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H₂O₂ mediates the crosstalk of brassinosteroid and abscisic acid in tomato responses to heat and oxidative stresses

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Abstract

The production of H₂O₂ is critical for brassinosteroid (BR)- and abscisic acid (ABA)-induced stress tolerance in plants. In this study, the relationship between BR and ABA in the induction of H₂O₂ production and their roles in response to heat and paraquat (PQ) oxidative stresses were studied in tomato. Both BR and ABA induced increases in RBOH1 gene expression, NADPH oxidase activity, apoplastic H₂O₂ accumulation, and heat and PQ stress tolerance in wild-type plants. BR could only induced transient increases in these responses in the ABA biosynthetic mutant notabilis (not), whereas ABA induced strong and prolonged increases in these responses in the BR biosynthetic mutant d⁷im compared with wild-type plants. ABA levels were reduced in the BR biosynthetic mutant but could be elevated by exogenous BR. Silencing of RBOH1 compromised BR-induced apoplastic H₂O₂ production, ABA accumulation, and PQ stress responses; however, ABA-induced PQ stress responses were largely unchanged in the RBOH1-silenced plants. BR induces stress tolerance involving a positive feedback mechanism in which BR induces a rapid and transient H₂O₂ production by NADPH oxidase. The process in turn triggers increased ABA biosynthesis, leading to further increases in H₂O₂ production and prolonged stress tolerance. ABA induces H₂O₂ production in both the apoplastic and chloroplastic compartments.

Key words: Abscisic acid; brassinosteroid; hydrogen peroxide; NADPH oxidase; Solanum lycopersicum; VIGS.

Introduction

Plants continuously face a myriad of biotic (i.e. fungi, bacteria, viruses, nematodes, and insects) and abiotic (e.g. extreme temperatures, drought, and salt) stresses in the natural environment. To survive such stresses, plants have evolved intricate defence mechanisms to increase their tolerance. Phytohormones, such as auxins, gibberellins, abscisic acid (ABA), cytokinins, salicylic acid, ethylene, jasmonates, brassinosteroids (BRs), and peptide hormones, are all involved in plant adaptation to biotic and abiotic stresses by mediating a wide range of adaptive responses (Lorenzo and Solano, 2005; Mauch-Mani and Mauch, 2005; Rubio et al., 2009; Xia et al., 2009). Moreover, reactive oxygen species (ROS) such as H₂O₂ are involved in the regulation of multiple plant responses to a variety of stresses (Neill et al., 2002; Xia et al., 2009; Cui et al., 2011). ROS may function as a second messenger in phytohormone signalings and other important biological

Abbreviations: ABA, abscisic acid; AC, Ailsa Craig; APX, ascorbate peroxidase; AsA, reduced ascorbate; BR, brassinosteroid; CAT, catalase; CR, Condine Red; DAB, 3,3′-diaminobenzidine; DHA, oxidized ascorbate; DMTU, dimethylthiourea; DPI, diphenylene iodonium; EBR, 24-epibrassinolide; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; NO, nitric oxide; PPFD, photosynthetic photon flux density; PQ, paraquat; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing.

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processes (Xiong et al., 2002; Chinnusamy and Zhu, 2009; Xia et al., 2009).

BRs are a class of plant steroid hormones involved in a broad spectrum of cellular and physiological processes (Choudhary et al., 2012). In addition to their role in plant growth and development, BRs have been implicated in the regulation of stress responses (Nakashita et al., 2003; Li et al., 2009; Xia et al., 2009). However, the majority of studies on the role of BRs in stress responses rely on exogenous application of BRs or their biosynthesis inhibitor since mutants defective in BRs biosynthesis and signalling often display severely dwarf phenotypes in model plants such as Arabidopsis (Nakashita et al., 2003; Liu et al., 2009). These mutants, therefore, may already be under intrinsic cellular stress with altered antioxidant activity (Cao et al., 2005). Accordingly, these mutants are hardly used for studies of the stress responses. As a result, there is a lack of genetic evidence for a role of BRs in plant stress responses. Recently, we found that BRs induce a transient increase in the Respiratory burst oxidase homolog 1 (RBOH1) transcript, NADPH oxidase activity, H2O2 in the apoplast and nitric oxide (NO). We have shown that BR-induced ROS production is important for BR-induced stress tolerance in cucumber and tomato (Xia et al., 2009, 2011; Cui et al., 2011; Nie et al., 2013).

ABA is another important plant hormone with a critical role in the regulation of stress responses (Leung and Giraudat, 1998; Rock, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000). Stresses such as drought induce accumulation of ABA, resulting in increased tolerance (Zhu, 2002). Several lines of evidence show that ABA induces H2O2 accumulation in the apoplast, which is dependent on NADPH oxidase RBOH genes and plays an important role in ABA signalling (Pei et al., 2000; Kwak et al., 2003). Recent studies have demonstrated the interconnections between ABA and other plant hormones such as auxins, cytokinins, gibberellins, ethylene, and BRs in a number of physiological processes (Gazzarrini and McCourt, 2001; Finkelstein et al., 2002; Finkelstein and Gibson, 2002). Several recent studies have also shown that exogenous BRs increased ABA accumulation, and the effect was more significant under stress conditions (Kurepin et al., 2008; Liu et al., 2009; Zhang et al., 2011). Other studies, however, have demonstrated that BRs and ABA displayed an antagonistic relationship in several physiological responses (Zhang et al., 2009). It is not clear whether there is an antagonistic interaction between BRs and ABA in the stress response.

The mechanisms by which BRs enhance plant stress tolerance have so far been largely unknown. Our previous studies have shown that BRs induce ROS production, which is critical for BR-induced stress tolerance in plants (Xia et al., 2009, 2011). We have further demonstrated that BRs induce elevated levels of NO, which appear to act downstream of ROS in BR-induced stress tolerance (Cui et al., 2011). Studies using detached shoots and cell suspensions have shown that chemical inhibition of ABA biosynthesis reduced BR-induced stress tolerance (Liu et al., 2009; Zhang et al., 2011), whilst studies in Arabidopsis showed that BRs increased the tolerance of mutant plants defective in ABA biosynthesis (Divi et al., 2010). Both ABA and BRs could induce NADPH oxidase RBOH genes and increase apoplastic H2O2 accumulation (Pei et al., 2000; Kwak et al., 2003; Xia et al., 2009). These results present a complex and conflicting picture about the possible relationship between BRs and ABA during the induction of plant stress tolerance. One possible scenario could be that BRs induce the production of ABA, which, in turn, induces apoplastic H2O2 accumulation and stress tolerance, but, if this is the case, one needs to account for how BRs increase ABA biosynthesis. It is also possible that BRs may themselves induce accumulation of apoplastic H2O2, which acts to increase biosynthesis of ABA, causing a further increase of H2O2 production and BR-induced stress responses. To test these possibilities, we compared mutants partially defective in BR biosynthesis with those defective in ABA biosynthesis and RBOH1-silenced plants for responses to BRs and ABA through analysis of the changes in stress tolerance, H2O2 and ABA accumulation, and transcript levels of stress-related genes and antioxidants in tomato.

Materials and methods

Plant materials, virus-induced gene silencing (VIGS) construct and Agrobacterium-mediated virus infection

Four tomato (Solanum lycopersicum L.) genotypes, Condine Red (CR) and its partially BR synthesis mutant d∗im, Ailsa Craig (AC) and its partially ABA-deficient mutant notabilis (not) were used. Seeds were germinated in a growth medium filled with a mixture of peat and vermiculite (7:3, v/v) in trays in a growth chamber. When the first true leaf was fully expanded, seedlings were transplanted into plastic pots (15 cm diameter×15 cm deep, one seedling per pot) containing the same medium and were watered daily with Hoagland nutrient solution. The growth conditions were as follows: a 14 h photoperiod, temperature of 25/20 °C (day/night), and photosynthetic photon flux density (PPFD) of 600 μmol m–2 s–1.

The tobacco rattle virus (TRV) VIGS construct used for the silencing of the tomato RBOH1 gene were generated by cloning a 311 bp RBOH1 cDNA fragment, which was PCR amplified using the forward primer (5'-ATACCGGAGCTCAAGAATGGGTTGATATTTG-3') and the reverse primer (5'-ATACCGGAGCTCCTGACGTTTATT CCTTAC-3') according to Liu et al. (2002). The amplified fragment was digested with SacI and Xhol and ligated into the same sites of pTRV2. The resulting plasmids were transformed into Agrobacterium tumefaciens GV3101. Agrobacterium-mediated virus infection was performed as described previously (Ekengren et al., 2003). Plants were then kept at 23±1 °C under 120 μmol m–2 s–1 PPFD for 30 d before they were used for experiments (Kandoth et al., 2007).

Stress treatments and analysis of chlorophyll fluorescence

To evaluate BR-induced tolerance to various stresses, tomato seedlings at the five-leaf stage were sprayed with 24-epibrassinolide (EBR) at 0, 30, 100, 200, 500, and 1000 nM or with ABA at 0, 10, 20, 50, 100, and 200 μM. After 3 or 24 h, the seedlings were exposed to heat-shock stress or paraquat (PQ) stress, respectively. For the heat-shock stress, the tomato seedlings were maintained at 42 °C under 800 μmol m–2 s–1 PPFD for 6 h. For the PQ stress, the seedlings were sprayed with 20 μM PQ and subsequently maintained at 600 μmol m–2 s–1 PPFD and 25 °C for 3 h. To determine the role of ROS from different sources, the plants were pre-treated with water, 50 μM diphenylethylidonium (DPI, an NADPH oxidase inhibitor) or 5 mM dimethyldithioreua (DMTU, an H2O2 scavenger) for 12 h and subsequently treated with 200 nM EBR or 50 μM ABA. At the end
H₂O₂ quantification, histochemical analysis, and cytotoxic analysis

H₂O₂ was extracted from leaf tissue according to Doulias et al. (1997) and measured as described in our earlier study (Xia et al., 2011). Histochemical staining of H₂O₂ in the plants was detected as described previously (Thordal-Christensen et al., 1997). Leaves from tomato plants treated with water, EBR, or ABA were detached and placed in a solution containing 1 mg ml⁻¹ of 3,3'-diaminobenzidine (DAB, pH 5.5) for 4 h after a brief vacuum infiltration. The leaf discs were boiled in 90% (v/v) ethanol for 10 min, stored in 50% glycerol, and photographed (BX61; Olympus Co., Tokyo, Japan). H₂O₂ was also visualized at the subcellular level using CeCl₃ for localization, as described previously (Xia et al., 2009; Zhou et al., 2012). The sections were examined using a transmission electron microscope (H7650; Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV to detect the electron-dense CeCl₃ deposits that were formed in the presence of H₂O₂.

NADPH oxidase and antioxidant analysis

For the determination of NADPH oxidase activity, leaf plasma membranes were isolated using a two-phase aqueous polymer partition system (Larsson et al., 1987). The NADPH-dependent O₂⁻-generating (EC 1.6.3.1) activity in isolated plasma membrane vesicles was determined as described previously (Xia et al., 2009; Zhou et al., 2012). The rates of O₂⁻ generation were calculated using an extinction coefficient of 21.6 mM⁻¹ cm⁻¹. Reduced (GSH) and oxidized (GSSG) glutathione were determined according to Rao and Ormrod (1995). Reduced (AsA) and oxidized (DHA) ascorbate was measured following the method of Tamura and Suzuki (1991).

For the antioxidant enzyme assays, leaf tissue (0.3 g) was ground with 2 ml of ice-cold buffer containing 50 mM PBS (pH 7.8), 0.2 mM EDTA, 2 mM AsA, and 2% (w/v) polyvinylpyrrolidone. The homogenates were centrifuged at 12 000 rpm for 20 min, and the resulting supernatants were used for the determination of enzyme activity. All steps were performed at 4 °C. The protein content was determined following the method of Bradford (1976) using bovine serum albumin as a standard. Superoxide dismutase (SOD) activity was assayed by the photochemical method described by Stewart and Bewley (1980). Ascorbate peroxidase (APX) was assayed according to Nakano and Asada (1981). The activity of catalase (CAT) was measured using the method of Cakmak and Marschner (1992). Glutathione reductase (GR) activity was measured following the protocol of Foyer and Halliwell (1976). All the spectrophotometric analyses were performed using a SHIMADZU UV-2401PC spectrophotometer (Shimadzu Co., Kyoto, Japan).

Measurement of endogenous ABA levels

For ABA extraction, 1 g of fresh leaves was ground in a mortar and homogenized in extraction solution (80% methanol, v/v). The extracts were centrifuged at 10 000g for 20 min. The supernatant was eluted through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) to remove the polar compounds and subsequently stored at −20 °C for an ELISA. The ELISA procedures were conducted following the manufacturer's instructions (China Agricultural University, Beijing, China). ABA was determined using the Multimode Plate Reader Label-free System (PerkinElmer, Boston, MA, USA).

QRT-PCR analysis

Total RNA was isolated from tomato leaves using Trizol reagent (Sangon, Shanghai, China), according to the manufacturer's recommendations. Genomic DNA was removed with an RNaseasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 µg) was reverse transcribed using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) following the manufacturer's instructions. Gene-specific RT-PCR primers were designed based on their cDNA sequences (Supplementary Table S1 at JXB online). Two of these genes encoded transcription factors (WRKY1 and WRKY72), and six were involved in stress responses: MAPK1 (encoding mitogen-activated protein kinase 1), HSP70 (encoding a 70 kDa heat-shock protein), Cu/Zn-SOD (encoding Cu/Zn-SOD), CAT1 (encoding catalase 1), and GRI (encoding glutathione reductase 1).

qRT-PCR was performed using a iCycler Q™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Each reaction (25 µl) consisted of 12.5 µl of SYBR Green PCR Master Mix (Takara, Chiga, Japan), 1 µl of diluted cDNA and 0.1 µM forward and reserve primers. The PCR cycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 58 °C for 45 s. The tomato Actin gene was used as an internal control. Relative gene expression was calculated according to Livak and Schmittgen (2001).

Results

Dynamic dependency of ABA biosynthesis in BR-induced tolerance

To study the relationship between ABA- and BR-induced stress tolerance, we compared BR-deficient Δbrm and ABA-deficient not mutants and their corresponding wild-type plants to determine their EBR- and ABA-induced tolerance to heat stress and PQ oxidative stress, respectively. Without heat or PQ treatment, all mutant and wild-type plants had similar Fv/Fm values (close to 0.83). Exposure to heat (Fig. 1a, c) and PQ (Fig. 1b, d) resulted in significant reductions in Fv/Fm at 6 and 3 h, respectively, and this decrease was more significant in the Δbrm and not mutants, suggesting that a defect in either BR or ABA accumulation reduced tolerance to heat and photo-oxidative stresses. Pre-treatment with EBR or ABA at 3 or 24 h prior to the exposure to heat and PQ significantly increased Fv/Fm in both wild-type plants. However, although ABA restored heat and PQ tolerance in the BR-deficient Δbrm mutant plants, EBR restored the heat and PQ tolerance of the not plants at 3 h but was ineffective at 24 h after its application (Fig. 1). We also analysed the changes in electrolyte leakage after exposure to heat-shock and photo-oxidative stress and similar results were observed (Supplementary Fig. S1 at JXB online). Thus, ABA was able to rescue the BR-deficient Δbrm mutant plants, but EBR could only partially and transiently rescue the defective...
stress tolerance of the ABA-deficient not mutant. This observation suggested that ABA might act downstream of BRs in plant stress responses. Furthermore, ABA-induced PQ tolerance was effectively blocked by pre-treatment with DMTU but not by DPI, whilst EBR-induced PQ tolerance in CR was abolished by both DPI and DMTU (Supplementary Fig. S2 at JXB online). These results suggested that non-apoplastic ROS were also responsible for the observed ABA-induced PQ tolerance.

Kinetics of EBR- and ABA-induced RBOH1 expression and $\text{H}_2\text{O}_2$ accumulation

*RBOH1*-NADPH oxidase plays an important role in BR- and ABA-induced stress responses in plants (Kwak et al., 2003; Xia et al., 2009). To determine whether the ABA dependence of BR-induced tolerance was related to the *RBOH1* transcript, which is important in stress responses (Zhou et al., 2012), we compared the BR-deficient *d*<sup>imm</sup> and ABA-deficient not mutants and their respective wild-type counterparts to determine EBR- and ABA-induced *RBOH1* transcription. *RBOH1* transcript levels were increased as early as 3h and remained elevated up to 24h after EBR or ABA treatment in CR and the BR-deficient *d*<sup>imm</sup> mutant (Fig. 2a). *RBOH1* was also rapidly elevated from 0.5 to 24h in AC after ABA or EBR treatment (Fig. 2b). ABA treatment increased the transcript levels of *RBOH1* in the ABA-deficient not mutant plants almost as rapidly and as strongly as in the wild-type plants (Fig. 2b). In contrast, EBR induced only a small and
transient elevation of RBOH1 transcripts at early time points after treatment in the not mutant. Thus, EBR is capable of inducing RBOH1 expression; however, strong and sustained induction of the gene by EBR is dependent on a sufficient ABA level.

A quantitative analysis of the H2O2 content supported the observation that EBR was almost as effective as ABA at inducing H2O2 in wild-type and BR-deficient dmm mutant plants, whilst it induced H2O2 accumulation only at 3 h and not at 24 h in the ABA-deficient not mutant plants (Fig. 3a, b). Similarly, both EBR and ABA increased NADPH oxidase activity in the wild-type plants at 3 and 24 h, whilst EBR increased NADPH oxidase activity only at 3 h and not at 24 h in the not mutant plants (Fig. 3c).

In situ DAB staining also revealed increased accumulation of H2O2 in the EBR- and ABA-treated wild-type plants (Supplementary Fig. S3 at JXB online). H2O2 accumulation was observed in the chloroplasts in the ABA-treated leaves but not in the EBR-treated leaves. Pre-treatment with DPI and DMTU abolished the EBR-induced H2O2 accumulation, whereas only DMTU could abolish the ABA-induced H2O2 accumulation (Supplementary Fig. S3b). Using CeCl3-based procedures, we further observed increased H2O2 accumulation in the apoplast and chloroplasts in response to ABA in the AC plants at 3 and 24 h (Fig. 3d). In comparison, increased H2O2 accumulation was observed only in the apoplast in the EBR-treated AC plants. Again, ABA induced H2O2 accumulation in the apoplast and chloroplasts in the not plants; in sharp contrast, EBR induced H2O2 accumulation in the apoplast but not in the chloroplasts at 3 h (Fig. 3d). However, no such apoplastic H2O2 accumulation was observed in the EBR-treated not plants at 24 h. Thus, it is plausible that ABA is able to activate another ROS production pathway that is not dependent on RBOH1-NADPH oxidase.

BR and ABA differentially trigger H2O2 accumulation in tomato plants

To determine the role of RBOH1 in BR- and ABA-induced H2O2 accumulation in the apoplast, we silenced RBOH1 using VIGS. As observed in our previous study (Nie et al., 2013), silencing of RBOH1 resulted in a 70–80% decrease in RBOH1 transcript levels in the leaves (data not shown). Importantly, EBR-induced NADPH oxidase activity was compromised in the RBOH1-silenced (pTRV-RBOH1) plants at both 3 and 24 h after EBR treatment (Fig. 4a). In comparison, ABA was also effective in inducing NADPH oxidase activity in pTRV-RBOH1 plants, although the increase was less significant compared with that observed in the non-silenced plants (pTRV) (Fig. 4a). At 3 and 24 h after EBR treatment, both the basal and EBR-induced H2O2 levels were significantly lower in the pTRV-RBOH1 than in the pTRV control plants (Fig. 4b). Additionally, ABA induced H2O2 production at 3 and 24 h in both pTRV control plants and pTRV-RBOH1 plants. However, EBR failed to significantly increase H2O2 staining in the RBOH1-silenced plants, whereas ABA substantially increased H2O2 staining (Fig. 3c).

It is possible that ABA is able to activate another ROS production pathway that is not dependent on RBOH1-NADPH oxidase. To confirm this hypothesis, we detected H2O2 accumulation in the chloroplasts and leaf discs of the RBOH1-silenced plants. Strong H2O2 accumulation was observed in the apoplast and chloroplasts of control and RBOH1-silenced leaves at 3 and 24 h after ABA treatment (Fig. 4e), and this accumulation could be abolished by DMTU pre-treatment (Fig. 4c, d). In contrast, no such H2O2 accumulation was observed in the chloroplasts of EBR-treated plants (Fig. 4d, e), and, importantly, EBR failed to induce H2O2 accumulation in the apoplast of RBOH1-silenced plants (Fig. 4c–e). These results suggested that, although EBR increased H2O2 accumulation almost exclusively in the apoplast through an RBOH1-dependent mechanism, ABA induced an additional ROS production pathway in the chloroplasts that was not dependent on NADPH oxidase in the apoplast. Our results also revealed that the EBR-induced sustained accumulation of H2O2 was largely dependent on ABA synthesis.

BR and ABA induce tolerance against photo-oxidative stress via different ROS generation pathways

To determine the role of RBOH1 in BR- and ABA-induced stress tolerance, pTRV and pTRV-RBOH1 plants were
exposed to PQ at 3 and 24 h after EBR or ABA treatment, respectively. EBR treatment alleviated the PQ-induced decrease in Fv/Fm in the pTR V control plants but not in the pTR V-RBOH1 plants. In comparison, ABA alleviated the Fv/Fm reduction in both the pTR V and pTR V-RBOH1 plants (Fig. 5a, b). The ABA-induced PQ tolerance was compromised by pre-treatment with DMTU (Fig. 5a, b). Furthermore, the pTRV-RBOH1 plants were not responsive to EBR at any of the concentrations tested. Similarly, the extent of ABA-induced tolerance was dependent on the applied concentration, although ABA also induced tolerance in the pTRV-RBOH1 plants (Fig. 5d). Thus, ABA-induced stress tolerance is independent of RBOH1-NADPH oxidase.

We further analyzed the effects of different concentrations of EBR and ABA on the tolerance to photo-oxidative stress at 3 h (Fig. 5c, d). EBR treatment increased Fv/Fm only marginally at a very low concentration (30 nM), whereas EBR increased Fv/Fm by approximately 20% at a higher concentration (200 nM) when compared with the untreated control in AC and pTRV plants (Fig. 5c). However, further increases in the EBR concentration resulted in a reduction in its beneficial effects. The most effective concentration of EBR was approximately 200 nM in the AC and pTRV plants; however, in ABA-deficient not plants, a higher EBR concentration (500 nM) was required to reach its maximum effects. Furthermore, the pTRV-RBOH1 plants were not responsive to EBR at any of the concentrations tested. Similarly, the extent of ABA-induced tolerance was dependent on the applied concentration, although ABA also induced tolerance in the pTRV-RBOH1 plants (Fig. 5d). Thus, ABA-induced stress tolerance is independent of RBOH1-NADPH oxidase.

Relationships among ABA biosynthesis, BR biosynthesis, and BR-induced apoplastic H$_2$O$_2$

To determine whether EBR treatment increases ABA biosynthesis and, if so, whether EBR-induced H$_2$O$_2$ accumulation in the apoplast is required for increased ABA accumulation, we analyzed the response of ABA to EBR and PQ in wild-type, BR$^{	ext{sim}}$, and pTRV-RBOH1 plants (Fig. 6). Interestingly, compared with the CR control plants, BR$^{	ext{sim}}$ plants exhibited reduced ABA contents. In both the CR and BR$^{	ext{sim}}$ plants, ABA accumulation was significantly increased after EBR treatment for the duration of the experiment (Fig. 6a). Similarly, EBR
induced ABA accumulation in the pTRV plants, and this increase was even higher after exposure to PQ stress (Fig. 6b). However, the ABA concentration in the pTRV-RBOH1 plants was increased only after PQ treatment and not after EBR treatment. These results strongly suggested that EBR treatment induced ABA biosynthesis, particularly under stress conditions.

**Fig. 4.** EBR- and ABA-induced H$_2$O$_2$ and NADPH oxidase activity is dependent on expression of RBOH1. (a) NADPH oxidase activity. (b) H$_2$O$_2$ accumulation. (c, d) In situ detection of H$_2$O$_2$ in leaves. The plants were pre-treated with 5 mM DMTU or water for 12 h and subsequently treated with 200 nM EBR or 50 µM ABA. After 24 h, DAB staining of leaf discs was performed. H$_2$O$_2$ accumulation was detected using an Olympus motorized system microscope (BX61; Olympus, Tokyo, Japan) at 2× (c) and 400× (d) magnification. (e) Cytochemical detection of H$_2$O$_2$. Black arrows indicate H$_2$O$_2$ on the membranes and white arrows indicate CeCl$_3$ precipitates in the chloroplasts.
conditions, and that this BR-induced ABA biosynthesis was dependent on RBOH1.

**Dynamics of defence-related gene transcription under BR or ABA treatment**

To analyse further the underlying molecular mechanisms of BR- and ABA-induced stress tolerance, we analysed the changes in defence-related genes at 3 and 24 h after EBR or ABA treatment (Fig. 7). Two of these genes are involved in gene transcription (WRKY1 and WRKY72) and have been identified as transcriptional regulators involved in defence stress responses (Bhattarai et al., 2010; Molan and El-Komy, 2010), and six are involved in stress responses (MAPK1, HSP70, CulZn-SOD, cAPX, CAT1, and GR1). As shown in Fig. 7, there was no significant difference in the expression of these genes between the wild-type and d^im plants, with the exception of CulZn-SOD, which was expressed at higher levels in the d^im plants than in the wild-type plants. In contrast, the transcript levels of MAPK1, WRKY72, WRKY1, HSP70, and GR1 were decreased and cAPX and CulZn-SOD transcripts were hyper-accumulated in the ABA not mutant when compared with the corresponding wild-type line. The expression levels of these genes did not differ in the pTRV-RBOH1 plants compared with the pTRV plants with the exception of HSP70, which was downregulated in the pTRV-RBOH1 plants.

EBR or ABA treatment increased the transcription of defence-related genes in the AC plants at 3 and 24 h. In the not plants, the transcription of these genes was also increased by ABA; however, EBR increased their transcription only at 3 h and not at 24 h. cAPX was downregulated by both BR and ABA in the not plants. In the pTRV-RBOH1 plants, these defence-related genes were induced by ABA but not by EBR at 3 and 24 h.

**Interplay of BR and ABA in the regulation of cellular redox homeostasis**

The activities of the antioxidant enzymes SOD, CAT, APX, and GR were increased at 24 h in the two wild-types and the d^im mutant after EBR or ABA treatment (Fig. 8a). In the not plants, ABA, but not EBR, increased the activities of SOD, CAT, and GR at 24 h after treatment. In comparison, APX activity was not affected by EBR treatment, but was decreased after ABA treatment. The total glutathione and ascorbate contents were not altered at 24 h after EBR or ABA treatment, although the ratios of GSH/GSSG and AsA/DHA were increased by the EBR or ABA treatment (Fig. 8b). Therefore, exogenous BRs induced plant responses to oxidative stress in an ABA-dependent manner at 24 h after treatment.

**Discussion**

An increasing body of evidence supports the role of BRs in stress and defence responses. In this study, we have presented evidence that BR-induced stress tolerance involves a positive...
feedback mechanism in which BRs induce a rapid and transient H₂O₂ production by NAPDH oxidase, which first triggers increases in ABA biosynthesis that lead to a further increase in H₂O₂ production. Consequently, prolonged stress tolerance is induced (Fig. 9).

**BR-induced stress tolerance is associated with changes in the cellular redox state**

We demonstrated that endogenous BR levels are closely related to stress tolerance (Fig. 1). Intriguingly, reduced BR levels in the BR biosynthetic mutant plants were associated with reduced ratios of GSH/GSSG and AsA/DHA, although exogenously applied BRs significantly increased the transcript levels of almost all defence-related genes, the activity of antioxidant enzymes, and the GSH/GSSG and AsA/DHA ratios (Fig. 8b). Several recent studies have revealed that stress-inducible genes are constitutively upregulated in the BR-insensitive Arabidopsis mutant bri1 and that heat-shock-induced oxidative stress depends on BR levels in tomato (Kim et al., 2010; Mazorra et al., 2011). In the present study, we observed increases in Cu/Zn-SOD expression and SOD activity in d'imm plants, suggesting that BR deficiency may induce partial oxidative stress in plants. In many cases, maintaining a reduced cellular redox state is important for plant growth and tolerance to biotic and abiotic stresses. Indeed, during the development of systemic acquired resistance, there is an initial ROS accumulation that perturbs the cellular redox state, which in turn activates the NPR1 pathway to activate the PRI gene (Mou et al., 2003). Most recently, we found that BR-induced photosynthesis and stress tolerance involves a H₂O₂-mediated increase in the GSH/GSSG ratio, which can positively regulate the synthesis and activation of redox-sensitive enzymes involved in carbon fixation (Jiang et al., 2012). Accordingly, BRs play an important role in the defence and stress responses and in the maintenance of a reduced cellular redox state in plants. Taken together, these studies support the involvement of BR in plant responses to various stresses.

**Crosstalk between BRs and ABA during H₂O₂ generation, defence responses, and stress tolerance**

In addition to BRs, ABA is well established as having positive effects on stress tolerance, and both BRs and ABA can trigger H₂O₂ production by NAPDH oxidase in the apoplast (Pei et al., 2000; Kwak et al., 2003; Hu et al., 2005; Xia et al., 2009; Cui et al., 2011). In the present study, we observed that both ABA and EBR increased the levels of RBOH1 transcripts in wild-type and BR-deficient mutant plants (Fig. 2a). However, EBR increased RBOH1 transcript levels at early time points but not at late time points after treatment in the ABA-deficient not mutant plants (Fig. 2b), which was consistent with the changes in H₂O₂ accumulation and antioxidative response (Figs 3, 7 and 8). Intriguingly, although ABA completely rescued the BR-deficient mutant in terms of stress tolerance, EBR rescued the stress tolerance of ABA-deficient mutant only at 3 h and not at 24 h after application (Fig. 1). The expression analysis of defence-related genes also provided further evidence that EBR could only transiently rescue the ABA-deficient mutant in the early stages after treatment (Fig. 7). These results suggest that the inability of EBR to induce prolonged stress tolerance in the not mutant plants was associated with the lack of sustained H₂O₂ accumulation in the late stage.

There are conflicting results regarding the relationship between BRs and ABA in stress tolerance. Several in vivo studies using chemical inhibitors have demonstrated that inhibition of ABA with a biosynthesis inhibitor compromises BR activity during the stress response, other studies have shown that EBR significantly improves the tolerance of an ABA-deficient mutant and that the effect of BRs on stress tolerance was ABA-independent (Divi et al., 2010; Liu et al., 2011; Zhang et al., 2011). These discrepancies could be explained by time-dependent changes in the RBOH1 transcript level, H₂O₂ accumulation, and defence responses (Figs 2 and 3). When plants were exposed to a continuous supply of BRs, RBOH1 induction and H₂O₂ production occurred...
continuously, without the aid of ABA. Taken together, these results strongly suggest that ABA biosynthesis plays an important role in sustained stress tolerance in BR-induced pathways in plants.

Different sources of H$_2$O$_2$ in EBR- and ABA-induced stress tolerance

Similar to ABA, BRs induce H$_2$O$_2$ accumulation by inducing/activating RBOH1-NADPH oxidase in the apoplast (Hu et al., 2005; Xia et al., 2009; Cui et al., 2011). Thus, a critical question is whether BR and ABA induce H$_2$O$_2$ production through the same pathway and whether BR-triggered H$_2$O$_2$ production is dependent on ABA. In the present study, we found that BRs could transiently induce H$_2$O$_2$ accumulation, which was independent of ABA biosynthesis; however, sustained induction was ABA dependent (Fig. 3). Interestingly, BR only induced H$_2$O$_2$ accumulation in the apoplast; however, ABA induced H$_2$O$_2$ accumulation not only in the apoplast but also in the chloroplasts (Figs 3 and S2). Apparently, BRs directly induced increases in RBOH1 transcripts, and RBOH1 silencing led to decreased H$_2$O$_2$ accumulation and compromised the effects of EBR on H$_2$O$_2$ accumulation and stress tolerance (Figs 4 and 5). Importantly, unlike in the wild-type and not plants, EBR was unable to induce tolerance to PQ in RBOH1-silenced plants (Fig. 5), even when applied at relatively high concentrations (up to 1000 nM). Therefore, our results showed that EBR-induced tolerance is dependent on H$_2$O$_2$ production in the apoplast by NADPH oxidase. In contrast, ABA was able to induce PQ tolerance at a wide range of concentrations (up to 50–100 μM; Fig. 5d) in RBOH1-silenced plants, and this ABA-induced PQ tolerance

Fig. 7. Effects of EBR and ABA treatments on the expression of stress-responsive genes. Leaf samples were collected at 3 and 24 h after foliar treatment with EBR (200 nM) or ABA (50 μM). qRT-PCR analysis was performed to examine the steady-state levels of mRNAs in the plants. The data are means of three replicates (±SD). Means denoted by the same letter do not differ significantly at P≤0.05 according to Turkey’s test. The expression level of genes in CR and pTRV plants was set to 1 for comparative expression analysis of the same genes in the not and pTRV-RBOH1 plants, respectively.
was compromised when DMTU was co-applied (Fig. 5a). These observations indicate that the induction of PQ tolerance by ABA at moderate concentrations is not dependent on RBOH1 because ABA could also induce H2O2 accumulation in the chloroplasts independent of RBOH1, which apparently plays an important role in ABA-induced tolerance. Therefore, ABA induces PQ tolerance by triggering H2O2 accumulation in the apoplast and chloroplasts.

Fig. 8. Changes in antioxidant enzymes, glutathione and ascorbic acid redox homeostasis after EBR or ABA treatment. Tomato plants with different levels of endogenous BRs and ABA were treated with 200nM EBR or 50 µM ABA and the activities of antioxidant enzymes and glutathione redox homeostasis were determined 24 h later. The values are means of three replicates (±SD). Means denoted by the same letter do not differ significantly at P<0.05 according to Turkey’s test.

Fig. 9. A proposed model for the induction of stress tolerance by BRs and ABA in tomato. Upon perception of a BR signal, plasma membrane-bound RBOH1-NADPH oxidase induces the production of apoplastic H2O2, which first induces the biosynthesis of ABA, in turn leading to a further increase in H2O2 production in both the apoplastic and chloroplastic compartments. Consequently, prolonged stress tolerance is induced.

Involvement of ROS in BR-induced ABA biosynthesis and prolonged stress tolerance

It remains to be clarified whether BR can induce ABA biosynthesis and, if so, whether ROS is required for this process. Several studies have shown that BR-mediated tolerance to high temperature, Phytophthora infestans infection, and water stress is associated with enhanced ABA accumulation.
(Krishna, 2003; Kurepin et al., 2008; Zhang et al., 2011). However, other studies have shown that pre-treatment with BR decreases stress-induced ABA accumulation (Shakirova and Bezrukova, 1998; Avalbaev et al., 2010). It is worth noting that all of these results were obtained in plants after long stresses and there is no genetic evidence to support the involvement of BRs in ABA biosynthesis. In the present study, we found that ABA levels were decreased in the BR biosynthetic mutant but could be increased by exogenous EBR application (Fig. 6a). Interestingly, the ABA content was reduced, and exogenous BR failed to increase ABA accumulation in the RBOHI-silenced plants, suggesting a role for H2O2 in EBR-induced ABA biosynthesis (Fig. 6b). Similarly, Zhang et al. (2011) reported that NO was involved in BR-induced ABA biosynthesis and similar to ROS, NO is a downstream signalling molecule of H2O2 in BR signalling (Cui et al., 2011). Further evidence for the role of H2O2 in ABA biosynthesis is based on the increase in ABA biosynthesis in plants exposed to PQ (Fig. 6b), which generates ROS in the chloroplasts during exposure to light. Similarly, Galvez-Valdivieso et al. (2009) found that exposure to high light stress induced the accumulation of both ROS and ABA in Arabidopsis. However, the EBR-induced increase in ABA observed in our study was much less than that observed in other studies (Liu et al., 2011; Zhang et al., 2011). In our study, the intact plants received only a single EBR treatment, whereas in other studies, in vivo shoots in BR solution or cell culture suspensions were continuously exposed to EBR (Liu et al., 2011; Zhang et al., 2011), which may lead to the continuous uptake of BRs and activation of RBOHI. Most recently, we found that a high level of BR could induce a prolonged increase in ROS, which formed a positive amplification loop with ABA signaling in stomatal closure (Xia et al., 2014). Consequently, higher levels of ROS may result in ABA accumulation.

In summary, we present genetic and molecular evidences for the dynamic interplay between BR- and ABA-induced H2O2 in tomato stress tolerances. Following the perception of a BR signal, RBOHI-NADPH oxidase is activated to produce H2O2. Increased ROS can then trigger increased ABA biosynthesis, which in turn causes a further increase in H2O2 production leading to prolonged stress tolerance. At concentrations effective for the induction of stress tolerance, ABA induced H2O2 production from two distinct sources (i.e. the apoplastic and chloroplastic compartments) (Fig. 9).

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