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Research article

Enhanced brassinosteroid signaling intensity via *SlBRI1* overexpression negatively regulates drought resistance in a manner opposite of that via exogenous BR application in tomato

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ABSTRACT

Brassinosteroids (BRs) regulate plant growth and stress responses. BRASSINOSTEROID-INSENSITIVE 1 (BRI1) is a BR receptor that perceives BRs and subsequently activates BR signaling. However, how BR contents and *BRI1* expression levels affect the drought resistance of tomato requires further investigation. Here, we exogenously applied 24-epibrassinolide (EBR) and brassinazole (Brz) to tomato plants and generated different transgenic tomato *SlBRI1* overexpression lines to study the drought stress response. Our results showed that EBR application 3 days before drought stress increased the contents of BRs and decreased abscisic acid (ABA) and reactive oxygen species (ROS), after which stomatal aperture and drought resistance eventually increased. Brz application reduced the drought resistance. Astonishingly, overexpression of *35S:SlBRI1*, which increased BR signaling intensity, led to slightly improved contents of ABA and ROS and ultimately reduced both stomatal aperture and drought resistance. Moreover, plants expressing *SlBRI1* driven by a stress-inducible promoter (*Atrd29A*) also exhibited reduced plant drought resistance. In all cases, enhancing the BR signaling intensity reduced antioxidant enzyme activity and reduced the expression of drought stress-related genes, ultimately compromising the drought resistance. Additionally, *SlBRI1* mutants with altered brassinolide sensitivity (*abs*), which was weak BR signaling, exhibited significantly increased drought resistance. Therefore, our results reveal that BR contents positively regulated tomato drought resistance and that BR signaling intensity via BRI1 was negatively related to the drought resistance. These imply that the increased drought resistance in response to BRs is a newly discovered BR signaling branch that is located downstream of BRs and that differs from that of BRI1.

1. Introduction

Drought stress is an extensive environmental adversity that affects the growth, yield and product quality of plants mainly by reducing plant photosynthetic capacity and water uptake (Talbi et al., 2015). Drought stress usually increases the production rate of reactive oxygen species (ROS), which can subsequently cause membrane lipid peroxidation, enzyme deactivation, and electrolyte leakage (Talbi et al., 2015). Plants have developed various self-defense systems that aid in resistance against drought-induced damage. To help prevent water loss, these systems facilitate reductions in stomatal aperture, root growth, antioxidant enzyme activities, and accumulations of compatible solutes (Farooq et al., 2012; Xu et al., 2015 Dong et al., 2016).

In plants, drought stress usually leads to increasing abscisic acid (ABA) content, which induces stomatal closure and upregulates the expression of drought stress-related genes (Seki et al., 2007). Thus, ABA plays an important role in drought stress tolerance. Stomata also play a crucial role in drought tolerance. Stomatal movements are controlled by environmental conditions and plant hormones such as ABA and brassinosteroids (BRs) (Ha et al., 2016). Via NADPH oxidase, ABA induces hydrogen peroxide (H_2O_2) production in guard cells, which subsequently regulates stomatal closure (Zhou et al., 2014b). In tomato, low concentrations of BRs promote stomatal opening, while high concentrations promote stomatal closure and can counteract ABA-induced

Abbreviations: BRs, Brassinosteroids; *BRI1*, Brassinosteroid-insensitive 1; ABA, Abscisic acid; ROS, Reactive oxygen species; Brz, Brassinazole; RWC, Relative water content; Pn, CO_2 assimilation rate; MDA, Malondialdehyde; Fv/Fm, Maximum photochemical efficiency of photosystem II (PSII); SOD, Superoxide dismutase; CAT, Catalase; POD, Peroxidase; APX, Ascorbate peroxidase

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stomatal closure (Ha et al., 2016).

BRs constitute a class of plant steroidal hormones that play important roles in the growth, development, and yield of plants as well as the quality of fruit (Clouse, 1996; Xia et al., 2015). During the past few decades, well-developed BR signal transduction models have been established in Arabidopsis. BRs are first perceived by the plasma membrane-localized receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) and the coreceptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), after which the BR signal is transduced to downstream components. An abundance of dephosphorylated forms of BES1 (bri1-EMS-Suppressor1) and BZR1 (Brassinazole-Resistant 1) ultimately accumulates in the nucleus to regulate the expression of thousands of BR-response genes (Wang et al., 2008; Liu et al., 2018). It has been well established that either overexpression of BRI1 or exogenous applications of BRs can improve BR signaling intensity in Arabidopsis, wheat and tomato (Li and Chory, 1997; Singh et al., 2016; Nie et al., 2017).

Many studies have recently shown that BRs can not only regulate plant growth and development but also increase plant resistance to abiotic and biotic stresses, such as drought, extreme temperature, and pathogen attack (Xia et al., 2009; Rajewska et al., 2016). Overexpression of the Arabidopsis BR biosynthesis gene *DWARF4* (*AtDWF4*) in *Brassica napus* ultimately increased resistance to drought stress (Sahni et al., 2016), and overexpression of the *Spinacia oleracea* BR biosynthesis gene *CYP85A1* (*SoCYP85A1*) increased both the CS content and drought stress tolerance (Duan et al., 2017). However, RNA interference transformants expressing *Brachypodium distachyon BdBRI1* exhibited increasing drought tolerance and upregulated expression of drought-induced genes (Feng et al., 2015). Furthermore, the Arabidopsis mutant *bes1-D* with increased BR signaling exhibited decreased drought resistance, while the mutant *bri1-301* with decreased BR signaling exhibited enhanced drought resistance (Chen et al., 2017; Ye et al., 2017). Regulating BR signal strength in different ways can result in contrasting responses in terms of plant drought resistance; in other words enhancing BR signaling probably either increases or decreases plant drought resistance. Recently, RESPONSIVE TO DESICCATION 26 (RD26), a NAC transcription factor, was reported to interact with BES1, forming a molecular link to coordinate BR signals and drought responses (Ye et al., 2017). Moreover, *WRKY46/54/70* positively regulate BR growth and negatively regulate drought responses (Chen et al., 2017). More recently, ABA INSENSITIVE1 (ABI1) and ABA INSENSITIVE2 (ABI2), which are negative regulators of ABA signaling, were reported to interact with BRASSINOSTEROID INSENSITIVE 2 (BIN2) and further regulate BR signaling, ultimately coordinating growth and stress responses in Arabidopsis (Wang et al., 2017). These investigations predicted that there were complex connections between BR signaling and drought resistance in plants.

To understand the relationship between BR signal strength and drought resistance in tomato, this study involved enhancing BR signal intensity via exogenous 24-epibrassinolide (EBR) applications and via *SlBRI1* overexpression to regulate drought resistance in tomato. The effects of both enhanced BR signaling methods on drought resistance were further studied via early applications of BRs (before drought stress) and via stress-inducible *BRI1* expression transgenic tomato lines (*Atrd29A:SlBRI1*) to determine whether enhancing BR signaling via exogenous BR applications improves plant drought resistance and whether BRI1 constitutive overexpression compromises plant drought resistance.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of the tomato cultivar Micro-Tom (used here as the wild type; WT), the tomato cultivar Money Maker (MM), and the tomato mutant *abs* (*SlBRI1* weak mutant) as well as seeds of T2-generation transgenic tomato plants were germinated. The germination method and seedling growth environment were in accordance with those described by Nie et al., (2017). The plants were imaged with a digital camera (Canon G15, Oita prefecture, Japan).

2.2. Constructs and plant transformation

Transgenic *35S:SlBRI1* overexpression tomato plants were obtained from Nie et al., (2017). The Arabidopsis rd29 A (Atrd29 A) DNA promoter from ecotype Colombia was first PCR-amplified (Yamaguchi-Shinozaki and Shinozaki, 1993). The Atrd29 A promoter sequence was cloned into the *Hin*dIII and *Xba*I sites of the binary pBI121-Flag vector (carrying the kanamycin resistance gene for bacterial and plant selection), and *SlBRI1* gene cDNA was then cloned into the pBI121-Atrd29 A-Flag vector at the *Kpn*I and *Xba*I sites. The construct was subsequently transformed into *Agrobacterium tumefaciens* strain GV3101, which was then transformed into tomato cotyledon explants (Park et al., 2003). Two homozygous transgenic tomato lines (*Atrd29A:SlBRI1-2* and *Atrd29A:SlBRI1-5*) were selected for subsequent drought stress experiments.

2.3. Experimental design

Solutions of EBR (Shanghai Yuanye Biotechnology Co. Ltd., Shanghai, China) and brassinazole (Brz; Tokyo chemical industry Co., Ltd., Tokyo, Japan) were applied to WT and transgenic *SlBRI1* overexpression plants.

2.3.1. Drought experiments

Solutions of 10 mM EBR and 10 mM Brz were prepared in ethanol. Aliquots of 0.1 μM EBR and 4 μM Brz were diluted with deionized water before use (Xia et al., 2009). The WT and transgenic plants were grown until the five-leaf stage. Uniformly sized WT, *35S:SlBRI1-6*, *35S:SlBRI1-37*, *Atrd29A:SlBRI1-2* and *Atrd29A:SlBRI1-5* seedlings were divided into three groups and sprayed with 5 ml of the different solutions prior to drought stress: deionized water that contained ethanol at the same concentration as EBR (control), 0.1 μM EBR (+EBR), and 4 μM Brz (+Brz). This pretreatment occurred daily for 3 successive days before drought stress. All plants were initially irrigated with water, after which point water was withheld. A total of 40 seedlings per treatment were used.

2.3.2. Drought experiments involving EBR applications to tomato seedlings at different growth stages

WT seedlings were divided into four groups, after which the seedlings were sprayed with EBR at different growth stages. The groups were as follows: control (deionized water that contained ethanol at the same concentration as that of EBR), WT + EBR-1 (EBR application 3 days before drought stress or, in other words, 30 days after sowing seeds), WT + EBR-2 (EBR application 13 days before drought stress or 20 days after sowing seeds) and WT + EBR-3 (EBR application 23 days before drought stress or 10 days after sowing seeds). The seedlings were sprayed once daily with 0.1 μM EBR solution for 3 successive days. All plants were initially irrigated, after which water was withheld for 18 days. A total of 40 seedlings per treatment were used.

2.3.3. Drought experiments involving MM and abs plants

Because *abs* plants grow slowly, they were sown 30 days earlier than were MM plants. The *abs* and MM plants were grown until the five-leaf stage. All plants were initially irrigated, after which water was withheld for 12 days. A total of 40 seedlings per treatment were used.

2.4. Determination of the relative water content (RWC), electrolyte leakage and malondialdehyde (MDA) content

The upper third and fourth fully expanded leaves were harvested for measuring the RWC, electrolyte leakage and MDA content. The

measurement methods followed those of Liu et al., (2015b).

2.5. Gas exchange and chlorophyll fluorescence parameters

The CO_2 assimilation rate (Pn) was measured in accordance with the method of Nie et al., (2017). The maximum photochemical efficiency (Fv/Fm) of photosystem II (PSII) was measured using a LI-6400 with an integrated leaf chamber fluorometer (LI-6400-40, LI-COR, Lincoln, NE, United States). After the plants were dark-adapted for 30 min, the minimal fluorescence (Fo) was measured under weak modulating irradiation ($< 0.1\ \mu mol\ m^{-2}\ s^{-1}$). A 600-ms saturating flash ($> 7000\ \mu mol\ m^{-2}\ s^{-1}$) was subsequently used to determine the maximum chlorophyll fluorescence yield (Fm) and to calculate the Fv/Fm, in which Fv = Fm – Fo.

2.6. Enzyme activity assays and histochemical detection of ROS

Approximately 0.2 g of ground leaf samples was homogenized in 2 ml of ice-cold 50 mM phosphate buffer (pH 7.8) that contained 1% polyvinylpyrrolidone and 0.1 mM EDTA. The homogenates were centrifuged at 12000 *g* for 15 min at 4°C, after which the supernatant was used to assay the activity of antioxidant enzymes. Superoxide dismutase (SOD) activity was measured as described by Zhang et al. A 3-ml reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitro blue tetrazolium (NBT), 2 mM riboflavin, 0.1 mM EDTA, and 0.2 ml of enzyme extract (the supernatant of the last step) was used. The reaction mixtures were irradiated for 20 min under a light intensity of $200\ \mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density, and the absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme that caused 50% of NBT photochemical reduction. Catalase (CAT) activity was evaluated by monitoring the reduction in absorbance at 240 nm to account for decomposition of H_2O_2 (an extinction coefficient of $39.4\ mM^{-1}\ cm^{-1}$ was used). A 1-ml reaction mixture that included 50 mM PBS (pH 7.0), 10 mM H_2O_2 and 30 µl of the supernatant was used. The reaction was initiated by adding H_2O_2 (Guo et al., 2012). Ascorbate peroxidase (APX) activity was measured by monitoring the oxidation of ascorbate (ASA) at 290 nm (an extinction coefficient of 2.8 mM was used). A 1-ml reaction mixture that included 50 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA-Na_2, 0.5 mM ASA, 1 mM H_2O_2 and 30 µl of enzyme extract was used (Guo et al., 2012). Peroxidase (POD) activity was measured at 470 nm (an extinction coefficient of 25.2 mM was used). A 1.0-ml reaction mixture that included 100 mM potassium phosphate buffer (pH 7.0), 16 mM guaiacol, 5 µl of 30% (w/v) H_2O_2, and 200 µl of enzyme extract was used. This reaction was started by adding the extract (Rao et al., 1996).

H_2O_2 accumulation in the leaves was visualized by staining with 3,3-diaminobenzidine (DAB) as described by Liu et al., (2015a). Superoxide (O_2^-) accumulation was visually measured using the NBT staining principle. Images were collected using a digital camera (Canon G15, Oita Prefecture, Japan), and the H_2O_2 content was quantified using detection kits (A064-1, Jiancheng, Nanjing, China). In principle, the H_2O_2 in the plant leaves could interact with molybdic acid to form a complex, which was measured at 450 nm.

2.7. Observations of leaf stomata via scanning electron microscopy (SEM)

Three leaves were collected per treatment after 0 and 10 days of drought treatment for SEM processing. The samples were prepared as described by Li et al., (2015). The lower leaf surface was observed and imaged using a JSM-6360LV microscope (JEOL Ltd., Tokyo, Japan). The stomatal density was measured at random within 16 visual fields, and a stomatal index was calculated for 16 random stomata using ImageJ software (Li et al., 2015).

2.8. Measurement of BR and ABA contents

Approximately 0.5 g of ground leaf samples was homogenized in 10 ml of extraction solution (which comprised 80% methanol and 1 mM tert-butyl-p-methylphenol) for 4 h at 4 °C. The homogenates were then centrifuged at 3500 *g* for 8 min, after which the pellet was added to 1 ml of extraction solution, which was subsequently incubated for 1 h at 4 °C and then centrifuged. Afterward, the solution and the supernatant were mixed twice. The samples were ultimately measured using enzyme-linked immunosorbent assays (Yang et al., 2001).

2.9. RNA extraction and qRT-PCR analysis

Total RNA was extracted and subsequently reverse-transcribed as described by Nie et al., (2017). The tomato *UBIQUITIN 3* (*UBI3*) gene was used as an internal control. The gene-specific qPCR primers used are listed in Table S1, Supporting Information.

2.10. Western blot analysis

Proteins were extracted from the young leaves (200 mg) of 25-day-old transgenic and WT plants with 2 × SDS gel loading buffer as described by Wang et al., (2016).

2.11. Statistical analysis

The data were analyzed using SPSS version 17.0 and the least significant difference (LSD) test. Means and standard errors were calculated to compare variables. Values for which $P < 0.05$ and $P < 0.01$ were considered statistically significant.

3. Results

3.1. Exogenous EBR application positively regulated the drought resistance of tomato

Exogenous EBR applications can increase drought resistance in some plant species. Our results showed that exogenous EBR applications significantly increased BR contents in tomato. Indeed, the BR content in the WT + EBR plants was 2.7 times greater than that in the WT plants (Fig. S1A). Although EBR application did not influence the transcript level of the *BRI1* gene, the expression levels of the BR biosynthesis genes *DWARF* and *CPD* significantly decreased in the WT + EBR plants (Figs. S1B–D). To confirm further whether BR contents are positively related to drought resistance of tomato seedlings, the WT and WT + EBR seedlings were not watered for 15 days. After 10 and 15 days of drought stress, the wilting degree of the WT + EBR plant leaves was less than that of the WT plant leaves (Fig. 2B and C). Under normal conditions, no significant differences in leaf RWC, electrolyte leakage, Pn or Fv/Fm were observed between the WT and WT + EBR plants (Fig. 1). The RWC and Pn markedly decreased in all plants after 10 days of drought stress, but the RWC and Pn were slightly higher in the WT + EBR plants than in the WT plants. The RWC and Pn of the WT + EBR plants were 6.7 and 38.7% greater, respectively, than those of the WT plants after drought stress (Fig. 1A, C). Drought stress significantly increased the electrolyte leakage in all plants, but the leakage was lower in the WT + EBR plants than in the WT plants (Fig. 1B). Drought stress also slightly reduced the Fv/Fm values in the tomato leaves, but the values were higher for the WT + EBR plants than for the WT plants (Fig. 1D). These results indicated that exogenous EBR application positively regulated the drought resistance of tomato seedlings.

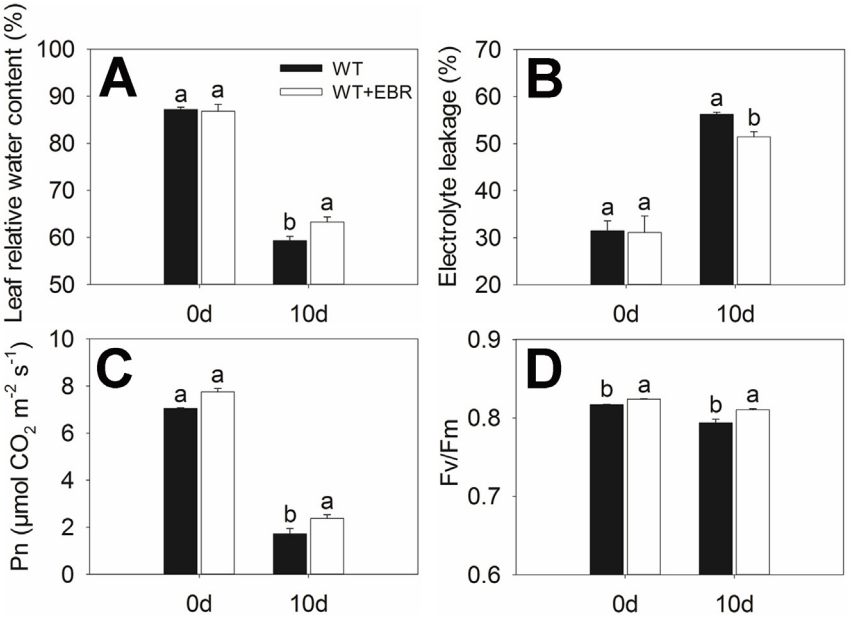

Fig. 1. Effects of 24-epibrassinolide (EBR) applications on wild-type (WT) plants under drought stress. (A) Leaf relative water content (RWC), (B) electrolyte leakage, (C) leaf CO_2 assimilation rate (Pn), and (D) the maximum photochemical efficiency of PSII (Fv/Fm) after 0 and 10 days of drought stress. The WT plants were pretreated with 5 ml of 0.1 μM EBR once daily for 3 successive days before drought stress. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

3.2. Overexpression of 35 S:SlBRI1 negatively regulated the drought resistance of tomato

The above results showed that exogenous EBR applications increased the BR content and further improved the drought resistance of tomato. To determine further whether increased BR signaling via overexpression of *SlBRI1* positively regulated drought resistance of tomato, we used the constitutive CaMV 35 S promoter to drive the expression of *SlBRI1* in transgenic tomato plants. These plants present significantly enhanced *SlBRI1* RNA and protein levels (Figs. S2A and B). The expression levels of *DWARF* and *CPD* significantly decreased in the transgenic plants (Figs. S2C and D). Neither WT nor *SlBRI1*-overexpressing tomato seedlings were watered for 15 days; after 10 and 15 days of drought stress, the degree of leaf wilting was more severe for the *35S:SlBRI1-6* and *35S:SlBRI1-37* plants than for the WT plants (Fig. 2A–C). Furthermore, the RWC and Pn of the transgenic plant leaves were significantly lower than those of the WT plant leaves after 10 days of drought stress. The RWC was 6.6 and 7.5% lower in the *35S:SlBRI1-6* and *35S:SlBRI1-37* leaves than in the WT leaves, respectively (Fig. 2D). Similarly, the Pn of the transgenic plant leaves was 27.2 and 44.6% lower than that of the WT leaves, respectively (Fig. 2F). Moreover, the electrolyte leakage in the transgenic plants was markedly higher than that in the WT plants (Fig. 2E), but the Fv/Fm values of the transgenic plants were slightly lower than those of the WT plants (Fig. 2G). The results showed that overexpression of *SlBRI1* increased the BR signal strength while negatively regulating the drought resistance of tomato.

To confirm the differences between the effects of EBR application and *SlBRI1* overexpression on drought resistance further, we measured the BR and ABA contents in different plants. Our results indicated that EBR application significantly increased BR contents but that overexpression of *SlBRI1* significantly reduced BR contents. Furthermore, compared with those in the WT plants, the BR and ABA contents in the *35S:SlBRI1-6* and *35S:SlBRI1-37* plants decreased by 35 and 25%, respectively (Fig. 3A). Drought stress reduced the BR contents in the WT and WT + EBR plants, while the BR contents in the transgenic plants

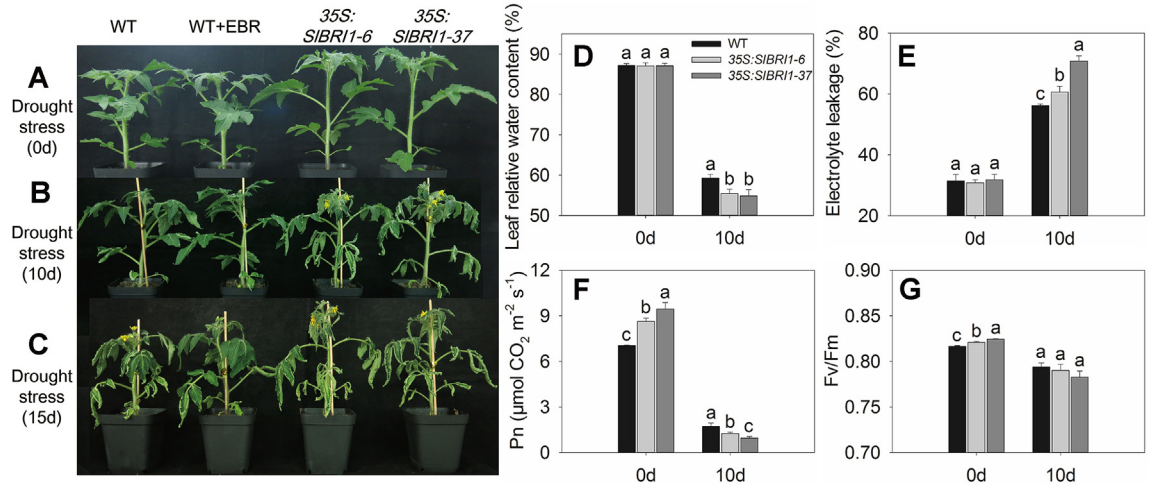

Fig. 2. Effects of 24-epibrassinolide (EBR) applications and *35S:SlBRI1* overexpression on plants under drought stress. Phenotypes of (A) wild-type (WT), (B) WT + EBR and (C) *35S:SlBRI1* overexpression plants after 0, 10 and 15 days of drought stress. The (D) leaf relative water content (RWC), (E) electrolyte leakage, (F) CO_2 assimilation rate (Pn), and (G) maximum photochemical efficiency of PSII (Fv/Fm) of WT, *35S:SlBRI1-6* and *35S:SlBRI1-37* plants after 0 and 10 days of drought stress. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

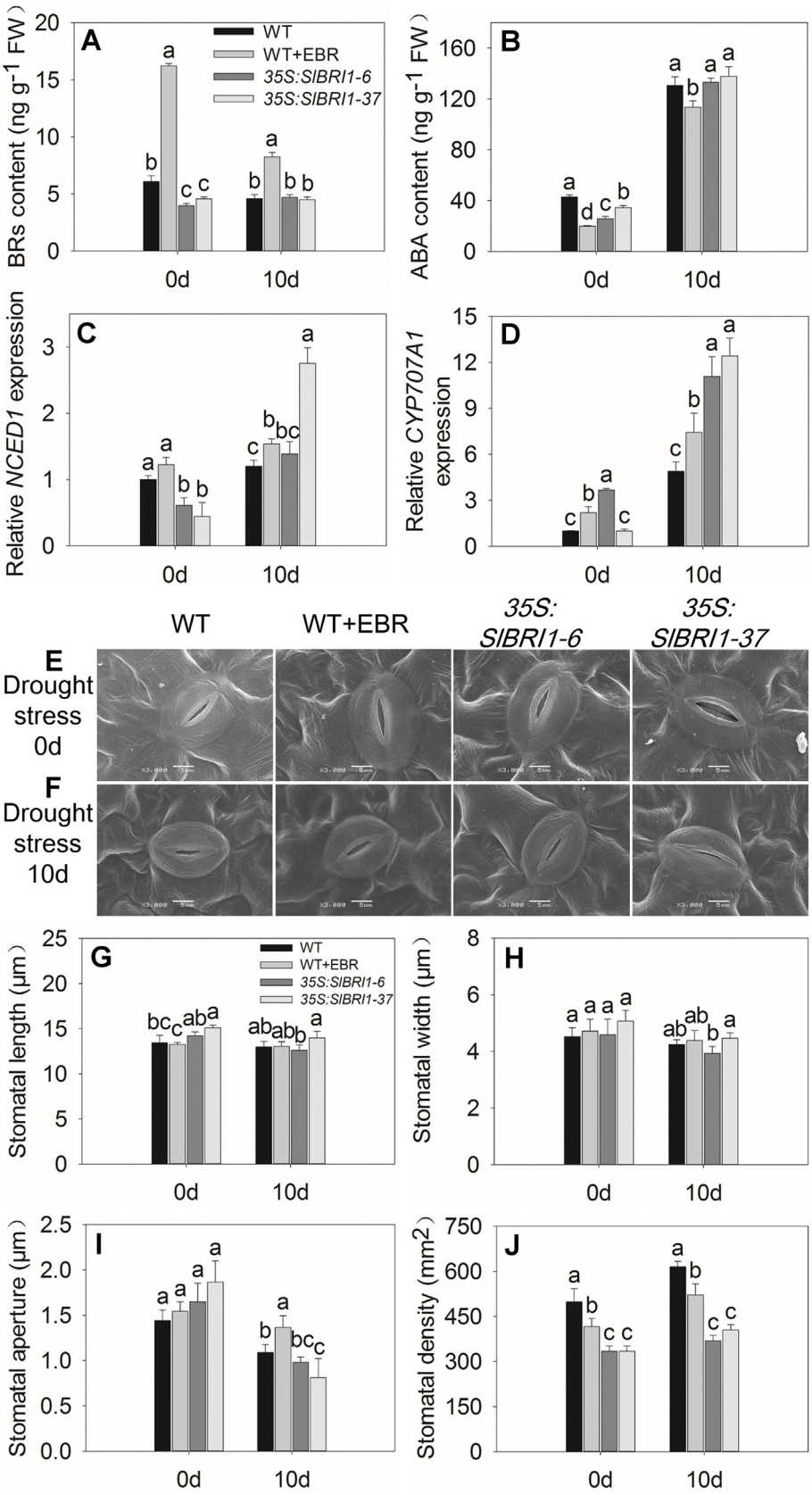

Fig. 3. Effects of 24-epibrassinolide (EBR) applications and *35S:SlBRI1* overexpression on hormones and stomatal changes within plants under drought stress. (A) Brassinosteroid (BR) content, (B) abscisic acid (ABA) content, (C) relative transcript levels of *NCED1*, and (D) relative transcript levels of *CYP707A1* in as well as the (E) phenotypes of stomata of WT, WT + EBR and *35S:SlBRI1* overexpression plants after 0 and 10 days of drought stress. (F) Phenotypes of stomata, (G) stomatal length, (H) stomatal width, (I) stomatal aperture, and (J) stomatal density after 10 days of drought stress. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

remained unchanged during drought stress (Fig. 3A). Under normal conditions, the ABA contents were 53.7, 39.8 and 19.6% lower in the WT + EBR, *35S:SlBRI1-6* and *35S:SlBRI1-37* plants than in the WT plants, respectively (Fig. 3B). Drought stress significantly increased the ABA content in all plants, but the ABA content was markedly lower in the WT + EBR plants than in the other plants under drought stress (Fig. 3B). To understand further why EBR application and *SlBRI1* overexpression led to different ABA contents under drought stress, the

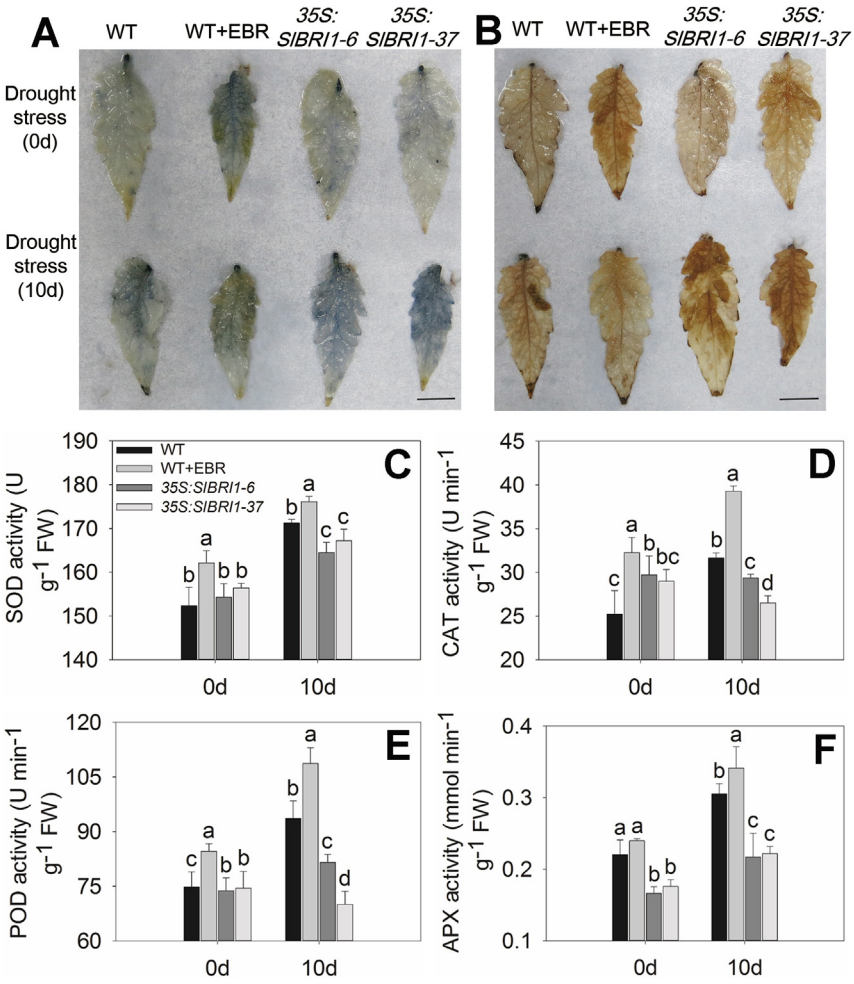

Fig. 4. Effects of 24-epibrassinolide (EBR) applications and *35S:SlBRI1* overexpression on the accumulation of reactive oxygen species (ROS) and antioxidant enzyme activity in plants under drought stress. Accumulation of (A) superoxide (O_2^-) and (B) hydrogen peroxide (H_2O_2) as well as activity of (C) superoxide dismutase (SOD), (D) catalase (CAT), (E) ascorbate peroxidase (APX), and (F) peroxidase (POD) in wild-type (WT), WT + EBR and *35S:SlBRI1* overexpression plants after 0 and 10 days of drought stress. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

expression of critical ABA biosynthesis and catabolism genes was analyzed via qRT-PCR. In the *SlBRI1* overexpression plants, transcripts of the ABA biosynthesis gene *NCED1* significantly decreased under normal conditions. However, drought stress increased the 9-cis-epoxycarotenoid dioxygenase (*NCED1*) expression levels in all plants. EBR application and *SlBRI1* overexpression further increased the *NCED1* expression levels (Fig. 3C). Transcripts of the ABA catabolism gene cytochrome P450, family 707, subfamily A, polypeptide 1 (*CYP707A1*) in both WT + EBR and *35S:SlBRI1-6* plants significantly increased under normal conditions. In addition, *CYP707A1* expression in all plants rapidly increased under drought stress. For example, after 10 days of drought stress, *CYP707A1* expression levels in the WT + EBR, *35S:SlBRI1-6* and *35S:SlBRI1-37* plants were 1.52-, 2.27- and 2.54-fold greater than those in the WT plants before drought stress, respectively (Fig. 3D). These results indicated that increased *CYP707A1* expression levels may result in reduced ABA contents in WT + EBR plants. To compare the differences between EBR application and *SlBRI1* overexpression on the stomatal index, SEM was used to observe the leaves of different plants. The abaxial surfaces of the leaf samples were scanned at 400 × and 3000 × . EBR application and *SlBRI1* overexpression slightly increased the stomatal width and aperture but significantly reduced the stomatal density (Fig. 3E–J). Drought stress caused stomata to be slightly shorter (Fig. 3F and G) and narrower (Fig. 3F, H), and their apertures were smaller than those under the control conditions (Fig. 3F, I). EBR application alleviated the reductions in stomatal length, width and aperture, while *SlBRI1* overexpression aggravated the reductions of those indices. Compared with that in the WT plants under drought stress, the stomatal aperture in the WT + EBR plants increased by 25.4%, but the stomatal aperture in the *35S:SlBRI1-6* and *35S:SlBRI1-37* plants decreased by 9.9 and 25.6%, respectively. EBR application and *SlBRI1* overexpression reduced the stomatal density, while drought stress increased the stomatal density in all plants (Fig. 3E–J).

On the basis of histochemical observations of leaves, we measured the accumulation of H_2O_2 and O_2^- to compare differences in the levels of ROS further. Under normal conditions, EBR applications resulted in H_2O_2 and O_2^- levels that were slightly greater than those in control plants. Drought stress resulted in increased staining in all plants. Lower levels of H_2O_2 and O_2^- accumulated in the WT + EBR plants than in the WT plants, and the *SlBRI1*-overexpressing plants accumulated greater levels of H_2O_2 and O_2^- after 10 days of drought stress (Fig. 4A and B). These results suggested that the *SlBRI1* overexpression plants suffered more oxidative stress damage under drought stress than did the WT plants.

To examine further whether EBR application and *SlBRI1* overexpression protected against cellular damage caused by drought-induced oxidative stress, we assayed the activities of major antioxidant enzymes, including SOD, CAT, APX and POD. Under normal conditions, the activities of SOD, CAT, and POD slightly increased in the plants in response to EBR application (Fig. 4C, D, F). Drought stress markedly increased the activities of all the studied enzymes. Under drought stress, the enzyme activities in the WT + EBR and *SlBRI1*-overexpressing plants were significantly greater and lower than those in the WT plants, respectively. For example, compared with those in the WT plants, the activities of SOD, CAT, APX, and POD in the WT + EBR plants increased by 2.8, 24.2, 11.9 and 16.1%, respectively, and those in the *35S:SlBRI1-37* plants decreased by 2.4, 16.2, 27.2 and 25.2%, respectively (Fig. 4C–F).

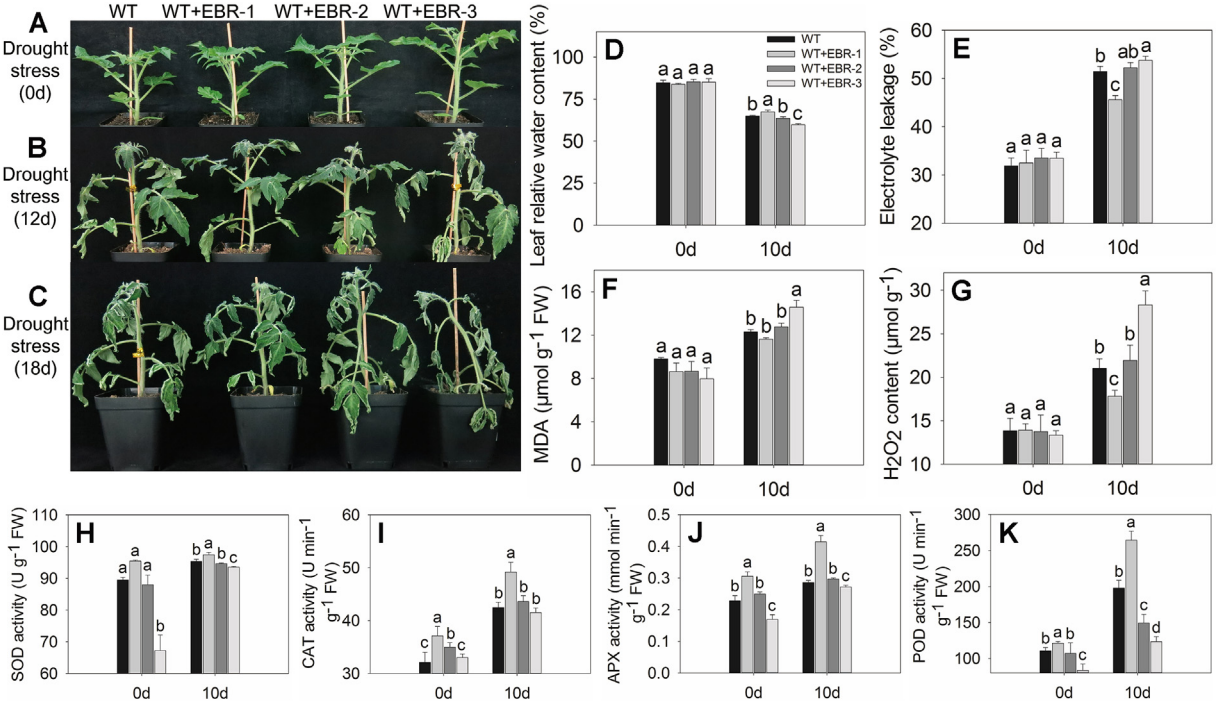

Fig. 5. Effects of 24-epibrassinolide (EBR) applications on wild-type (WT) plants under different durations of drought stress. Phenotypes of WT, WT + EBR-1, WT + EBR-2 and WT + EBR-3 plants after (A) 0, (B) 12 and (C) 18 days of drought stress. (D) Leaf relative water content (RWC), (E) electrolyte leakage, (F) malondialdehyde (MDA) content, (G) and hydrogen peroxide (H_2O_2) content as well as activity of (H) superoxide dismutase (SOD), (I) catalase (CAT), (J) ascorbate peroxidase (APX), and (K) peroxidase (POD) in WT, WT + EBR-1, WT + EBR-2 and WT + EBR-3 plants after 0 and 10 days of drought stress. WT + EBR-1 (EBR application 3 days before drought stress), WT + EBR-2 (EBR application 13 days before drought stress) and WT + EBR-3 (EBR application 23 days before drought stress) plants were pretreated with 5 ml of 0.1 μM EBR once daily for 3 successive days. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

3.3. Exogenous early EBR application negatively regulated the drought resistance of tomato

To confirm further whether early exogenous EBR applications before drought stress affected the drought resistance of tomato seedlings, we exogenously applied EBR at 3 days (WT + EBR-1), 13 days (WT + EBR-2) and 23 days (WT + EBR-3) before drought stress. The expression levels of *DWARF* and *CPD* significantly decreased in all plants treated with EBR (Figs. S3A and B). The WT seedlings treated with EBR at different times before drought stress were not watered for 18 days. After 12 and 18 days of drought stress, the degree of wilting of the WT + EBR-1 plants was less than that of the WT plants, while the degree of wilting of the WT + EBR-3 plants was the most severe (Fig. 5A–C). There were no significant differences in the degree of wilting between the WT and WT + EBR-2 plants (Fig. 5A–C).

Furthermore, the leaf RWC in the WT + EBR-1 plants was significantly greater than that in the plants of the other treatments. Furthermore, the leaf RWC in the WT + EBR-3 plants was markedly lower than that in the plants of the other treatments after 10 days of drought stress (Fig. 5D). Under normal conditions, no significant differences in electrolyte leakage or MDA and H_2O_2 contents were observed among the different treatments. The electrolyte leakage and MDA and H_2O_2 contents in the WT + EBR-1 plants were 11.4, 5.5 and 15.2% lower than those in the WT plants after 10 days of drought stress, respectively; by contrast, the same contents in the WT + EBR-3 plants were 4.4, 18.6 and 14.7% higher, respectively (Fig. 5E–G). Compared with those in the WT plants, the antioxidant enzyme activities of SOD, CAT, APX, and POD in the WT + EBR-1 plants and WT + EBR-3 plants under drought stress were greater and lower, respectively; moreover, the activities in the WT + EBR-1 plants increased by 2.2, 15.7, 44.8, and 33.7%, respectively, but decreased by 1.9, 2.4, 5.1, and 37.7%, respectively, in the WT + EBR-3 plants. These results indicated that early applications of BRs before drought stress reduced the drought resistance of tomato seedlings (Fig. 5H–K).

3.4. Atrd29 A:SlBRI1 transgenic plants negatively regulated the drought resistance of tomato

To determine further whether the reduced drought resistance observed in plants constitutively expressing *SlBRI1* was caused by a previous enhancement of BR signaling, similar to what was observed with early exogenous BR applications, we used the stress-induced Atrd29 A promoter to control *SlBRI1* expression in transgenic plants. These plants presented elevated expression levels of *SlBRI1* after 4 days of drought stress, and the expression levels further increased at the later stage of drought stress (Fig. 6A). WT and *Atrd29A:SlBRI1* transgenic seedlings were not watered for 18 days. After 12 and 18 days of drought stress, the degree of wilting of the *Atrd29A:SlBRI1* transgenic plants was more severe than that of the WT plants (Fig. 6B). Furthermore, the leaf RWC in the WT seedlings was slightly greater than that in the Atrd29 A*:SlBRI1* transgenic seedlings after 10 days of drought stress (Fig. 6C). In contrast, the electrolyte leakage and MDA and H_2O_2 contents were significantly higher in the transgenic seedlings than in the WT seedlings (12.7, 36.5 and 32.9% greater, respectively; Fig. 6D–F). After 10 days of drought stress, the antioxidant enzyme activities of SOD, CAT, APX and POD in the transgenic Atrd29 A*:SlBRI1-5* plants were significantly lower than those in the WT plants (the activities decreased by 14.6, 9.1, 10.4 and 15.2%, respectively; Fig. 6G–J). These results indicated that simultaneously increasing *SlBRI1* expression and further improving BR signaling still negatively regulated the drought resistance of tomato seedlings.

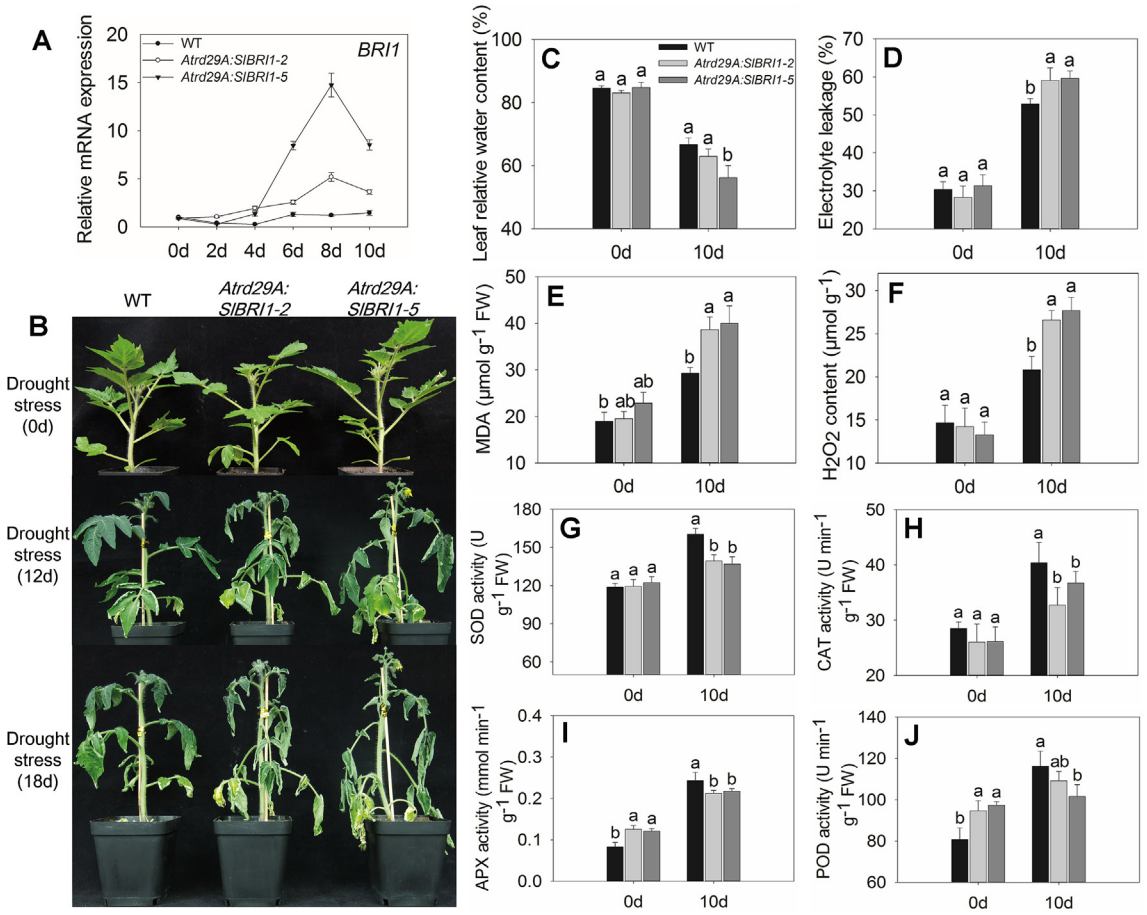

Fig. 6. Effects of drought stress on *Atrd29A:SlBRI1* overexpression plants. (A) Relative change in *SlBRI1* transcript levels in plants under drought stress for different days. (B) Phenotypes of wild-type (WT) and *Atrd29A:SlBRI1* transgenic plants after 0, 12 and 18 days of drought stress. (C) Leaf relative water content (RWC), (D) electrolyte leakage, (E) malondialdehyde (MDA) content, and (F) hydrogen peroxide (H_2O_2) content as well as activity of (G) superoxide dismutase (SOD), (H) catalase (CAT), (I) ascorbate peroxidase (APX), and (J) peroxidase (POD) in WT and *Atrd29A:SlBRI1* transgenic plants after 0 and 10 days of drought stress. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

3.5. Expression of drought stress-related genes in plants treated with EBR and in transgenic plants

To gain further insight into the molecular mechanisms governing drought resistance under different treatments and in different transgenic plants, qRT-PCR was used to analyze the expression levels of antioxidant-related and stress-responsive genes and of transcription factors. Under normal conditions, the expression of the antioxidant-response genes *CAT* and *APX* increased in the WT + EBR, *35S:SlBRI1-6*, *35S:SlBRI1-37*, WT + EBR-2, and WT + EBR-3 plants, and the expression of the *GSH1*, *RBOH1*, *LOX*, and *WRKY72* genes also increased in the WT + EBR plants (Fig. 7). Drought stress markedly increased the expression of all analyzed stress-responsive genes. Furthermore, EBR application further increased the transcription levels of those genes, especially the proline biosynthesis gene pyrroline-5-carboxylate synthetase (*LeP5CS*), whose expression after drought stress was hundreds of times greater than that in the WT plants (Fig. 7). The *35S:SlBRI1-6*, *35S:SlBRI1-37*, WT + EBR-2, WT + EBR-3, *Atrd29A-SlBRI1-2*, and *Atrd29A-SlBRI1-5* plants also presented increased transcription levels of the *RBOH1*, *LeP5CS* and *EDR2* genes, while the expression of *WRKY1* and *WRKY72* significantly decreased in those plants. Overall, compared with those in control plants, the expression levels of all analyzed stress-responsive genes in plants treated with EBR were greater, while the expression levels of most stress-responsive genes decreased in the WT + EBR-2 and WT + EBR-3 plants as well as in all the transgenic plants (Fig. 7).

3.6. Brz application negatively regulated the drought resistance of tomato, whereas bri1 mutants displayed positive regulation of drought resistance

To determine further whether relatively low endogenous BR contents negatively regulated drought resistance, tomato BR synthesis was inhibited by applications of Brz, which can inhibit a crucial BR biosynthesis gene (cytochrome P450) and ultimately reduce the BR content in plants (Ahammed et al., 2013). WT and WT + Brz plants were not watered for 15 days. Under normal conditions, no significant differences in leaf RWC, electrolyte leakage, Pn or Fv/Fm were observed between the WT and WT + Brz plants (Fig. 8A–D). However, the RWC, Pn and Fv/Fm of the WT plants were significantly greater than those of the WT + Brz plants, and the electrolyte leakage in the WT plants under drought stress was markedly lower than that in the WT + Brz plants (Fig. 8A–D). Therefore, the results showed that decreased endogenous BR contents negatively regulated the drought resistance of tomato. To confirm whether reduced BR signaling intensity positively regulated drought resistance further, we withheld the watering of both the tomato *bri1* weak mutant (*abs*) and its WT MM for 12 days. After 10 and 12 days of drought stress, the MM plant leaves exhibited marked wilting and yellowing, while the *abs* plant leaves exhibited a weak degree of wilting (Fig. 8E). Furthermore, the soil water content under the *abs* plants and the leaf RWC of the *abs* plants were significantly greater than the soil water content under and the leaf RWC of the MM plants, respectively, after 10 days of drought stress. For example, under drought stress, the leaf RWC of the MM plants was only 51.3%, but that

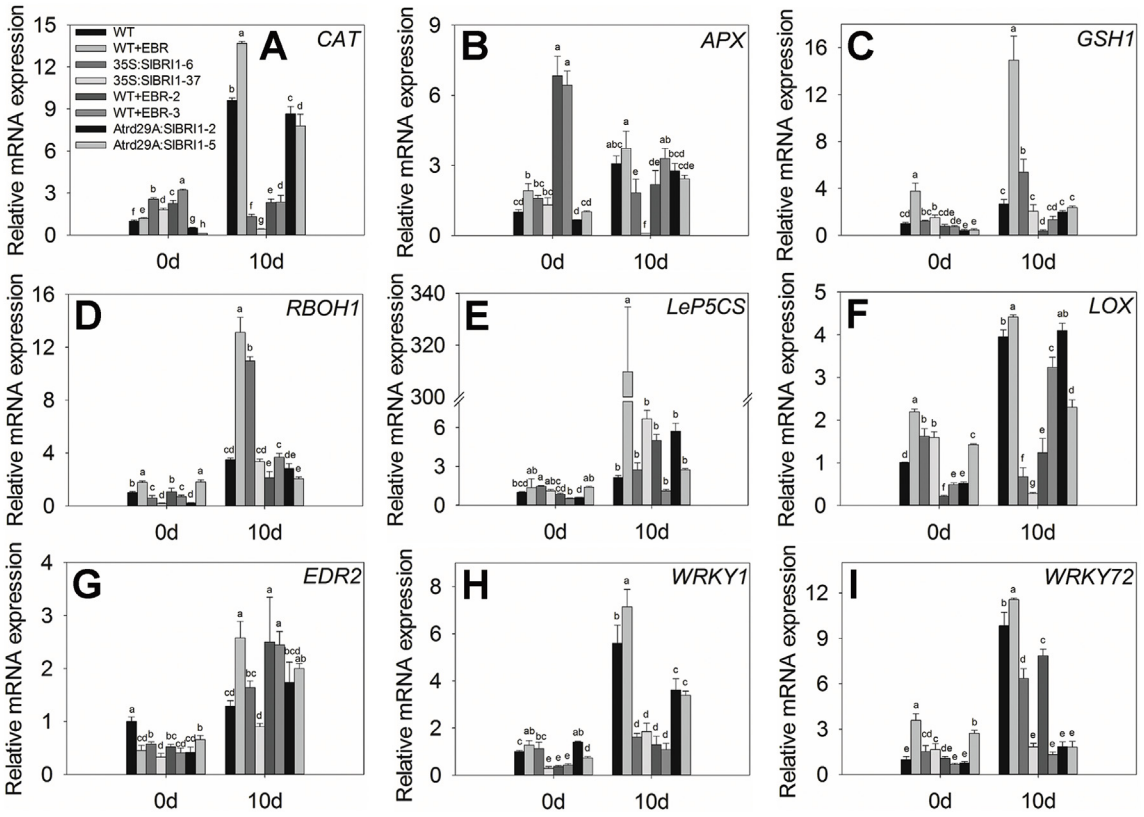

Fig. 7. Relative expression levels of (A) *CAT*, (B) *APX*, (C) *GSH1*, (D) *RBOH1*, (E) *LeP5CS*, (F) *LOX*, (G) *EDR2*, (H) *WRKY1*, and (I) *WRKY72* in wild-type (WT), WT + EBR, *35S:SlBRI1-6*, *35S:SlBRI1-37*, WT + EBR-2, WT + EBR-3, *Atrd29A:SlBRI1-2* and *Atrd29A:SlBRI1-5* plants. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

of the *abs* plants reached 86.5% (Fig. 8F and G). Moreover, after drought stress, compared with those in the MM plants, the electrolyte leakage and MDA content in the *abs* plants were 36.4 and 44.1% lower, respectively (Fig. 8H and I). These results demonstrated that the tomato BR signaling *bri1* mutant was significantly more drought resistant than was MM.

4. Discussion

The role of BRs and BR signaling in plant growth, development, yield and fruit quality is well established in Arabidopsis and tomato (Zhu et al., 2013; Nie et al., 2017). Furthermore, many studies have shown that BRs can increase the stress resistance of plants under biotic and abiotic stress, including extreme temperature, drought and salinity stress as well as pathogen attack (Xia et al., 2009; Rajewska et al., 2016). Usually, enhancing plant stress resistance comes at a cost of reduced plant growth and development. Thus, it is unclear how exogenous applications of BRs could not only promote plant growth and development but also synchronously increase plant stress resistance.

Recent studies have indicated that the Arabidopsis mutant *bes1-D* exhibits increased BR signaling but reduced drought resistance, while the mutant *bri1-301*, which is a weak *bri1* mutant that presents substantially reduced BR signaling, exhibits increased drought resistance (Chen et al., 2017; Ye et al., 2017). These results were verified by our multiple observations in Arabidopsis and tomato but obviously contrast with previous studies reporting that increased BR signaling via exogenous applications of BRs increases stress resistance. The study of the mechanism that underlies these contrasting results was the starting point of our investigation. Our study further revealed that enhancing BR signaling with BR applications could positively regulate tomato drought stress resistance; however, enhancing BR signaling with *BRI1* overexpression negatively regulated tomato drought stress resistance. To eliminate the effects of rapid growth on the drought resistance of *SlBRI1* transgenic plants, EBR and Brz were exogenously applied on different days before drought stress, and transgenic *SlBRI1* tomato plants in which *SlBRI1* was driven by the Atrd29 A stress-induced promoter were generated and analyzed.

ABA plays an important role in plant tolerance to abiotic stress. ABA can inhibit BR signaling output; exogenous ABA rapidly upregulates the expression of *DWF4* and *CPD* and increases the phosphorylation of BES1 (Zhang et al., 2009; Gui et al., 2016). ABA inhibits seed germination and postgerminative growth, but BRs promote seed germination and seedling growth (Chung et al., 2014). Therefore, ABA and BRs play antagonistic roles in multiple physiological processes in Arabidopsis (Cai et al., 2014). Our results indicated that EBR application significantly increased the BR content, BR signaling intensity and drought resistance of tomato; the expression levels of the BR biosynthesis genes *DWARF* and *CPD* were markedly inhibited (Fig. S1) (He et al., 2005), while applications of Brz, a crucial BR biosynthesis inhibitor, significantly reduced the drought resistance of tomato (Fig. 8A–D). Overexpression of *35S:SlBRI1* increased the BR signaling intensity, which reduced the transcription of the *DWARF* and *CPD* genes and ultimately reduced the BR contents and drought resistance of tomato (Fig. S2). In addition, our results indicated that, compared with the WT MM, the *abs* tomato mutant, which has lost the main activity of BRI1 (causing reduced BR signaling), was more drought resistant (Fig. 8E–I). Furthermore, EBR application and *SlBRI1* overexpression significantly upregulated the expression of *CYP707A1* (which is involved in ABA catabolism), which may reduce the ABA content (Fig. 3B, D). Drought stress upregulated the expression of *NCED1* (which is involved in ABA biosynthesis) and *CYP707A1*, which eventually increased the ABA content in all plants. However, the expression of *CYP707A1* was significantly greater in the plants treated with EBR than in the WT plants, and ultimately, lower ABA contents were detected in the EBR-treated

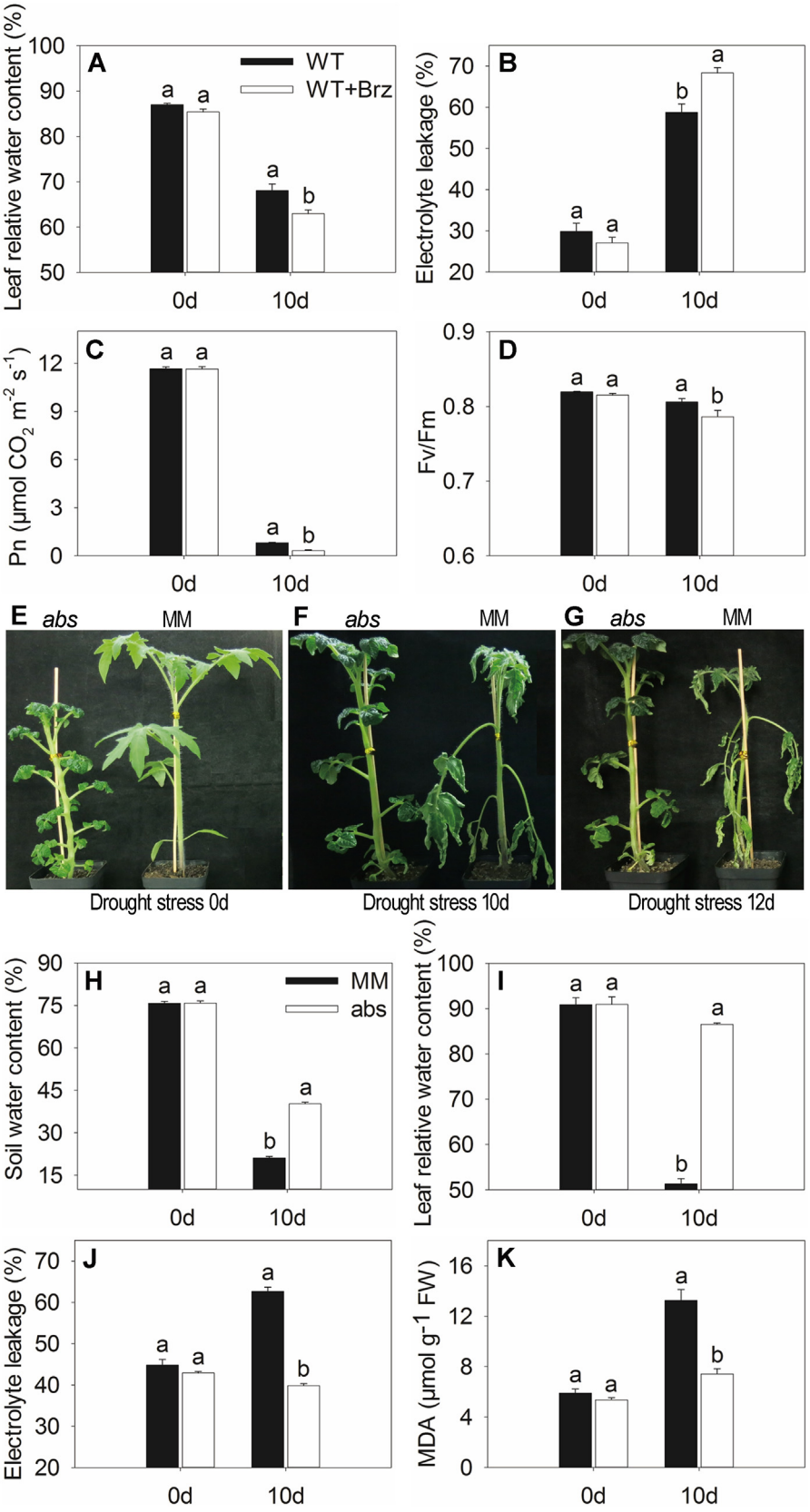

Fig. 8. Effects of brassinazole (Brz) applications and performance of the *SlBRI1* weak mutant (*abs*) under drought stress. (A) Leaf relative water content (RWC), (B) electrolyte leakage, (C) CO_2 assimilation rate (Pn), and (D) the maximum photochemical efficiency of PSII (Fv/Fm) in wild-type (WT) and WT + Brz plants after 0 and 10 days of drought stress. (E) Phenotypes of Money Maker (MM) and *SlBRI1* weak mutant *abs* plants after 0, 10 and 12 days of drought stress. (F) Soil RWC, (G) leaf RWC, (H) electrolyte leakage, and (I) malondialdehyde (MDA) content in MM and *abs* plants after 0 and 10 days of drought stress. The WT plants were pretreated with 5 ml of 4 μM Brz once daily for 3 successive days before drought stress. The data values are the means ± SDs of three independent biological samples. The different letters indicate significant differences according to the least significant difference (LSD) test ($P < 0.05$).

plants (Fig. 3B, D). Although *35S:SlBRI1* overexpression also upregulated *CYP707A1* expression, the ABA content in those plants was slightly greater than that in the WT plants under drought stress (Fig. 3C and D). Exogenous EBR applications markedly improved the ABA content of plants of the tomato cultivar AC under drought stress (Yuan et al., 2010). These differences could probably be attributed to different experimental conditions.

Stomata play a crucial role in drought tolerance. Stomatal

movement is controlled by environmental conditions and plant hormones such as ABA and BRs (Ha et al., 2016). Via NADPH oxidase, ABA induces H_2O_2 production, and H_2O_2 further regulates stomatal closure in guard cells (Zhou et al., 2014b). In tomato, low concentrations of BRs promote stomatal opening; however, high concentrations promote stomatal closure and can counteract ABA-induced stomatal closure (Ha et al., 2016). In the present study, EBR application and *35S:SlBRI1* overexpression slightly increased stomatal width and aperture but significantly reduced stomatal density (Fig. 3E–J). These results correspond to previous results in which mutants defective in both BR signaling and biosynthesis exhibited improved stomatal density but reduced stomatal size (Haubrick et al., 2006). Furthermore, these data were consistent with slight increases in the Pn and stomatal conductance (g_s) recorded for plants treated with EBR and overexpressing *SlBRI1* (Fig. 2F). However, compared with control conditions, drought stress conditions caused the stomata to be slightly shorter and narrower, and they had smaller apertures; however, the stomatal density was greater (Fig. 3E–J). Under drought stress, apple and watermelon stomatal indices have also shown analogous changes (Mo et al., 2015; Talbi et al., 2015). In the present study, the BR content in the EBR-treated plants started to decrease under drought stress, but the BR content was still greater in those plants than in the WT plants, which displayed increased stomatal aperture (Fig. 3A, B, I). However, by directly increasing the ABA content or indirectly reducing the BR content, *35S:SlBRI1* overexpression decreased the stomatal aperture. These results indicated that increased stomatal aperture was not related to BR signaling intensity but was possibly related to the BR content in the plants.

Drought stress usually leads to increased ABA content, which accelerates the production of ROS and ultimately results in membrane lipid peroxidation, enzyme deactivation, electrolyte leakage, and reduced photosynthetic capacity (Talbi et al., 2015). EBR application caused relatively less H_2O_2 to accumulate, whereas the *35S:SlBRI1* plants, early-stage EBR-treated plants and Atrd29 A-SlBRI1 plants accumulated greater amounts of H_2O_2 than did the WT plants under drought stress (Fig. 4A and B, 5G, 6F). Two reasons may have caused the difference in H_2O_2 contents between EBR-treated and BRI1 transgenic plants. First, EBR application increased ABA degradation, therefore reducing the ABA content and consequently reducing the H_2O_2 accumulation in those plants compared with the WT plants. Second, EBR application increased the SOD, CAT, APX and POD enzyme activities, which in turn could effectively remove any excess H_2O_2 (Fig. 4C–F). However, the 35 S:*SlBRI1* plants, early-stage EBR-treated plants and Atrd29 A-*SlBRI1* plants presented reduced activities of those enzymes, which led to a comparatively greater accumulation of H_2O_2 than that in the WT plants under drought stress (Fig. 4C–F, 5H-K, 6G-J). Therefore, increasing the stomatal aperture and reducing ABA and H_2O_2 contents under drought stress could synergistically improve the drought tolerance of plants, which was reflected by the reduced degree of wilting, drought index, electrolyte leakage and MDA content, as well as the increased leaf RWC and photosynthetic parameters.

The application of BRs can increase the transcript levels of defense-related genes and increase the activity of antioxidant enzymes (Zhou et al., 2014a). The Arabidopsis BR-insensitive mutant *bri1* exhibits upregulated expression of stress-inducible genes (Kim et al., 2010). In our study, EBR application also increased the expression of most antioxidant- and stress-responsive genes. However, the *35S:SlBRI1* plants, early-stage EBR-treated plants and *Atrd29A:SlBRI1* plants presented transcript levels that were lower than those in the WT plants under drought stress (Fig. 7A–I). Recent studies have revealed that Arabidopsis and *Brachypodium distachyon* mutants that exhibit reduced BR signaling intensity present enhanced drought tolerance and increased expression of drought-regulated genes. Moreover, mutants that exhibit increased BR signaling intensity present reduced drought tolerance and transcript levels of drought-induced genes, whereas the expression of growth-regulated genes increased (Feng et al., 2015; Chen et al., 2017). Our results further indicated that the *35S:SlBRI1* plants, early-stage EBR-treated plants and *Atrd29A-SlBRI1* plants presented increased BR signaling intensity, improved expression of growth-regulated genes, and reduced expression of stress-responsive genes. In addition, our results showed that the *abs* tomato mutant (*BRI1* weak mutant), which exhibits very weak BR signaling, displays strong drought resistance (Fig. 8E–I). Logical analysis of the current available evidence implies that exogenously applied BRs can improve plant drought resistance possibly via an unknown branch that exists downstream of BRs and that differs from BRI1 signaling cascades.

5. Conclusion

Our results demonstrated that applications of EBR increased BR contents, which led to reduced ABA and H_2O_2 contents and further increased the stomatal aperture, antioxidant enzyme activity and expression of drought stress-related genes, ultimately improving the drought resistance of tomato. The application of Brz, a BR biosynthesis inhibitor, reduced the BR content and drought resistance. However, increased BR signaling intensity by increasing *SlBRI1* expression levels resulted in increased H_2O_2 contents and further reduced the stomatal aperture, antioxidant enzyme activity and expression of drought stress-related genes, ultimately reducing the drought resistance of tomato. In contrast, reduced endogenous BR signaling intensity in the tomato *SlBRI1* weak mutant *abs* improved drought resistance. This study will pave the way for identifying the unknown components that work downstream of BRs and that differ from those of BRI1 cascades to regulate plant drought resistance.

Author contributions

S.M.N. and X.F.W. contributed to the conception of the study and wrote the main manuscript; S.M.N., S.H.H., and S.F.W. performed the experiments; and S.M.N., Y.J.M., J.W.L., and R.L.M. analyzed the data and provided constructive discussions. All the authors gave their final approval of the submission of the manuscript.

Conflict of interest

Authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.014.

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