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Brassinosteroid and brassinosteroid-mimic differentially modulate *Arabidopsis thaliana* fitness under drought

Lucia Sandra Pérez-Borroto[1,2] · Laila Toum[2] · Atilio Pedro Castagnaro[2] · Justo Lorenzo González-Olmedo[1] · Francisco Coll-Manchado[3] · Esteban Mariano Pardo[2] · Yamilet Coll-García[3]

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Abstract

Brassinosteroids (BRs) are widely used to promote plant growth/development and alleviate environmental stresses' adverse effects. However, its low stability in the field precludes large-scale application, challenging research, and more stable and cost-effective analogues. The most commonly used is 24-Epibrassinolide (EBL), yet, due to its high production cost, the study of cheaper molecules with similar/higher activity constitutes a priority. In this study, we analyzed, under drought, the effects of EBL and DI-31, a synthetic functional analogue, through a physiological and biochemical approach in *Arabidopsis thaliana* wild-type plants. Additionally, differential BRs/ABA interactions were detected and further analyzed in abscisic acid (ABA) mutants, assays in stomata with ABA-closure inhibitors, and analysis of ABA-stress-responsive genes expression via qRT-PCR. Similar to EBL, DI-31 induced dose-responsive growth and stomatal closure curve. Compared to EBL, DI-31 induced oxidative burst in a stronger but delayed manner; and increased biomass and foliar area under drought, preventing more effectively the relative water content fall under stress. Although both, EBL and DI-31, enhanced drought-response, the DI-31 action was more effective, durable, and differed in regulating several ABA/stress-response indicators. DI-31/ABA interactions under drought were confirmed in ABA-mutants, where the analogue compromised the activation of ABA-regulated proteins. Moreover, the analogue mediates stomatal closure through paths partially alternative to the ABA-controlled and specifically repressed stress-responsive genes regulated by AREB/ABF transcriptional factors. These findings confirm the DI-31 practical value as growth-promoter and defence-enhancer, with stronger and longer-term activity than EBL, constituting an environmentally-friendly and cost-effective alternative to increase plant fitness under drought, precluding large biomass penalty.

Keywords *Arabidopsis* · Brassinosteroid analogue · DI-31 · Drought tolerance · 24-epibrassinolide

Introduction

Brassinosteroids (BRs) possess beneficial pleiotropic effects due to their broad and highly coordinated cell modulation capacity (Sahni et al. 2016). They influence various developmental processes and modulate agronomical traits (González-Olmedo et al. 2004; Gonzalez-Olmedo et al. 2005; Vriet et al. 2012). Moreover, researchers have established a direct link between BR levels' modulation and stress adaptation to major abiotic stresses such as water deprivation (Kaur and Pati 2019; Li et al. 2020). Thus, BRs exogenous application and the genetic manipulation of its endogenous levels have been used as strategies also directed to increase crop performance under drought. However, the BR's low stability in the field precludes the large-scale application (Sakai et al. 1999), challenging research and development of more cost-effective

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✉ Yamilet Coll-García
yaqsaida@gmail.com

[1] Centro de Bioplantas, Ciego de Ávila University "Máximo Gómez Báez", Road to Morón 9 ½ Km, Ciego de Ávila, Cuba

[2] Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA), Estación Experimental Agroindustrial Obispo Colombres (EEAOC)/Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), William Cross St. No. 3150, Las Talitas, Tucumán, Argentina

[3] Centro de Estudios en Productos Naturales (CEPN), Faculty of Chemistry, Havana University, Zapata and G St., 10400 Vedado, Havana, Cuba

analogues with higher biological activity and field average life (Sasse 2003). The use of BR's analogues with a predominantly growth-promoting effect in a wide range of plant species constitutes an alternative to improving crop yields (Ali et al. 2017). More than 80 natural BRs and over 130 structurally and functionally analogues have been characterized in the course of BRs studies (Liu et al. 2017; Kvasnica et al. 2019). The most commonly used is 24-Epibrassinolide (EBL) despite its high production cost (Liu et al. 2017; Moreno-Castillo et al. 2018), so the study of cheaper molecules with similar/higher activity constitutes a promising alternative.

In this research, we used EBL and the synthetic spirostanic brassinosteroid (BR) analogue (25R)-3β,5α-dihydroxyspirostan-6-one (DI-31), the active ingredient of the commercial formulation BIOBRAS 16. The DI-31, characterized by a spiroketalic ring instead of the typical BR side chain (Coll et al. 1995; Mazorra et al. 2002), was reported to exhibit BR-like activity similar to other analogues such as MH5 and BB6 (Mazorra et al. 2002, 2004). The compound has an epoxyoximic polar group, one of its major conformations, that interacts with the BRI1 (BRASSINOSTEROID-INSENSITIVE1) BRs plant receptor with a similar affinity and binding energy than the EBL (Moreno-Castillo et al. 2018), therefore having a greater potential activity. Researchers reported that foliar applications of DI-31 stimulated the photosynthetic rate and yield of greenhouse-grown pepper (*Capsicum annuum* L.) (Serna et al. 2012) and endive (*Cichorium endivia* L.) plants (Serna et al. 2013). Both the application of BIOBRAS 16 (Núñez et al. 2003) and DI-31 (Serna et al. 2015) attenuated the adverse effects of NaCl stress. Moreover, compared with EBL treatments, foliar applications of BIOBRAS 16 induce greater protection against the fungal pathogen *Colletotrichum acutatum* in strawberry (Furio et al. 2019).

However, neither single nor comparative studies yet reported the regulatory effect of DI-31 on the growth/defence trade-off under drought, the most severe abiotic stress in worldwide agriculture (Chai et al. 2016). Therefore, this work aimed to comparatively analyze, under water scarcity, the effects of EBL and DI-31 in *Arabidopsis thaliana* (L.) Heynh. Markers of growth, water management, respiratory burst, stomatal movement, and stress-response were assessed. Moreover, DI-31/ABA interactions were detected and further evaluated in drought-response experiments with ABA-mutants, stomatal movement assays with specific inhibitors, and relative expression analysis of ABA/stress-responsive genes.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana seedlings (Col-0 ecotype) were used as the wild-type plant in all experiments. Also, *pp2ca*-1 (Rubio et al. 2009) and *SnRK2.6*/*ost1* (SALK_008068) (Gonzalez-Guzman et al. 2012) single ABA mutants seeds were used in this research (Col-0 background). Seeds were sterilized with a mixture of commercial bleach/distilled water/ethanol (1:1:8 v/v) for 5 min, washed three times with 96% ethanol, and seeded in MS medium plates (Murasnige and Skoog 1962), containing 1% (w/v) sucrose, 0.5 g/L of MES pH 5.8 and 0.8% (w/v) agar. Seeded plates were stratified in the dark for 3 days at 4 °C. All plants were grown in a growth chamber (22 ± 2 °C and 16-h photoperiod) in plates, or 110 mL pots with commercial substrate Grow Mix MULTIPRO (Terrafertil S.A., Argentina), according to the experimental requirements. Plants were irrigated with Hoagland Complete Solution (Hoagland and Arnon 1950).

Chemicals

The DI-31 was produced in the CEPN Synthesis Laboratory (Faculty of Chemistry, Havana University of Cuba). The EBL, agar, ABA, DPI and SHAM were obtained from Sigma-Aldrich (USA), while the MES was obtained from Duchefa (Holland). Stock solutions of DI-31 (22.3 mM) and EBL (4.1 mM) were prepared in ethanol 50%, while the working solutions used for the experiments were diluted with distilled water (DW) and used immediately after preparation.

DI-31 dose–response assay in plates

The influence of DI-31 in growth promotion was corroborated in two independent experiments using 5-day-old wild-type plants (10 plants per treatment) transferred to plates supplemented with three different analogue doses. First, the fresh MS medium was sterilized by autoclaving at 120 °C, then the DI-31 was filter-sterilized and added when the medium had cooled to below 40 °C. The following treatments were defined: (i) MS medium as control, (ii) MS medium + DI-31 (22 nM; 0.01 mg/L), (iii) MS medium + DI-31 (112 nM; 0.05 mg/L) and (iv) MS medium + DI-31 (223 nM; 0.1 mg/L). The Col-0 plants were transferred to plates with the different DI-31 concentrations and growth for 5 days in a growth chamber. On the sixth day, plants were photographed and sampled. The following indicators were measured: (i) the number of leaves, (ii) foliar area, (iii) root length, (iv) root area and (v) biomass. Area and length measurements were performed digitally using the ImageJ Software (version 1.52) (Schneider et al. 2012). Fresh weight was determined immediately after collecting the plants. Dry weight was determined after drying the plants for 2 days at 70 °C. Biomass was calculated and expressed in terms of dry weight.

EBL and DI-31 dose–response assay in stomata

The effects of EBL and DI-31 in stomatal aperture (Gudesblat et al. 2009) were measured in 4-week-old wild-type plants (non-flowered). Epidermal peels were placed in wells plates with 500 µL of a 10:10 buffer solution (10 mM KCl and 10 mM MES-KOH, pH 6.15) under the normal culture conditions for 2 h. Then, treatments were applied, and the epidermises were incubated for an additional 1.5 h. The following treatments were defined: (i) untreated buffer solution as opening control, (ii) buffer solution + ABA (20 µM) as closing control, (iii) serial dilutions of EBL and (iv) DI-31 (0.1; 0.5; 1; 5; 10 and 20 µM). The opening of 40 stomata per treatment was measured at 400X in two independent experiments.

EBL and DI-31-mediated oxidative burst

The effect of EBL (1 µM) and DI-31 (2.23 µM) applications in superoxide radical production was measured in an independent experiment using 3-week-old wild-type seedlings. Plants grown in Grow Mix MULTIPRO were sprayed with DW, DI-31 and EBL. Three plants were collected for each treatment and harvest timing (total of 45 plants). The oxidative burst was assessed by NBT (Nitroblue tetrazolium) staining protocol (Doke 1983). After 3, 6, 12, 24, and 48 h of DI-31, EBL or DW treatments, the sixth and seventh fully expanded leaves (counting from the youngest) were detached and immersed in NBT solution, submitted twice to a 1-min vacuum (100 mm Hg), kept in the dark for 1 h, washed with 96% ethanol, clarified with lactic acid/glycerol/H_2O (3:3:4 v/v) and then photographed. The percentage of blue formazan spots formed over time was digitally quantified using the QUANT Software (version 1.0.2) (do Vale et al. 2003).

EBL and DI-31 effects under drought

The effects of EBL (1 µM) and DI-31 (2.23 µM) applications in wild-type plant growth/ water–management and stress-response under drought were assessed in two independent experiments using 3-week-old plants grown in Grow Mix MULTIPRO. Seedlings were grouped in the following treatments: (i) well-irrigated plants + DW as control, (ii) well-irrigated plants + DI-31, (iii) well-irrigated plants + EBL, (iv) drought-stressed plants + DW, (v) drought-stressed plants + DI-31 and (vi) drought-stressed plants + EBL. At the beginning of the experiment, the irrigation was withheld, and EBL and DI-31 were sprayed on the entire foliar region. The drought was maintained for 8 days, and whole plants were sampled at 0, 2, 4, and 8 days after stress and compounds application. Ten plants were used for each treatment and harvest timings (a total of 240 plants). Changes in the foliar area were non-destructively measured using ImageJ Software (version 1.52) (Schneider et al. 2012). The fresh, turgor and dry weights were determined in half of the plants collected per treatments and time to calculate the relative water content (RWC) (Weatherley 1950) and the amount of biomass produced in terms of dry weight (van Halsema and Vincent 2012). Fresh weights were determined immediately after collecting the plants, whereas dry weights were measured after drying the plants for 5 days at 70 °C. The rest of the sampled plants were ground under liquid nitrogen and used for physiological and biochemical determination. A uniform extraction (Liu et al. 2010) and protein content quantification (Bradford 1976) were made. Then, the activities of superoxide dismutase (SOD, EC 1.15.1.1) (Li 2012), catalase (CAT, EC 1.11.1.6) (Chance and Maehly 1955), ascorbate (APX, EC 1.11.1.11) (Nakano and Asada 1987) and phenol (POX, EC 1.11.1.7) (Kar and Mishra 1976) peroxidases were measured. The content of total chlorophylls (Porra 2002), carotenoids (Riemann 1978), malondialdehyde (MDA) (Hodges et al. 1999), and free proline (Bates et al. 1973) were assessed. Spectrophotometric readings were triplicated.

DI-31/ABA cross-talk in drought-response regulation

The effect of DI-31 in plant antioxidant activity under drought was evaluated in wild-type and the ABA-mutants *pp2ca-1* (high production of endogenous ABA) and *SnRK2.6/ost1* (reduced ABA synthesis). An independent experiment was conducted using 3-week-old seedlings grown in Grow Mix MULTIPRO. Plants were grouped in the following treatments: (i) well-irrigated plants + DW as control, (ii) well-irrigated plants + DI-31 (2.23 µM), (iii) drought-stressed + DW and (iv) drought-stressed plants + DI-31 (2.23 µM). Drought and DI-31 treatments and the sampling procedure are described in the previous section: "*EBL and DI-31 effects under drought*". The activity of SOD, CAT, APX, and POX enzymes and the accumulation of MDA and free proline was assessed 4 days after treatments. Ten plants per genotype and treatment were sampled (a total of 120 plants).

DI-31/ABA interactions in stomatal movement

The effect of specific inhibitors in DI-31 and ABA-mediated stomatal closure was measured in two experiments using 4-week-old wild-type plants (non-flowered) and following the procedures described in the section "EBL and DI-31 dose–response assay in stomata". The Diphenyleneiodonium (DPI) and Salicylichydroxamic acid (SHAM) were used as NADPH-oxidases and cell-wall peroxidases inhibitors. The defined treatments

were: (i) untreated buffer solution as opening control, (ii) buffer solution + ABA (20 µM) as closing control, (iii) buffer solution + DI-31 (10 µM) as BR control, (iv) DPI (10 µM) + ABA (20 µM), (v) DPI (10 µM) + DI-31 (10 µM), (vi) SHAM (2 mM) + ABA (20 µM) and (vii) SHAM (2 mM) + DI-31 (10 µM). Epidermal peels were placed in wells plates with buffer solution under the normal culture conditions for 2 h. For the ABA and BR controls, the epidermises were incubated for another 1.5 h after the compound's application. The DPI and SHAM were applied for the inhibitor's treatments, and the epidermises were incubated for 30 min before DI-31 and ABA application. A total of 40 stomata per treatment were measured at 400X.

Quantitative RT-PCR

The effect of DI-31 (2.23 µM) on the relative expression of the stress-responsive genes *AtP5CS1* (DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1; AT2G39800), *AtRAB18* (DROUGHT-INDUCED 8; RESPONSIVE TO ABA 18; AT5G66400), *AtRD22* (RESPONSIVE TO DESICCATION 22; AT5G25610), and *AtRD29A* (RESPONSIVE TO DESICCATION 29A; AT5G52310) was assessed in two independent experiments using 7-day-old wild-type seedlings stressed with mannitol (300 mM) in MS liquid medium. The defined treatments were: (i) untreated MS medium as control, (ii) MS medium + DI-31, (iii) MS medium + mannitol and (iv) MS medium + mannitol + DI-31. After 2 days of acclimatization in the liquid medium, plants from the corresponding treatments were transferred to MS liquid medium supplemented with mannitol. After 1 h, the DI-31 was added, and 21 plants per treatment were collected after 1, 3, and 24 h. Samples were immediately ground in liquid nitrogen and stored at − 85 °C until RNA extraction. RNA purification was performed by TRIzol (TRI reagent) method (Rio et al. 2010). The extracted RNA quality was determined spectrophotometrically, and its integrity was confirmed by electrophoresis in Agarose gel (1%). Quantitative RT-PCRs were performed with primers designed for the ELONGATION FACTOR 1 (*ef1; At1g18070*) *gene* coding as an internal reference. The relative expression values were automatically generated by the iCycler iQTM associate software (Real-Time Detection System Software, version 3.0). Relative expression was calculated by ΔΔCt method (Livak and Schmittgen 2001). Data were reported as fold relative expression transformed to $\log_2$. Primers were designed in Primer3 4.1 program (Koressaar and Remm 2007; Untergasser et al. 2012) and are provided in the Supplementary Table 1.

Statistical analysis

Statistical analysis was realized using InfoStat software (Di Rienzo et al. 2018). Data describing stomatal apertures were subjected to a one-way ANOVA. Statistical analyses for ABA-mutants' experiments were performed over the raw data, and results were expressed as the natural logarithm (ln) of the ratio stressed/control. Gene expression data were analyzed by Duncan's Multiple Range Test (DMRT). The remaining data were analyzed through ANOVA with post hoc contrast by Tukey's test. Means were considered significantly different at $p < 0.05$ and presented ± SE (standard error).

Results

Dose-dependent growth

To assess the overall growth effects of the DI-31 application, we measured the number of leaves, foliar area, root length and area, and biomass increase of wild-type plants treated with three different doses (Fig. 1). Results showed a significant dose-responsive effect on the plant phenotype (Fig. 1a) and all the parameters evaluated (Fig. 1b–f) that increased proportionally to the DI-31 applied doses. Plants treated with the two highest concentrations (112 and 223 nM) respectively increased their number of leaves by ~ 30% and ~ 37% (Fig. 1b), and their foliar area in ~ 59% and ~ 62% (Fig. 1c). When analyzing the root length and area (Fig. 1d, e) and the biomass produced (Fig. 1f), no statistical differences were found between the two highest doses of DI-31. However, plants treated with the maximum concentration showed a more homogeneous root growth, with numerous lateral roots.

Stomatal movement

To corroborate whether DI-31 stimulates respiration in a BR-like manner, we performed stomatal closure trials comparing the analogue effects with the EBL ones in wild-type plants (Fig. 2). The stomatal movement results demonstrate that EBL and DI-31 regulate stomatal closure in a very similar dose-responsive manner (Fig. 2a). Here, BR doses above 0.5 µM significantly closed stomata; however, as expected, neither EBL nor DI-31 treatments statistically exceeded the closure induced by ABA treatment.

Oxidative burst

To comparatively assess the DI-31 and EBL effects on respiratory burst, an assay was performed using wild-type plants (Fig. 2b, c). Here, we observed that EBL triggered

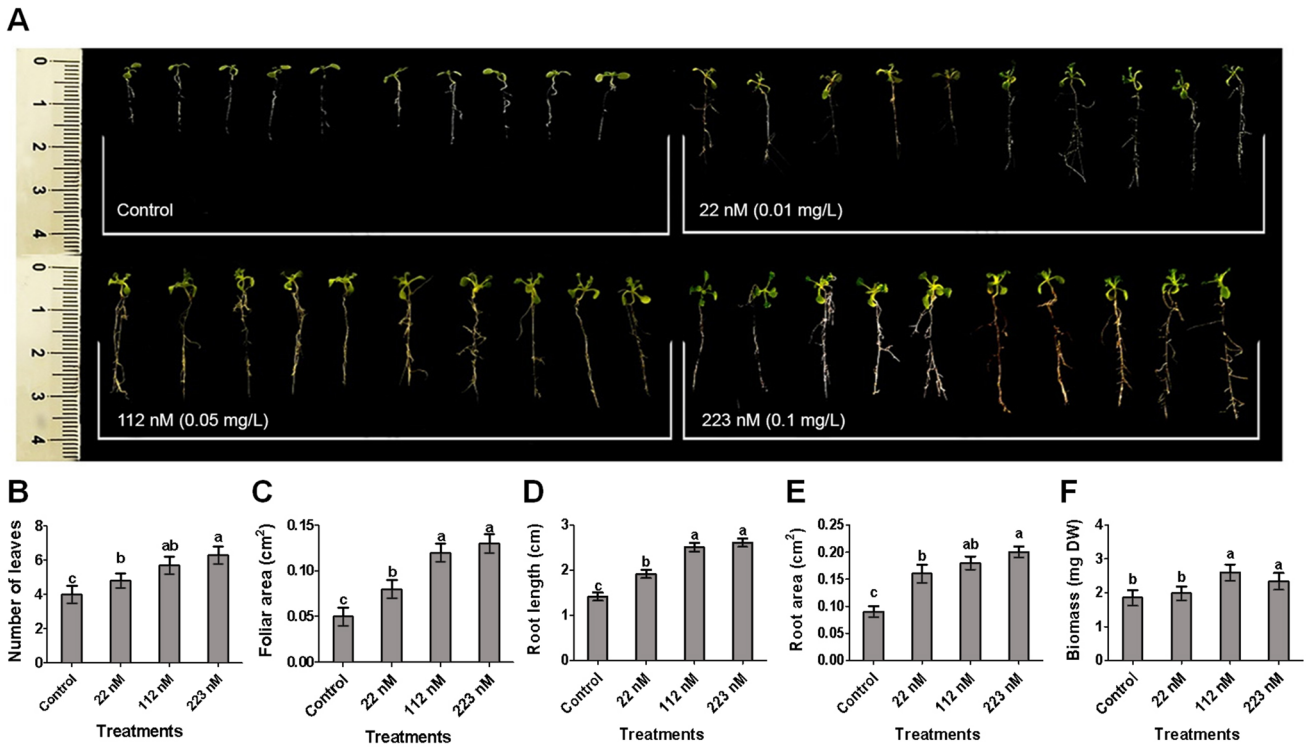

Fig. 1 Effect of three doses of DI-31 in *A. thaliana* wild-type plants growth-promotion. **a** Representative image of plants growing in a dose-responsive manner. Morphological parameters such as **b** number of leaves, **c** foliar area, **d** root length, **e** root area and **f** biomass were measured in 5-day-old plants grown in plates with MS medium (Control) and supplemented with three different doses of DI-31: 22 nM (0.01 mg/L); 112 nM (0.05 mg/L); 223 nM (0.1 mg/L) for 5 days. Ten biological samples per treatment were collected and analyzed. Data are presented in means ± SE of two independent experiments (n = 40). Different letters indicate significant differences, $p < 0.05$, ANOVA with post hoc contrasts by Tukey's test

the oxidative burst after 3 h, while the DI-31 did it after 6 h (Fig. 2b). Moreover, we observed a noticeable difference between the rates of blue formazan formation in the leaves treated with BRs (Fig. 2c). Here, the DI-31 application increased the formazan generation a ~ 53% and ~ 46% more than EBL after 24 and 48 h, respectively.

Drought-response

The DI-31 and EBL action in drought-response induction was comparatively analyzed in trials using wild-type plants (Fig. 3). Here, we decided to sprayed EBL and DI-31 at 1 µM and 2.23 µM, respectively, being concentrations that induced partial stomatal closure (~ 32%). The wild-type stressed plants exhibited changes in the rosette morphology (Fig. 3a) and physiological parameters of growth/water-management like biomass (Fig. 3b), foliar area (Fig. 3c) and RWC (Fig. 3d) over time. BRs application, as expected, stimulates biomass production and foliar area in well-watered plants. After 8 days of drought, only plants treated with distilled water (DW) showed elongated and lanceolate leaves as typical drying symptoms, as well as lower biomass, leaf area, and a drop in RWC of ~ 29%. In comparison, stressed plants treated with BRs exhibited no leaf morphology alterations and less biomass and leaf area penalties. Moreover, plants treated with EBL under drought showed a ~ 22% RWC reduction, while those treated with DI-31 presented a ~ 16% fall.

On the other hand, we also determined several physiological and biochemical markers associated with stress-response in the wild-type plants submitted to drought and BRs treatments (Fig. 4). Here, stressed plants treated with DW increased SOD, CAT, APX, and POX activities over time (Fig. 4a–d), also showing a significant chlorophyll loss (Fig. 4e), and accumulation of carotenoids (Fig. 4f), MDA (Fig. 4g), and proline (Fig. 4h). BRs also stimulated the antioxidant response, independently of drought effects, although the EBL and DI-31 effects significantly differed. Under well-irrigation conditions, the BRs foliar applications increased SOD, CAT, APX, POX activities, chlorophyll, carotenoids, and free proline synthesis. However, under drought, DI-31-treated plants showed higher SOD activity, while EBL ones exhibited the lowest. Concerning CAT, APX, and POX activities under stress, the EBL treatment showed a stimulating effect, whereas the DI-31 application decreased CAT and APX activities and did not affect POX. The chlorophyll

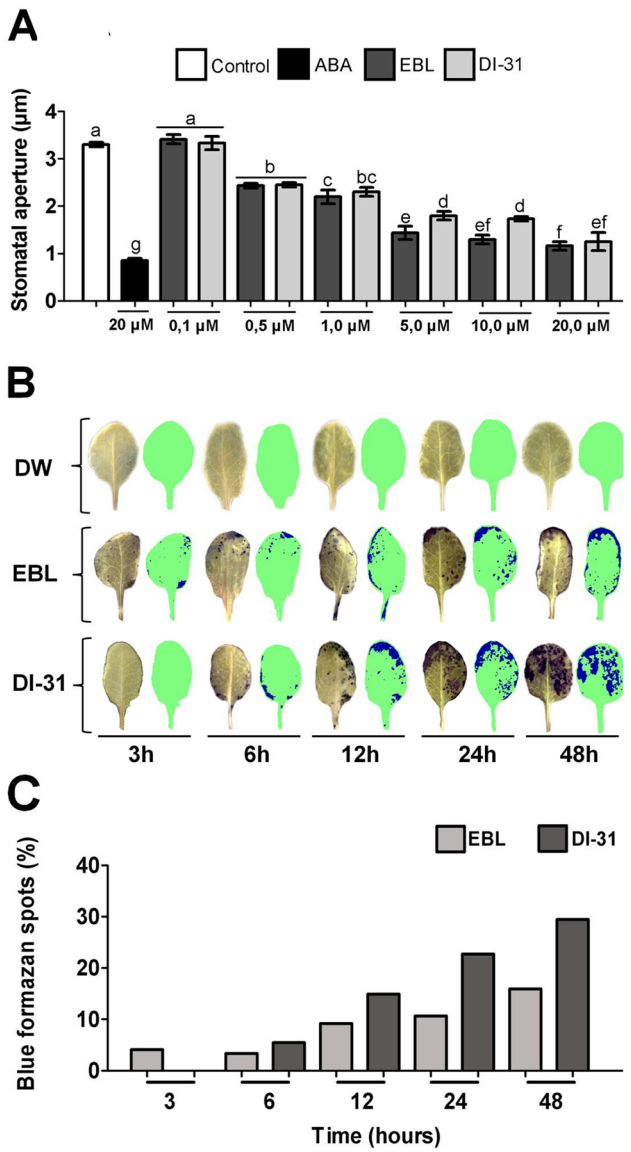

Fig. 2 Effect of DI-31 and 24-epibrassinolide (EBL) in *A. thaliana* wild-type plants stomatal closure and oxidative burst. **a** Stomatal aperture of 4-week-old plants (non-flowered) treated with KCl-MES-KOH buffer as opening control, ABA (20 µM) as closing control and serial dilutions of EBL and DI-31 (0.1; 0.5; 1; 5; 10 and 20 µM). **b** Scanned and colour-segmented image of the oxidative burst; and **c** the percentage of blue formazan spots formed in 3-week-old plants sprayed with distilled water (DW), EBL (1 µM) or DI-31 (2.23 µM). For the stomatal closure assay, the opening of 40 stomata per treatment was measured in two independent experiments (n = 560). For the oxidative burst assay, three biological samples per treatment were collected and submitted to NBT staining after 3, 6, 12, 24, and 48 h of treatments (n = 45). Data are presented in means ± SE. Different letters indicate significant differences, $p < 0.05$, one-way ANOVA

loss under stress was lower in DI-31 treated plants compared to EBL ones. Regarding carotenoids accumulation, drought-stressed plants treated with DI-31 showed the highest levels while the EBL ones exhibited the lowest. Both BRs attenuated the MDA accumulation under drought, although DI-31-treated plants showed the lowest values. Besides this, plants treated with DI-31 showed the highest accumulation of free proline under stress.

The DI-31 and EBL showed differences in stress-response indicators regulation and also in the action time. Here, EBL seems to affect plant physiology until the fourth day after application, while the DI-31 did it until the eighth day.

DI-31/ABA cross-talk regulation

During drought-response trials, we detected that DI-31, unlike EBL, decreased the action of ABA-regulated antioxidants like CAT and APX peroxidases whereas increased the accumulation of carotenoids. Thus, we decided to investigate possible DI-31/ABA interactions through drought-response experiments with the ABA mutant's *pp2ca-1* (high production of endogenous ABA) and *SnRK2.6/ost1* (reduced ABA synthesis). Also, stomatal assays with specific inhibitors of ABA-mediated paths and relative expression analysis of ABA/stress-responsive genes were performed.

When analyzing the drought/control ratio of the indicators evaluated in plants sprayed with DW and DI-31, we found substantial differences between wild-type plants and ABA mutants after 4 days of treatment (Fig. 5). Under DW treatment, drought/control ratios indicated that stress significantly increased SOD activity in wild-type plants (Fig. 5a). In contrast, the higher increases in CAT and APX activities were quantified in *pp2ca-1* plants (Fig. 5b, c). The lowest increments of SOD, CAT, and APX were detected in *SnRK2.6/ost1* mutants. Concerning the POX enzyme, drought only increased its activity in wild-type and *pp2ca-1* plants (Fig. 5d). Moreover, MDA and free proline accumulation were higher in ABA-mutants (Fig. 5e, f). About DI-31 effects under drought, 4 days after application, wild-type and *pp2ca-1* plants showed higher increments in SOD activity. As expected, CAT, APX, and POX activities were reduced by DI-31 action in stressed wild-type plants. In *pp2ca-1* mutants, those enzymes activities were massively reduced due to the BR analogue compared to drought and DW-treated plants. Contrariwise, in stressed *SnRK2.6/ost1* plants, CAT, APX, and POX activities were increased by DI-31 treatment. The application of DI-31 reduced MDA production under drought. Here, wild-type plants showed the lowest levels, while the *pp2ca-1* plants exhibited the highest. Similarly, the free proline content under drought was also enhanced by DI-31 action, mainly in ABA-mutants.

On the other hand, we found substantial differences in the effect of NADPH oxidases and cell-wall peroxidase inhibitors on ABA and DI-31-mediated stomatal movement (Fig. 6). Here, ABA application closed the stomata of wild-type plants in a ~ 68%; yet, treatments with SHAM and DPI inhibited the ABA closure completely (for

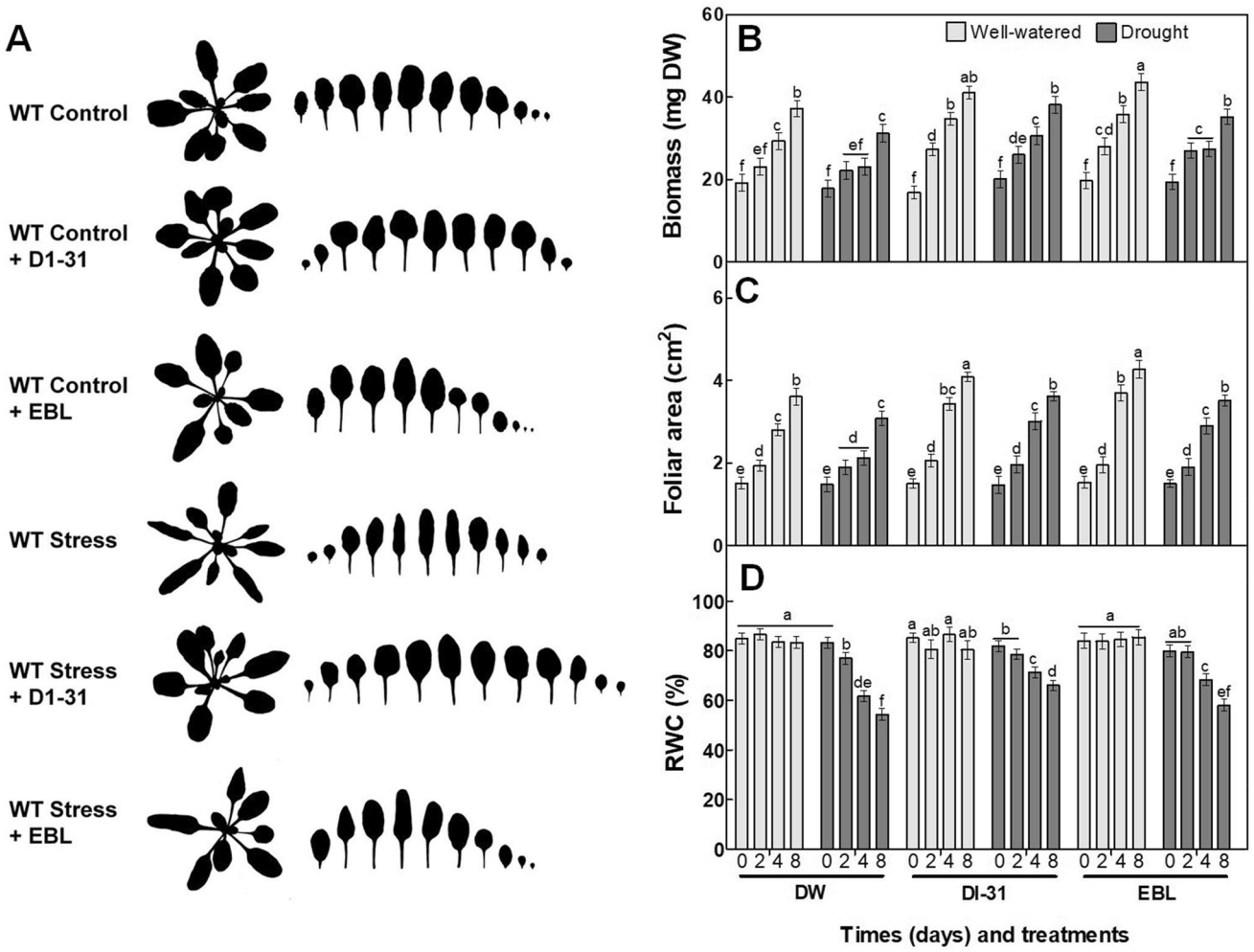

Fig. 3 Effect of DI-31 and 24-epibrassinolide (EBL) in *A. thaliana* wild-type plant growth and water–management under drought. **a** Scanned and colour-segmented image of well-watered and drought-stressed plants after distilled water (DW), DI-31 (2.23 µM), and EBL (1 µM) treatment. Physiological parameters such as **b** biomass, **c** foliar area and **d** relative water content (RWC) were assessed in 3-week-old plants sprayed with DW, DI-31 or EBL and submitted to 8 days of drought. Five biological samples per treatment were collected and analyzed at 0, 2, 4, and 8 days after stress and compounds application. Data are presented in means ± SE of two independent experiments (n = 240). Different letters indicate significant differences, $p < 0.05$, ANOVA with post hoc contrasts by Tukey's test

SHAM) and in a ~ 98% (for DPI) (Fig. 6a). Meanwhile, the DI-31 closed the stomata a ~ 48%, and treatments with SHAM and DPI inhibited the closure effect an ~ 18% and ~ 90%, respectively.

Finally, possible DI-31 effects in ABA/stress-responsive genes regulation were measured by qRT-PCR in wild-type plants submitted to stress induced by mannitol (Fig. 6b). The DI-31 significantly up-regulated the expression of the *AtP5CS1* stress-responsive gene over time. Meanwhile, the *AtRAB18*, *AtRD22,* and *AtRD29A* ABA/stress-responsive genes were up-regulated over time in mannitol-treated plants and repressed due to DI-31 action, with or without mannitol, where the greater repression was observed under combined mannitol and DI-31 treatments.

Discussion

BRs play a pivotal role in plant growth (Kaur and Pati 2019), participating in many developmental processes, such as cell elongation-division and assimilate translocation (Müssig 2005), increasing shoot fresh and dry weights, plant height, leaves size, and number (Anjum et al. 2011). This study corroborates that the DI-31, a BR functional analogue, increased *A. thaliana* biomass accumulation, leaves formation, and foliar area expansion in a dose-responsive manner; a result that confirms the analogue promoting effect in photosynthetic area development and growth rate. Interestingly, the DI-31 application

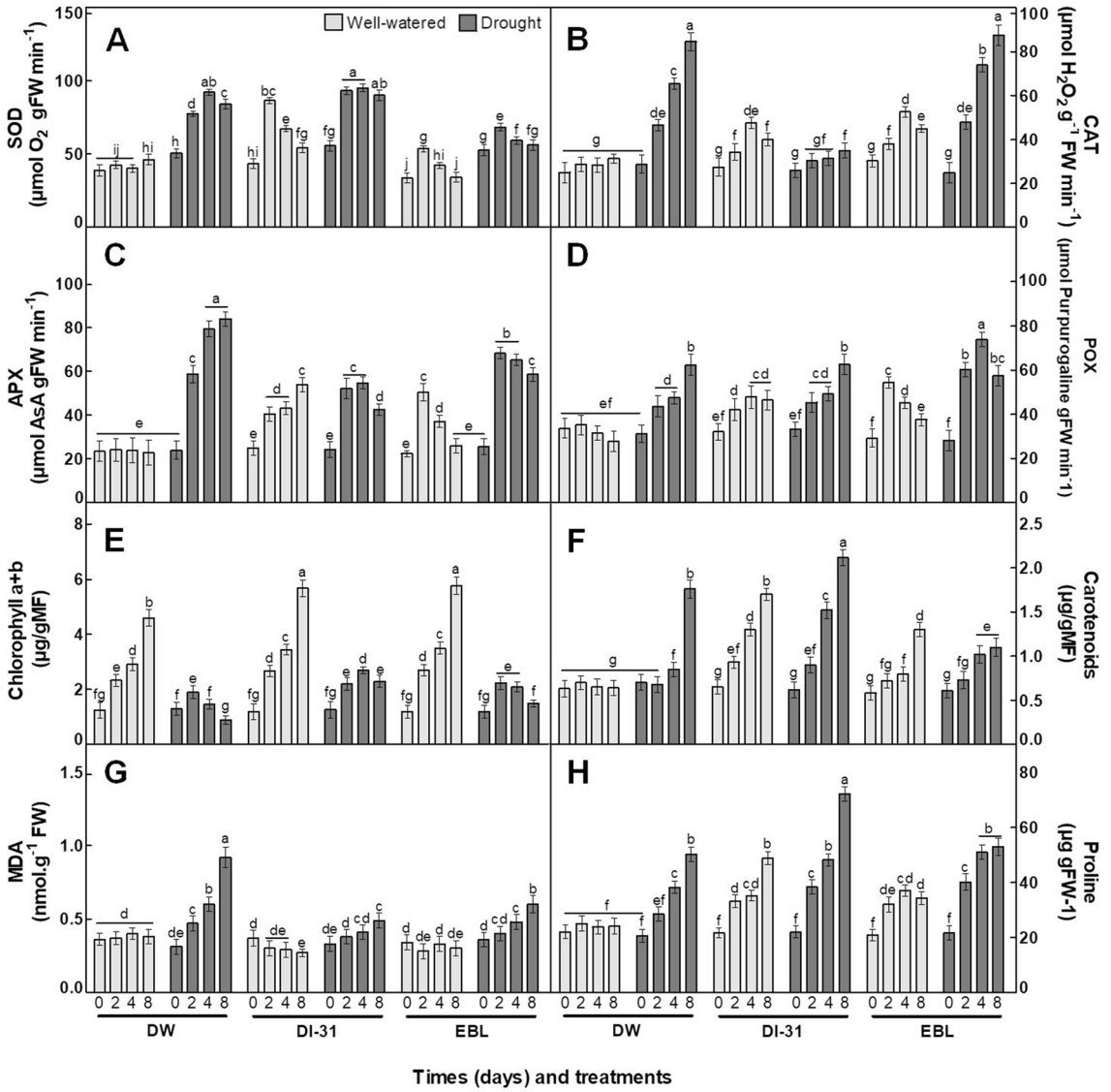

Fig. 4 Effect of DI-31 and 24-epibrassinolide (EBL) in the drought-response of *A. thaliana* wild-type plants. Physiological and biochemical parameters such as **a** superoxide dismutase (SOD) activity, **b** catalase (CAT) activity, **c** ascorbate peroxidase (APX) activity, **d** phenol peroxidase (POX) activity, **e** total chlorophyll content, **f** total carotenoids content, **g** malondialdehyde (MDA) content and **h** free proline content were measured in 3-week-old plants sprayed with distilled water (DW), DI-31(2.23 µM), or EBL (1 µM), and submitted to 8 days of drought. Five biological samples per treatment were collected and analyzed at 0, 2, 4, and 8 days after stress and compounds application. Data are presented in means ± SE of two independent experiments (n = 240). Different letters indicate significant differences, $p < 0.05$, ANOVA with post hoc contrasts by Tukey's test

also increased plants water-nutrient absorption capacity by enhancing the primary and lateral root's development. In this regard, several studies on *Arabidopsis* seedlings in the light and treated with higher concentrations of BRs showed an inhibited development of the primary root (Clouse et al. 1993; Szekeres et al. 1996; Müssig et al. 2003). However, roots responses to BRs applications are dose-dependent and tissue-specific (Nolan et al. 2020); thus, it is possible to observe a growth-promoting effect for subinhibitory concentrations and growth retardation in response to higher doses. In agreement, (Chaiwanon and Wang 2015) reported that *Arabidopsis* roots treated with Brassinolide (BL) initially showed an increased growth, which ceases later, under higher doses or prolonged exposure. These findings demonstrate that culture conditions, particularly the mode, concentration, and time of exposure to BRs, may explain the BR-mediated promotion/inhibition of root growth.

BRs also modulate many stress-responsive pathways (Kaur and Pati 2019), although the mechanisms through

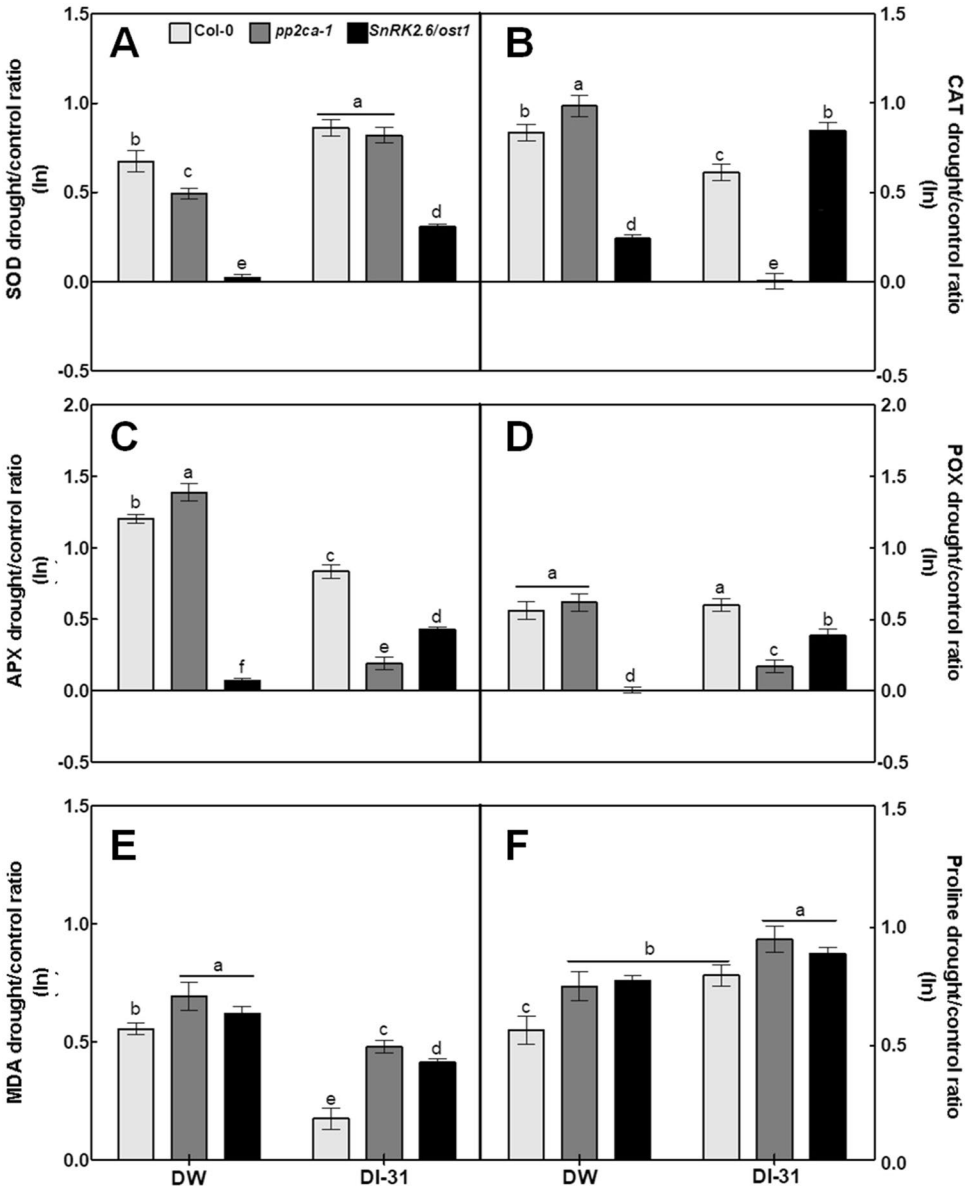

Fig. 5 Effect of DI-31 in *A. thaliana* wild-type and ABA-mutants *pp2ca-1* and *SnRK2.6/ost1* drought-response. Physiological and biochemical parameters such as **a** superoxide dismutase (SOD) activity, **b** catalase (CAT) activity, **c** ascorbate peroxidase (APX) activity, **d** phenol peroxidase (POX) activity, **e** malondialdehyde (MDA) content and **f** free proline content were measured in 3-week-old plants sprayed with distilled water (DW) or DI-31 (2.23 µM), submitted to 4 days of drought. Ten biological samples per treatment were collected and analyzed 4 days after stress and compounds application. Data are presented as the natural logarithm (ln) of the ratio stressed/control of two independent experiments (n = 120). Different letters indicate significant differences, $p < 0.05$, ANOVA with post hoc contrasts by Tukey's test

which they confer adaptation to abiotic stresses remain partially elusive. It is known that BRs regulate the metabolism of plant oxidation radicals, mediating their responses to stresses, such as drought, salinity, and heat (Tang et al. 2016). BR analogues can stimulate reactive oxygen species (ROS) generation, second messengers in several processes like respiration, photosynthesis, and stress tolerance/resistance (Tripathy and Oelmüller 2012; Tang et al. 2016). To confirm those observations, we evaluated the potential effect of DI-31 on plants' stomatal movement and respiratory burst. Our findings demonstrate that the analogue induced dose-responsive stomatal closure and triggered the superoxide radicals' (O_2^-) formation over time, similar to the EBL-mediated. Although, when analyzing the O_2^- accumulation induced by DI-31, we observed a delayed onset of the respiratory burst but a higher rate of O_2^- generation over time compared with EBL. These findings suggest that both BRs caused physiological responses that differ in magnitude and duration.

Regarding stomatal movement, the DI-31 can close the stomata at the same level of EBL, only at higher concentrations; hence, lower levels of the analogue are likely to induce partial stomatal closure. The stomata mainly regulate CO_2 exchange and transpiration (Shi et al. 2015); thus, the DI-31 could modulate plant gas exchange and water loss. Plants tend to regulate their stomatal aperture for acclimatization under stress conditions (Kaur and Pati 2019); therefore, the DI-31 effect could be beneficial, especially under unfavourable environments, where plant

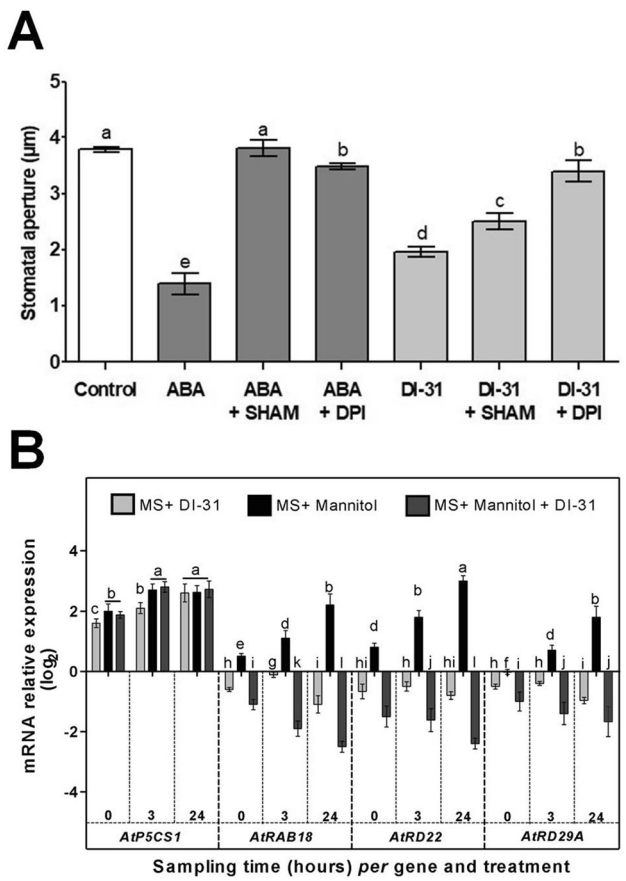

Fig. 6 Effect of DI-31 in ABA-mediated stomatal closure and the relative expression of ABA/stress-responsive genes in *A. thaliana* wild-type plants. **a** Stomatal aperture of 4-week-old plants (non-flowered) treated with KCl-MES-KOH buffer as opening control, ABA (20 µM) as closing control, DI-31 (10 µM) and combine applications of DPI (10 µM) and SHAM (2 mM) inhibitors. **b** Relative expression (log_2 transform) of *AtP5CS1* (AT2G39800), *AtRAB18* (AT5G66400), *AtRD22* (AT5G25610) and *AtRD29A* (AT5G52310) genes in 5-day-old plants. For stomatal closure assay, the epidermal peels were incubated in buffer solution under the normal culture conditions for 2 h; for ABA and BR controls, the epidermises were incubated for another 1.5 h after the compound's application, while for the inhibitor's treatments, the epidermises with DPI or SHAM were incubated for 30 min before DI-31 and ABA application. Here, the opening of 40 stomata per treatment was measured, and data is presented in means ± SE of two independent experiments (n = 560). For the gene expression assay, plants were acclimatized in liquid MS medium (control) for 2 days, treated with mannitol and incubated for 1 h, then the DI-31 (2.23 µM) was added. 21 biological samples per treatment were collected at 1, 3, and 24 h after the compound application. Graph represents relative expression ratio of each gene in MS medium + DI-31, MS medium + mannitol and MS medium + mannitol + DI-31 treatments, respect to MS medium control treatment. Data obtained from qRT-PCR were analyzed using the ΔΔCt method and normalized using the *ef1* gene. Different letters indicate significant differences, $p < 0.05$, one-way ANOVA (stomatal closure) and DMRT test (qRT-PCR)

transpiration could be significantly reduced without necessarily having a large gas exchange penalty.

Among the abiotic stresses, water deficit has the most severe effect in worldwide agriculture and shows detrimental effects on plants' stomatal morphology, photosynthesis, respiration rates, and oxidation–reduction balance (Chai et al. 2016). In response, plants can activate/increase a major defence mechanism composed, among others, by enzymatic antioxidants (Sajedi et al. 2011). Consequently, in many crops, a high antioxidant capacity has been linked with an increased tolerance to environmental stresses (Sharma et al. 2012).

One of the first effects of drought is the significant and transient increase of cell damage by ROS (Van Oosten et al. 2016). To tightly control ROS levels, cells regulate a scavenger system that comprises non-enzymatic and enzymatic antioxidants like SOD and a wide range of peroxidases, among which stands APX, CAT, and POX enzymes (Sharma et al. 2012). Foliar applications of BRs, natural or analogues, up-regulate the plant antioxidant activity, protein synthesis, compatible solutes accumulation, and change the level of other phytohormones (Liu et al. 2017). Hence, BR's ability to mitigate oxidative injuries is a highly desirable trait that brings a huge prospect for agriculture (Gill et al. 2017; Hussain et al. 2020). Here, the DI-31 showed similar effects to EBL in plant growth regulation under drought, maintaining biomass production, and foliar development. However, the analogue regulates water management more effectively than EBL, probably due to their partial stomatal closure effect. Moreover, even if both compounds, EBL and DI-31, alleviated drought effects, the DI-31 showed a better regulation of plant stress-response mechanisms. Compared with EBL, the analogue decreased chlorophylls loss and increased carotenoid and free proline production more efficiently, preventing large MDA amounts.

Up to this point, our results showed that the DI-31 efficiently regulates plant water management and stress-response under drought, having a more effective and longest-term action than EBL. However, the DI-31 action on some components of the antioxidant system, carotenoids, CAT, APX, and POX enzymes, significantly differed from the effects generated by drought and EBL treatments. Under optimal watering conditions, the DI-31, like EBL, regulated O_2^-, hydrogen peroxide (H_2O_2), and phenols levels. Conversely, under water deficit, where large amounts of H_2O_2 and phenols are generated, the analogue precluded these compounds' degradation via the Asa-GSH cycle, CAT and POX. These results indicate that the DI-31 regulates H_2O_2 accumulation through alternative pathways, suggesting a possible interaction between the analogue and these enzymes' regulatory mechanisms, known as AREB/ABF (ABA-RESPONSIVE BINDING PROTEIN/ FACTOR) (Kavitha et al. 2008). In agreement, some authors report

that APX, POX, and CAT coding genes were up-regulated by BR deficiency in *A. thaliana Det2* BR-mutant (Müssig et al. 2002; Goda et al. 2002).

Addressing whether DI-31 overlaps or antagonizes with ABA-responsive mechanisms, we found that the analogue drastically reduced the APX, CAT, and POX activities in the *pp2ca-1* mutant, with high production of endogenous ABA (Kuhn et al. 2006). Contrarily, in *SnRK2.6/ost1* plants, with reduced ABA synthesis, especially under abiotic stress (Umezawa et al. 2009), the DI-31 stimulated these peroxidases' activity under drought. These results suggest a possible DI-31/ABA antagonistic cross-talk, although such interaction does not affect the stress-response quality. In agreement with our finding, some authors reported that negative regulators of BRs signalling such as the BIN2 kinase (BRASSINOSTEROID-INSENSITIVE2) activates positive regulators of ABA pathway like SnRK2 kinases (Wang et al. 2018).

Furthermore, we evaluated the DI-31 action in mechanisms controlled mainly by ABA, as the activation of the stomatal closure and stress-responsive genes. Although the BR/ABA stomatal regulation remains partially unclear, ABA plays a significant role (Daszkowska-Golec and Szarejko 2013) in activating ROS-producing NADPH-oxidases (Xia et al. 2014). Meanwhile, BRs like EBL control stomatal closure through the combined action of NADPH-oxidases and cells-wall peroxidases also ABA-regulated (Xia et al. 2009, 2014). Here, DPI and SHAM's application, which inhibits the O_2^- (Kwak et al. 2003) and H_2O_2 (Khokon et al. 2011) generation, allowed us to identify that DI-31 regulates stomatal closure through ROS-producing paths, strongly dependent on O_2^- and partially independent of the H_2O_2 produced by the cells-wall peroxidases. This result suggests that DI-31 regulates O_2^- levels through pathways also controlled by ABA, while the H_2O_2 production seems to be regulated in a partially ABA-independent manner. In agreement, in a previous study in *Brassica napus* plants submitted to salt stress, the authors reported that EBL influences stomatal movement partly via an ABA-alternative respiratory pathway (Derevyanchuk et al. 2017).

Under drought, two major transcriptional networks are activated in *A. thaliana*: an ABA-dependent signalling path and an ABA-independent one (Van Oosten et al. 2016). It is known that PYR/PYL/RCAR receptors, combined with SnRK2 and PP2Cs proteins, and AREB/ABF transcriptional factors (TFs), formed the core of the ABA-dependent pathway (Valliyodan and Nguyen 2006). Altogether, directly control the expression of several genes like the RAB (RESPONSIVE TO ABA), RD (DESICCATION/DEHYDRATION), and several peroxidases genes that have ABRE/ABF cis-elements in their promoter regions (Yoshida et al. 2010; Singh and Laxmi 2015). Thus, to corroborate if the DI-31 affects specifically ABRE/ABF-regulated elements, we assessed the analogue's effect on a small gene set that comprised the ABRE/ABF-independent *AtP5CS1* gene and the ABRE/ABF-dependent *AtRAB18*, *AtRD22* and *AtRD29A* genes, widely known as ABA/stress-responsive markers (Cao et al. 2006).

As expected, the DI-31 up-regulated the stress-inducible *AtP5CS1* gene, involved in the first rate-limiting step of proline synthesis under drought (Signorelli and Monza 2017). This result agrees with our findings and those reported in several crops treated with different EBL concentrations (Tanveer et al. 2019). However, even if ABRE/ABF cis-elements do not regulate the *AtP5CS1* gene expression, it is known that, under stress, it is controlled by endogenous ABA (Takahashi et al. 2020). Meanwhile, a strong repression effect was detected when observing the DI-31 action on the ABRE/ABF-dependent genes *AtRAB18*, *AtRD22* and *AtRD29A*. The *AtRAB18* gene is induced by ABA accumulation, constituting an ABA signalling and synthesis indicator, especially under drought (Cao et al. 2006). Meanwhile, *AtRD22* and *AtRD29A* are drought-inducible genes involved in the ABA-mediated response to dehydration and mannitol (Shinozaki and Yamaguchi-Shinozaki 1997; Kasuga et al. 1999; Cao et al. 2006). Our results strongly suggest that DI-31 negatively interacts with specific ABRE/ABF-responsive elements, explaining the peroxidases inactivation detected during the drought-response assays. Moreover, the repression of these ABA-inducible genes could indicate, to some extent, the occurrence of DI-31/ABA antagonistic interactions under drought. Findings that agree with several studies that describe the effect of key downstream elements of BRs signalling pathway like BES1 TF (bri1-EMS-SUPPRESSOR1) that repress drought-inducible genes regulated by ABA accumulation (Wang et al. 2018; Nolan et al. 2020).

The role of ABA/BR cross-talk in stress tolerance regulation remains partially unclear. Some authors support the hypothesis that BRs and ABA control plant stress-responses in a combined manner (Haubrick et al. 2006; Zhang et al. 2011; Ha et al. 2018), while others reported antagonistic interactions (Steber and McCourt 2001; Seo et al. 2009; Xue et al. 2009; Shang et al. 2016). Previous studies have shown that under stress, these hormones synergically controlled many processes such as signal output and gene expression regulation (Nemhauser et al. 2006; Zhang et al. 2009), suggesting that BRs may enhance oxidative stress tolerance via ABA accumulation (Zhang et al. 2011). However, recent studies demonstrate the antagonistic interaction between these hormones (Wang et al. 2018; Nolan et al. 2020). It is known that, in the BR signalling pathway, the ABA/BR regulation occurs downstream the BRI1 receptor complex by several TFs such as BES1 or the AREB/ABF ABI3 or ABI5 (Ryu et al. 2014; Yang et al. 2016).

Our results showed that DI-31, unlike EBL, particularly stimulates carotenoids gathering during drought. These are

well-known precursors of ABA synthesis (Ruiz-Sola and Rodríguez-Concepción 2012); thus, its accumulation due to DI-31 action could positively modulate ABA synthesis and, therefore, increase their endogenous levels. However, our findings also showed that even if DI-31 and ABA shared some regulatory pathways during stomatal closure control, the analogue cancelled the action of genes and proteins regulated by specific AREB/ABF TFs. Hence, it is possible to consider a partially antagonistic interaction between DI-31 and ABA on stress-response regulation.

Another feature that must be highlighted is the DI-31 effect on plant water management and stress-response, which, compared to EBL, was stronger and more durable. This result increased the practical use of DI-31, but the compound's high stability could also represent a challenge for environmentally-friendly agriculture. In this regard, previous ecotoxicological studies reported the BIOBRAS 16 innocuity in mammals, fish (*Oreochromis niloticus* L.), mosquitos (*Aedes aegypti* L.) and a cladoceran (*Daphnia pulex* L.), also being considered non-ecotoxicant for agricultural purposes after trials with several crops such as garlic, rice, onion, corn, tomato and potato (Pérez-Davison et al. 2002).

The most active BR discovered is BL (Tang et al. 2016; Souri et al. 2020; Bajguz et al. 2020); yet, its low content in natural sources and expensive isolation/synthesis precludes large-scale applications. Consequently, the most widely used BR is EBL, a synthetic stereoisomer of BL (Moreno-Castillo et al. 2018). Numerous studies corroborate the effectiveness of the exogenous application of EBL in drought tolerance enhancement (Kagale et al. 2007; Anjum et al. 2011; Lima and Lobato 2017; Ha et al. 2018; Tanveer et al. 2019). However, EBL production is also cost-limited (Liu et al. 2017); thus, it is not practical for agricultural uses. Consequently, the use of more affordable and readily available analogues has great practical significance. Among BR's analogues with higher potential activity, those possessing spirostanol structure are more economically achieved (Liu et al. 2017). Hence, the DI-31, as a cheaper molecule with more effective and longer-term activity than EBL, constitutes an environmentally friendly alternative to overcoming drought-derived impacts.

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Author contributions All authors contributed to the study conception and design. LSP-B designed and performed the experiments, compiled the data and first draft of the manuscript. LT performed the stomata analysis. APC and JLG-O conceived the project; FC-M, EMP and YC-G designed and supervised the experiments. All authors commented on previous versions of the manuscript, read and approved the final manuscript.

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