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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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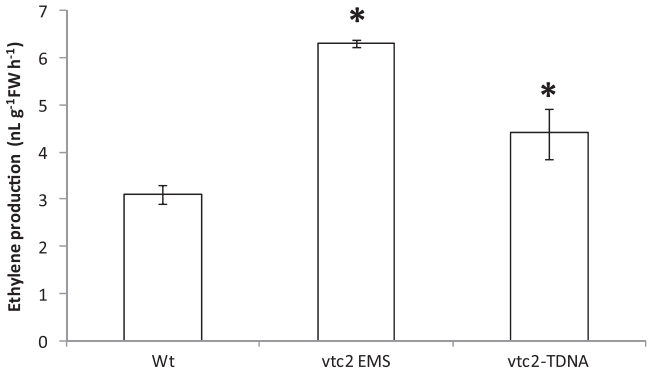

Fig. 1. Ethylene production displayed by above ground tissues of wt, *vtc2* EMS and *vtc2* T-DNA Arabidopsis plants. The asterisk indicates statistical differences with wt (ANOVA, $P < 0.05$). Data represent the mean ± SEM.

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),

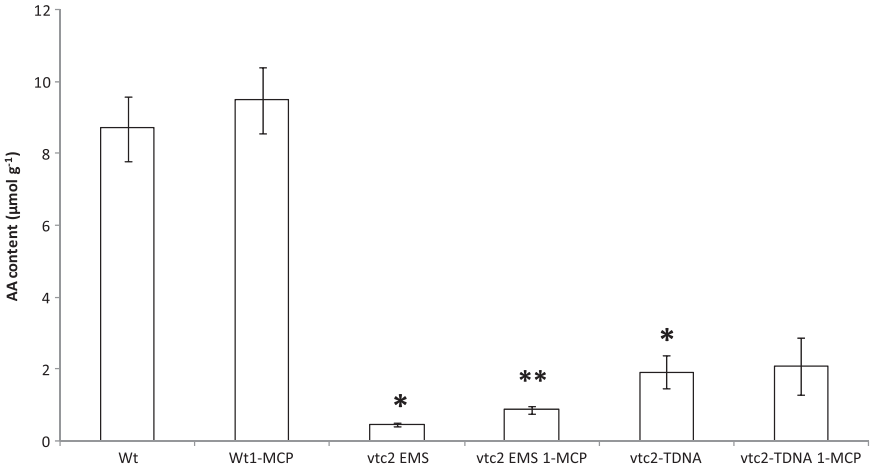

Fig. 2. Concentration of leaf AA in untreated or 1-MCP treated wt, *vtc2* EMS and *vtc2* T-DNA Arabidopsis plants. One asterisk indicates statistical differences with wt (without 1-MCP treatment) and two asterisks indicate statistical differences with 1-MCP treatment for the same genotype (ANOVA, $P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

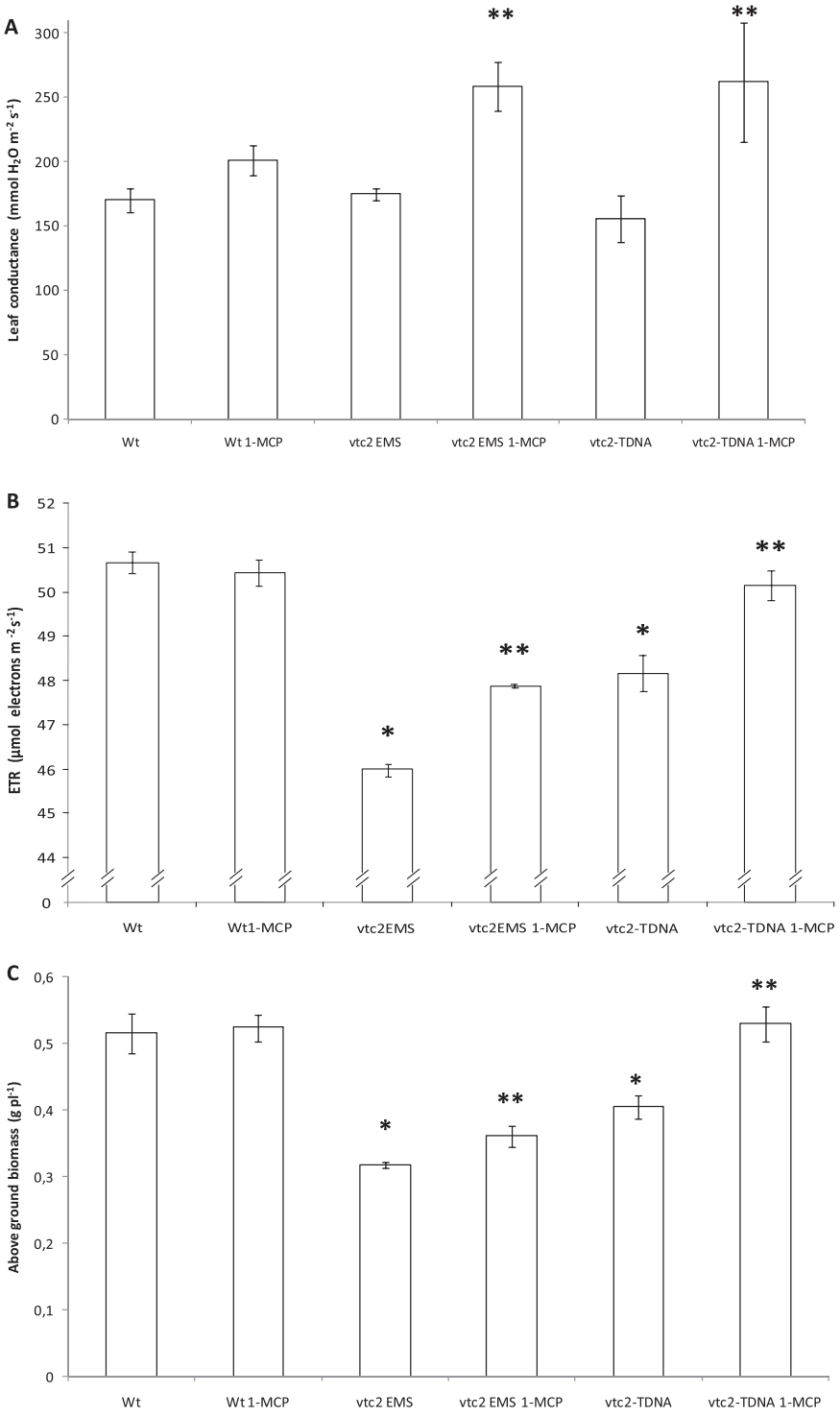

Fig. 3. Leaf gas exchange, photosynthesis and growth displayed by untreated or 1-MCP treated wt, *vtc2* EMS and *vtc2* T-DNA plants. A) Leaf conductance; B) Photosynthetic ETR and C) Biomass accumulation. One asterisk indicates statistical differences with wt (without 1-MCP treatment) and two asterisks indicate statistical differences with 1-MCP treatment for the same genotype (ANOVA, $P < 0.05$). Data represent the mean ± SEM.

and were tested for enrichment of GO terms related to phytohormones using topGo (v2.22) and GO annotations processed by PlantRegMap (http://plantregmap.cbi.pku.edu.cn).

2.8. Statistical analysis

Differences between means obtained from at least three independent experiments were statistically analyzed using a single-factor analysis of variance (ANOVA, $P < 0.05$). Six plants per treatment were included in each experiment.

3. Results and discussion

3.1. Ethylene production in plants with low concentration of AA

Both *vtc2* mutants produce higher amounts of ethylene than wt plants (Fig. 1). This high level of volatile hormone production coincides with the increased levels of transcripts encoding proteins associated

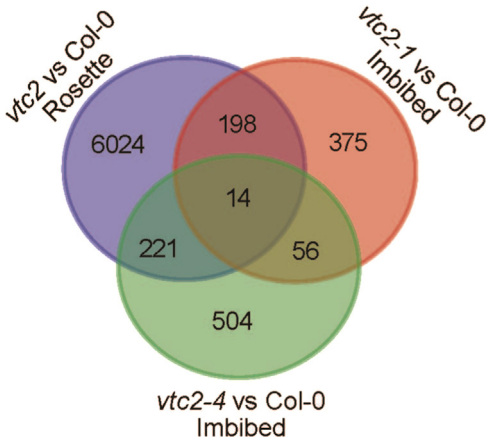

Fig. 4. A Venn diagram comparison of *vtc2-1* and *vtc2-4* seedlings with the available datasets for *vtc2-1* rosette leaves, showing the overlap between transcripts that are significantly differentially expressed between *vtc2* and wt.

with ethylene signaling [12]. The synthesis of ethylene is an autocatalytic process occurring at the last stages of plant growth in order to accelerate plant senescence [15]. The increased synthesis of this hormone during the early stages (i.e. plant growth before flowering) may affect physiological processes such as photosynthesis leading to the inhibition of vegetative growth. Hence, the consequence of ethylene augmented production was further studied.

3.2. Effect of ethylene in the content of AA

The relationship between ethylene and AA was previously reported. Spinach leaves treated with ethylene decrease AA concentration and the activities of the enzyme catalyzing the last step in AA synthesis and in the recovery from oxidized forms (L-galactono-1,4-Lactone dehydrogenase and dehydro- and monodehydro ascorbate reductases, respectively) [22]. Furthermore, mutant Arabidopsis plants with inhibited ethylene signaling show higher AA concentration and increased activities of the enzymes catalyzing both synthesis and recycling of oxidized forms [22]. Leaves of dwarf tomato mutants present lower AA content and L-galactono-1,4-Lactone dehydrogenase activity (and higher ethylene production) than wt plants but both increase after specifically inhibition of ethylene signaling with 1-MCP [23]. We used 1-MCP in the present work to investigate the phenotype modifications provoked by enhanced ethylene production in *vtc2* plants further. The AA content of wt leaves was higher than that of the leaves of both *vtc2* mutant lines (Fig. 2) and AA levels were not changed in wt leaves by the inhibition of ethylene signaling. However, the content of AA in the *vtc2* EMS but not in *vtc2* T-DNA leaves treated with 1-MCP increased to values that were almost twice those of untreated plants. Although inhibition of ethylene signaling stimulates AA synthesis in one of the mutants (largely far from the wt level) the lack of complete recovering of AA concentration indicates the presence of a “bottle neck” imposed by the loss of VTC2 function in both mutant lines. The oxidized/reduced AA ratio was not altered by 1-MCP in either genotype, the AA pool being around 15% oxidized in all cases indicating that ethylene enhanced production did not lead to oxidation of the leaf AA pool.

3.3. Effect of ethylene in the photosynthetic activity

Since ethylene is implicated in the stomatal closure [24] leaf stomatal conductance was determined. No differences in stomatal conductance values were observed comparing leaves of wt with both *vtc2* mutant lines in the absence of the inhibitor; however, the addition of 1-MCP produced increases in leaf conductance of about 48% and 68% for *vtc2* EMS and *vtc2* T-DNA plants, respectively (Fig. 3A). These results show that ethylene is limiting the gas diffusion in *vtc2* leaves. It was recently reported that feeding *vtc2* EMS plants with L-galactone-1,4-Lactone increased stomatal conductance as well as leaf AA content [25]. These data suggest that an elevation of AA in *vtc2* plants counteracts the closing effect of ethylene on stomata. It is important to mention that *vtc2* leaves have higher stomatal density and rubisco content than wt plant [25] suggesting compensation processes for achieving normal gas exchange.

The leaves of both AA deficient mutants had lower photosynthetic ETRs compared to wt plants (Fig. 3B) [26]. However, CO_2 assimilation rates were similar in *vtc2* EMS and wt plants [12,25]. The rate of AA synthesis is dependent on photosynthetic ETR [27]. An increase in photosynthetic ETR was observed following 1-MCP application in both *vtc2* mutants, but not in wt leaves (Fig. 3B). This observation shows that AA and ETR interact in the regulation of gas exchange with the potential to modulate leaf carbon metabolism.

Leaf respiration, measured as O_2 uptake, was the same in *vtc2* EMS and wt plants [25]. In agreement with this observation, CO_2 evolution was similar in wt and *vtc2* EMS plants (Suppl Fig. S1). The effect of 1-MCP on respiration was therefore not analyzed further.

Ethylene has many physiological roles in plants. In particular, this stress hormone induces dormancy, senescence and an inhibition of cell elongation [16]. Both *vtc2* mutants showed lower shoot biomass accumulation than wt plants but the application of 1-MCP produced increases in the dry weight of around 13% and 31% for the *vtc2* EMS and *vtc2* T-DNA plants, respectively (Fig. 3C). It is worth mentioning that no genotypic differences in flowering time were observed relative to the treatments under the growth conditions used here (Suppl Fig. S2). The *vtc2-4* but not the *vtc2-1* mutant attained a similar biomass to the wt plants after 1-MCP treatment. This result indicates, as in the previous observations of [17], that additional physiological constraints (e.g. photosynthetic ETR) may prevent the growth of the *vtc2-1* plants. However, 1-MCP-dependent increases in shoot biomass accumulation were observed in both lines, demonstrating that shoot growth is restrained by low AA accumulation in a hormone-dependent manner.

Taken together, these findings show that AA and ethylene have opposite effects on plant biomass accumulation. While AA increases leaf conductance [25,28], allowing optimization of photosynthesis and leaf cell expansion [1], ethylene inhibits growth [15] and AA synthesis and accumulation [22].

3.4. Effect of low AA concentration in the expression of genes involved in the hormone pathways that control growth

An analysis of gene expression in *vtc2* plants was performed to better understand the linkage between AA levels and growth. A comparison of available datasets for *vtc2-1* rosette leaves and *vtc2-1* and *vtc2-4* seedlings (imbibed germinated seeds) show many more transcripts are differentially expressed in AA-deficient leaves compared to the wild type than are found in AA-deficient seedlings compared to the wild type (Fig. 4). The significantly different genes between the alleles are shown in Supplementary Table S1.

An analysis of the effects of AA on the hormones that regulate growth however, might be considered to be more precise on seedlings than on leaves because of the absence of the added complication of photosynthesis. This analysis reveals that low AA specially altered the abundance of transcripts encoding proteins involved in the synthesis/signaling/responses to the following phytohormones: auxin, cytokinins, abscisic acid (ABA), brassinosterioids, ethylene and salicylic acid. Although there are some variations in the overall numbers of transcripts that are differentially changed in the *vtc2-1* and *vtc2-4* seedlings compared to the wild type, some clear trends in AA (redox)-mediated phytohormone patterns can be distinguished (Fig. 5).

For example, the *vtc2-1* and *vtc2-4* seedlings show significant increases in transcripts encoding proteins involved in auxin and cytokinin synthesis and/or responses/signaling (Fig. 5). Transcripts that encode genes involved in ethylene biosynthesis and signaling were increased in abundance in *vtc2-4* seedling, whereas greater numbers of components

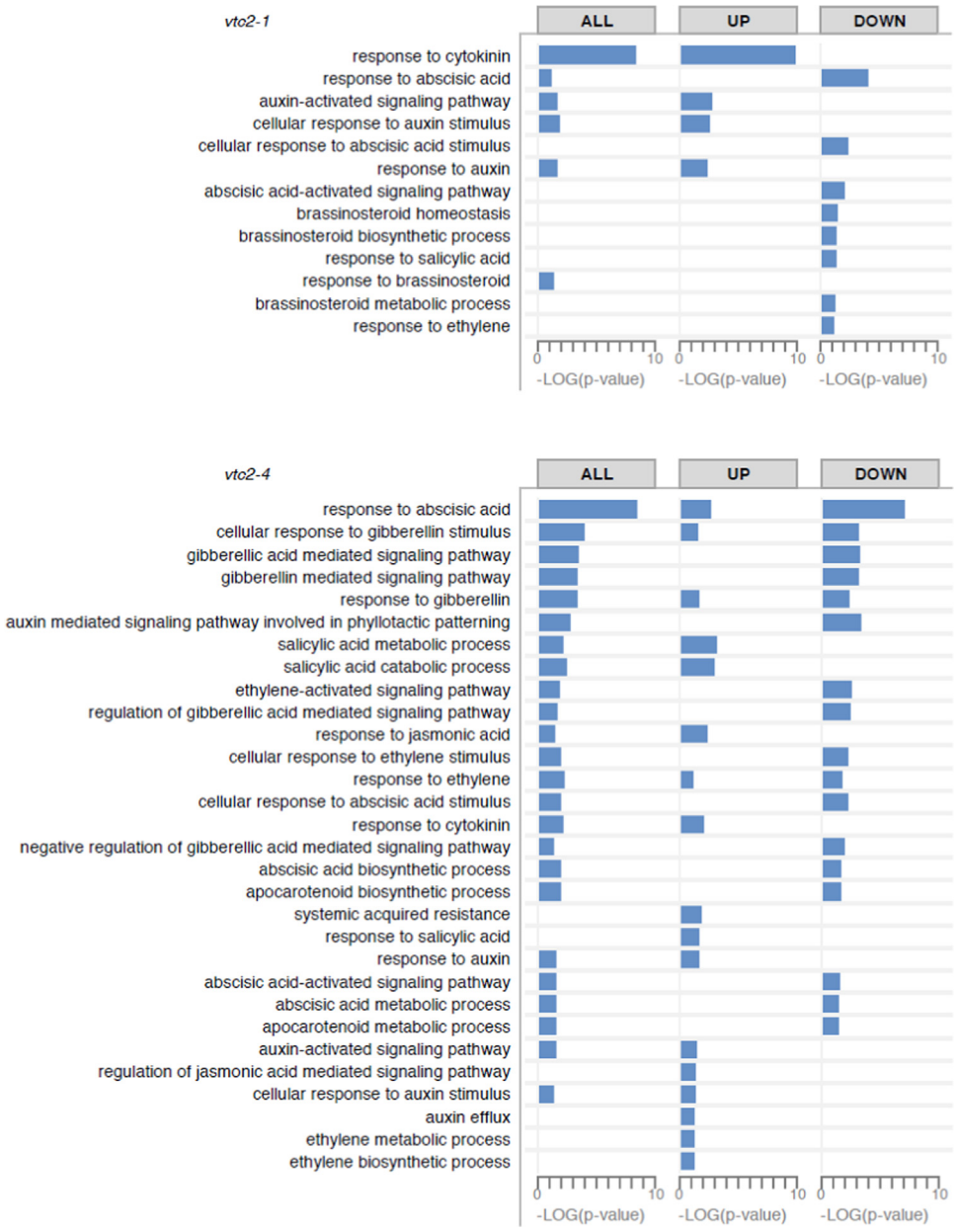

Fig. 5. Gene Ontology enrichment analysis of significantly differentially expressed transcripts in *vtc2-1* and *vtc2-4* mutant seedlings vs wt for terms related to metabolism, signaling, transport or response to phytohormones. Bars show the negative log10 of the p-value for the Fisher's exact test of genes annotated with the ontology terms, for the set of up-regulated, down-regulated and the combined set of differentially regulated transcripts.

involved in ethylene-mediated signaling pathways were decreased in abundance rather than increased (Fig. 5).

Previous work on *vtc2-1* seedlings has shown that they have a higher ABA contents than the wild type [12,13] but the data in Fig. 5 show that transcripts involved in responses to ABA were decreased in abundance rather than increased in the *vtc2-1* and *vtc2-4* seedlings. The closer scrutiny of the phytohormone-related transcripts that showed the greatest changes in abundance, shows that many are transcription factors (Fig. 6). For example, *ABI4* transcripts are lower in *vtc2-1* mutants while *ABI3* and *ABI5* transcripts are increased in *vtc2-4* seedlings relative to the wild type. Moreover, marker genes of ET-regulated pathways are differentially changed in the *vtc2-1* and *vtc2-4* seedlings relative to the wild type, some transcripts increased in abundance while others are decreased in abundance (Fig. 6). The data presented in Figs. 5 and 6 reveal the complexity of the redox-dependent changes in hormone-mediated pathways that control growth. Unraveling this crosstalk, which is likely to encompass multiple points of reciprocal control, hence requires a systematic analysis of the relative effects of each hormone, such as that described here. The significantly different transcripts between the alleles for imbibed seeds are shown in Supplementary Table S2.

Vast literature evidence suggests that ethylene-dependent regulation of plant growth is exerted through extensive crosstalk with other hormones and related redox signaling pathways [29,30]. For example, brassinosteroid-deficient mutants showed increased ethylene production and an extreme dwarf phenotype. The application of 1-MCP reverted this phenotype and additionally, increased AA concentrations to wt levels [23]. Moreover, the functions and/or stability of DELLA proteins, which are transcription factors that inhibit plant growth, are regulated by different plant hormones. Gibberellins promote growth specifically by targeting these proteins for degradation. In contrast, ethylene enhances DELLA functions to reduce growth under abiotic

Fig. 6. Summary of the most differentially expressed transcripts annotated as being involved in the metabolism, signaling, transport or response of ABA, GA and Ethylene in the Gene Ontology for both the *vtc2-1* and *vtc2-4* mutants compared to wt. Bars show the fold-change induction (blue) or repression (red) in *vtc* mutants with the FDR corrected p-value associated with the moderated t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stress conditions [31,32]. Steady state levels of reactive oxygen species (ROS) are lower when DELLA proteins are present, probably through the regulation of antioxidant gene expression [33]. Roots exposed to abiotic stresses show a range of typical responses including decreased elongation and increased branching. These changes are mediated by ethylene/auxin/redox interactions [34]. Such findings demonstrate that oxidative metabolism/signaling is a key component of phytohormone signal transduction pathways. Similarly, the enhanced sensitivity to oxidation caused by lower antioxidant capacity, as observed here, alters the transcriptome signature and related hormonal signaling pathways [35]. Taken together, the data presented here highlight the close inter-relationships between hormonal and redox signaling networks.

4. Conclusions

The results presented here demonstrate that AA deficiency modulates ethylene emissions and signaling leading to alterations in gas diffusion and photosynthesis in *vtc2* leaves (Fig. 7). Taking in consideration that 1-MCP does not substantially affect AA concentration, ethylene effects on leaf conductance is exerted by non-dependent AA-mechanisms. Since AA is a major cellular antioxidant the findings reported here are entirely consistent with the hypothesis that oxidative signals and ethylene converge at the redox signaling hub to control plant biomass accumulation. The role of this interaction might be studied in other plant species of human interest to demonstrate its usefulness for the improvement of crop biomass production.

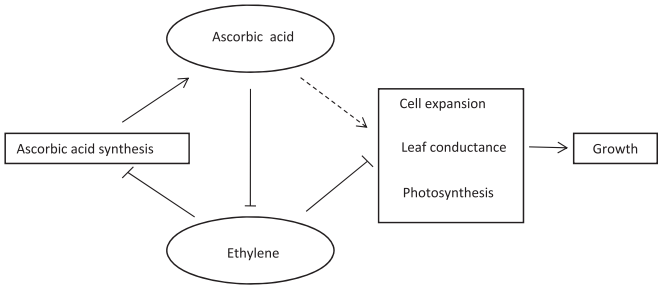

Fig. 7. Diagram indicating proposed interactions between AA and ethylene. While high AA inhibits ethylene production low antioxidant concentrations lead to increased hormone production and growth reduction. In turn, high ethylene production decreases AA synthesis and accumulation. Dashed line indicates the participation of AA in growth through non-ethylene dependent processes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2018.01.032.

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