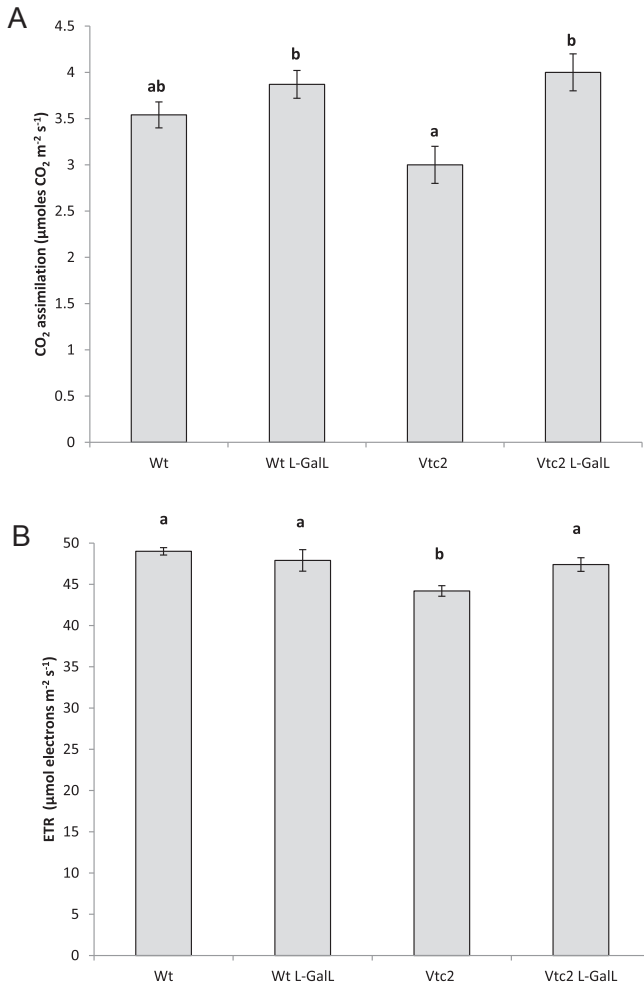


Fig. 3. Effects of different AA content/L-Gall treatments on photosynthesis of wt and *vtc2* Arabidopsis plants: A, CO₂ assimilation, and B, ETR. Results with the same letters indicate statistical differences (ANOVA, P < 0.05).

form (Gergoff and Bartoli, 2014). For this reason L-Gall treatment was used here to achieve increments of AA levels through its normal way of synthesis in mitochondria (Bartoli et al., 2000).

Results presented in this work illustrate plant responses to AA deficiency and recovery: modifications observed in *vtc2* plants constitute a long term adaptation to low AA and on the other hand, some of them can be reverted in the short-term by L-Gall treatment.

4.1. Effect of AA metabolism on respiration

AA is synthesized in mitochondria through the oxidation of L-Gall feeding electrons into the mitochondrial electron transport chain (Bartoli et al., 2000). However, little information is recorded in the literature studying the effect of AA synthesis and/or accumulation on mitochondrial metabolism. Oxygen uptake, malic and citric acids and NADH contents were measured to assess the effects of AA deficiency and recovery on leaf mitochondrial metabolism. No changes were observed in neither of these parameters suggesting that the limitation of L-Gall in *vtc2* plants does not lead to an alteration in plant respiration, at least under the conditions used here. In contrast with this conclusion, *vtc1* with an intermediate AA level between wt and *vtc2*, showed a decrease of around 25% on leaf O₂ uptake (Talla et al., 2011). It is important to keep in mind that

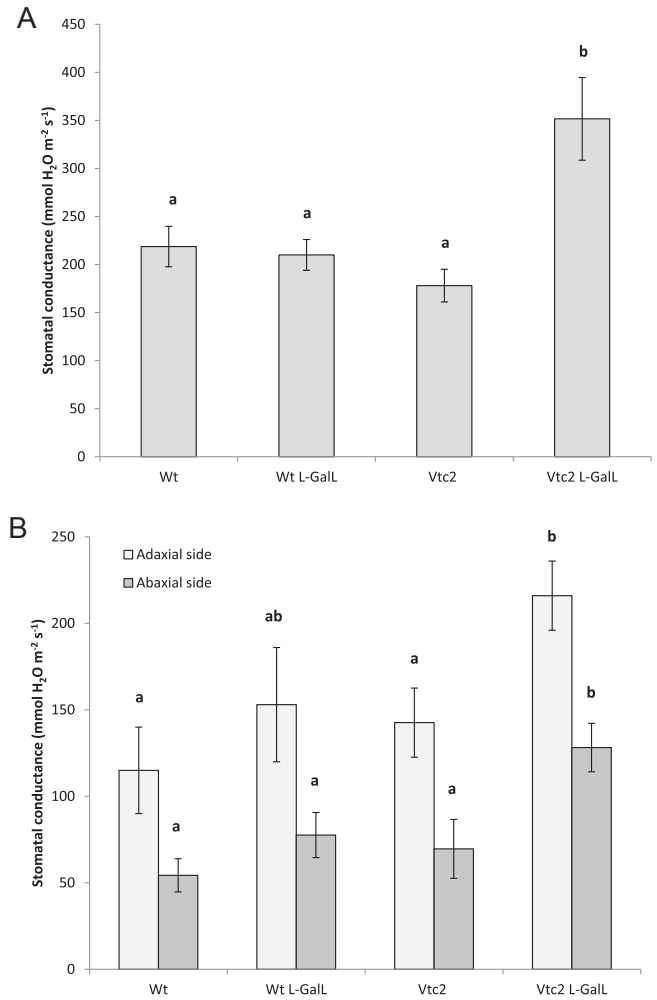


Fig. 4. Effects of different AA content/L-Gall treatments on stomatal conductance: A, total stomatal conductance, and B, adaxial and abaxial leaf sides stomatal conductances of wt and *vtc2* Arabidopsis plants. Results with the same letters indicate statistical differences (ANOVA, P < 0.05).

other aspects of mitochondria metabolism might be affected by L-Gall oxidation, like the increase in NAD observed here, or other not

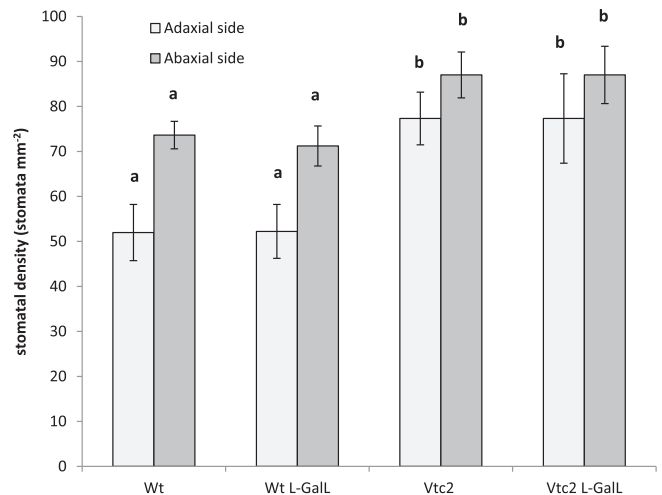


Fig. 5. Effects of different AA content/L-Gall treatments on stomatal density in both leaf sides of wt and *vtc2* Arabidopsis plants. Results with the same letters indicate statistical differences (ANOVA, P < 0.05).

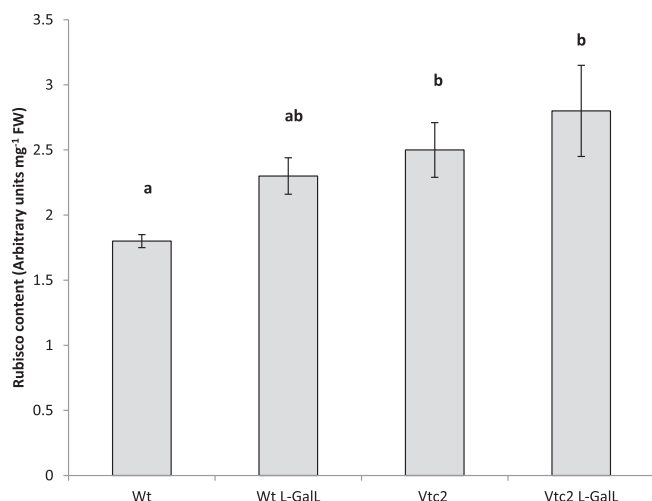


Fig. 6. Effects of different AA content/L-Gall treatments on rubisco accumulation in leaves of wt and *vtc2* Arabidopsis plants. Results with the same letters indicate statistical differences (ANOVA, $P < 0.05$).

registered in this study.

4.2. Effect of AA metabolism on photosynthesis

Some studies have reported the effects of very low AA content on photosynthetic activity using *vtc2* mutants (Kerchev et al., 2011; Müller-Moulé et al., 2004); however, the consequences of altered AA content on CO₂ assimilation are far from being completely understood. AA is synthesized by plant mitochondria and actively accumulated in chloroplast where it participates in the optimization of photosynthesis. This suggests that AA might be a signal connecting the metabolisms of both organelles (Nunes-Nesi et al., 2008). In that sense, modifications in AA contents and/or L-Gall oxidation might affect photosynthesis.

The present work shows that plants deficient in AA do not present different CO₂ assimilation rates but they increase CO₂ uptake when AA is enhanced to normal concentration. This response is observed in *vtc2* but not in wt plants suggesting that the low AA level/L-Gall oxidation in the mutant restricts its potential photosynthetic activity. In addition, this observation suggests that some physiological and/or morphological modifications take place in *vtc2* plants allowing them to get normal photosynthesis with only 10% of AA and to actively respond when AA is restored. Consequently, some mechanisms possibly involved in this photosynthetic response were studied.

The gas diffusion from the ambient air to the chloroplasts is an important process regulating photosynthesis (Evans and Loreto, 2000). This leaf gas exchange providing CO₂ for photosynthesis is maintained through the modifications on the dynamic of stomatal aperture and/or on the stomatal density (Farquhar and Sharkey, 1982).

Over-expression of dehydroascorbate reductase (DHAR) produces an increase in AA content with a highly reduced redox state

(Chen et al., 2003). High AA content may lead to active H₂O₂ consumption in guard cells, a ROS that induces closure of stomata. Consequently, decreased H₂O₂ concentration keeps the stomata pores open increasing leaf gas diffusion (Chen et al., 2003). Plants with suppressed DHAR activity have normal AA content but with more oxidized redox state associated with lower stomatal conductance. These data lead to the conclusion that AA redox state is the regulator of stomatal aperture (Chen and Gallie, 2004). In contrast with the previous studies, *vtc2* leaves responded to different concentrations of reduced AA but without changes in its redox state. Taken together all these evidences suggest that the concentration of AA regulates the stomatal conductance and more experiments would be done to unravel the role of its redox state.

Considering AA as an inducer of stomatal aperture, deficient mutants may compensate a putative lower pore aperture increasing the amount of stomata. Consequently, the higher stomatal density observed in *vtc2* than in wt leaves may be a compensation trait to keep normal gas diffusion. As a result of this modification, the L-Gall supplement produces a higher effect in *vtc2* than in wt leaves leading to an increase in stomatal conductance of both leaf sides, and consequently, in CO₂ assimilation. Other possibility, as AA plays a crucial role in division and elongation of plant cells (Potters et al., 2000; Kato and Esaka, 1999), the low AA in *vtc2* mutants would lead to the modification in stomatal density and this trait would not be related to an acclimation of photosynthetic activity. In other words, it cannot be discarded that changes in stomatal density in *vtc2* could be a pleiotropic effect of the mutation.

Other modifications in mitochondria metabolism affect photosynthesis through stomata aperture dynamics; down-regulation of succinate dehydrogenase (Araújo et al., 2011) or fumarase (Nunes-Nesi et al., 2007) lead to changes in the accumulation of organic acids (i.e. malate or fumarate) decreasing the stomatal aperture and consequently, CO₂ uptake. However, no changes in intermediates of Krebs cycle were reported here indicating no participation of them in the different response of *vtc2* stomatal aperture.

It was previously observed that *vtc2* plants present normal CO₂ assimilation with increasing CO₂ levels (Kerchev et al., 2011) suggesting that photosynthetic machinery is not limiting the process. Furthermore, the data presented here show that *vtc2* plants present an increased amount of rubisco that would involve a photosynthetic adaptation to keep normal CO₂ assimilation. In contrast, *vtc1* mutant presents a smaller amount of rubisco but with highly activated state compensating photosynthetic activity (Pastori et al., 2003). Further studies on rubisco activation would be important to characterize *vtc2* photosynthetic activity.

In addition, it is worth noting that this effect of AA enhancing stomatal conductance was observed in AA deficient but not in wt plants. This different behavior indicates that concentration of this antioxidant in wt plants is enough to achieve normal photosynthesis, at least under the growth conditions used in this study.

4.3. Effect of AA changes on chloroplast redox network

Impairment of photosynthetic ETR in *vtc2* plants was reported

Table 1

Effects of L-Gall treatments on nucleotide pyridines and GSH accumulation in leaves of wt and AA deficient plants.

	NADH	NAD ⁺	NADPH	NADP ⁺	GSH	GSSG
	(μmol g ⁻¹ FW)					
Wt H ₂ O	0.50 ± 0.1a	0.17 ± 0.04a	0.77 ± 0.19a	0.75 ± 0.19a	0.30 ± 0.05a	0.076 ± 0.03a
Wt L-Gall	0.62 ± 0.1a	0.26 ± 0.05a	1.07 ± 0.15b	1.4 ± 0.17b	0.38 ± 0.04ab	0.043 ± 0.007a
Vtc 2H ₂ O	0.6 ± 0.05a	0.095 ± 0.01a	0.92 ± 0.03a	0.46 ± 0.17a	0.53 ± 0.08b	0.081 ± 0.004a
Vtc 2 L-Gall	0.72 ± 0.06a	0.35 ± 0.05b	1.18 ± 0.03b	1.7 ± 0.32b	0.41 ± 0.06b	0.070 ± 0.026a

Results with similar letters indicate non-statistical differences (ANOVA, $P < 0.05$).

by Müller-Moulé et al. (2004) and here it is demonstrated that normal activity may be restored with the addition of L-GallL. Low photosynthetic ETR may impact the generation of reducing power like NADPH in chloroplast affecting further photosynthetic reactions. Pyridine nucleotides, GSH and AA are highly interlinked forming a redox network integrating metabolic signals influencing the growth and development of plants under different ambient conditions (Foyer and Noctor, 2011). These metabolites cooperate for the detoxification of ROS and for the optimization of photosynthesis through the water–water cycle and the thermal dissipation (Asada, 1999; Gilmore, 1997). The different relative accumulations of AA, GSH and pyridine nucleotides observed in this work point out the flexibility of plant metabolism. *Vtc* mutants have increased GSH concentration (Kerchev et al., 2011) but here no further modification was observed after AA recovery. In contrast NADP(H) concentration is not changed by AA deficiency and it is increased in both genotypes after L-GallL treatment. These results suggest that relative levels of these metabolites do not determine the *vtc2* phenotype.

5. Concluding remarks

The evidences presented here demonstrate that AA synthesized by mitochondria participates in the regulation of photosynthesis through specific processes, mainly through modifications in the leaf gas diffusion. In addition, the absence of any change in mitochondrial parameters in either *vtc2* or wt leaves after L-GallL supplementation indicates that AA synthesis (or accumulation) does not have an influence on plant respiration.

Contributions

María E Senn and Gustavo Gergoff Grozoff carried out several experiments especially measuring photosynthesis and respiration. They also participated with statistical analysis of the data.

Matías Alegre and Franco Barrile collaborated with several lab determinations and with the cultivation of the plants.

Carlos Bartoli coordinated the work participating in the design of the experiments, lab determinations and writing the manuscript.

Mario De Tullio collaborated planning the experiments and writing the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2016.03.012>.

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