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

Luis Miguel Mazorra, María Eugenia Senn, Gustavo Gergoff Grozeff, Diego Darío Fanello, Cristian Antonio Carrión, Miriam Núñez, Gerard J. Bishop, Carlos Guillermo Bartoli

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

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4 Luis Miguel Mazorra^{¥£}, María Eugenia Senn[¥], Gustavo Gergoff Grozoff[¥], Diego
5 Darío Fanello[¥], Cristian Antonio Carrión[¥], Miriam Núñez[£], Gerard J. Bishop^{Φ&},
6 Carlos Guillermo Bartoli[¥].

7

8 [£] National Institute of Agricultural Sciences. Carretera a Tapaste Km 3½. Habana.
9 Cuba

10

11 [¥] Plant Physiology Institute (INFIVE), Schools of Agronomy and Natural
12 Sciences, National University of La Plata, CCT-CONICET La Plata
13 cc 327 (1900), La Plata, Argentina.

14

15 ^Φ Division of Biology, Department of Life Sciences, Imperial College London,
16 South Kensington Campus, London SW7 2AZ, UK.

17

18 [&] Current address East Malling Research, East Malling, Kent, ME19 6BJ, UK.

19

20 **Corresponding author at:** Plant Physiology Institute (INFIVE),
21 National University of La Plata, CCT-CONICET La
22 Plata.

23 cc 327 (1900) La Plata, Argentina

24 Fax: 54-2214233698

25 E-mail address: carlos.bartoli@agro.unlp.edu.ar

26

1 Abstract

2

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

1 **Keywords**

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

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1 **1. Introduction**

2 Plants are sessile organisms and consequently, they need to be able to rapidly
3 respond to changing environmental conditions especially those that cause
4 oxidative damage. Ascorbic acid (AA) and glutathione (GSH) interact to form the
5 central antioxidant system in plant cells, called the AA-GSH cycle [1] that helps
6 prevent oxidative damage in plants. In addition, AA has several physiological
7 roles including the regulation of photosynthesis, stomata closure and cell growth
8 [2,3]. AA plays an important role in the detoxification of reactive oxygen species,
9 including reducing hydrogen peroxide to water [4]. This reaction is catalysed by
10 the AA peroxidase (APX, EC 1.11.1.11) producing monodehydroascorbate
11 radicals (MDA), which can be reduced by NAD(P)H in a reaction catalysed by
12 MDA reductase (EC 1.1.5.4) [5]. MDA radicals are able to spontaneously
13 disproportionate to dehydroascorbate (DHA) and AA [6]. DHA reduction may
14 occur via either a non-enzymatic reaction with GSH or enzymatically by DHA
15 reductase (DHAR, EC 1.8.5.1) leading to the production of AA [7]. Recycling of
16 GSH is catalysed by glutathione reductase (GR, EC 1.6.4.2) that reduces
17 glutathione disulphide (GSSG) by using NADPH [2].

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

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

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

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

9 The combined utilization of BR-related mutants and ET-related chemicals serve
10 as complementary tools to elucidate the outputs of the signalling crosstalk
11 between these hormones. The tomato *Dwarf* gene encodes a cytochrome P450
12 enzyme controlling a key step in BR biosynthesis. The null d^x mutation [20,21]
13 and the d^x complemented line over-expressing *Dwarf* [21] leads to the respective
14 lines with deficiency or accumulation of endogenous BRs. 1-methylcyclopropene
15 (1-MCP) is an ET signalling inhibitor that specifically binds to ET receptors [22].
16 Using both the BR-related mutants and 1-MCP, this work tests the hypothesis that
17 BR- and ET- signalling interact to control AA synthesis in tomato leaves.

18

19

1 **2. Results**

2

3 *2.1. Modifications of the AA-GSH cycle in leaves of BR mutants*

4

5 Key antioxidant enzyme activities and metabolite concentrations were determined in
6 leaf tissues from plants with BR-deficiency or those over-accumulating BRs. Altered
7 chloroplastic APX activities were observed in plants with modified BR levels (Fig.
8 1A-B). Stromal APX (sAPX) activity was highly reduced in d^x and $35S:D$ plants
9 (~70 % lower than in the wt) but thylakoid APX (tAPX) slightly increased in both
10 BR-modified plants. In contrast, cytosolic APX (cytAPX) and DHAR activities were
11 similar in all genotypes (Fig. 1C-D). GR activity decreased in d^x plants, whereas, it
12 was similar in $35S:D$ plants to that in wt (Fig. 1E).

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

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

27

28 *2.2. Effect of BRs on the AA content and synthesis from leaves of the d^x mutant and* 29 *wt plants.*

30

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

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

7

8 2.3. ET production and the control of AA accumulation and synthesis in BR mutant 9 leaves

10

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

17 To verify that ET action is involved in these changes in AA accumulation, the ET
18 signaling inhibitor 1-MCP was used at a concentration 1.0 $\mu\text{L L}^{-1}$. Leaves of d^x
19 mutant plants treated with the ET inhibitor showed increases of 70 % and 114 %, in
20 total AA content and *in vivo* L-GaLLDH activity, respectively. Such increases reached
21 similar AA values to those observed in the wt leaves (Table 2). On the contrary, the
22 capacity of AA synthesis in *35S:D* leaves (Approximately 60 % higher than in wt
23 leaves) was decreased by 1-MCP treatment to a similar wt value (Table 2). The
24 application of 1-MCP to d^x and *35S:D* plants did not produce any major visible
25 alterations in leaf morphology. Treatment with 100 μM ACC (Aminocyclopropane-
26 1-carboxylic acid, an ET biosynthesis precursor) significantly reduced both the leaf
27 AA content and the *in vivo* L-GaLLDH activity in leaves of wt plants. However, ACC
28 application on 1-MCP treated wt plants did not show a response (Supplementary
29 Table S1), indicating that 1-MCP effectively blocks ET-mediated AA response in
30 tomato leaves.

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

1 amount of L-GalLDH in mitochondrial extracts was only different between wt and d^x
2 without 1-MCP and was unaltered among the rest of the treatments (Table 3). Thus
3 this indicates a lack of correlation between L-GalLDH level and AA synthesis
4 capacity as previously observed [24].
5 Since the amount of L-GalLDH did not explain the modified AA synthesis, it was
6 thought that other processes such as the photosynthetic and respiratory electron
7 transport rates could have an involvement. No differences were observed in
8 photosynthesis measured as ETR, for any genotype with or without 1-MCP treatment
9 (Table 4). Respiratory activity was however lower in dwarf plants but similar in
10 *35S:D* compared with wt plants. Moreover, 1-MCP decreased the oxygen uptake
11 rates of wt and d^x leaves but, surprisingly, increased respiration of the BR-over-
12 accumulating plants (Table 4), showing opposite effects as those seen for L-GalLDH
13 activities. A complementary assay detecting mito-traker red[®] fluorescence by
14 confocal microscopy, showed decreases on the number of active mitochondria in
15 both wt and d^x leaves due to the inhibition of ET signalling pathway (Supplementary
16 Figure S2). However, mitotracker fluorescence emission was not modified in 1-MCP
17 treated *35S:D* leaves suggesting that their increment in the oxygen uptake rate is not
18 due to changes in the amount of active mitochondria (Supplementary Figure S2).
19 Modifications in mitochondria activity may be also linked to alterations in the
20 respiratory substrate flux.

21

22 *2.4. Effects of BRs on AA content of leaves with suppression of ET action.*

23

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

27 Total AA content and *in vivo* L-GalLDH activity were not affected by EBL
28 applications in 1-MCP pre-treated wt plants (Table 5). However, 1-MCP pre-treated
29 d^x plants showed a marked increase of AA content and synthesis when treated with
30 1.0 μ M EBL. Interestingly, EBL treatment increased foliar respiration in 1-MCP pre-
31 treated d^x plants but resulted ineffective in wt plants (Table 5).

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

1 3. Discussion

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

9 10 3.1. BRs control the synthesis capacity of AA at the final biosynthetic step

11 As AA content was highly affected by BR concentration our experiments focused on
12 gaining better understanding of the regulation of AA synthesis by BRs. This analysis
13 utilized tomato leaves from wt and d^x genotypes that were supplemented with BRs.
14 EBL-dependent increments of AA level and *in vivo* L-GalLDH activity in d^x tissues
15 (Fig. 3C-D) indicate that EBL stimulates the AA synthesis pathway. In contrast, EBL
16 treatment does not affect AA content in wt leaves (Fig. 3A) suggesting that BR levels
17 are not limiting the accumulation of this antioxidant in wt plants. The EBL
18 stimulation of AA synthesis in d^x leaves might be related with the increment in the
19 AA precursor formation. Elevation of L-GalL pool in d^x leaves may be linked to the
20 BR-dependent increase of hexose levels as previously observed by Lisso [25] in
21 fruits after BR application to leaves. However, Lisso [25] did not measure hexose
22 levels in BR-treated leaves. Goetz [26] showed that BR treatment increase sucrose
23 uptake providing carbohydrates for supporting the stimulated growth of
24 *Lycopersicon peruvianum* cells. Furthermore, BR treatments increase photosynthetic
25 activity in other plant species [27]. Research has shown that the over-expression of
26 enzymes of the AA biosynthetic pathway enables a large accumulation of AA [28].
27 However, it is unknown whether BRs are implicated, directly or indirectly, in the
28 regulation of the L-GalL level in steps upstream, prior to its final conversion to AA.
29 The highly oxidized AA redox state in dwarf leaves after EBL treatments (Fig. 3)
30 indicates that the synthesis but not the recovery from oxidized forms is controlled by
31 BRs. Recycling of DHA to AA is a vital function that takes place in different plant
32 cell organelles using the reducing equivalents generated in the chloroplast and
33 mitochondria (i.e. NADP(H)). This recovery process is important for the availability
34 of AA for both quenching ROS and dissipation of an excessive energy load in

1 chloroplasts, known as water-water cycle [4]. High oxidation rates may lead to
2 increased oxidized/reduced ratio and decreased accumulation of reduced forms of
3 antioxidants [1]. The higher oxidized state found in leaves with deficient or excessive
4 BR levels suggests that AA recycling might be impaired. Conversion of oxidized AA
5 to reduced forms depends on the activity of DHAR and GR, which are in different
6 cell compartments [2,3]. Under the growth conditions used in this study GSH content
7 (Fig. 2D-F) and DHAR activity (Fig. 1D) were similar in all genotypes suggesting
8 that they are not limiting DHA reduction. On the other hand, the reduction in sAPX
9 isoenzyme activity shown in the BR-deficient mutant and in the *35S:D* leaves (Fig.
10 1A) might contribute to increased susceptibility of plants to oxidative stress [29].
11 Enhanced oxidative stress has been detected in BR-related mutants in Arabidopsis
12 [30] and tomato [31].

13

14 3.2. BRs and ET show opposite effects on the regulation of AA synthesis.

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

24 ET decreases the accumulation of AA lowering its synthesis in both Arabidopsis and
25 spinach leaves [16] (and tomato, see Supplementary Table S1). In addition defective
26 BR homeostasis leads to altered AA metabolism that it has increased sensitivity to 1-
27 MCP treatment. The opposite effect of mutant plants treated with 1-MCP (Table 2)
28 on *in vivo* L-GalLDH activity suggests that BRs antagonize ET response. These data
29 demonstrate that BR-ET signaling pathways interact antagonistically which is
30 consistent with the negative BR-ET interrelationship observed for growth responses
31 [19]. However, these results contrast with synergism reported by De Grauwe [18]
32 who found that BRs and ET synergistically interact stimulating the elongation of
33 Arabidopsis hypocotyls and that BR application has shown to induce ET production

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

3 In addition to the negative BR-ET interaction for regulating AA synthesis in mutants
4 with altered BR content, our data is also consistent with both hormones acting
5 independently of each other. The response of BR-deficient mutant to 1-MCP (Tables
6 2, 4) indicates that endogenous steroidal hormones are not essential for ET action.
7 Similarly, the response of 1-MCP treated d^x plants to BR supplementation (Table 5)
8 suggests that ET-signaling is not required for BR's effect on AA synthesis.

9

10 3.3. Association between changes in respiratory activity and AA synthesis

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

21 In contrast, changes in *in vivo* L-GalLDH activity were opposite to respiratory
22 activity in BR deficient and over-accumulating leaves when ET signalling was
23 blocked (Tables 2 and 4). 1-MCP produced inverse changes in O₂ uptake rates (i.e.
24 decreasing respiration in d^x and increasing in *35S:D* leaves). Interestingly, this
25 opposite behavior between the respiration and the *in vivo* L-GalLDH activity was
26 lacking in wt plants in response to 1-MCP. Moreover, the relationship was also not
27 observed in d^x plants pre-treated with 1-MCP and then sprayed with EBL (Table 5).

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

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

3

4 *3.4 Summary*

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

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1 **4. Materials and methods**

2

3 *4.1. Plant material and growth conditions*

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

10 Experiments analysing the metabolite levels and enzyme activities of the AA-
11 GSH cycle in BR mutant and wt leaves were performed using two month-old
12 plants grown in the glasshouse during spring. Plants received a maximum of 1200
13 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PPFD) with a temperature regime of $\sim 30/20^\circ\text{C}$ (day/night). Samples
14 were taken approximately at 5 h into the photoperiod. Analysis of BR and ET
15 participation in AA synthesis and accumulation was carried out with two week-
16 old plants grown in chambers at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ (PPFD), $25 \pm 2^\circ\text{C}$ and a 16/8 hs
17 light/dark period.

18

19 *4.2. Treatment with BRs*

20

21 Leaves of two week-old plants from genotypes d^x and wt were sprayed with 0.01,
22 0.1 and 1 μM EBL (*PhytoTechnology Laboratories*[®]). Approximately 5 mL of
23 solution was applied to each plant so leaves were completely wetted with the
24 solution. Leaves of control plants were sprayed with distilled water containing 0.5
25 % (v/v) ethanol and 0.01 % (v/v) Tween 80 that were also present in the EBL
26 solution. Two applications of EBL were carried out (day 0, day 3) and
27 measurements were taken at day 7.

28

29 *4.3. Treatment with 1-MCP*

30

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

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

2

3 *4.4. Quantification of antioxidant levels*

4

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

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

17

18 *4.5. Enzyme activity measurements*

19

20 APX activities were determined according to Miyake and Asada [37]. Leaves
21 were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing
22 0.1 mM EDTA, 0.5 mM AA and 0.1 % (w/v) phenylmethanesulfonyl fluoride and
23 then the homogenate was centrifuged at 13 000 x g for 15 min. The soluble
24 fraction containing sAPX and cytAPX and the pellet containing the tAPX were
25 used for the enzyme activity assays. The supernatant was added to N₂-bubbling
26 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM H₂O₂ in the
27 absence of AA for the chloroplastic APX inactivation. A control assay without
28 H₂O₂ was used to measure the non-inactivated reaction. The residual APX activity
29 in the reaction mixtures was then assayed by adding H₂O₂ (0.1 mM). APX activity
30 was measured spectrophotometrically following changes at 290 nm ($\epsilon = 2.8 \text{ mM}^{-1}$
31 cm^{-1}). The 13 000 x g-membrane fraction was washed with 50 mM potassium
32 phosphate buffer (pH 7.0) containing 0.5 mM AA and suspended in a similar
33 buffer including 1 % (v/v) Triton X-100 for tAPX activity.

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

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

2

3 *4.6. Oxidative damage*

4

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

9

10 *4.7. Ethylene synthesis*

11

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

19

20 *4.8. Photosynthesis and respiration*

21

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

30

31 *4.9. In vivo L-GalLDH activity and immunochemical detection*

32

33 Detached leaves were incubated in 2 mM L-GalL (precursor of AA biosynthesis)
34 or distilled water at room temperature and 100 μ mol m⁻² s⁻¹ (PPFD) for 3 h. AA

1 concentration was then measured and its accumulation was considered as leaf *in*
2 *vivo* L-GalLDH activity, an estimation of the maximum potential of *in vivo* AA
3 synthesis in leaves [9]. AA synthesis capacity of isolated mitochondria was also
4 evaluated. The isolation of mitochondria, the incubation in the presence or
5 absence L-GalL and the AA quantification was performed as previously described
6 [34].

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

10

11 *4.10. Statistical analysis*

12

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

15

1 **The following data are also available on line:**

2 Supplementary Figure S1

3 Supplementary Figure S2

4 Supplementary Figure S3

5 Supplementary Table S1

6

7

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ACCEPTED MANUSCRIPT

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1 **Legends to figures**

2

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

9 Letters indicate statistically homogenous groups, n = 4 (ANOVA $P \leq 0.05$).

10

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

17 Letters indicate statistically homogenous groups, n = 4 (ANOVA $P \leq 0.05$).

18

19 Figure 3. Effect of exogenous supplementation with EBL on total AA and *in vivo*
20 L-GalLDH activity in d^x and wt tomato leaves. The assays were performed using
21 leaves from two weeks-old tomato plants. Four independent experiments were
22 analyzed for: **A) wt total AA content; B) wt *in vivo* L-GalLDH activity; C) d^x total**
23 **AA content and D) d^x *in vivo* L-GalLDH activity.** The redox state of AA was not
24 affected by EBL treatments and kept around 13 and 27 % for wt and d^x ,
25 respectively. Wt= wild type (Ailsa Craig), EBL = 24-epibrassinolide, d^x = BR
26 biosynthesis mutant *extreme dwarf*, *35S:D* = over-expression line of the tomato
27 *Dwarf* gene. Letters indicate statistically homogenous groups, n = 4 (ANOVA
28 $P \leq 0.05$) for each graph.

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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 ($\mu\text{L g}^{-1}\text{FW h}^{-1}$)	0.24±0.08 a	0.6±0.17 b	0.27±0.07 a
Capacity of ET synthesis ($\mu\text{L g}^{-1}\text{FW h}^{-1}$)	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 ($\mu\text{mol g}^{-1}$ FW)	0.73 \pm 0.11 a	0.90 \pm 0.09 a	0.41 \pm 0.06 b	0.69 \pm 0.06 a	0.91 \pm 0.15 a	1.03 \pm 0.09 a
<i>In vivo</i> L-GalLDH activity ($\mu\text{mol g}^{-1}$ FW h $^{-1}$)	0.80 \pm 0.12 a	0.91 \pm 0.07 a	0.37 \pm 0.07 b	0.80 \pm 0.06 a	1.31 \pm 0.14 c	0.91 \pm 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 ($\mu\text{mol } \bar{e} \text{ m}^{-2} \text{ s}^{-1}$)	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 ($\text{nmol O}_2 \text{ g}^{-1} \text{ FW min}^{-1}$)	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-MCP)	d^x _(1-MCP)	Wt _(1-MCP)	d^x _(1-MCP)
	- EBL		+ EBL	
Total AA ($\mu\text{mol.g}^{-1}$ FW)	0.94 \pm 0.24 ab	0.86 \pm 0.06 b	1.14 \pm 0.14 ab	1.42 \pm 0.05 c
<i>In vivo</i> L-GalLDH activity ($\mu\text{mol.g}^{-1}$ FW h ⁻¹)	0.75 \pm 0.15 a	0.90 \pm 0.04 a	0.53 \pm 0.06 a	1.13 \pm 0.02 b
Respiration (nmol O ₂ g ⁻¹ FW min ⁻¹)	53.87 \pm 1.97 a	45.46 \pm 1.38 b	53.91 \pm 3.1 a	53.31 \pm 0.8 a

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

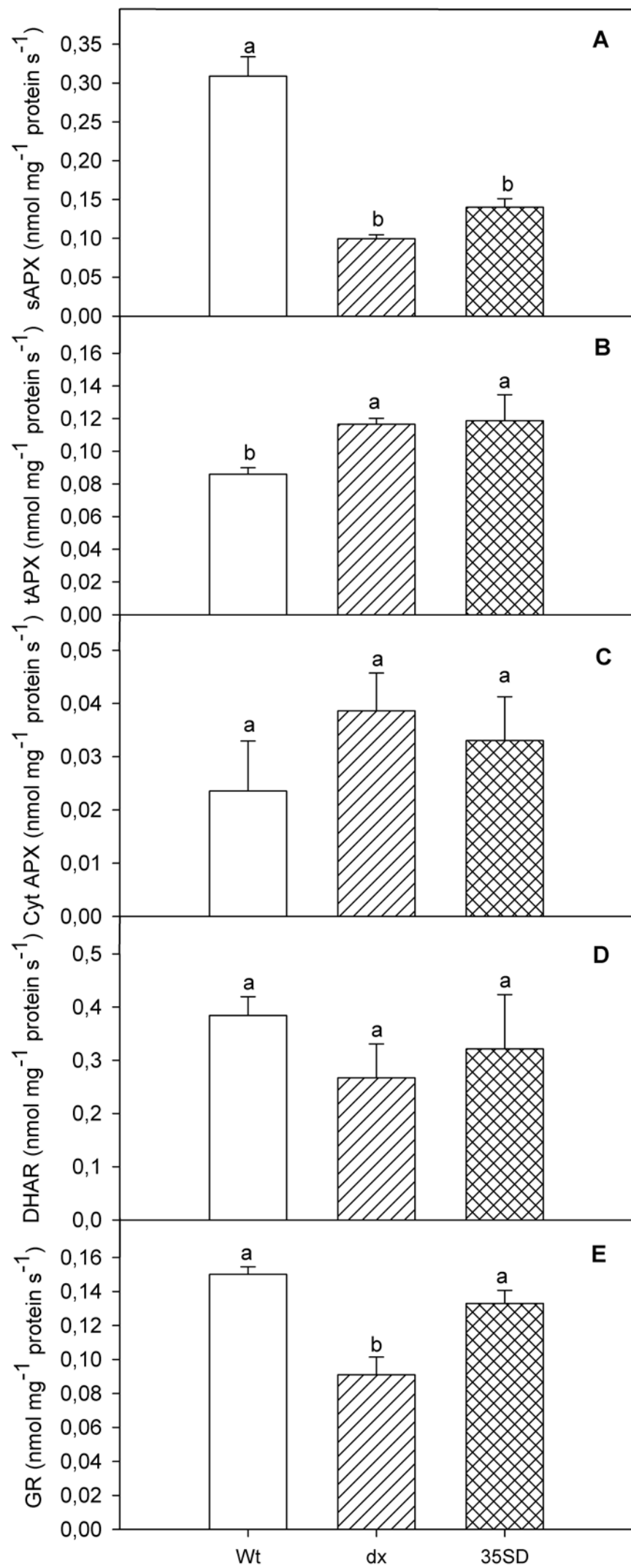


Figure 1

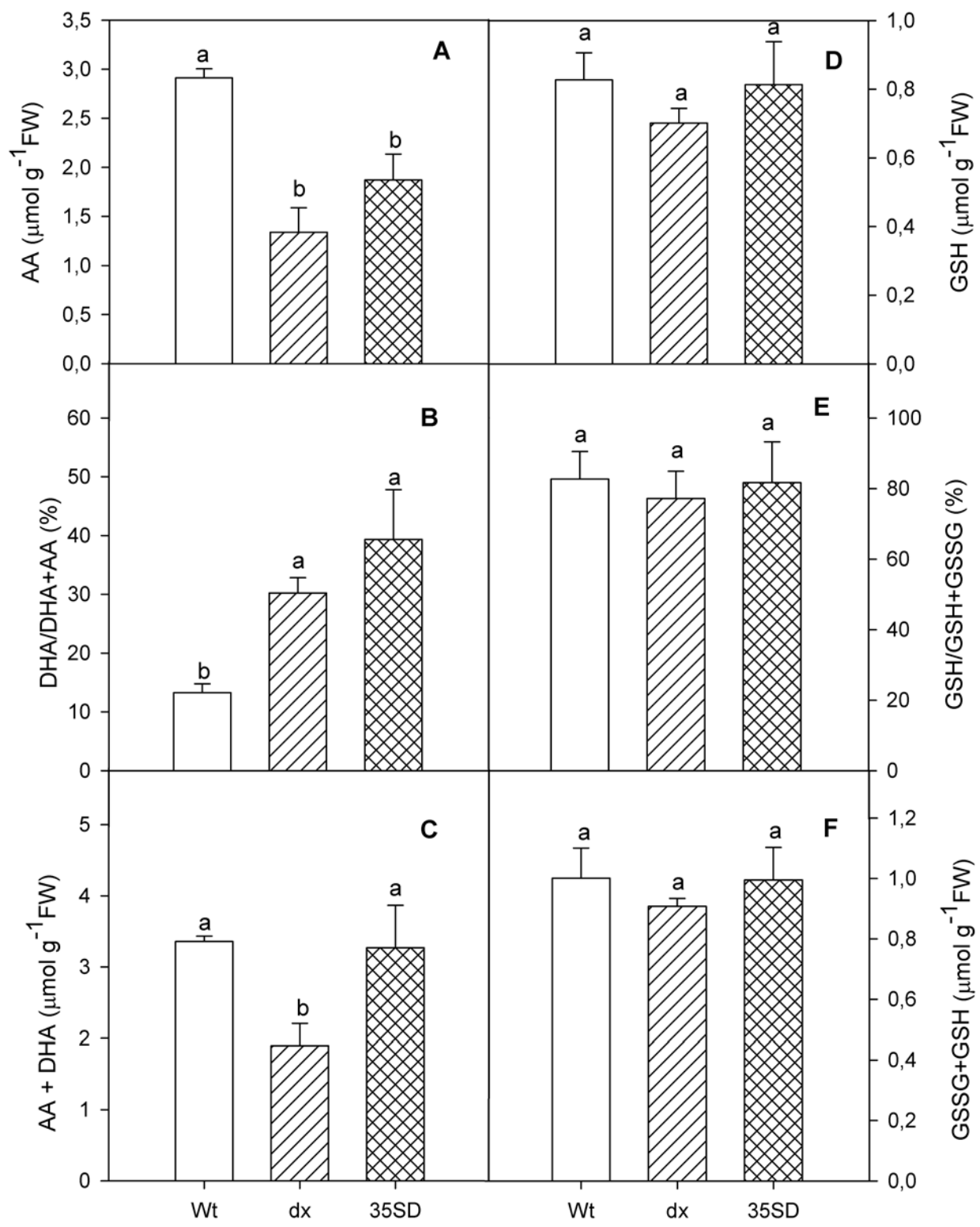


Figure 2

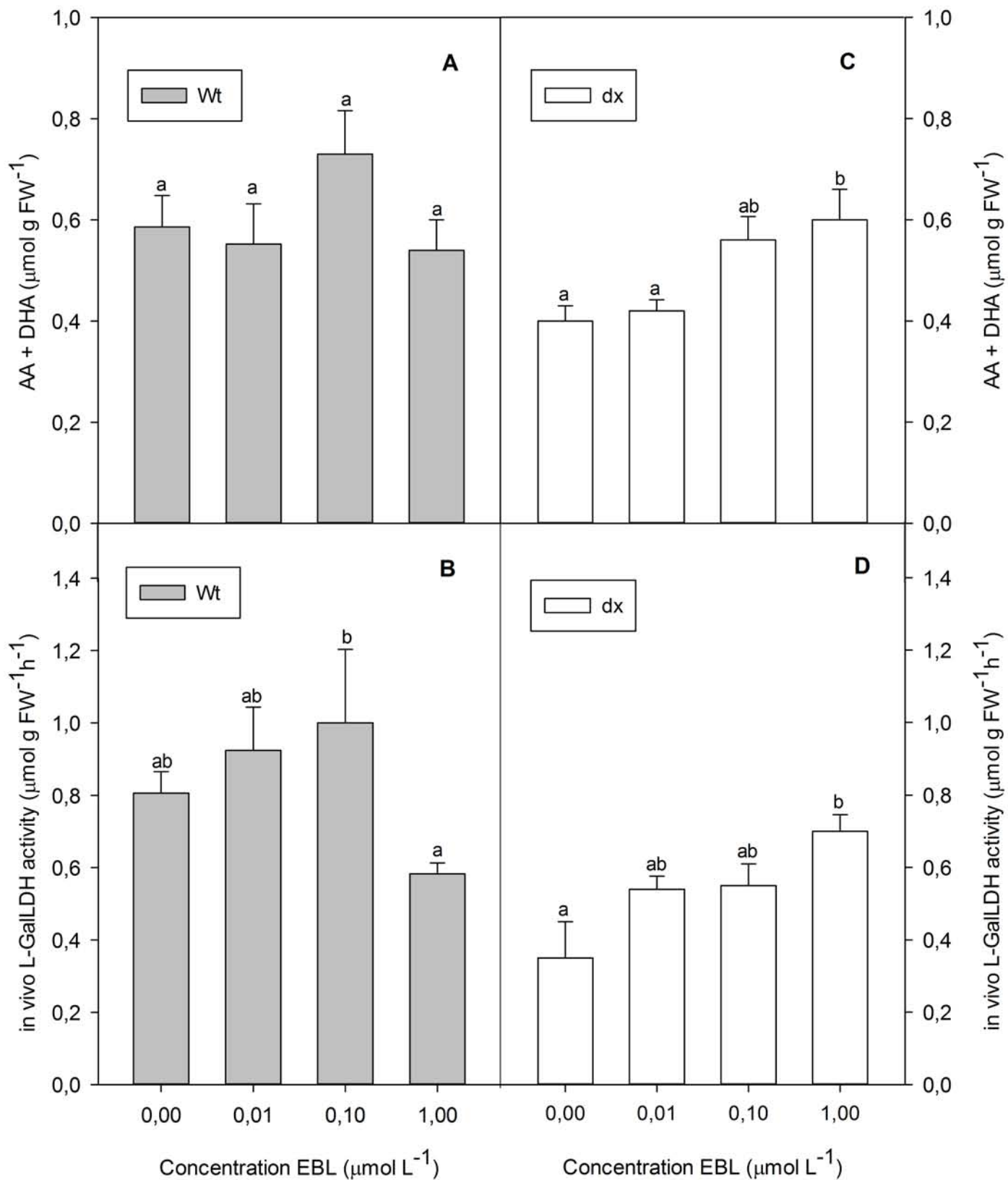


Figure 3

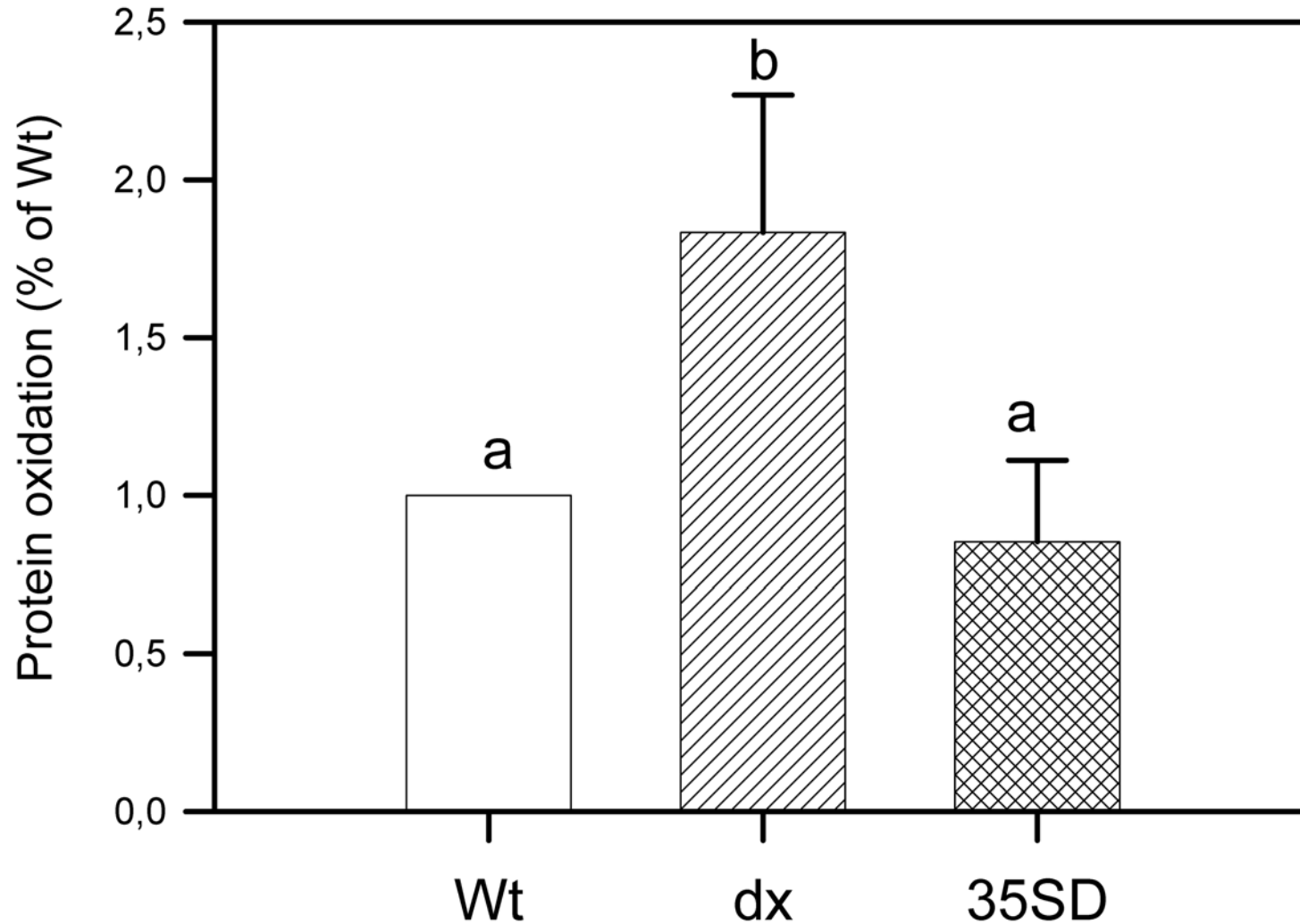
Highlights

Brassinosteroids (BRs) promote AA accumulation in tomato leaves.

BRs control L-GalLDH activity affecting AA formation.

BRs deficiency leads to increase ethylene production.

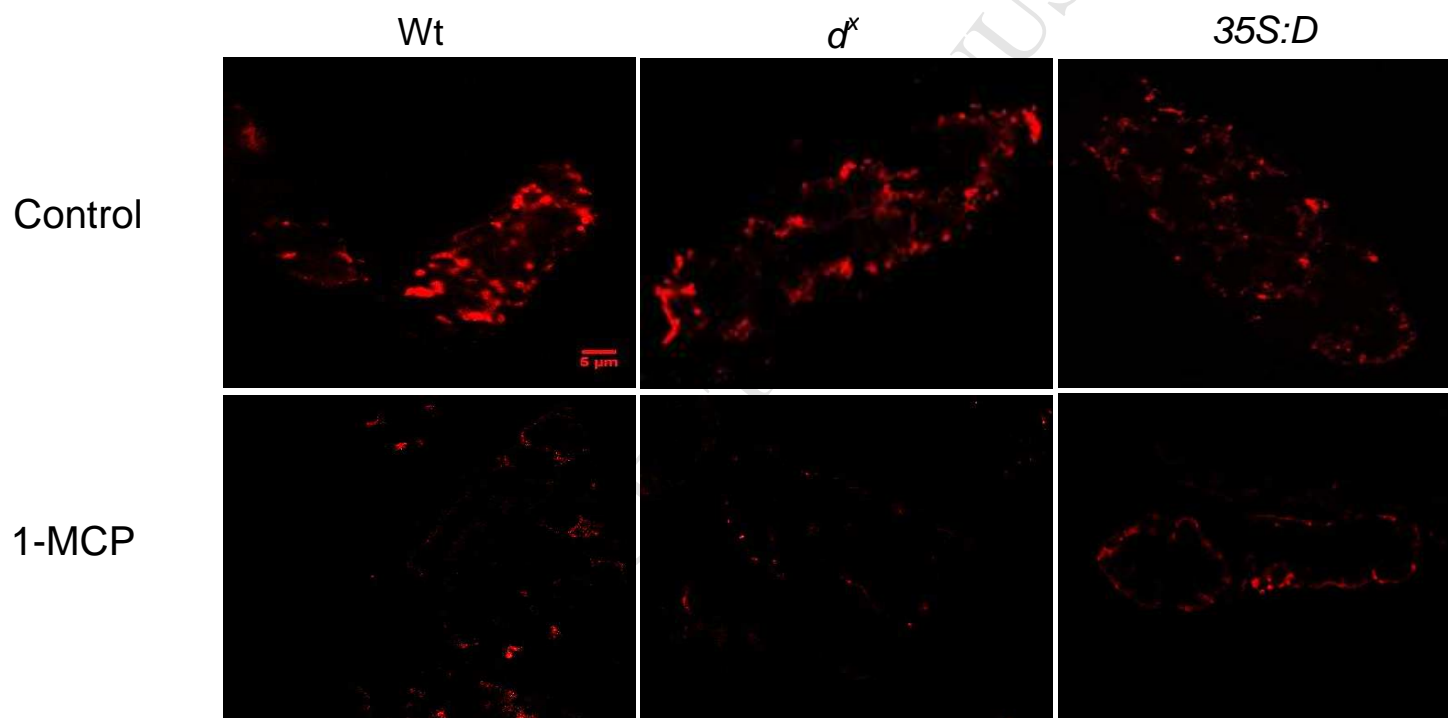
Ethylene shows an antagonistic BR response decreasing AA content and synthesis.

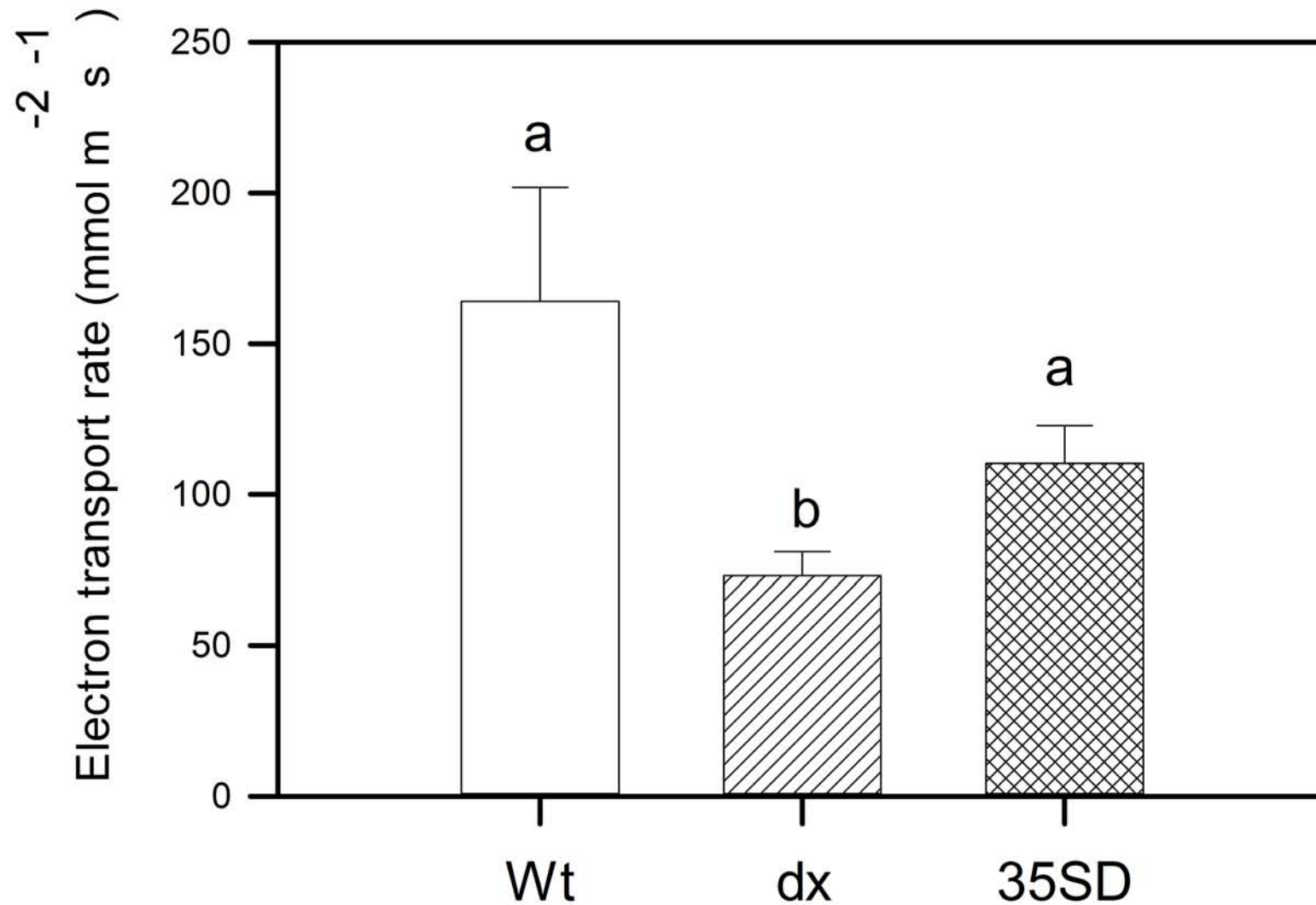


Supplementary Figure S1 Protein oxidative damage in the first expanded leaf from the apex of two month-old tomato plants. Four independent experiments using three leaves of different plants were analyzed. WT= Wild Type (Ailsa Craig), d^x = BR biosynthesis mutant *extreme dwarf*, 35S:D = overexpression 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 μ m.





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), *dx* = 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 $\mu\text{L L}^{-1}$ 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 ($\mu\text{mol.g}^{-1}$ FW)	<i>in vivo</i> L-GalLDH activity ($\mu\text{mol.g}^{-1}$ FW)
Wt + Control	0.82 \pm 0.061 a	0.82 \pm 0.004 a
Wt + ACC	0.57 \pm 0.04 b	0.54 \pm 0.001 b
Wt + 1-MCP	0.83 \pm 0.11 a	0.73 \pm 0.014 a
Wt + 1-MCP + ACC	0.95 \pm 0.07 a	0.88 \pm 0.002 a

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