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Ethylene signaling triggered by low concentrations of ascorbic acid regulates biomass accumulation in *Arabidopsis thaliana*

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ABSTRACT

Ascorbic acid (AA) is a major redox buffer in plant cells. The role of ethylene in the redox signaling pathways that influence photosynthesis and growth was explored in two independent AA deficient *Arabidopsis thaliana* mutants (*vtc2-1* and *vtc2-4*). Both mutants, which are defective in the AA biosynthesis gene GDP-L-galactose phosphorylase, produce higher amounts of ethylene than wt plants. In contrast to the wt, the inhibition of ethylene signaling increased leaf conductance, photosynthesis and dry weight in both *vtc2* mutant lines. The AA-deficient mutants showed altered expression of genes encoding proteins involved in the synthesis/responses to phytohormones that control growth, particularly auxin, cytokinins, abscisic acid, brassinosterioids, ethylene and salicylic acid. These results demonstrate that AA deficiency modifies hormone signaling in plants, redox-ethylene interactions providing a regulatory node controlling shoot biomass accumulation.

1. Introduction

Ascorbic acid (AA) participates of many physiological processes in plants. It has a central function in plant antioxidant defenses, in the elongation and cell division and in the optimization of photosynthesis [1]. The concentration of AA changes during plant development presenting high levels in young and actively growing tissues and declining during senescence [2]. Since *Homo sapiens* like other primates has lost the capacity to synthesize AA, the accumulation of this antioxidant to high levels in edible plant organs is of paramount interest to human nutrition [3].

Glucose is the primary precursor for AA synthesis in different organisms [4]. However, L-galactose is considered the first metabolite exclusively committed to this pathway in plants [5]. GDP-L-galactose phosphorylase (VTC2/VTC5) catalyzes the formation of L-galactose from GDP-L-galactose. Mutant plants deficient in VTC2 still have an active homologue VTC5 protein. The reduced activity of VTC5 leads to a small contribution to this pathway and consequently *vtc2* plants have very low concentration of AA [6]. Mutants with low activity of VTC2/VTC5 are very useful to study the specific role of AA in plant biology.

Phenotype modifications due to low AA were analyzed in a collection of Arabidopsis deficient mutants [7]. AA-deficient mutants are highly susceptible to the oxidative stress caused by ozone [8] but show a high level of pathogen resistance [8,9]. In addition these AA-deficient mutants have a smaller rosette size than the wild type, altered root architecture and gravitropism and flowering time [10,11]. AA deficient plants also show alterations in hormone metabolism and/or signaling. Higher concentrations of abscisic acid were observed in *vtc* mutant leaves [12]. Furthermore, an increased expression of genes associated with abscisic acid signaling was reported in AA-deficient mutants [13], together with altered expression of salicylic acid [14] and ethylene-associated genes [12]. Ethylene is an important stress hormone in plants, which inhibits growth and promotes senescence in different organs [15,16]. While it has been suggested that the reduced growth observed in *vtc2-1* mutants might segregate independently of the *vtc2-1* mutation [17], this has not been substantiated in other studies using different growing conditions. Consequently, redox-dependent changes in phytohormone pathways may be responsible for some of the characteristics of *vtc2* phenotype, especially those leading to slower plant growth. In the following studies, we investigated how low redox buffering capacity in two independent *vtc2* mutant lines that have very low AA contents interacts with ethylene signaling to regulate

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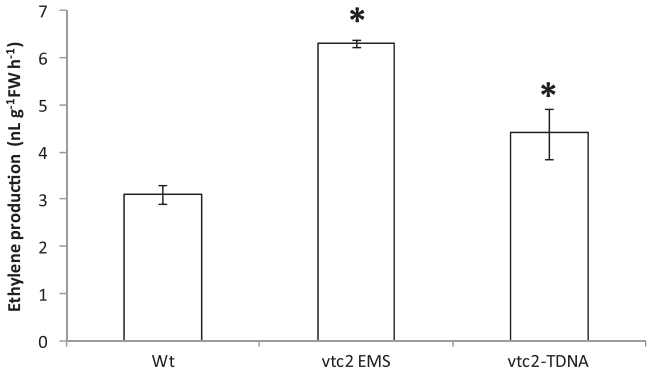

Fig. 1. Ethylene production displayed by above ground tissues of wt, *vtc2* EMS and *vtc2* T-DNA Arabidopsis plants. The asterisk indicates statistical differences with wt (ANOVA, $P < 0.05$). Data represent the mean ± SEM.

photosynthesis and rosette development.

2. Material and methods

2.1. Plant material and treatments

Experiments were carried out with wild type *Arabidopsis thaliana* (L.) Heynh. (Ecotype Columbia 0, wt) and AA deficient plants (*vtc2-1* and *vtc2-4*). Seeds of the wt and *vtc2-4* T-DNA were obtained from the Nottingham Arabidopsis Stock Centre and *vtc2-1* EMS from Dr Robert Last [18]. Plants were grown in a chamber under a PPFD of 120 μmol m^{-2} s^{-1}, at 23 °C and a photoperiod of 10/14 h light/dark, respectively. After one month, plants were transferred to another chamber with similar conditions but with a longer photoperiod (16/8 hs light/dark, respectively) to induce flowering.

Plants were placed in sealed 40 L chambers including or not the ethylene inhibitor 1-methyl cyclopropene (1-MCP, Smart Fresh^{SM}, 1 μL L^{-1}) overnight [19]. Treatments with 1-MCP were applied four times once a week starting at the fourth week (i.e. at the beginning of the last week under short photoperiod).

2.2. Concentration of AA

AA was measured in leaves 48 h after receiving the first 1-MCP treatment by HPLC as previously described [20].

2.3. Ethylene and CO_2 production

These determinations were made in one month-old plants without 1-MCP treatment. Ethylene was analyzed placing above ground tissues in 50 mL tubes for 2 h. 250 μL were injected in a GC system equipped with Carboxen Supelco Column (30 m × 0.33 mm), using the following conditions: "carrier" flux at 9 mL min^{-1}, injector at 200 °C, flame ionization detector at 300 °C and the oven at 170 °C.

CO_2 was measured in the same samples and with the same equipment but detected with a TCD at 250 °C.

2.4. Photosynthesis

Photosynthesis was determined in plants after receiving the second 1-MCP treatment and before transferring them to the long photoperiod chamber. Electron transport rate (ETR) was used to measure photosynthetic activity with a Portable chlorophyll fluorescence meter (FMSII, Hansatech, UK) and calculated according to [21]. Determinations were performed under the growth conditions mentioned above.

2.5. Leaf conductance

The same plants used for photosynthesis determination were used for leaf conductance estimation. Measurements of both sides of the leaves were measured and added to obtain total leaf conductance. Determinations were done with a steady state diffusion leaf poromoter (SC-1, Decagon Devices).

2.6. Plant biomass accumulation

Determination of plant growth was done in two-month old plants (Four weeks after transferred to the long photoperiod condition). Plant growth was estimated by the dry mass of above ground plant organs. For these measurements samples were collected, placed at 68 °C for at least 48 h and then the weight was recorded.

2.7. RNA seq analysis

This analysis was performed on three biological replicates of imbibed seeds of the genotypes wt, *vtc2-1*, and *vtc2-4*, as described previously [18]. All RNAseq data from this article are available at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-5103. Transcripts found to be significantly differentially regulated in comparisons of *vtc2-1* and *vtc2-4* mutants vs wt were annotated against the GO term annotations from (http://www.geneontology.org/page/download-annotations, v10-5–2017-TAIR),

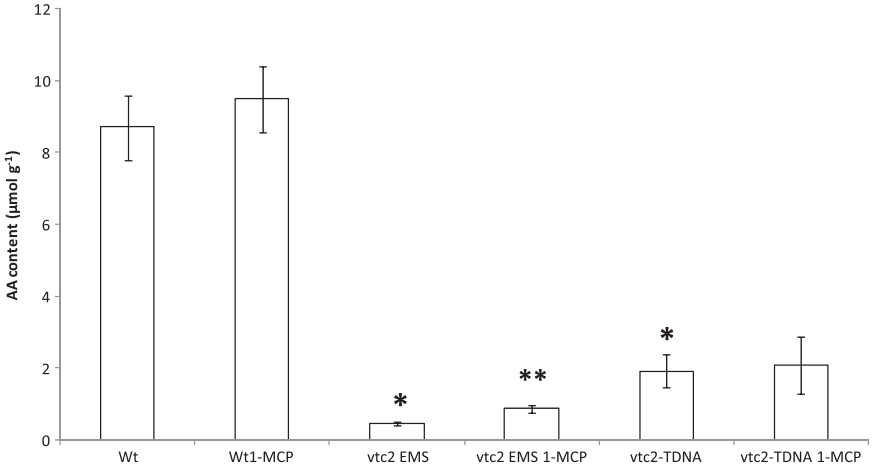

Fig. 2. Concentration of leaf AA in untreated or 1-MCP treated wt, *vtc2* EMS and *vtc2* T-DNA Arabidopsis plants. One asterisk indicates statistical differences with wt (without 1-MCP treatment) and two asterisks indicate statistical differences with 1-MCP treatment for the same genotype (ANOVA, $P < 0.05$). Data represent the mean ± SEM.