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Constitutive gibberellin response in grafted tomato modulates root-to-shoot signaling under drought stress

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ABSTRACT

Plants are sessile organisms that must perceive and respond to various environmental constraints throughout their life cycle. Among these constraints, drought stress has become the main limiting factor to crop production around the world. Water deprivation is perceived primarily by the roots, which efficiently signal the shoot to trigger drought responses in order to maximize a plant's ability to survive. In this study, the tomato (*Solanum lycopersicum* L.) mutant *procera* (*pro*), with a constitutive response to gibberellin (GA), and its near isogenic line cv. Micro-Tom (MT), were used in reciprocal grafting under well-watered and water stress conditions to evaluate the role of GA signaling in root-to-shoot communication during drought stress. Growth, oxidative stress, gene expression, water relations and hormonal content were measured in order to provide insights into GA-mediated adjustments to water stress. All graft combinations with *pro* (i.e. *pro/pro*, MT/*pro* and *pro*/MT) prevented the reduction of growth under stress conditions without a reduction in oxidative stress. The increase of oxidative stress was followed by upregulation of *SlDREB2*, a drought-tolerance related gene, in all drought-stressed plants. Scions harboring the *pro* mutation tended to increase the abscisic acid (ABA) content, independent of the rootstock. Moreover, the GA sensitivity of the rootstock modulated stomatal conductance and water use efficiency under drought stress, indicating GA and ABA crosstalk in the adjustment of growth and water economy.

1. Introduction

Water stress is one of the main constraints for crop production around the world. In addition, there are predictions that this will worsen in the next years due to global warming and climate changes (Dai, 2011; Bornman et al., 2015; Trnka et al., 2015). Climate changes can strongly impact rainfall regime, which is one of the greatest limitations to crop expansion in agricultural systems (Skirycz and Inzé, 2010; Dai, 2011; Sardans and Peñuelas, 2013; Wheeler and Von Braun, 2013). Under this climatic changing context, plants would be more vulnerable to severe drought conditions (Dai, 2012). Water stress adversely affects many aspects of the physiology of plants by reducing stomatal conductance to maintain leaf water status and, consequently, result in lower leaf internal CO_2 concentrations that negatively impact photosynthesis and plant growth under stress conditions (Granier and Tardieu, 1999; Skirycz and Inzé, 2010; Tramontini et al., 2013; Ollas et al., 2015). These modifications are coordinated by an intricate network of molecular and biochemical signals (González et al., 2013; Meyer et al., 2014; Qazi et al., 2014; Sellin et al., 2014). The expression of various genes with functions in the water stress responses has been identified in many species (Guo and Wang, 2011; González et al., 2013; Blum, 2014; Ober et al., 2014). In addition, the involvement of general physiological processes associated with drought-responsive gene expression include oxidative stress molecules production (Ashraf and Foolad, 2007; Ahmed et al., 2014; Tesfaye et al., 2014) and plant hormone biosynthesis and signaling (Pospíšilová, 2003; Colebrook et al., 2014; Cui et al., 2015; Ollas and Dodd, 2016).

The plants take up water from the soil by the roots. Therefore, the

Abbreviations: *A*, CO_2 assimilation; ABA, abscisic acid; DAS, days after sowing; DW, dry weight; *E*, water transpiration; FW, fresh weight; GAs, gibberellins; GID1, GIBBERELLIN-INSENSITIVE DWARF 1; *gs*, stomatal conductance; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase; ROS, reactive oxygen species; RWC, relative water content; TW, turgid weight; WUE_i, intrinsic water use efficiency

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reduction in water availability in the soil is readily sensed by plant roots to respond to local moisture (Holbrook et al., 2002; Tramontini et al., 2013; Martorell et al., 2015). Thus, in order to limit water loss during soil drying, plants can control the stomata aperture to reduce water transpiration even before the water status declines in the root and shoot (Gollan et al., 1986; Stoll et al., 2000; Augé and Moore, 2002; Holbrook et al., 2002; Osakabe et al., 2014) which implicates root-to-shoot communication to modulate shoot response to drought. This suggests the existence of a biochemical signal from the roots that triggers adaptive mechanisms in the shoot. There is compelling evidence that the abscisic acid (ABA) plays an important role for long-distance signaling considering that the levels of this water-stress associated hormone increases in both xylem sap and leaves controlling stomata closure under stress (Jacobsen et al., 2009; Carvalho et al., 2011; Vijayalakshmi et al., 2014; Ollas and Dodd, 2016). However, several experiments have demonstrated that, under drought conditions, the stomata close can occur independently of the ABA biosynthesis by the roots (Stoll et al., 2000; Augé and Moore, 2002; Holbrook et al., 2002). For instance, grafting experiments with tomato (*Solanum lycopersicum* L.) mutants with reduced ABA biosynthesis (*flacca* and *sitiens*) revealed that stomatal closure occurred independently of ABA production by the roots, but rather ABA biosynthesis in the leaves represents a key signal for stomatal behavior (Holbrook et al., 2002). Thus, the nature of the root-derived systemic signal induced by water stress has remained elusive. Recent investigation indicates a crosstalk mechanism between ABA and gibberellins (GAs) during water-limited conditions, in which ABA biosynthesis and the control of stomatal conductance were regulated by the soluble receptor for GA, GIBBERELLIN-INSENSITIVE DWARF 1 (GID1) under water stress (Du et al., 2015). The *gid1* rice (*Oryza sativa* L.) mutant, which impairs GA signaling, showed reduced levels of ABA and increased stomatal conductance in comparison to wild-type plants under drought stress (Du et al., 2015).

The phytohormone GA is involved in the adaptive response to various abiotic stresses such as cold, salinity, heat, flooding and drought (Achard et al., 2008; Colebrook et al., 2014; Khan et al., 2015). However, the role of GAs during drought stress adaptation is still unclear. Reduction of GAs levels in maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and ramie [*Boehmeria nivea* (L.) Gaud] have been described during drought conditions (Wang et al., 2008; Coelho Filho et al., 2013; Liu et al., 2013a, 2013b). Moreover, a GA application could recover plant growth under stress conditions, providing greater growth and maintenance of photosynthesis, as well as oxidative stress reduction (Kaya et al., 2006; Akter et al., 2014). On the other hand, there is a range of studies demonstrating that reduced sensitivity to GAs may induce a greater tolerance to water stress. For instance, wheat *Rht8*, *Rht-1b* and *Rht-D1b* mutants, with reduced GA sensitivity, were more tolerant to drought stress compared to the wild-type (Landjeva et al., 2008; Alghabari et al., 2014; Alghabari et al., 2016). Likewise, plants with reduced levels of active GAs, such as the mutants of *Arabidopsis thaliana* (*ga20ox1/2* and *ga3ox1/2*) and the transgenic tomato overexpressing *AtGAMT1*, a gene from Arabidopsis that encodes an enzyme that induces GA deactivation, induce greater tolerance to water-limiting conditions (Colebrook et al., 2014; Nir et al., 2014). However, the involvement of GAs signaling in root-to-shoot communication to coordinate growth and development at the whole-plant level in response to drought stress is largely unexplored.

Furthermore, the recent discovery of GA_{12} transported by vascular bundles (Regnault et al., 2015) allows us to raise the following questions: i) Do GAs act in the perception of water deprivation by the roots? ii) Are GAs the biochemical signal transported to long-distance from the roots to the shoot controlling drought stress responses? iii) If so, is the role of GAs during drought stress negative or positive? To provide insights into these questions, we used the tomato mutant *procera* (*pro*), which has a constitutive response to GA (Carrera et al., 2012), and its near isogenic line cv. Micro-Tom (MT) in reciprocal grafting under well-watered and water stress conditions.

2. Material and Methods

2.1. Plant material and grafting

Seeds of tomato (*Solanum lycopersicum* L.) mutant *procera* (*pro*), which exhibits constitutive GA response due to a point mutation in the gene encoding DELLA protein (Bassel et al., 2008), and a near isogenic line cv. Micro-Tom (MT) were germinated in boxes containing a mixture of 1:1 (v/v) commercial pot mix (BioPlant, Brazil) and vermiculite. Fifteen days after sowing (DAS), the plants were transferred to pots filled with the same sowing mixture, and grafting was performed by splice method combining MT and *pro* in reciprocal grafting (MT/MT, *pro/pro*, MT/*pro*, *pro*/MT; the first genotype indicates the scion, and the second genotype indicates the rootstock). Immediately, the grafted plants were placed in a floating moist chamber and were kept until complete healing of the grafting region (*c.* 15 days), and then were transferred to a glasshouse.

2.2. Water stress conditions

All plants were watered daily until the beginning of water stress. To establish the stress treatment, irrigation was suspended for a seven-day period in the grafted plants (37 DAS). As a control, plants were daily watered by maintaining water availability close to the capacity of the potting mix. After seven days under the respective growth conditions (well-watered and drought stress), plants (45 DAS) were taken for analysis as described below.

2.3. Growth analysis

Plant height was obtained using a graduated ruler. The leaf area was measured using an Image Analysis System (Delta-T Devices, Cambridge, UK), whereas the root area was measured using a Hewlett Packard 125C scanner; the image of each plant was analyzed by Delta-T Scan software. Subsequently, the weights of both the roots and shoot fresh mass were recorded. Afterwards, they were oven-dried at 60 °C for 72 h, and the dry weight was determined using an analytical balance (Denver Instrument Company AA-200).

2.4. Chlorophylls and carotenoids contents

The pigments were extracted from the third fully expanded leaf as described by Alves et al. (2017) and were determined spectrophotometrically at 661.6 nm (Chlorophyll *a*), 644.8 nm (Chlorophyll *b*) and 470 nm (Carotenoids), and the concentration of each pigment was estimated by the equations of Lichtenthaler (1987).

2.5. Lipid peroxidation and H_2O_2 content

Lipid peroxidation was estimated by the content of thiobarbituric acid reactive substances (TBARS). Malondialdehyde (MDA) was estimated by measurements at 535 and 600 nm, and the concentration was calculated using an extinction coefficient of 1.55×10^{-5} mol^{-1} cm^{-1} (Gratão et al., 2012). MDA content was expressed in nmol g^{-1} fresh weight.

The content of hydrogen peroxide (H_2O_2) was determined by a reaction with potassium iodide, as described by Alexieva et al. (2001). The absorbance was read at 560 nm, and the H_2O_2 content for all samples was determined using a known H_2O_2 concentration curve as a standard. H_2O_2 content was expressed in mol g^{-1} fresh weight (Alves et al., 2017).

2.6. Peroxidase activity (POD EC 1.11.1.7)

Approximately 500 mg of plant tissue were macerated in the presence of liquid nitrogen and homogenized with 50 mM potassium

phosphate buffer (pH 6.7) containing 2 mM ethylenediaminetetraacetic, 5 mM 2-mercaptoethanol and 20% polyvinylpolypyrrolidone. Then, the homogenate was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant obtained from each sample was collected to determine enzymatic activity. The activity of POD was determined according to Lima et al. (1999). The reaction system was composed of 30 μL of enzymatic extract, 50 mmol L^{-1} potassium phosphate buffer at pH 6.5, 20 mmol L^{-1} 7-1 pyrogallol at pH 7.8, and 5 mmol L^{-1} H_2O_2, totalizing a volume of 1.0 mL. The reaction was carried out at 30 °C for 5 min and quenched by adding 2 mL of absolute ethyl alcohol. The purpurogallin formation was measured in a spectrophotometer at 505 nm, and its molar extinction coefficient (2.5 mmol L^{-1} cm^{-1}) was used to calculate the specific activity of the enzyme, which was expressed in μmol of H_2O_2 min^{-1}.

2.7. RNA extraction, cDNA synthesis and quantitative RT-qPCR

Total RNA was extracted from leaves of grafted plants using the Trizol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. RNA integrity was evaluated by the 260/280 and 260/230 ratios. RNA samples from three independent biological replicates were used for each comparison. For each sample, the total RNA (1 μg) were treated with DNase I (Life Technologies) to remove DNA contamination according to the manufacturer's protocol and 20 U of Ribolock (Sinapse Biotechnology) was added prior cDNA synthesis. cDNA was synthesized using SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. RT-qPCR reactions were performed with FastStart SYBR Green Master (Roche), cDNA (v/v 2:10) and 0.3 μM of each gene-specific primers (Appendix S1). Three-step PCR cycles in the RotorGene-6000 (Qiagen) were used to determine the quantitative PCR analysis. The reference gene, *SolyACTN* (gene bank: BT013524) was used as internal controls for normalizing gene expression and two technical replicates per samples were done. After amplification, melting curves were determined between 72 °C and 95 °C.

2.8. Leaf temperature and water potential

Leaf temperature was recorded using an infrared thermometer (Fluke 59 Max®) on the terminal leaflet of the third fully expanded leaf at 1:00 P.M. The leaf water potential (Ψ_{leaf}) was determined in the morning (7:00–9:00 A.M.) using the third fully expanded leaf with a pressure chamber (Model m670) (Pms Instrument Co., Albany, USA) (Scholander et al., 1964).

2.9. Stomatal measurements

The measurements of stomatal number and opening were obtained from paradermal sections of the abaxial epidermis using "super-glue" imprints of the third leaf of the plants of each genotype placed on glass microscope slides and using an optical microscope (Martin and Stimart, 2005).

2.10. Leaf gas exchange and relative water content

The leaf gas exchange, including net CO_2 assimilation (*A*) and H_2O transpiration (*E*), were measured using a gas exchange system (LCpro, Analytical Development Co., Hoddesdon, UK), assisted by a light source with a luminous intensity of 1400 μmol m^{-2} s^{-1}. These measurements were taken on the third completely expanded leaf in the morning between 8:00–11:00 A.M. In addition, stomatal conductance (*gs*) was measured on the abaxial surface in the central portion of the terminal leaflet of the third leaf using a diffusion porometer (Model AP4; Delta-T Devices Ltd.) between 8:00–9:00 A.M. The intrinsic water use efficiency (WUE_i) was also calculated via the relation between *A* and *gs* (Larcher, 2003).

The relative water content (RWC) was determined in leaf discs according to the following equation: (FW-DW)/(TW-DW) × 100 previously described in Turner (1981).

2.11. Hormonal content

The lyophilized tissues of the upper and lower portions of the stem and the leaves were used to quantify hormonal contents. The samples were macerated in liquid nitrogen with the addition of 1.8 mL of the extraction solution (80% methanol, 1% acetic acid and 19% distilled water), following the addition of the deuterated analogue of the respective hormones (Oilchemim Ltd, Olomuc, Czech) to be quantified [30 μL of a mixture containing GAs, ABA, auxins, jasmonic acid (JA) and salicylic acid (SA)]. Then, this mixture was shaken for 1 h at 4 °C; afterwards, the samples were centrifuged at 10000*g* at 4 °C for 4 min. The supernatant was removed and conditioned in a 2 mL tube for 24 h at −20 °C for precipitation of the proteins, and the samples were centrifuged again at 10000*g* at 4 °C for 4 min; the supernatant was transferred to 5 mL glass tubes, and the samples were concentrated in a rotovapor (Thermos Scientific®) for 3 h. The concentrated samples were finalized with 1 mL of 1% acetic acid, and after a rapid shaking, the mixture was filtered in Oasis HLB® columns (reverse phase). The hormones were recovered by applying 1 mL of 95% methanol. Again, the samples were dried in the rotovapor and subsequently dissolved with 150 μL of 5% acetyl nitrile (ACN) + 1% acetic acid. Finally, the readings were performed in a spectrometer coupled to a UHPLC and an autosampler (Accucore RP-MS column 2.6 μm, 50 × 2.1 mm; ThermoFisher Scientific) (Seo et al., 2011).

2.12. Water loss measurements in detached leaves

Fully expanded third leaf of 45-day-old plants was used to determine water loss. The petiole was placed in 2 mL microtubes containing an artificial xylem sap solution (AX) previously described in Carvalho et al. (2011). ABA was added in final concentration of 10 μM or polyethylene glycol 6000 to reach −0.6 MPa. Detached leaves were then maintained overnight at room temperature and the leaves were further weighed every 2 h over 12 h after treatment.

2.13. Statistical analyses

The experimental design was completely randomized, with three replications in a 4 × 2 factorial scheme; there were four combinations of grafting (MT/MT, *pro/pro*, MT/*pro* and *pro*/MT) and two water conditions (well-watered and drought stress). Analyses of variance (ANOVA) was performed all data, and the means were compared using Tukey's test (at $P \leq 0.05$) in AgroEstat software (www.agroestat.com).

3. Results

3.1. Growth parameters

Well-watered plants (45 DAS) exhibited similar development, although *pro* scions grafted onto MT rootstocks resulted in reduced area, fresh and dry weight of the leaves and roots compared to *pro/pro* (Fig. 1). During drought conditions, MT/MT plants suffered with leaf and root area reductions, whereas there were no significant differences in root area among reciprocal or self-grafted plants (Fig. 1A). On the other hand, plant height was not affected by drought stress exposure (Fig. 1B). Despite this, we verified that *pro* scions induced an increase in plant height, but the height improvement was rootstock-dependent since the scions of *pro* grafted onto MT rootstocks showed an intermediate plant height (Fig. 1B). In addition, a similar pattern was observed for fresh and dry weight among grafted plants (Fig. 1C, D) in which MT/MT was negatively affected by stress, showing fresh and dry weight inhibition for shoot and roots compared to the corresponding well-watered samples. Despite the reduced plant growth for *pro* scions

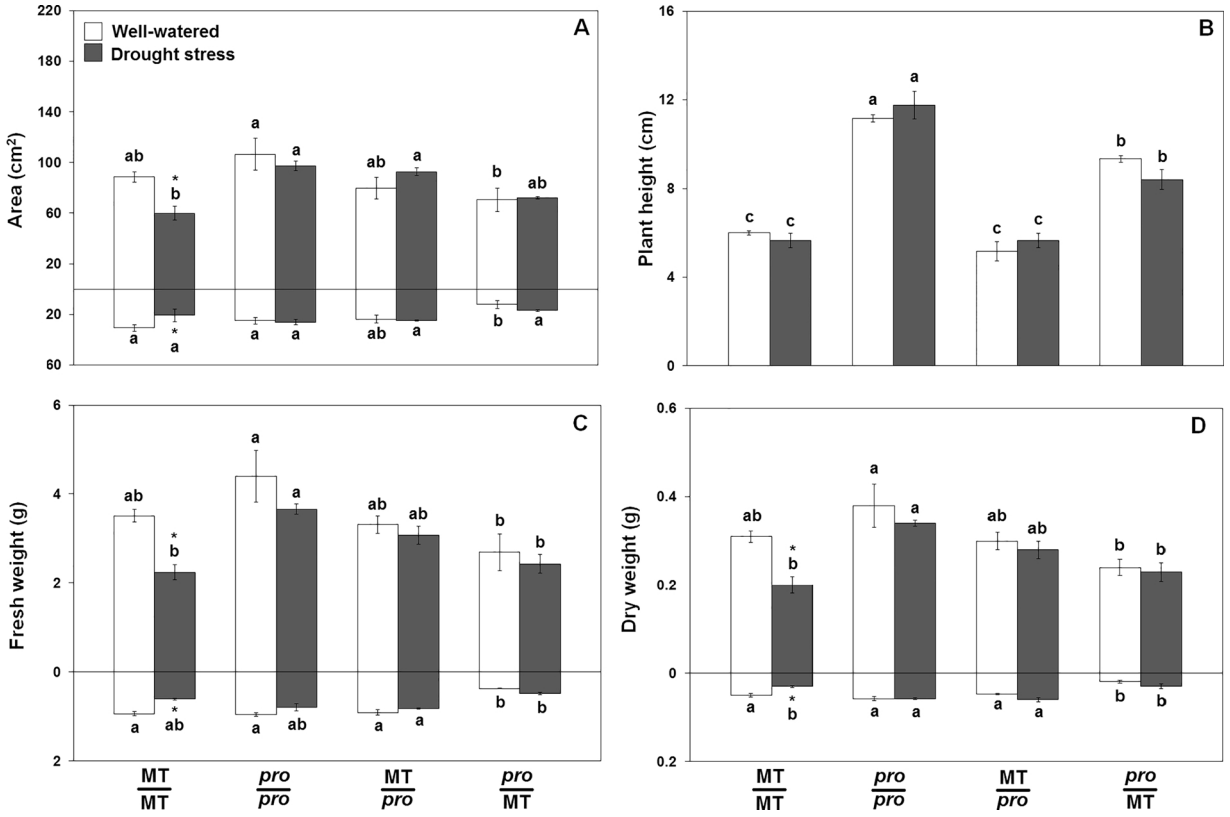

Fig. 1. Growth parameters of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. (A) in the coordinate axis, values above and below 0 correspond to leaf and root area, respectively; (B) plant height; (C) in the coordinate axis, values above and below 0 correspond to fresh weight of the shoot and roots, respectively; (D) values above and below 0 correspond to dry weight of the shoot and roots, respectively. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

grafted onto MT rootstocks, drought stress did not affect plant fresh and dry weight (Fig. 1C, D). These results indicate that constitutive GA response in *pro* mutant in either self-grafted or heterografted plants causes more resistant than wild-type self-grafted plants to growth reduction caused by water stress.

3.2. Oxidative stress

Concerning lipid peroxidation, expressed as MDA content, we verified a higher MDA content in the shoot of *pro*/MT was found under irrigated conditions, compared to all grafting combinations (Fig. 2A). In addition, drought stress induced a pronounced MDA accumulation in both the shoot and roots, irrespective of grafting combination (Fig. 2A). Furthermore, it was observed that *pro* scions grafted onto MT rootstock exhibit higher MDA levels in both the shoot and root tissues (Fig. 2A). Unlike MDA, there was no difference of H_2O_2 content in the shoot or roots of irrigated plants (Fig. 2B). On the other hand, *pro* rootstocks induced higher H_2O_2 accumulation in the shoot tissue under drought

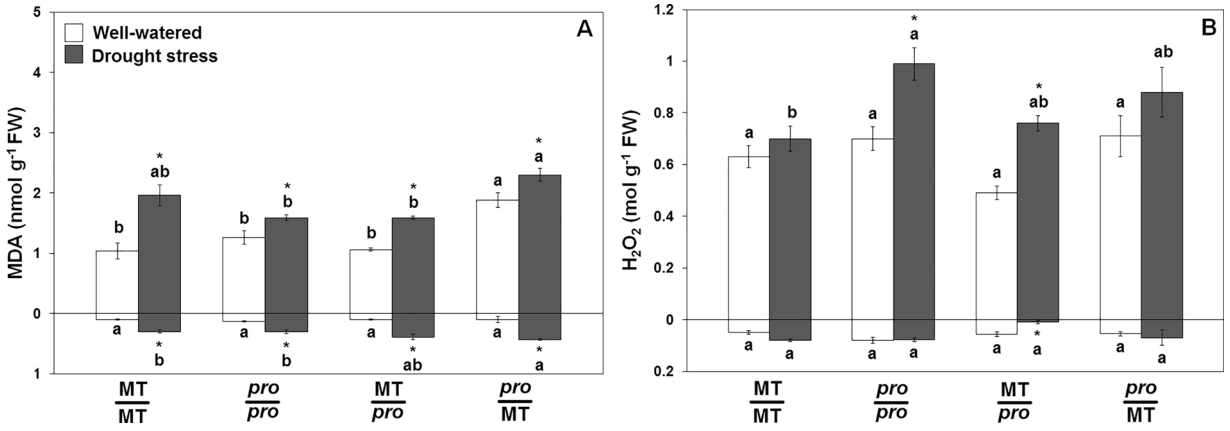

Fig. 2. Oxidative stress analysis of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. (A) in the coordinate axis, values above and below 0 correspond to the shoot and roots malondialdehyde content, respectively; (B) in the coordinate axis, values above and below 0 correspond to the shoot and roots hydrogen peroxide content, respectively. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

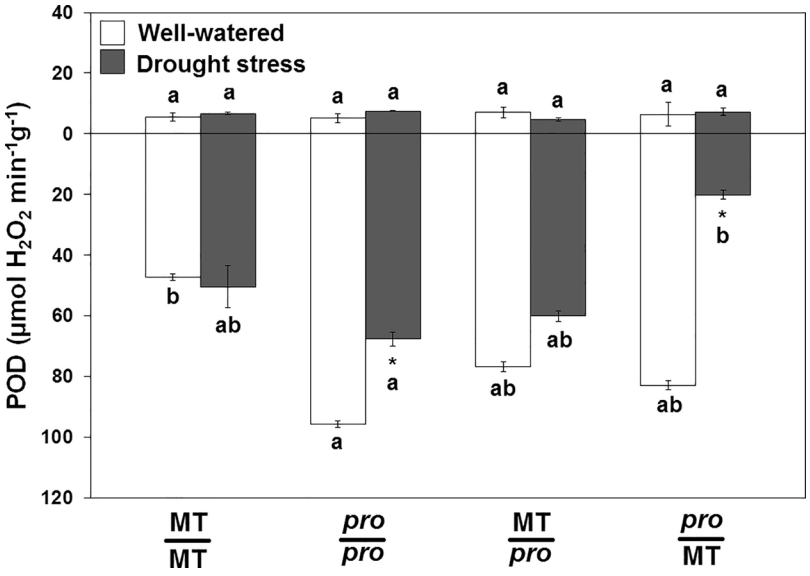

Fig. 3. Peroxidase (POD) activity in the leaves (values above 0 in the coordinate axis) and roots (values below 0 in the coordinate axis) of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

stress conditions, independent of the scion genotype (i.e. *pro/pro* and MT/*pro*) (Fig. 2B). In roots, there was no influence of stress on H_2O_2 content, except for MT/*pro*, which showed a reduction of H_2O_2 content during drought (Fig. 2B).

There was no difference of peroxidase activity (POD) in the shoots of irrigated or non-irrigated plants (Fig. 3). On the other hand, we observed great variability of POD activity in the root tissue in well-watered conditions; the highest and lowest levels of POD activities was observed in self-grafted *pro/pro* and MT/MT, respectively (Fig. 3). During drought, an inhibition of the root POD activity was found in the plants grafted onto *pro* rootstock, such as *pro/pro* and *pro*/MT, where the latter showed the lowest POD activity in the roots (Fig. 3).

3.3. Relative SlDREB2 and SlDELLA genes expression

In well-watered condition, the relative expression of the *SlDREB2* gene was similar among all grafting combinations (Fig. 4A). As expected, *SlDREB2* expression was up-regulated in plants under water stress, but the lowest induction levels were observed in MT scions grafted onto *pro* rootstock (Fig. 4A). In turn, *SlDELLA* expression was dependent of grafting combination (Fig. 4B). The self-grafted *pro* plants exhibited the highest relative expression of *SlDELLA* when compared to MT/MT and MT/*pro* in well-watered condition, whereas *pro*/MT showed intermediate expression of *SlDELLA* (Fig. 4B). Under drought stress, inhibition of *SlDELLA* expression was observed only in *pro* scions (i.e. *pro/pro* and *pro*/MT), but still showed higher *SlDELLA* expression compared to MT/MT and MT/*pro* (Fig. 4B).

3.4. Water relations

Leaf temperature was increased under stressful conditions, but leaf temperature appeared to be less affected in the stressed MT/MT plants (Fig. 5A). Likewise, well-watered plants initially exhibited an elevated and similar RWC (Fig. 5B). However, the RWC was severely reduced in non-irrigated plants, except for the MT/MT, which exhibited a not significant reduction of RWC under drought stress (Fig. 5B). In addition, all plants showed a reduction of Ψ_{leaf} under water deprivation. Furthermore, Ψ_{leaf} reduction was more pronounced in *pro*/MT plants (Fig. 5C).

3.5. Stomatal measurements, leaf gas exchange and water loss in detached leaves

In well-watered plants, stomata density was higher in self-grafted plants *pro/pro* compared to MT/MT, while heterografted MT/*pro* and *pro*/MT exhibited intermediate stomata density (Fig. 6A). Under water

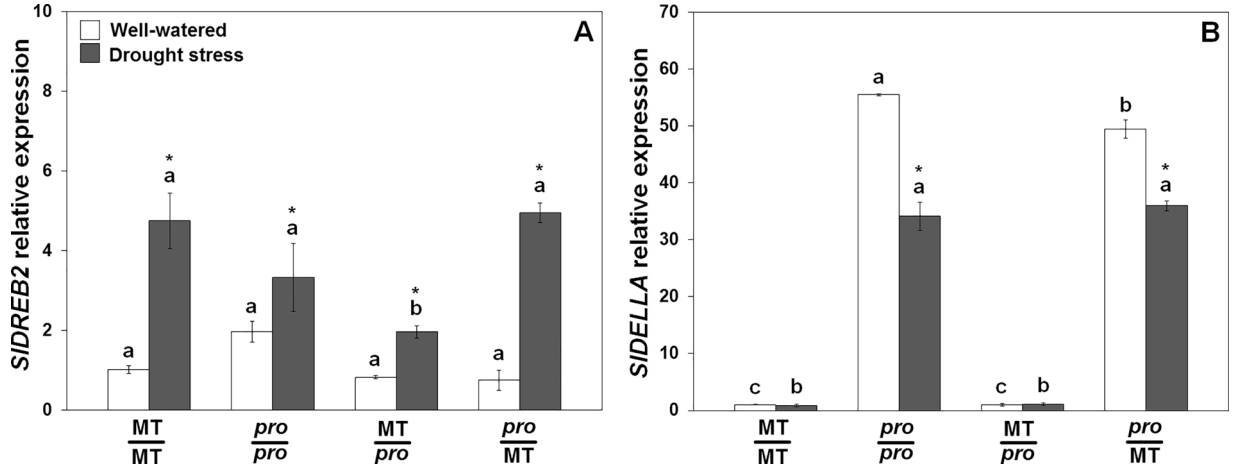

Fig. 4. Relative expression of *SlDREB2* (A) and *SlDELLA* (B) of leaves of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

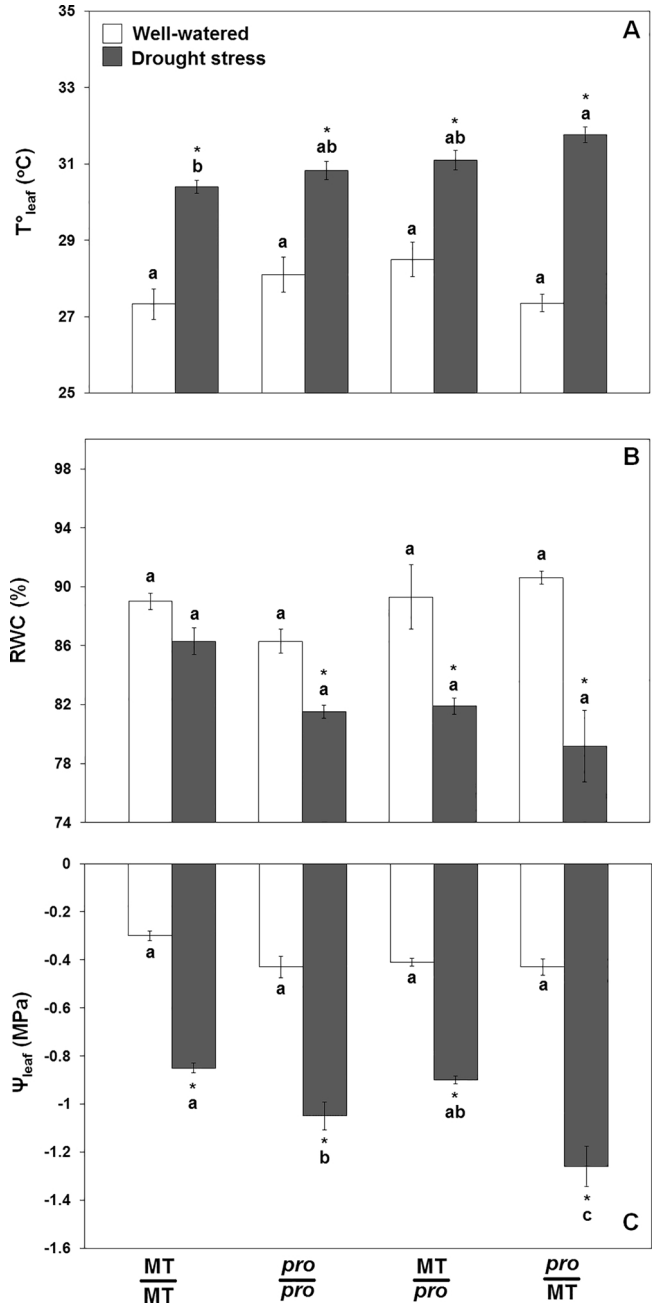

Fig. 5. Water relations of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. (A) leaf temperature; (B) relative water content; (C) leaf water potential. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

stress, only MT scions were responsive to the stress conditions showing greater stomata density in both MT/MT and MT/*pro* (Fig. 6A). In addition, as expected, drought stress caused severe stomata closure (Fig. 6B).

Under well-watered conditions, we observed the existence of significant variance in *gs* among the grafted plants, which was greater in MT/*pro* (Fig. 7A). On the other hand, drought stress induced a strong reduction in *gs* in all grafted plants when compared to the well-watered plants. Furthermore, self-grafted *pro/pro* exhibited a pronounced *gs* reduction by water deprivation (Fig. 7A). The irrigated plants had similar *E*, independent of grafting combination. However, under stressful conditions, although the combinations with *pro* exhibited a reduction of *E*, mainly *pro/pro* plants, self-grafted MT/MT did not suffer a significant inhibition of the transpiration rate under stress conditions (Fig. 7B).

Regarding *A*, in non-stressful conditions, there was no difference among the grafted plants (Fig. 7C). However, after seven days of water stress, we verified a strong reduction of *A*, which was greater in *pro/pro* plants (Fig. 7C). The WUE_i differed among the grafting combinations according to the water availability (Fig. 7D). In well-watered plant, the *pro* rootstock clearly induced a lower WUE_i, irrespective of the scion genotype, but mainly in MT scions (i.e. MT/*pro*). Conversely, water restriction caused an improvement of WUE_i, except for that in self-grafted MT/MT plants (Fig. 7D).

MT and *pro* detached leaves showed similar water loss over the evaluation period (Fig. 8). However, ABA treatment caused a more pronounced reduction in water loss in *pro* when compared to MT detached leaves (Fig. 8). In order to simulate the drought stress condition, water losses were measured in MT and *pro* detached leaves with supply of polyethylene glycol (−0.6 MPa). Under this condition, *pro* leaves exhibited reduced water loss than MT leaves (Fig. 8).

3.6. Hormone content

Regarding the hormone content, MT/MT and *pro/pro* exhibited similar content of active GAs (GA_1 and GA_4), but reduced GA_{12} levels were found in *pro/pro* compared to MT/MT plants in well-watered conditions (Fig. 9). In addition, it is remarkable that the largest and smallest values of GAs were in the leaves of MT/*pro* and *pro*/MT, respectively, indicating a key role of the rootstock on GA content in the shoot (Fig. 9). On the other hand, GA_1 levels were similar among all genotypes under well-watered conditions, except for MT/*pro,* which, as commented above, showed increased GA_1 content and exhibited a reduction of GA_1 in the leaves under water stress (Fig. 9A). In contrast, GA_4 and GA_{12} were tightly regulated by stress conditions, in which GA_4 and GA_{12} were lowered by drought, except in *pro/pro* plants which showed an increase of GA_{12} in the leaves (Fig. 9B, C). In addition, drought stress enhanced ABA leaf content in all grafting combination, while the *pro* scions (i.e. *pro/pro* and *pro*/MT) exhibited greater ABA in the leaves in both well-watered and stressful conditions (Fig. 9D). Thus irrespective of the water availability, *pro* scions have more ABA content in leaves.

4. Discussion

GA regulates plant growth and integrates with various hormonal signals during plant development (Davière and Achard, 2016). In addition, recent studies have evidenced GA involvement in the responses to various abiotic stresses, especially in drought stress (Colebrook et al., 2014). However, the underlying mechanisms to GA signaling in adaptive responses or a possible role of GA in the root-to-shoot communication during water-limited conditions remains elusive (Akter et al., 2014; Nir et al., 2014). Therefore, we used a constitutive GA response tomato mutant and its near isogenic line (MT) in self- and reciprocal grafting combinations under contrasting water availability conditions in to gain further insight into GA mediated long-distance signaling during drought stress.

These experiments revealed that during drought primarily self-grafted MT/MT plants are affected by stress, as shown by reductions of leaf and root area and as a consequence lower fresh and dry weight accumulations for shoot and roots (Fig. 1). In contrast, self- or reciprocal-grafted plants with *pro* (*pro/pro*, MT/*pro* and *pro*/MT) did not exhibit growth inhibitory effects by drought stress when compared to well-watered plants (Fig. 1). In fact, it has been demonstrated that a GA application under water-limiting conditions can induce plant growth recovery to levels similar or close to well-watered conditions (Kaya et al., 2006; Akter et al., 2014). In the referred studies, the pigments were maintained, and oxidative stress was inhibited (Kaya et al., 2006;

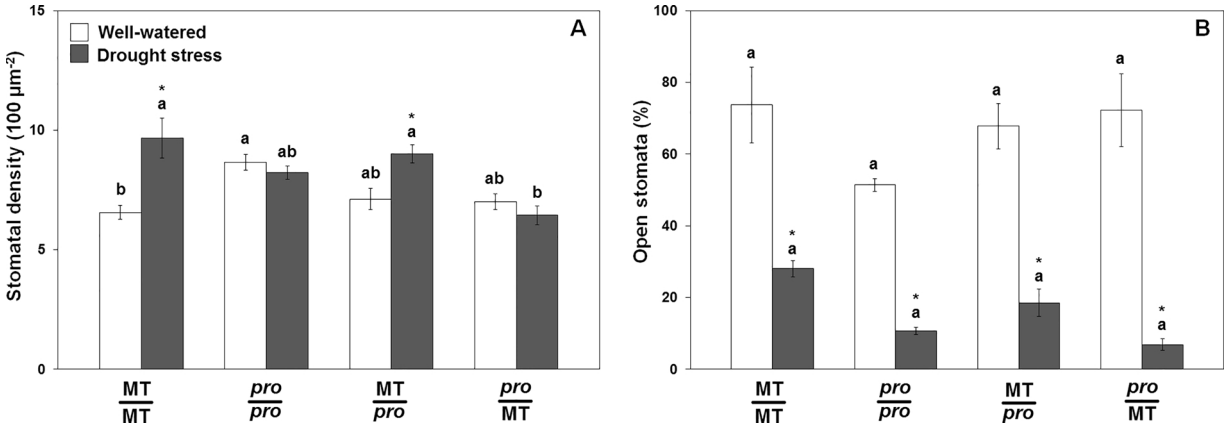

Fig. 6. Stomatal analysis of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. (A) stomatal density; (B) open stomata. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

Akter et al., 2014). However, in this experiment, there was no reduction of photosynthetic pigments after exposure to drought stress (Appendix S2). Meanwhile, grafted water-stressed plants increased the lipid peroxidation, expressed as MDA content, in both the shoot and root tissues in comparison to well-watered conditions (Fig. 2A), whereas the H_2O_2 production, which is another indicator of oxidative stress (Gratão et al., 2005; Żamojć et al.,2016), enhanced specifically in the leaves of plants grafted onto *pro* rootstock (i.e. *pro/pro* and MT/*pro*) under drought stress (Fig. 2B). The enhanced H_2O_2 response in *pro/pro* and MT/*pro* could be related to the reduced activity of POD, which has a role in cellular scavengers of H_2O_2 (Passardi et al., 2005). However, there was no difference in POD activity in the leaves of the grafted plants under contrasting water availability conditions. Conversely, rootstocks of *pro/pro* and *pro*/MT plants exhibited a significant reduction in root POD activity during drought with no direct effect in root H_2O_2 accumulation (Figs. Figure 2B, Figure 3).

It has been shown that POD and ROS, particularly H_2O_2, are important players in the cellular adjustment mechanisms to abiotic

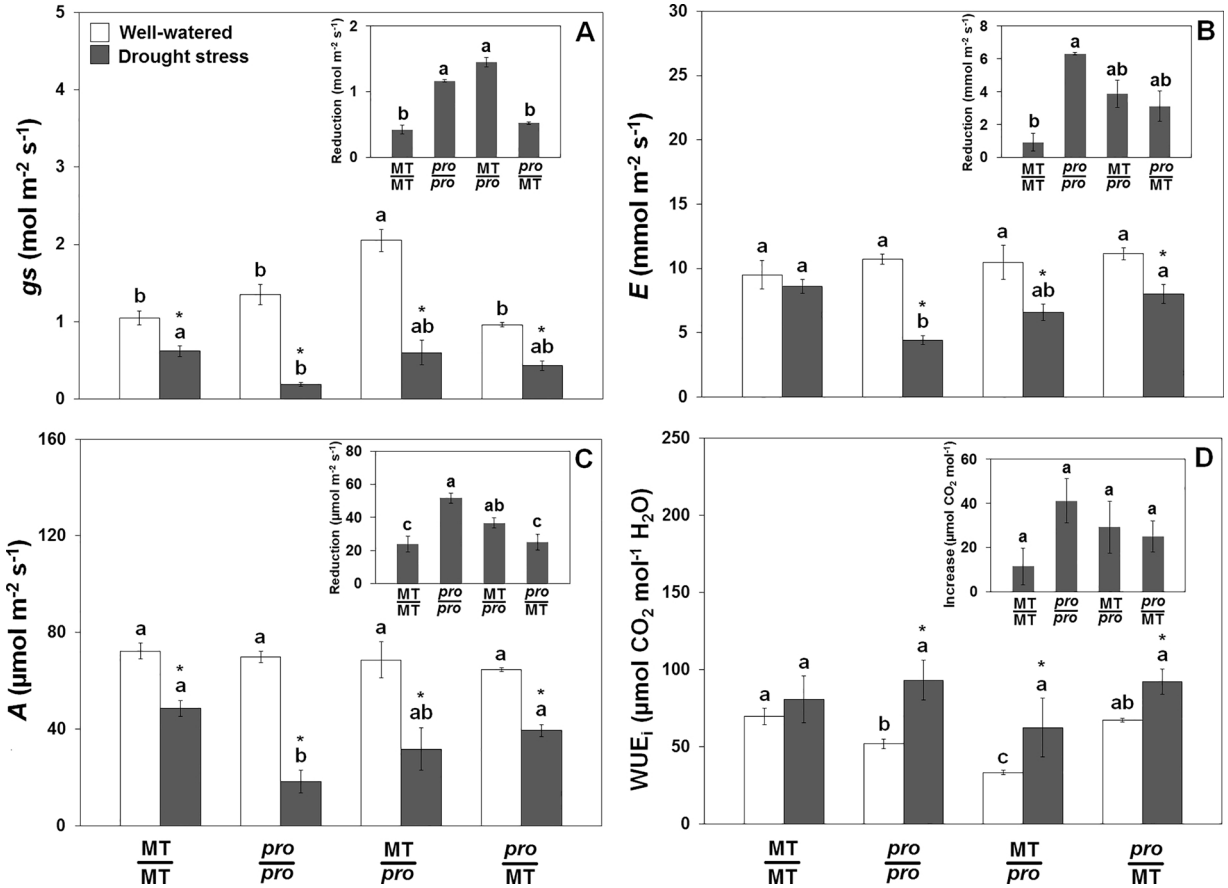

Fig. 7. Gas exchange of the third leaf fully expanded of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. (A) stomatal conductance; (B) rate transpiration; (C) CO_2 assimilation; (D) intrinsic water use efficiency. Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

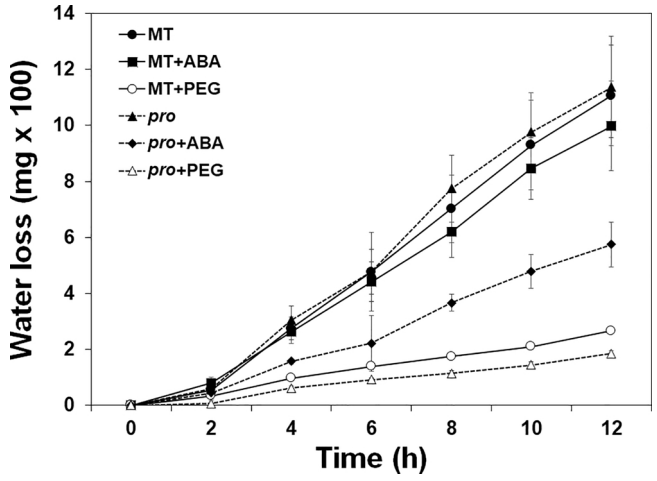

Fig. 8. Water loss from detached MT and *pro* leaves with petiole immersed in artificial xylem solution, which was add with 10 µM abscisic acid or polyethylene glycol 6000 (-0.6 MPa). The values correspond to the means of each treatment (n = 4), followed by the standard error.

stresses (Fan et al., 2006; Pandey and Shukla, 2015). For example, due to a high H_2O_2 permeability via the plasma membrane, this molecule mediates cell-to-cell communication playing a central role during cell wall remodeling in response to water stress (Rhee et al., 2010; Wang et al., 2016). Therefore, cell wall stiffening limits cell expansion in order to cope with water-limiting conditions, thus favoring increased mechanical stability as well as cellular turgor (Passardi et al., 2005; Tenhaken, 2015). Interestingly, we observed that *pro* rootstock increased H_2O_2 levels in the grafted scions shoot when subjected to drought stress (Fig. 2B), which might indicate a DELLA effects in oxidative stress response mediated by root-to-shoot communication (Fig. 4B). For example, transcriptional up-regulation of genes encoding ROS-detoxification enzymes is dependent of DELLA activity in roots under abiotic stress conditions (Achard et al., 2008). However, the link between H_2O_2 alteration and water stress resistance in *pro* is still elusive. So far, we can speculate that the constitutive GA response rootstock in grafted plants (*pro/pro* and MT/*pro*) provided the most efficient water stress perception or signaling to the shoot, triggering cell wall remodeling and increased mechanical stability, thus permitting growth maintenance under reduced water availability. This adaptive response likely involves GA mediating a complex regulatory system during acclimation to drought stress, while the lack of drought-mediated growth inhibition on *pro*/MT plants is likely due to the reduced growth in well-watered conditions (Fig. 1).

As expected, drought stress induces leaf temperature rise, as well as RWC and Ψ_{leaf} reduction in all grafting combinations (Fig. 5). The RWC was negatively affected by stress in most grafted combinations, whereas MT/MT was slightly reduced with no significant difference between well-watered and water-limited plants (Fig. 5B). On the other hand, the constitutive GA response mutant scion onto MT rootstock exhibited a great reduction in Ψ_{leaf} under water stress conditions (Fig. 5C). Thus, the steady RWC and the reduction of Ψ_{leaf} likely explain the higher and lower leaf temperatures in *pro*/MT and MT/MT, respectively (Fig. 5A). Furthermore, in response to drought stress, reduction in RWC and Ψ_{leaf} is strictly related to stomatal closure (Fig. 6B) and *gs* reduction (Daszkowska-Golec and Szarejko, 2013; Golldack et al., 2014; Blatt, 2016; Fig. 7A). In fact, we noticed a strong reduction in stomatal opening and *gs* in all plants (Figs. Figure 6B, Figure 7A). The stomatal opening and *gs* reductions were followed by *A* inhibition in grafted plants, but mainly in self-grafted *pro/pro* under drought conditions

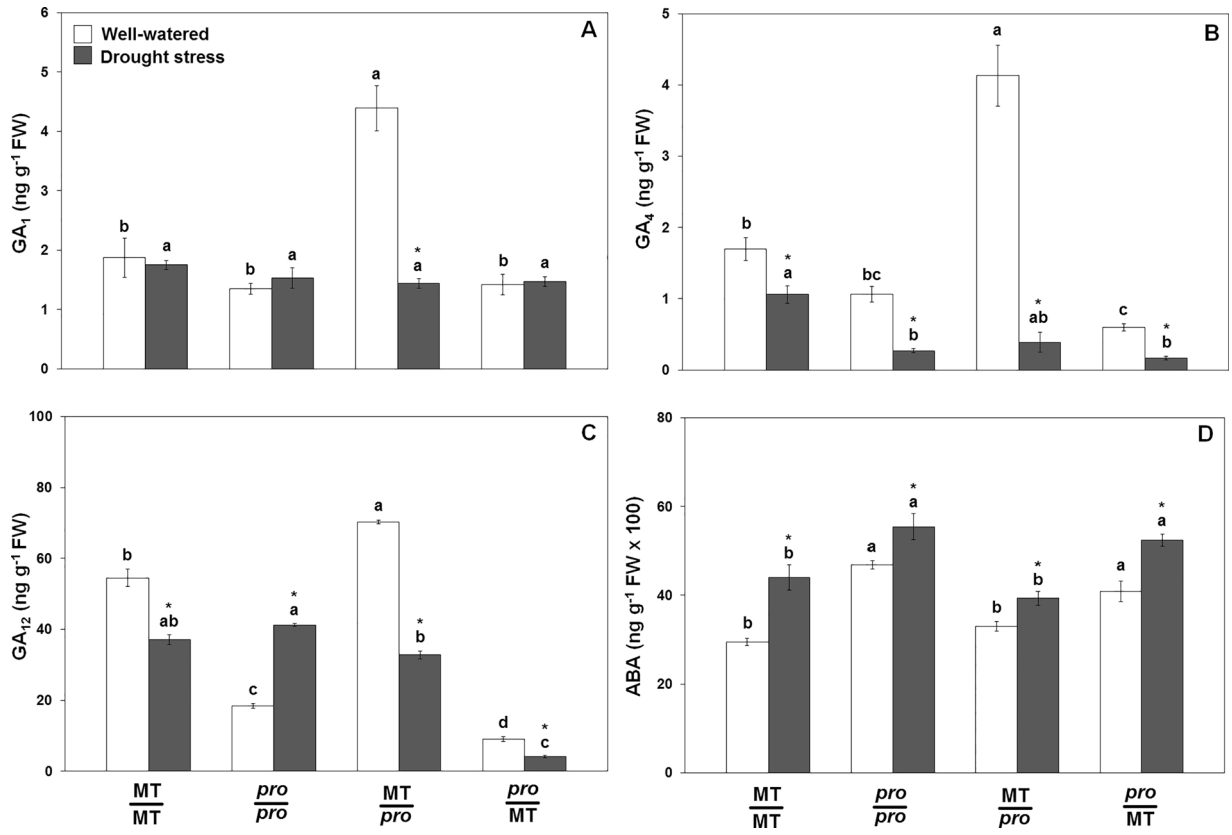

Fig. 9. Leaf hormone content of self- and reciprocal-grafted tomato MT and *pro* grown under well-watered or drought stress conditions. Bioactive gibberellins, GA_1 (A) and GA_4 (B); xylem-mobile gibberellin, GA_{12} (C) (Regnault et al., 2015); abscisic acid (D). Values are the means of each treatment (n = 3), and the bars are the standard error of each treatment. Letters above the bars represent the differences in the means among the grafting treatments within each condition, and asterisks mark differences between conditions within the same grafting combination; both were calculated using Tukey's test at 5%.

(Fig. 7A, C). This response was followed by inhibition of *E,* as well as WUE_i enhancement, occurred in self- and reciprocal-grafted *pro* (*pro/pro*, MT/*pro* and *pro*/MT) (Fig. 7B, D). These results indicated that although in well-watered conditions *pro* rootstock induced a lower WUE_i, the rootstocks with enhanced GA sensitivity exposed to a water stress have a more efficient signaling to maintain leaf turgor (Appendix S3), which might indicates a root-to-shoot GA-dependent signaling modulating water stress adaptive responses.

Moreover, there are various studies demonstrating that water relations traits are ordinarily controlled by signals from the root system during water stress conditions (Tramontini et al., 2013; Martorell et al., 2015; Visentin et al., 2016). Currently, ABA is the primary hormone that modulates stomatal movement under stress (Holbrook et al., 2002; Daszkowska-Golec and Szarejko, 2013; Golldack et al., 2014; Verma et al., 2016) and therefore, it has long been thought that ABA could be the signal transported from roots to shoot to control the water stress responses (e.g. *gs* reduction). Here, we observed that drought stress caused *gs* reduction and increased ABA content in the leaves of all grafted scions (Figs. Figure 7A, Figure 9D). Interestingly, plants grafted onto *pro* rootstocks (*pro/pro* and MT/*pro*) exhibited the highest reductions of *gs* (85.93 and 70.74%, respectively), *E* (58.74 and 37.12%, respectively) and *A* (73.72 and 53.49%, respectively) under drought stress (Fig. 7). Thus, the constitutive GA response in *pro* rootstocks under drought stress induced a marked reduction of *gs*, *E* and *A,* independent of the scions genotype. In addition, water loss from detached leaves indicates that the *pro* mutant lost water significantly slower than the MT with exogenous application of ABA, which suggests an enhanced ABA sensitivity in the *pro* mutant than in the MT (Fig. 8). Thus, the increased drought-induced ABA accumulation and sensitivity in *pro* grafted plants likely conferred the drought tolerant phenotypes (Fig. 1) and is the result of a GA constitutive response induced by this mutation.

Regarding GA involvement in stomatal physiology, recent studies have found that GA levels or signal can modulate both stomatal development and stomatal response to drought stress (Nir et al., 2014; Du et al., 2015; Plaza-Wuthrich et al., 2016). For example, Teff plants [*Eragrostis tef* (Zucc.) Trotter] treated with paclobutrazol (PBZ), an inhibitor of GA biosynthesis, showed a reduction of *gs* and stomatal density (Plaza-Wuthrich et al., 2016). Indeed, an altered signaling response to GA in *pro/pro* induced greater stomata density than MT/MT under well-watered conditions (Fig. 6A). Nonetheless, the *gid1* rice mutant, presenting low GA sensitivity, exhibited lower stomatal closure and leaf rolling under water stress, which are key mechanisms to cope with drought linked to *gs* and transpiration area reduction, respectively (Du et al., 2015). This response in the *gid1* mutant was correlated with reduced ABA accumulation triggered by drought (Du et al., 2015). The authors showed that stomatal closure was more responsive to ABA application in the *gid1* mutant, suggesting that the higher sensitivity to ABA was probably due to a GA signaling pathway disruption in *gid1* (Du et al., 2015). Hence, responses to drought stress were accompanied by ABA accumulation and GA content reduction, especially GA_4, in the leaves of the grafted plants, but mainly in *pro* self- and reciprocal-grafted plants (Fig. 9). In fact, it had been observed that *pro* mutant reduced GA levels (Jones, 1987; Van Tuinen et al., 1999), which showed that the phenotype of *pro* cannot be explained by GA overproduction. On the other hand, the reduction of GA levels can also be associated with the enhanced expression of the *SlDREB2* gene under drought stress, which encodes the transcription factor DREB2 (Fig. 4A). DREB2 has been shown to be involved in regulation of drought response mechanisms as well as inhibition of key genes for GA biosynthesis (Li et al., 2012; Hichri et al., 2016).

Furthermore, Pearson's correlation factor (*p*) among *gs,* GA_4 and GA_9, a precursor of GA_4, was 0.85 and 0.89, respectively, indicating a strong correlation between both, while the *p* value between *gs* and ABA was only −0.57, indicating a moderate correlation (Appendix S4). In addition, we observed that the relationship between ABA and active GAs in the leaves was greater in plants with *pro* as the scion, and this relationship increased under drought, which was possibly an attempt to achieve hormonal homeostasis (Appendix S5). Together, these results fall into the classic interaction between ABA and GA during plant growth (See review Golldack et al., 2013; Chiang et al., 2015; Liu et al., 2016), but a more molecular detailed analysis in *pro* is required to further clarify this event, especially in the modulation of stress responses.

5. Conclusion and future perspectives

In this work, we found that the constitutive GA response in tomato rootstock induced a fine adjustment in stomatal responses (Fig. 7, Appendix S4), as well as a considerable tolerance to water stress (Fig. 1). This indicates complex regulatory mechanisms by which GA acts upon, which includes the oxidative stress system (Fig. 2B), hormonal homeostasis by the regulation of the GA biosynthesis route (Appendix S6, S7, S8, S9), and the levels of other hormones such as auxin, JA and SA (Appendix S10). However, the molecular basis of the root-to-shoot communication from *pro* still remains to be explored. Recently, evidenced has been raised demonstrating the complexity of GA responses to endogenous and environmental cues (Schwechheimer, 2012; Wang and Deng, 2014; Davière and Achard, 2016). This intricate complexity lies on the variable number and quantity of the bioactive GAs content dependent on the organ, plant age and species as well as the existence of DELLA-dependent and −independent responses (Van Tuinen et al., 1999; Carrera et al., 2012; Livne et al., 2015). In addition, GA signal transduction can act integrating several other hormones classes through the DELLA protein (Davière and Achard, 2016). So far, it is known that the differential responses to GA in *pro* is due to the lack of a functional GA-repressor DELLA protein, which has often been associated with responses to drought stress. For instance, there is strong evidence that DELLA proteins induce ABA synthesis via the positive regulation of *XERICO*, an inducer of ABA synthesis (Ko et al., 2006; Ariizumi et al., 2013), and can have its mRNA transported from the roots to the shoot by xylem sap (Wang et al., 2012). This could partially explain why *pro* scions grafted on MT rootstock under water-limiting conditions exhibited a *gs*, *E* and *A* response pattern closer to MT/MT than *pro/pro* (Fig. 7). On the other hand, although self-grafted *pro/pro* plants have exhibited high relative expression of the *SlDELLA* gene, the produced mRNA generates non-functional DELLA proteins (Fig. 4B). However, our results provide more conclusively evidence that a more noticeable induction of tolerance by means of GA amplification from rootstock is dependent on ABA signaling (Fig. 10). In the other words, GA signal amplification from root-to-shoot, at last non-dependent on enhanced GA biosynthesis, can be an interesting alternative to provide for the necessary balance between growth and water economy during drought stress.

Author contributions

L.A.G. and R.F.C. planned and designed the research. L.A.G., C.C.M., F.J.R.C., D.R.R., I.L.D., E.C.B. and J.E.L. carried out the experiments. L.A.G. and R.F.C. analyzed the data. L.A.G., L.E.P.P. and R.F.C. wrote the manuscript. J.E.L., L.E.P.P. and R.F.C. revised the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest

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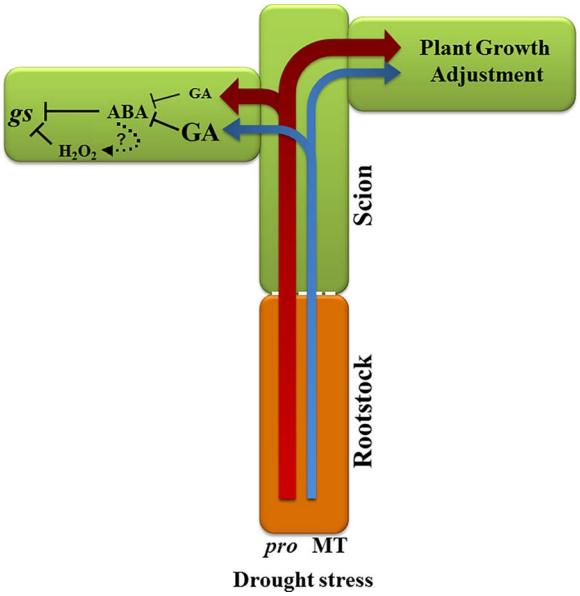

Fig. 10. Schematic representation of gibberellin (GA) modulating the root-to-shoot signaling during drought stress events. Our results indicated that *procera* (*pro*) mutant rootstock, with a constitutive gibberellin response, provided an amplification of signaling (indicated by the thicker red arrow) from the roots to the shoot due to soil drying. Therefore, under drought stress conditions, *pro* rootstock induced a strong reduction in the content of GAs, an increase of abscisic acid (ABA) in the leaves, and consequently, a reduction of stomatal conductance (*gs*), irrespective of the scion (Figs. A,). In addition, the GA regulation of the drought responses in plants grafted onto *pro* could be mediated by hydrogen peroxide (H_2O_2), which can act downstream of ABA in stomatal closure (An et al., 2016; Niu and Liao, 2016). These modifications led to the activation of adjustment mechanisms and greater water economy as well as maintenance of growth under drought stress conditions. On the other hand, the use of the wild-type (Micro-Tom, MT) as rootstock apparently induced a less effective communication regarding water deprivation (indicated by the thinner blue arrow), thus inducing deficient regulation of the GA levels and a lower ABA/GA relation in the leaves, which caused a lower reduction of *gs* and as consequence greater rate transpiration, mainly when self-grafted (MT/MT) (Figs. 7 and 9, and Appendix S5). Together, these results demonstrated that *pro* rootstock could increase drought stress tolerance in a scion-independent way. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jplph.2017.12.003.

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