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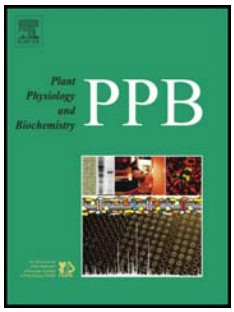

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Impact of brassinosteroids and ethylene on ascorbic acid accumulation in tomato leaves.

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Abstract

Plant steroid hormones brassinosteroids (BRs) and the gaseous hormone ethylene (ET) alter the ascorbic acid - glutathione (AA-GSH) levels in tomato (*Solanum lycopersicum L.*) plants. The interaction of these hormones in regulating antioxidant metabolism is however unknown. The combined use of genetics (BR-mutants) and chemical application (BR/ET-related chemicals) shows that BRs and ET signalling pathways interact, to regulate leaf AA content and synthesis. BR-deficient (d^x) leaves display low total AA but BR-accumulating (*35S:D*) leaves show normal total AA content. Leaves with either BR levels lower or higher than wild type plants showed a higher oxidised AA redox state. The activity of L-galactono-1,4-lactone dehydrogenase (L-GalLDH), the mitochondrial enzyme that catalyses the last step in AA synthesis is lower in d^x and higher in *35S:D* plants. BR-deficient mutants show higher ET production but it is restored to normal levels when BR content is increased in *35S:D* plants. Suppression of ET signalling using 1-methylcyclopropene in d^x and *35S:D* plants restored leaf AA content and L-GalLDH activity, to the values observed in wild type. The suppression of ET action in d^x and *35S:D* leaves leads to the respective decreasing and increasing respiration, indicating an opposite response compared to AA synthesis. This inverse relationship is lacking in ET suppressed d^x plants in response to external BRs. The modifications in the *in vivo* activity of L-GalLDH activity do not correlate with changes in the level of the enzyme. Taken together, these data suggest that ET suppresses and BRs promote AA synthesis and accumulation.

Keywords

Antioxidants; ascorbic acid; brassinosteroids; ethylene; leaves; respiration; tomato.

1. Introduction

Plants are sessile organisms and consequently, they need to be able to rapidly respond to changing environmental conditions especially those that cause oxidative damage. Ascorbic acid (AA) and glutathione (GSH) interact to form the central antioxidant system in plant cells, called the AA-GSH cycle [1] that helps prevent oxidative damage in plants. In addition, AA has several physiological roles including the regulation of photosynthesis, stomata closure and cell growth [2,3]. AA plays an important role in the detoxification of reactive oxygen species, including reducing hydrogen peroxide to water [4]. This reaction is catalysed by the AA peroxidase (APX, EC 1.11.1.11**)** producing monodehydroascorbate radicals (MDA), which can be reduced by NAD(P)H in a reaction catalysed by MDA reductase (EC 1.1.5.4) [5]. MDA radicals are able to spontaneously disproportionate to dehydroascorbate (DHA) and AA [6]. DHA reduction may occur via either a non-enzymatic reaction with GSH or enzymatically by DHA reductase (DHAR, EC 1.8.5.1) leading to the production of AA [7]. Recycling of GSH is catalysed by glutathione reductase (GR, EC 1.6.4.2) that reduces glutathione disulphide (GSSG) by using NADPH [2].

The activity of the AA-GSH cycle described above strongly depends on both AA levels and its redox state. The last step of AA biosynthesis in plants is catalyzed by the mitochondrial flavoenzyme L-galactono-1,4-lactone dehydrogenase (L-GalLDH; EC 1.3.2.3) [8]. L-GalLDH is located in the mitochondrial inner membrane and mediates the two-electron oxidation of L-galactono-1,4-lactone (L-GalL) into AA with the concomitant reduction of cytochrome *c* feeding electrons in the mitochondrial electron transport chain, most likely between mitochondrial complexes III and IV [9].

Brassinosteroids (BRs) are plant steroid hormones that control cell elongation, growth, photosynthesis and stress responses [10]. BRs regulate, directly or indirectly, enzymes and metabolites of AA-GSH cycle although discrepancies have been observed. For example exogenous BR application decreased AA content in tomato fruits [11,12] or shifted both AA/DHA and GSH/GSSG to a more oxidised state in *Brassica napus* embryos [13]. In contrast, BR treatment increased the content of AA and GSH in suspension cultured cells or in seedlings under stress conditions [14,15]. These discrepancies suggest the participation of

different and yet to be identified factors controlling BR-mediated antioxidant regulation.

Previous research has shown that leaf AA synthesis and content are down-regulated by the gaseous hormone ethylene (ET) [16]. BRs are known to induce ET production and modulate ET-mediated growth response [17,18]. Both antagonistic or synergistic BR and ET responses have been observed in previous studies [18,19]. However, how BR and ET signalling regulates the content and biosynthesis of AA is unknown.

The combined utilization of BR-related mutants and ET-related chemicals serve as complementary tools to elucidate the outputs of the signalling crosstalk between these hormones. The tomato *Dwarf* gene encodes a cytochrome P450 enzyme controlling a key step in BR biosynthesis. The null d^x mutation [20,21] and the d^x complemented line over-expressing *Dwarf* [21] leads to the respective lines with deficiency or accumulation of endogenous BRs. 1-methylcyclopropene (1-MCP) is an ET signalling inhibitor that specifically binds to ET receptors [22]. Using both the BR-related mutants and 1-MCP, this work tests the hypothesis that BR- and ET- signalling interact to control AA synthesis in tomato leaves.

2. Results

2.1. Modifications of the AA-GSH cycle in leaves of BR mutants

Key antioxidant enzyme activities and metabolite concentrations were determined in leaf tissues from plants with BR-deficiency or those over-accumulating BRs. Altered chloroplastic APX activities were observed in plants with modified BR levels (Fig. 1A-B). Stromal APX (sAPX) activity was highly reduced in d^x and *35S:D* plants (~70 % lower than in the wt) but thylakoid APX (tAPX) slightly increased in both BR-modified plants. In contrast, cytosolic APX (cytAPX) and DHAR activities were similar in all genotypes (Fig. 1C-D). GR activity decreased in d^x plants, whereas, it was similar in *35S:D* plants to that in wt (Fig. 1E).

To discern whether the redox status of AA-GSH cycle may vary in leaves with altered BR levels, the relative amount of the reduced and oxidised forms of AA and GSH in both BR-deficient and BR-over-accumulating mutants was measured. Significant decrease in the AA content and an increase in its oxidised state were observed in both d^x and *35S:D* leaves as compared with those of wt (Fig. 2A-B). However, total AA content was lower in d^x but similar in *35S:D* leaves to those observed in wt (Fig. 2C). GSH content and redox state were similar in all genotypes (Fig. 2D-F).

A more oxidised antioxidant redox state may indicate a raise in the oxidative stress load [23]. To verify this, oxidative damage was measured as oxidised protein content in leaves of BR mutants. Dwarf plants with reduced BR content showed higher amounts of carbonylated proteins (Supplementary Figure S1) as compared with wt. However, *35S:D* plants had lower leaf protein oxidative damage with levels similar to wt.

2.2. Effect of BRs on the AA content and synthesis from leaves of the d^x mutant and wt plants.

To gain more insight into how AA content and redox state were severely affected in the BR altered lines, further characterization of AA metabolism was performed after BR application. No differences were observed in total AA leaf content in wt after supplementation of leaves with 24-epibrassinolide (EBL, Fig. 3A). AA biosynthesis

capacity measured as *in vivo* L-GalLDH activity increased upon increasing EBL concentrations up to 0.1 µM. However, at the highest EBL concentration (1.0 µM), enzyme activity was inhibited (Fig. 3B). In contrast, both total AA content and the *in vivo* L-GalLDH activity increased when leaves of d^x plants were sprayed with increasing concentrations of EBL (Fig. 3C-D). EBL did not modify the oxidised state of AA in either wt or d^x leaves (Fig. 3).

2.3. ET production and the control of AA accumulation and synthesis in BR mutant leaves

The altered AA accumulation and synthesis in BR-mutant leaves may potentially be linked to ET production, and thus ET synthesis was measured in tomato plants with altered BR levels. BR-deficient leaves showed higher ET production and higher capacity of ET synthesis than wt plants (Table 1). These hormonal characteristics were lower in *35S:D* leaves compared with d^x mutant but similar to those of wt plants (Table 1).

To verify that ET action is involved in these changes in AA accumulation, the ET signaling inhibitor 1-MCP was used at a concentration 1.0 µL L^{-1}. Leaves of d^x mutant plants treated with the ET inhibitor showed increases of 70 % and 114 %, in total AA content and *in vivo* L-GalLDH activity, respectively. Such increases reached similar AA values to those observed in the wt leaves (Table 2). On the contrary, the capacity of AA synthesis in *35S:D* leaves (Approximately 60 % higher than in wt leaves) was decreased by 1-MCP treatment to a similar wt value (Table 2). The application of 1-MCP to d^x and *35S:D* plants did not produce any major visible alterations in leaf morphology. Treatment with 100 µM ACC (Aminocyclopropane-1-carboxylic acid, an ET biosynthesis precursor) significantly reduced both the leaf AA content and the *in vivo* L-GalLDH activity in leaves of wt plants. However, ACC application on 1-MCP treated wt plants did not show a response (Supplementary Table S1), indicating that 1-MCP effectively blocks ET-mediated AA response in tomato leaves.

As the last reaction of AA synthesis in plant cells takes place in mitochondria an analysis of AA production by this organelle was carried out. 1-MCP treatment regulated AA synthesis in isolated mitochondria with induction of AA production in the d^x mutant and repressing production in *35S:D* plants (Table 3). However, the

amount of L-GalLDH in mitochondrial extracts was only different between wt and d^x without 1-MCP and was unaltered among the rest of the treatments (Table 3). Thus this indicates a lack of correlation between L-GalLDH level and AA synthesis capacity as previously observed [24].

Since the amount of L-GalLDH did not explain the modified AA synthesis, it was thought that other processes such as the photosynthetic and respiratory electron transport rates could have an involvement. No differences were observed in photosynthesis measured as ETR, for any genotype with or without 1-MCP treatment (Table 4). Respiratory activity was however lower in dwarf plants but similar in *35S:D* compared with wt plants. Moreover, 1-MCP decreased the oxygen uptake rates of wt and d^x leaves but, surprisingly, increased respiration of the BR-over-accumulating plants (Table 4), showing opposite effects as those seen for L-GalLDH activities. A complementary assay detecting mito-traker red® fluorescence by confocal microscopy, showed decreases on the number of active mitochondria in both wt and d^x leaves due to the inhibition of ET signalling pathway (Supplementary Figure S2). However, mitotracker fluorescence emission was not modified in 1-MCP treated 35S:D leaves suggesting that their increment in the oxygen uptake rate is not due to changes in the amount of active mitochondria (Supplementary Figure S2). Modifications in mitochondria activity may be also linked to alterations in the respiratory substrate flux.

2.4. Effects of BRs on AA content of leaves with suppression of ET action.

To further explore the ET role in the BR-mediated effects on AA accumulation, we pre-treated plants with the ET inhibitor and then evaluated AA content and *in vivo* L-GalLDH activity following foliar spraying with 1.0 µM EBL.

Total AA content and *in vivo* L-GalLDH activity were not affected by EBL applications in 1-MCP pre-treated wt plants (Table 5). However, 1-MCP pre-treated d^x plants showed a marked increase of AA content and synthesis when treated with 1.0 µM EBL. Interestingly, EBL treatment increased foliar respiration in 1-MCP pre-treated d^x plants but resulted ineffective in wt plants (Table 5).

These data suggest that the response of L-GalLDH activity to external BRs could be associated with respiratory oxygen consumption and further AA accumulation is achieved when ethylene perception was suppressed in the BR-deficient mutant.

3. Discussion

BRs are plant steroidal hormones with diverse roles in plant growth and development, including the regulation of antioxidant responses. AA has a central role in the plant antioxidant defence participating in the detoxification of ROS in many cell compartments. In addition the amount and redox state of AA are important factors influencing gene expression [2]. Here, two novel functions for BRs in AA metabolism were found: i) BRs control L-GalLDH activity affecting leaf AA formation, and ii) BRs promote AA accumulation in tomato leaves.

3.1. BRs control the synthesis capacity of AA at the final biosynthetic step

As AA content was highly affected by BR concentration our experiments focused on gaining better understanding of the regulation of AA synthesis by BRs. This analysis utilized tomato leaves from wt and d^x genotypes that were supplemented with BRs. EBL-dependent increments of AA level and *in vivo* L-GalLDH activity in d^x tissues (Fig. 3C-D) indicate that EBL stimulates the AA synthesis pathway. In contrast, EBL treatment does not affect AA content in wt leaves (Fig. 3A) suggesting that BR levels are not limiting the accumulation of this antioxidant in wt plants. The EBL stimulation of AA synthesis in d^x leaves might be related with the increment in the AA precursor formation. Elevation of L-GalL pool in d^x leaves may be linked to the BR-dependent increase of hexose levels as previously observed by Lisso [25] in fruits after BR application to leaves. However, Lisso [25] did not measure hexose levels in BR-treated leaves. Goetz [26] showed that BR treatment increase sucrose uptake providing carbohydrates for supporting the stimulated growth of *Lycopersicon peruvianum* cells. Furthermore, BR treatments increase photosynthetic activity in other plant species [27]. Research has shown that the over-expression of enzymes of the AA biosynthetic pathway enables a large accumulation of AA [28]. However, it is unknown whether BRs are implicated, directly or indirectly, in the regulation of the L-GalL level in steps upstream, prior to its final conversion to AA.

The highly oxidized AA redox state in dwarf leaves after EBL treatments (Fig. 3) indicates that the synthesis but not the recovery from oxidized forms is controlled by BRs. Recycling of DHA to AA is a vital function that takes place in different plant cell organelles using the reducing equivalents generated in the chloroplast and mitochondria (i.e. NADP(H)). This recovery process is important for the availability of AA for both quenching ROS and dissipation of an excessive energy load in

chloroplasts, known as water-water cycle [4]. High oxidation rates may lead to increased oxidized/reduced ratio and decreased accumulation of reduced forms of antioxidants [1]. The higher oxidized state found in leaves with deficient or excessive BR levels suggests that AA recycling might be impaired. Conversion of oxidized AA to reduced forms depends on the activity of DHAR and GR, which are in different cell compartments [2,3]. Under the growth conditions used in this study GSH content (Fig. 2D-F) and DHAR activity (Fig. 1D) were similar in all genotypes suggesting that they are not limiting DHA reduction. On the other hand, the reduction in sAPX isoenzyme activity shown in the BR-deficient mutant and in the *35S:D* leaves (Fig. 1A) might contribute to increased susceptibility of plants to oxidative stress [29]. Enhanced oxidative stress has been detected in BR-related mutants in Arabidopsis [30] and tomato [31].

3.2. BRs and ET show opposite effects on the regulation of AA synthesis.

Different physiological responses or sensitivity to BRs may be due to the interaction of BR-signaling with the signaling of other hormonal compounds, for example ET. BR deficiency leads to the up-regulation of ET production and the over-accumulation of BRs in the d^x background down regulates ET emission (Table 1). The respective inhibitory and stimulatory effects of ACC and BR application on AA synthesis suggest opposite effects of ET and BR on regulating AA levels. However, the effect of higher EBL concentration on reducing *in vivo* L-GalLDH activity was absent when BR was applied on 1-MCP pretreated wt plants (Table 5), indicating the need for normal ET signaling to enable the BR induced reduction.

ET decreases the accumulation of AA lowering its synthesis in both Arabidopsis and spinach leaves [16] (and tomato, see Supplementary Table S1). In addition defective BR homeostasis leads to altered AA metabolism that it has increased sensitivity to 1-MCP treatment. The opposite effect of mutant plants treated with 1-MCP (Table 2) on *in vivo* L-GalLDH activity suggests that BRs antagonize ET response. These data demonstrate that BR-ET signaling pathways interact antagonistically which is consistent with the negative BR-ET interrelationship observed for growth responses [19]. However, these results contrast with synergism reported by De Grauwe [18] who found that BRs and ET synergistically interact stimulating the elongation of Arabidopsis hypocotyls and that BR application has shown to induce ET production

in wild plants [17,32]. This highlights the complexity of the BR-ET interaction in plant tissues.

In addition to the negative BR-ET interaction for regulating AA synthesis in mutants with altered BR content, our data is also consistent with both hormones acting independently of each other. The response of BR-deficient mutant to 1-MCP (Tables 2, 4) indicates that endogenous steroidal hormones are not essential for ET action. Similarly, the response of 1-MCP treated d^x plants to BR supplementation (Table 5) suggests that ET-signaling is not required for BR's effect on AA synthesis.

3.3. Association between changes in respiratory activity and AA synthesis

AA levels are highly dependent on the chloroplastic and mitochondrial electron transport chains [33,34]. Consequently, modifications in photosynthesis and respiration may lead to alteration in AA levels. Exogenous application of BRs is known to increase the rate of photosynthesis [26]. Conceivably the BR-ET effect on AA synthesis capacity is associated with respiration but not with photosynthesis (Table 4). This is because the similar photosynthetic activity, measured as photosynthetic electron transport rate (ETR), observed in BR modified plants does not explain differences in AA accumulation and *in vivo* L-GalLDH activity. It is worth noting however that older d^x plants grown at high irradiance present lower ETR than wt (Supplementary Figure S3).

In contrast, changes in *in vivo* L-GalLDH activity were opposite to respiratory activity in BR deficient and over-accumulating leaves when ET signalling was blocked (Tables 2 and 4). 1-MCP produced inverse changes in O_2 uptake rates (i.e. decreasing respiration in d^x and increasing in *35S:D* leaves). Interestingly, this opposite behavior between the respiration and the *in vivo* L-GalLDH activity was lacking in wt plants in response to 1-MCP. Moreover, the relationship was also not observed in d^x plants pre-treated with 1-MCP and then sprayed with EBL (Table 5).

L-GalLDH biosynthetic capacity displayed differences in both intact leaves and isolated mitochondria from leaves, without modification in the amount of L-GalLDH (Tables 2, 3). This discrepancy suggests that 1-MCP may affect the availability of oxidised cytochrome *c* that is crucial for L-GalL oxidation [34]. Alternatively, it is plausible that a modulation of respiratory rates by other plant hormones or metabolites may explain differences in AA levels. Treatments with gibberellins

decrease respiration and increase AA synthesis in isolated mitochondria from Arabidopsis leaves [35].

3.4 Summary

Taken as a whole the data presented here suggest that BR and ET signaling pathways are acting antagonistically in altering the capacity of AA synthesis leading to changes in AA content in plant tissues. The antagonistic regulation of AA accumulation by ET and BRs is however occurring by independent mechanisms with endogenous BRs not being critical for ET action, and normal ET signaling is not required for BR effects on AA content.

4. Materials and methods

4.1. Plant material and growth conditions

The experiments were carried out using the first fully expanded leaf from the apex of tomato (*Solanum lycopersicum L.*) plants. Plants from the extreme dwarf BR-deficient mutant (d^x) and the *Dwarf* over-expressing transgenic line (*35S:D*) have been previously described [21]. *35S:D* plants show accelerated growth as a result of the constitutive over-expression of the *Dwarf* gene in the d^x background [21]. Mutant plants were obtained on the Ailsa Craig cultivar background.

Experiments analysing the metabolite levels and enzyme activities of the AA-GSH cycle in BR mutant and wt leaves were performed using two month-old plants grown in the glasshouse during spring. Plants received a maximum of 1200 µmol m^{-2} s^{-1} (PPFD) with a temperature regime of ~30/20ºC (day/night). Samples were taken approximately at 5 h into the photoperiod. Analysis of BR and ET participation in AA synthesis and accumulation was carried out with two week-old plants grown in chambers at 300 µmol m^{-2} s^{-1} (PPFD), 25±2 ºC and a 16/8 hs light/dark period.

4.2. Treatment with BRs

Leaves of two week-old plants from genotypes d^x and wt were sprayed with 0.01, 0.1 and 1 µM EBL (*Phyto*Technology Laboratories®). Approximately 5 mL of solution was applied to each plant so leaves were completely wetted with the solution. Leaves of control plants were sprayed with distilled water containing 0.5 % (v/v) ethanol and 0.01 % (v/v) Tween 80 that were also present in the EBL solution. Two applications of EBL were carried out (day 0, day 3) and measurements were taken at day 7.

4.3. Treatment with 1-MCP

Two week-old potted plants from d^x, *35S:D* and wt genotypes were placed in sealed tight 40 L container for the treatment or not with 1.0 µL L^{-1} 1-MCP, an inhibitor of ET action, over 12 h. Afterwards, treated and untreated leaves were used for the assays. When indicated, plants incubated with 1-MCP were sprayed

with EBL as described above and then used for the assays.

4.4. Quantification of antioxidant levels

AA concentration was measured using a HPLC (Shimadzu LC-10ATvp solvent delivery module) fitted with a C-18 column (Varian Chromsep 100mm×4.6mm) and detection carried out at 265 nm (Shimadzu UV–vis SPD-10Avp detector), as previously reported [34]. The oxidised form (DHA) content was calculated as the difference between the content of total ascorbate (DHA+AA) and AA after reducing DHA with DTT.

GSH and GSSG were measured following Griffith [36]. Briefly, leaf tissue was ground in 0.5 mL of TCA (3 % w/v), centrifuged at 17 000 x *g* for 10 min and the supernatant used for the assays. Total glutathione (GSH + GSSG) and GSSG were determined spectrophotometrically before and after derivatization with 2-vinylpiridine. GSH was calculated as the difference between the contents of total and oxidised form.

4.5. Enzyme activity measurements

APX activities were determined according to Miyake and Asada [37]. Leaves were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5 mM AA and 0.1 % (w/v) phenylmethanesulfonyl fluoride and then the homogenate was centrifuged at 13 000 x *g* for 15 min. The soluble fraction containing sAPX and cytAPX and the pellet containing the tAPX were used for the enzyme activity assays. The supernatant was added to N_2-bubbling 50 mM potassium phosphate buffer (pH 7.0) containing 10 µM H_2O_2 in the absence of AA for the chloroplastic APX inactivation. A control assay without H_2O_2 was used to measure the non-inactivated reaction. The residual APX activity in the reaction mixtures was then assayed by adding H_2O_2 (0.1 mM). APX activity was measured spectrophotometrically following changes at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). The 13 000 x *g*-membrane fraction was washed with 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM AA and suspended in a similar buffer including 1 % (v/v) Triton X-100 for tAPX activity.

DHAR and GR activities were carried out as reported by Bartoli [24].

The protein content was quantified by the Bradford method [38].

4.6. Oxidative damage

Oxidative damage was estimated by the determination of leaf protein carbonylation. Proteins were analysed by western blotting and carbonyl groups detected after their derivatization with 2,4-dinitrophenylhydrazine as reported by Levine [39].

4.7. Ethylene synthesis

About 1 g of tomato leaves from two week-old plants was placed in a 10 mL flask sealed with a rubber septum for 2 h. Then 1 mL of air from the flask head space was taken for ethylene determination. Measurements were carried out using a gas chromatograph equipped with an alumina column and a flame ionization detector. Ethylene synthesis capacity was estimated as *in vivo* ACC oxidation activity. To carry out these measurements leaves were immersed in 1mM ACC and 30mM AA solutions for 2 h and then ethylene production was determined.

4.8. Photosynthesis and respiration

ETR was measured through the analysis of chlorophyll fluorescence with a Fluorescence Modulated System (FMS-2, Hansatech Instruments Ltd., Norfolk, UK). ETR was calculated as described by Genty [40]. Respiration was determined placing dark adapted leaves in an air-tight chamber and following their oxygen uptake with a Clark type oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) [34]. Active mitochondria were detected by confocal microscopy incubating cells with 5 µM MitoTracker Red® and detected at 543/585-615 nm, excitation/emission wavelengths.

4.9. In vivo L-GalLDH activity and immunochemical detection

Detached leaves were incubated in 2 mM L-GalL (precursor of AA biosynthesis) or distilled water at room temperature and 100 µmol m^{-2} s^{-1} (PPFD) for 3 h. AA

concentration was then measured and its accumulation was considered as leaf *in vivo* L-GalLDH activity, an estimation of the maximum potential of *in vivo* AA synthesis in leaves [9]. AA synthesis capacity of isolated mitochondria was also evaluated. The isolation of mitochondria, the incubation in the presence or absence L-GalL and the AA quantification was performed as previously described [34].

The accumulation of L-GalLDH was quantified by western blot as previously reported [24] and presented as arbitrary units per mass mitochondria protein. Parallel gels were run for sample protein quantification and blotting analysis.

4.10. Statistical analysis

The means from four independent experiments were statistically analysed by the Duncan test, significance determined at $P \leq 0.05$.

The following data are also available on line:

Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S3

Supplementary Table S1

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Legends to figures

Figure 1. Effect of BR levels on APX isoenzymes, GR and DHAR activities in tomato leaves. Samples were taken from the first expanded leaf from the apex of two months-old plants. Four independent experiments were analyzed for: **A)** sAPX; **B)** tAPX; **C)** cytAPX; **D)** DHAR and **E)** GR activities. Wt= wild type (Ailsa Craig), d^x = BR biosynthesis mutant *extreme dwarf*, *35S:D* = over-expression line of the tomato *Dwarf* gene.
Letters indicate statistically homogenous groups, n = 4 (ANOVA P≤0.05).

Figure 2. Effect of BR levels on AA and GSH contents and redox states in tomato leaves. Samples were taken from the first expanded leaf from the apex of two months-old plants. Four independent experiments were analyzed for: **A)** AA content; **B)** AA redox state; **C)** total AA content; **D)** GSH content; **E)** GSH redox state and **F)** total GSH content. Wt= wild type (Ailsa Craig), d^x = BR biosynthesis mutant *extreme dwarf*, *35S:D* = over-expression line of the tomato *Dwarf* gene.
Letters indicate statistically homogenous groups, n = 4 (ANOVA P≤0.05).

Figure 3. Effect of exogenous supplementation with EBL on total AA and *in vivo* L-GalLDH activity in d^x and wt tomato leaves. The assays were performed using leaves from two weeks-old tomato plants. Four independent experiments were analyzed for: A) wt total AA content; B) wt *in vivo* L-GalLDH activity; C) d^x total AA content and D) d^x *in vivo* L-GalLDH activity. The redox state of AA was not affected by EBL treatments and kept around 13 and 27 % for wt and d^x, respectively. Wt= wild type (Ailsa Craig), EBL = 24-epibrassinolide, d^x = BR biosynthesis mutant *extreme dwarf*, *35S:D* = over-expression line of the tomato *Dwarf* gene. Letters indicate statistically homogenous groups, n = 4 (ANOVA P≤0.05) for each graph.

Table 1

ET production and ET synthesis capacity in dwarf BR-deficient mutant (d^x), BR-overaccumulating (*35S:D*) and wt tomato leaves. ET synthesis capacity was measured in leaves previously incubated in 1mM ACC for 2h.

	Wt	d^x	35S:D
ET production ($\mu L\ g^{-1}FW\ h^{-1}$)	0.24±0.08**a**	0.6±0.17**b**	0.27±0.07**a**
Capacity of ET synthesis ($\mu L\ g^{-1}FW\ h^{-1}$)	19.7±3.64**a**	32.4±4.6**b**	22.8±6.0**a**

Data were obtained from at least 4 independent experiments. Results with the same letter are not statistically different (ANOVA, $P \leq 0.05$).

Table 2

Effects of 1-MCP on total AA (AA+DHA) content and AA synthesis capacity (measured as *in vivo* L-GalLDH activity) in dwarf BR-deficient mutant (d^x), BR-overaccumulating (*35S:D*) and wt tomato leaves. Leaves were incubated in 2 mM L-GalL for 3 h under light and then AA accumulation was measured for the estimation of AA synthesis capacity.

	wt	wt + 1-MCP	d^x	d^x + 1-MCP	*35S:D*	*35S:D* + 1-MCP
Total AA ($\mu mol\ g^{-1}$ FW)	0.73±0.11**a**	0.90±0.09**a**	0.41±0.06**b**	0.69±0.06**a**	0.91±0.15**a**	1.03±0.09**a**
In vivo L-GalLDH activity ($\mu mol\ g^{-1}$ FW h^{-1})	0.80±0.12**a**	0.91±0.07**a**	0.37±0.07**b**	0.80±0.06**a**	1.31±0.14**c**	0.91±0.094**a**

Data were obtained from at least 4 independent experiments. Results with the same letter are not statistically different (ANOVA, $P \leq 0.05$).

Table 3

Effects of 1-MCP on AA synthesis capacity of mitochondria isolated from dwarf BR-deficient mutant (*d*^{x}), BR-overaccumulating (*35S:D*) and wt tomato leaves. Isolated mitochondria were incubated in 2 mM L-GalL for 30 min and then AA accumulation was measured for the estimation of AA synthesis capacity.

	wt	wt + 1-MCP	*d*^{x}	*d*^{x} + 1-MCP	*35S:D*	*35S:D* + 1-MCP
Mitochondria AA synthesis capacity (nmol mg^{-1} prot)	1.97±0.16**a**	2.11±0.34**ab**	1.74±0.17**a**	3.67±0.56**b**	2.80±0.26**b**	1.67±0.58**a**
L-GalLDH content (AU mg^{-1} prot)	6.0±0.6**a**	5.5±0.2**ab**	4.2±0.3**b**	4.7±0.5**ab**	5.2±0.7**ab**	4.6±0.6**ab**

Data were obtained from at least 4 independent experiments. Results with the same letter are not statistically different (ANOVA, $P \leq 0.05$).

Table 4

Effects of 1-MCP on photosynthetic electron transport rate (ETR) and respiration in dwarf BRs-deficient mutant (d^x), BRs-overaccumulating (*35S:D*) and wt tomato leaves.

	Wt	Wt + 1-MCP	d^x	d^x + 1-MCP	*35S:D*	*35S:D* + 1-MCP
ETR (μmol ē m^{-2} s^{-1})	65.5±3.1**a**	65.2±1.7**a**	67.1±1.6**a**	65.6±2.9**a**	62.6±1.4**a**	61.1±1.6**a**
Respiration (nmol O_2 g^{-1} FW min^{-1})	99.57±6.52**a**	66.63±12.71**b**	68.05±15.3**b**	40.16±10.83**c**	98.14±17.95**a**	127.92±10.51**d**

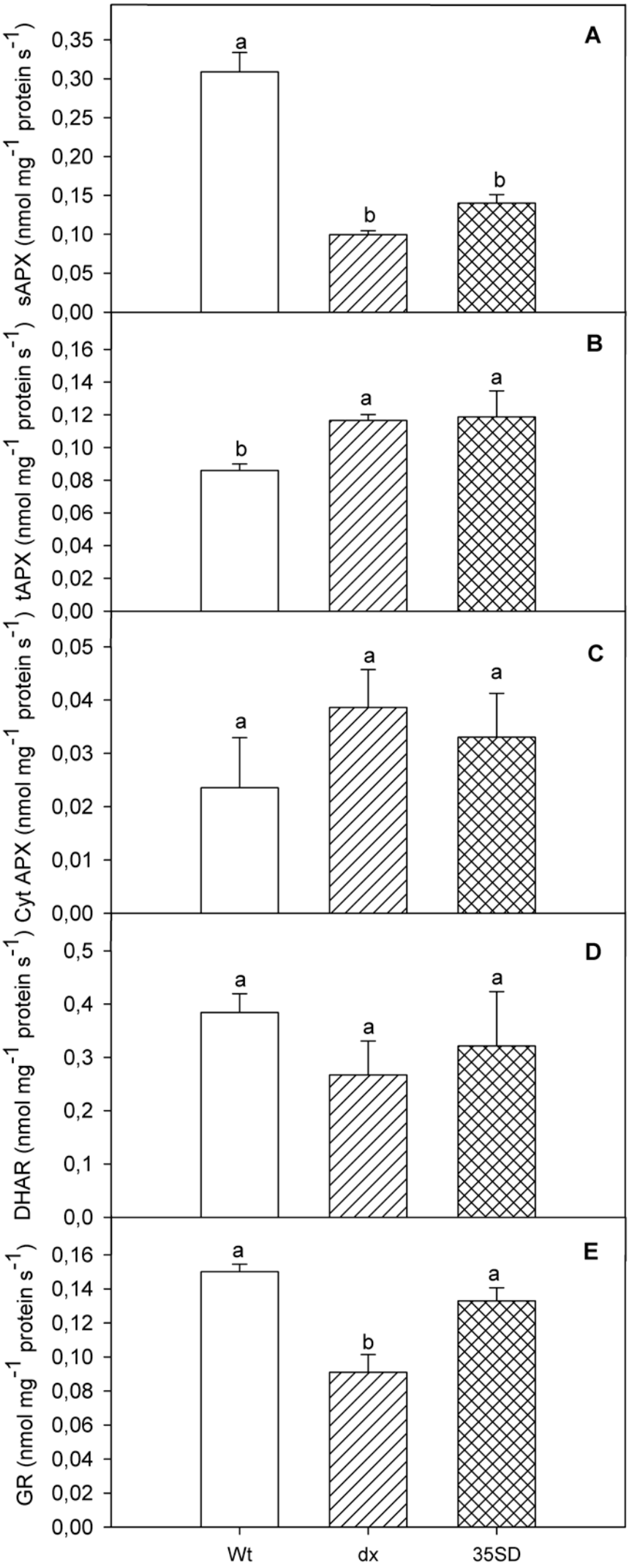
Data were obtained from at least 4 independent experiments. Results with the same letter are not statistically different (ANOVA, $P \leq 0.05$).

Table 5

Effect of exogenous application of 1 µM EBL on the content of total AA, *in vivo* L-GalLDH activity and respiration in wt and d^x leaves previously treated with 1-MCP. Leaves were incubated in 2 mM L-GalL under light for 3 h and then AA was measured for the quantification of *in vivo* L-GalLDH activity.

	$Wt_{(1\text{-}MCP)}$	$d^x{}_{(1\text{-}MCP)}$	$Wt_{(1\text{-}MCP)}$	$d^x{}_{(1\text{-}MCP)}$
	- EBL		+ EBL	
Total AA ($\mu mol.g^{-1}$ FW)	0.94±0.24**ab**	0.86±0.06**b**	1.14±0.14**ab**	1.42±0.05**c**
In vivo L-GalLDH activity ($\mu mol.g^{-1}$ FW h^{-1})	0.75±0.15**a**	0.90±0.04**a**	0.53±0.06**a**	1.13±0.02**b**
Respiration (nmol O_2 g^{-1} FW min^{-1})	53.87±1.97**a**	45.46±1.38**b**	53.91±3.1**a**	53.31±0.8**a**

Data were obtained from at least 4 independent experiments. Values with different letters indicate statistical differences among treatments (ANOVA, $P \leq 0.05$).


A
sAPX (nmol mg-1 protein s-1)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
a
b
b
B
tAPX (nmol mg-1 protein s-1)
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
b
a
a
C
Cyt APX (nmol mg-1 protein s-1)
0,05
0,04
0,03
0,02
0,01
0,00
a
a
a
D
DHAR (nmol mg-1 protein s-1)
0,5
0,4
0,3
0,2
0,1
0,0
a
a
a
E
GR (nmol mg-1 protein s-1)
0,16
0,14
0,12
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0,08
0,06
0,04
0,02
0,00
a
b
a
Wt
dx
35SD

Figure 1

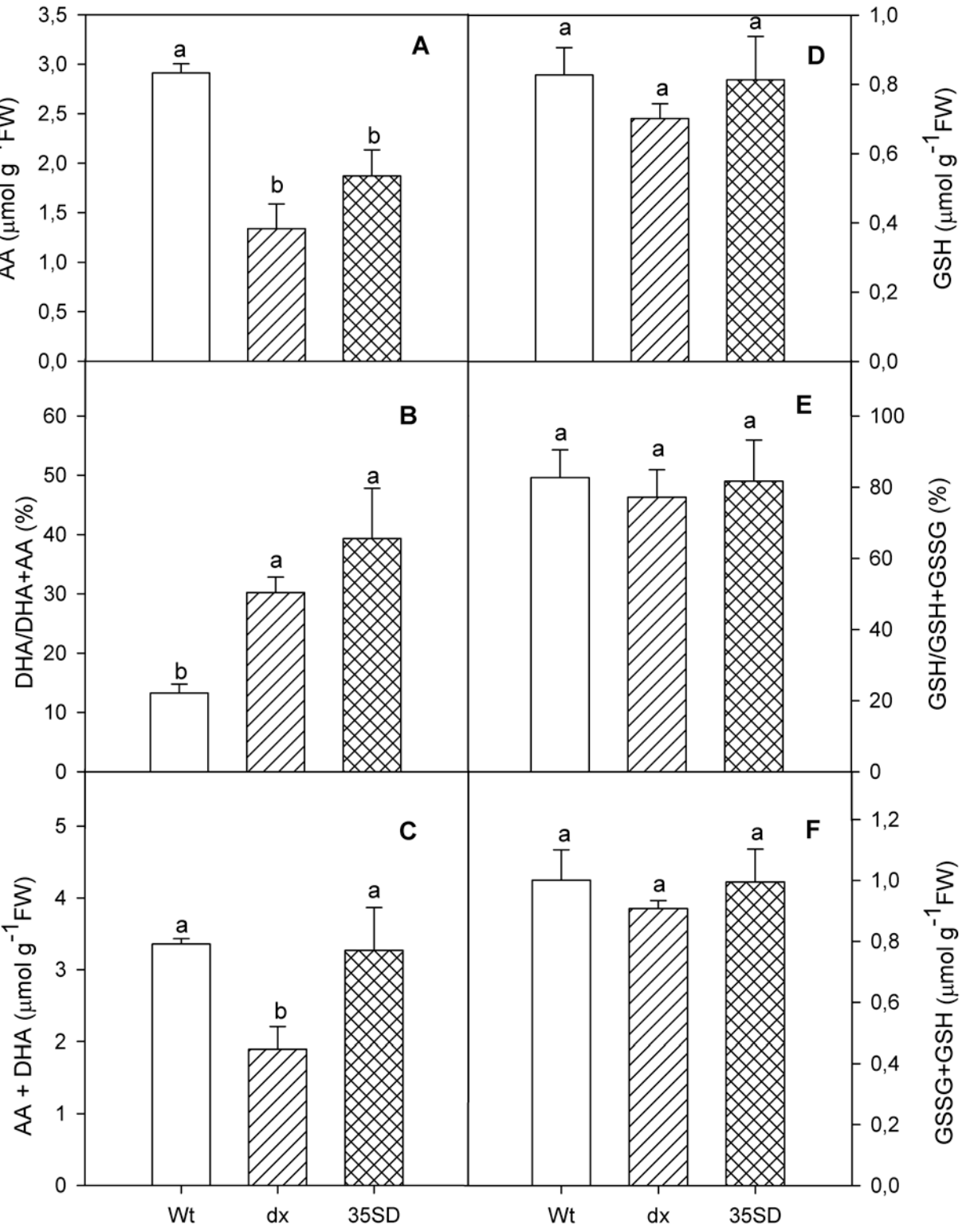

A
B
C
D
E
F
AA (µmol g-1FW)
DHA/DHA+AA (%)
AA + DHA (µmol g-1FW)
GSH (µmol g-1FW)
GSH/GSH+GSSG (%)
GSSG+GSH (µmol g-1FW)
Wt
dx
35SD

Figure 2

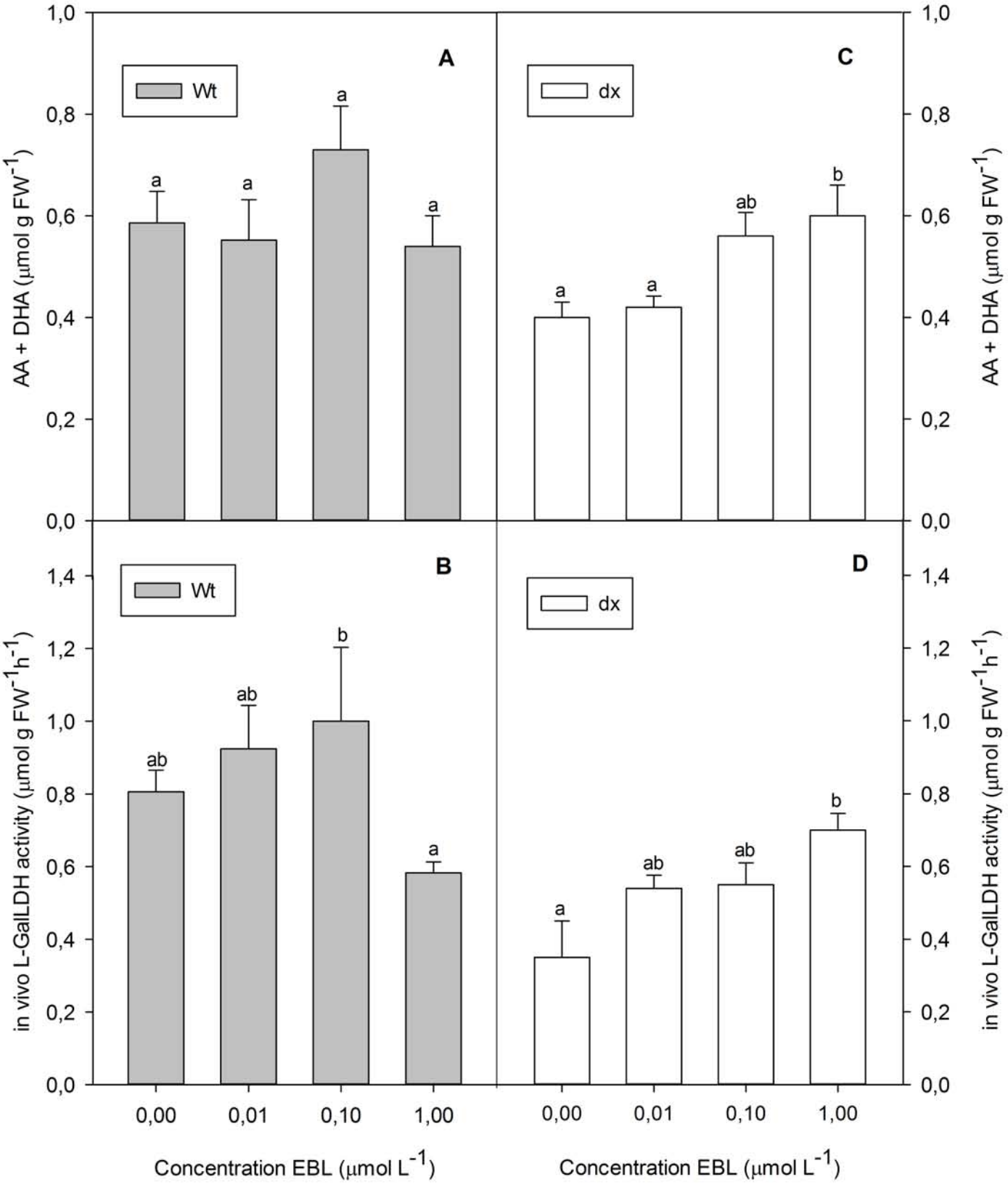

A
B
C
D
Wt
dx
AA + DHA (µmol g FW-1)
in vivo L-GalLDH activity (µmol g FW-1h-1)
1,0
0,8
0,6
0,4
0,2
0,0
1,4
1,2
a
ab
b
0,00
0,01
0,10
1,00
Concentration EBL (µmol L-1)

Figure 3

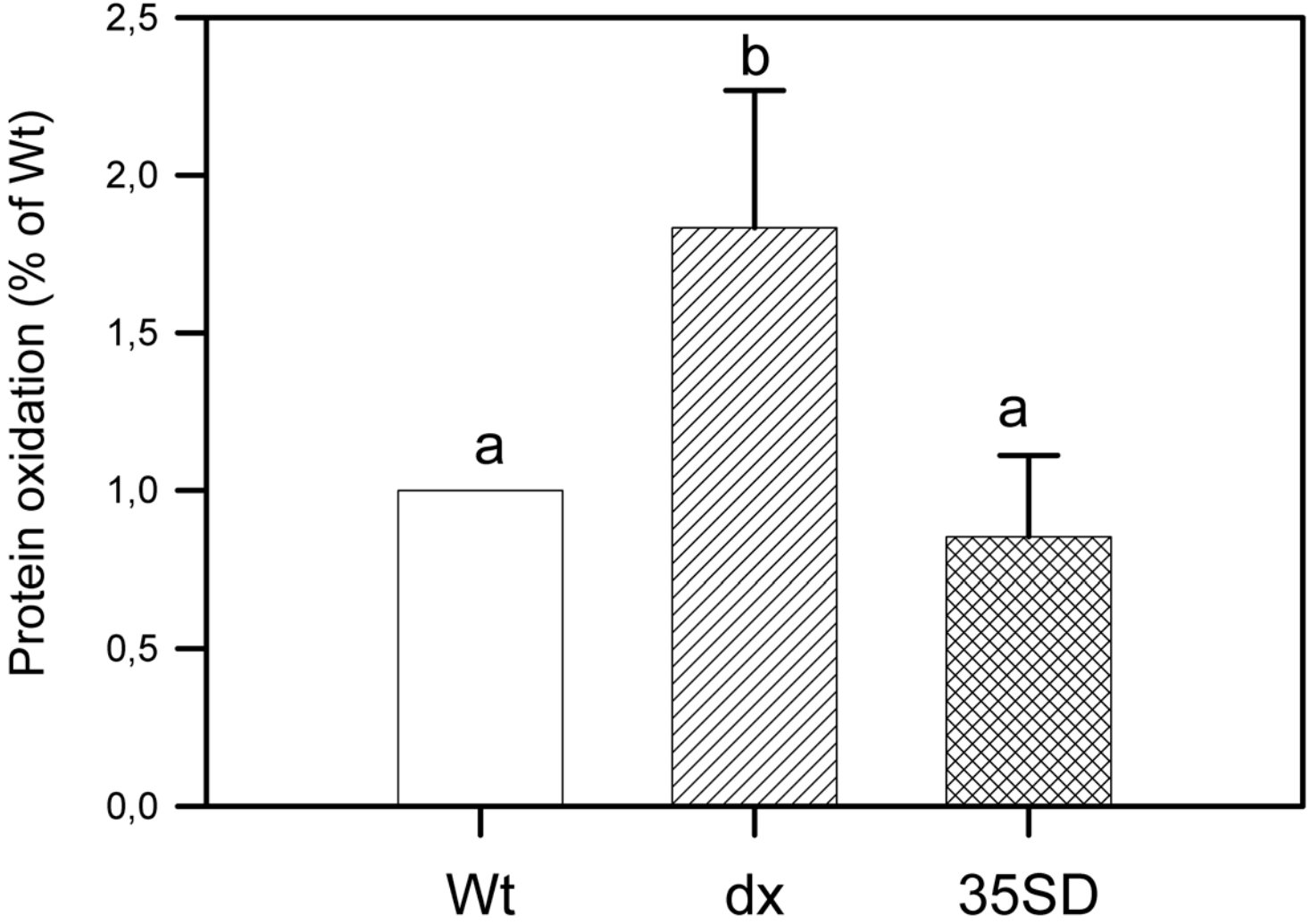
Highlights

Brassinosteroids (BRs) promote AA accumulation in tomato leaves.

BRs control L-GalLDH activity affecting AA formation.

BRs deficiency leads to increase ethylene production.

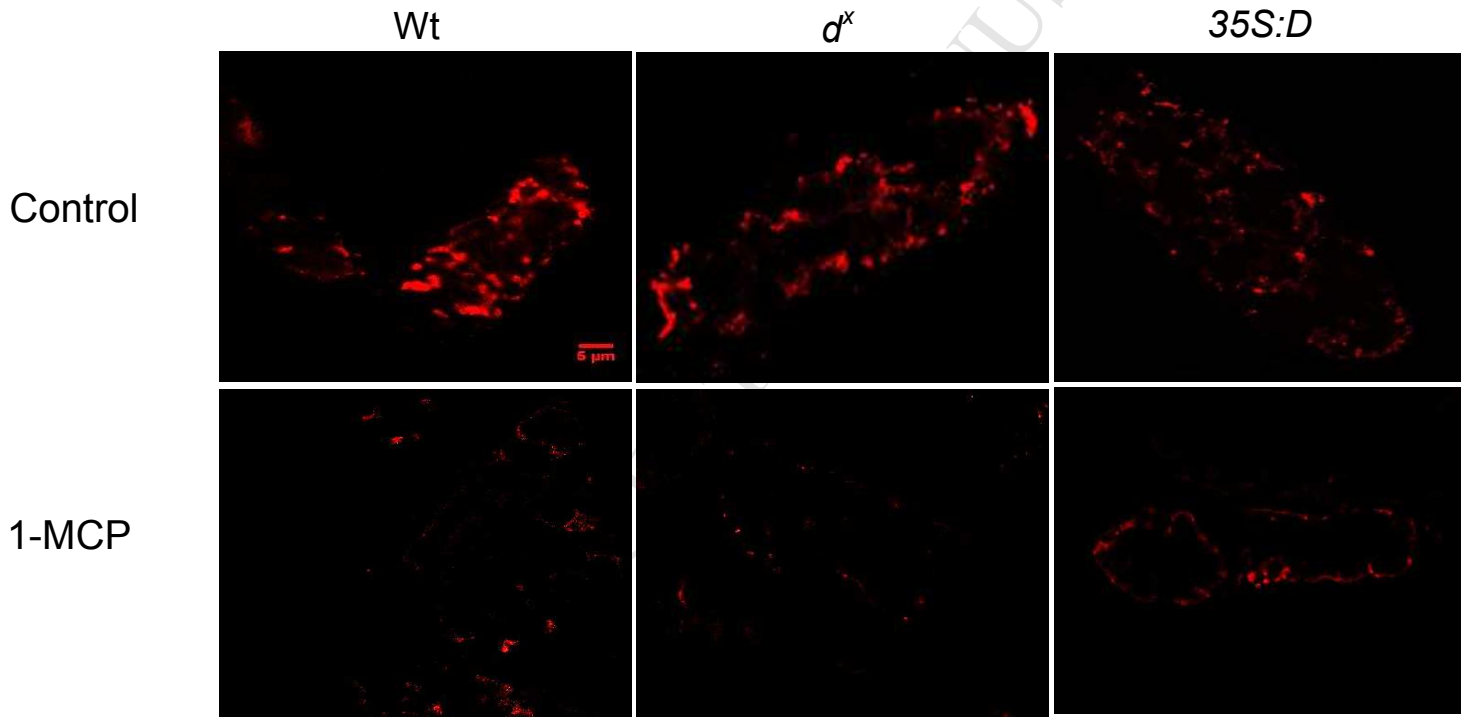
Ethylene shows an antagonistic BR response decreasing AA content and synthesis.

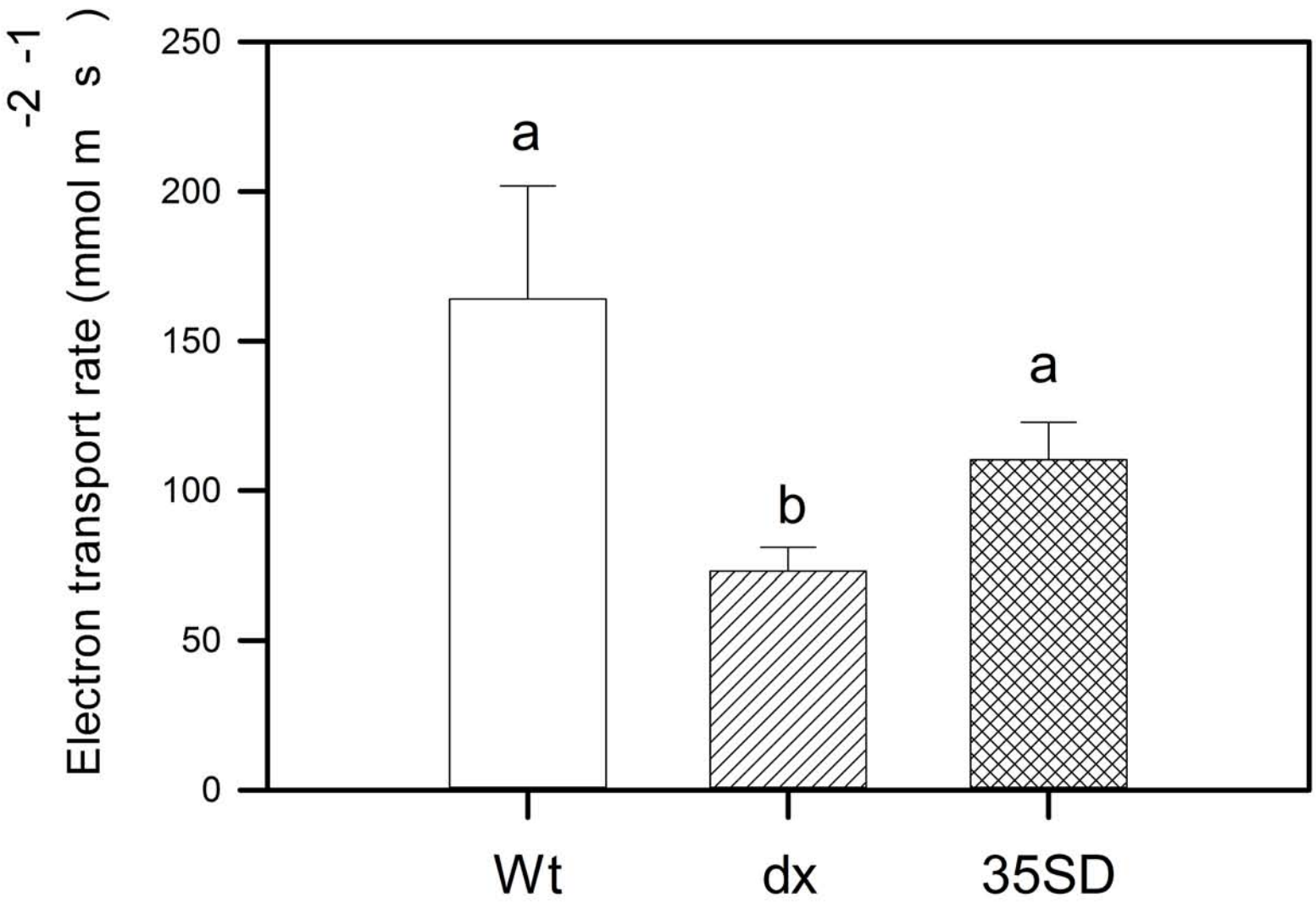


Supplementary Figure S1 Protein oxidative damage in the first expanded leaf from the apex of two month-old tomato plants. Four independent experiments using three leaves of different plants were analyzed. WT= Wild Type (Ailsa Craig), d^x = BR biosynthesis mutant *extreme dwarf*, *35S:D* = overexpression line of the tomato *Dwarf* gene. Letters indicate statistically homogenous groups, n=4 (ANOVA P=0.05).

Supplementary Figure S2.

Detection of active mitochondria in isolated cells obtained from tomato leaves with normal (Wt), deficient (d^x) or over-accumulating (*35S:D*) levels of brassinosteroids treated or not with 1-methylcyclopropene (1-MCP, an inhibitor of ethylene action). Active mitochondria were detected with MitoTracker Red® (543/585-615 nm) shown as pseudo-colored confocal images. Scale barr: 5 μm.





Supplementary Figure S3 Photosynthetic electron transport rate (ETR) in the first expanded leaf from the apex of two month-old tomato plants. Four independent experiments using three leaves of different plants were analyzed. WT= Wild Type (Ailsa Craig), d^x = BR biosynthesis mutant *extreme dwarf*, *35S:D* = overexpression line of the tomato *Dwarf* gene.

Supplementary Table S1.

Effect of spraying 100 µM ACC on total AA and synthesis capacity (measured as *in vivo* L-GalLDH activity) of wt leaves treated or not with 1 µL L^{-1} 1-MCP over 12 hours. Treated and untreated plants were immediately sprayed with the ET precursor (ACC) and evaluations were done 24 hours after spraying. AA synthesis capacity was measured in leaves incubated in 2 mM L-GalL under light for 3 h and then AA content measured.

Treatments	Total AA (µmol.g^{-1} FW)	*in vivo* L-GalLDH activity (µmol.g^{-1} FW)
Wt + Control	0.82±0.061**a**	0.82±0.004**a**
Wt + ACC	0.57±0.04**b**	0.54±0.001**b**
Wt + 1-MCP	0.83±0.11**a**	0.73±0.014**a**
Wt + 1-MCP + ACC	0.95±0.07**a**	0.88±0.002**a**

Data were obtained from at least 4 independent experiments. Results with the same letter are not statistically different (ANOVA, $P \leq 0.05$).