Brassinosteroids regulate root growth by controlling reactive oxygen species homeostasis and dual effect on ethylene synthesis in *Arabidopsis*

Bingsheng Lv¹, Huiyu Tian¹, Feng Zhang¹, Jiajia Liu¹, Songchong Lu¹, Mingyi Bai¹, Chuanyou Li², Zhaojun Ding¹*

¹ The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, College of Life Science, Shandong University, Jinan, People’s Republic of China, ² State Key Laboratory of Plant Genomics, National Centre for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, People’s Republic of China

* dingzhaojun@sdu.edu.cn

Abstract

The brassinosteroids (BRs) represent a class of phytohormones, which regulate numerous aspects of growth and development. Here, a *det2-9* mutant defective in BR synthesis was identified from an EMS mutant screening for defects in root length, and was used to investigate the role of BR in root development in *Arabidopsis*. The *det2-9* mutant displays a short-root phenotype, which is result from the reduced cell number in root meristem and decreased cell size in root maturation zone. Ethylene synthesis is highly increased in the *det2-9* mutant compared with the wild type, resulting in the hyper-accumulation of ethylene and the consequent inhibition of root growth. The short-root phenotype of *det2-9* was partially recovered in the *det2-9/acs9* double mutant and *det2-9/ein3/eil1-1* triple mutant which have defects either in ethylene synthesis or ethylene signaling, respectively. Exogenous application of BR showed that BRs either positively or negatively regulate ethylene biosynthesis in a concentration-dependent manner. Different from the BR induced ethylene biosynthesis through stabilizing ACSs stability, we found that the BR signaling transcription factors BES1 and BZR1 directly interacted with the promoters of *ACS7*, *ACS9* and *ACS11* to repress their expression, indicating a native regulation mechanism under physiological levels of BR. In addition, the *det2-9* mutant displayed over accumulated superoxide anions (O$_2^-$) compared with the wild-type control, and the increased O$_2^-$ level was shown to contribute to the inhibition of root growth. The BR-modulated control over the accumulation of O$_2^-$ acted via the peroxidase pathway rather than via the NADPH oxidase pathway. This study reveals an important mechanism by which the hormone cross-regulation between BRs and ethylene or/and ROS is involved in controlling root growth and development in *Arabidopsis*.
Author summary

Both brassinosteroids (BRs) and ethylene have been known to control root growth and development. ROS have been also reported to play an important role in root development. However, the relationship between BRs and ethylene or ROS in root growth and development was not addressed before. In this study, a det2-9 mutant defective in BR synthesis was identified from an EMS mutant screening, displaying a short-root phenotype which is result from the hyper-accumulation of ethylene and superoxide anions (O$_2^-$). Exogenous BR apply showed that BRs either positively or negatively regulate ethylene biosynthesis in a concentration-dependent manner. Different from the BR induced ethylene biosynthesis through stabilizing ACSs stability, we found that the BR signaling transcription factors BES1 and BZR1 interacted with promoters of ACS7, ACS9 and ACS11 to repress their expression, indicating a native regulation mechanism under physiological levels of BR. The BR-modulated control over the accumulation of O$_2^-$ acted via the peroxidase pathway rather than via the NADPH oxidase pathway. This study provides new insights into how brassinosteroids control root growth through the cross-regulation with ethylene synthesis and ROS.

Introduction

Roots are important plant ground organs, which absorb water and nutrients to control plant growth and development. In higher plants, root growth is maintained by coordinating cell proliferation and differentiation [1–3]. Plant hormones have been known to play a crucial role in the regulation of root growth [4]. Recent studies in the Arabidopsis root have shown that different hormones control organ growth by regulating specific growth processes such as cell proliferation, differentiation or expansion in distinct tissues. Plant hormones such as auxin, cytokinin, abscisic acid, brassinosteroids, ethylene and gibberellins have been shown to be involved in root growth through a range of complex interactions. The activities of these hormones during root growth progression depend on cellular context and exhibit either synergistic or antagonistic interactions. For example, ethylene enhances inhibition of root cell elongation through upregulating the expression of ASA1 and ASB1 to enhance auxin biosynthesis in Arabidopsis seedlings [5]. Furthermore, ethylene regulated root growth was also mediated through modulating the auxin transport machinery [6]. In addition, cytokinin was also found to control root growth through transcriptional regulation of the PIN genes and thus influencing auxin distribution [7]. The balance between auxin and cytokinin signaling is crucial during root growth. In Arabidopsis, cell division and cell differentiation largely determines root meristem size, which is under the control of cytokinin and auxin through an ARR1/SHY2/PIN circuit [1]. All these studies suggest that hormonal cross-talk plays a pivotal role in the regulation of root growth.

The brassinosteroids (BRs) represent a class of phytohormones involved in a wide variety and developmental processes including root development [8–12]. BR, detected by the BRI1 receptor, activates the transcription factors BES1 and BZR1, which in turn govern the transcription of a large number of genes [13–16]. BRs are known to participate in root growth and development, because mutants impaired with respect to either the synthesis or signaling of BR develop foreshortened roots [17, 18]. However, excessive application of bioactive BR hampered normal development of plants [19]. Therefore, a finely tuned cellular regulation of BR levels is important for the development of plant. It has been found that BR deficient conditions elicit the expression of BR biosynthesis genes, while increase in endogenous BR concentration...
lead to feedback regulation of the expression of BR metabolic genes to maintain the homeostasis of BR [20]. Recent studies demonstrate that BR interacts with plant hormones such as abscisic acid, gibberellins, auxin and cytokinin to regulate plant growth and development [21–23]. BR interacts with ethylene to regulate the gravitropic response of the shoot, and is involved in ethylene-controlled processes in the hypocotyl of both light- and dark-grown seedlings [24, 25]. Exogenously supplied BR enhances the stability of type 2 of the enzymes 1-aminocyclopropane-1-carboxylate synthase (ACS5 and ACS9) and thus increasing ethylene production, thereby modulating the hypocotyl growth of etiolated seedlings [26]. Though both BRs and ethylene have been reported to regulate root growth and development, it is still unknown if there is a cross-regulation between BRs and ethylene in this process.

In addition to plant hormones, the regulation of root growth has also been tightly linked to reactive oxygen species (ROS). Root growth is profoundly affected by endogenously generated ROS. While ROS were initially believed to merely represent a damaging by-product of the plant’s stress response [27], they have been now recognized as signaling molecules [28]. For example, ROS have been shown to be important for balancing cell proliferation and differentiation during root growth, and have been proposed to adopt a signaling role during lateral root formation [29, 30]. It has been reported that ROS produced in mitochondria of root tip cells in response to the hormone abscisic acid (ABA) are responsible for regulating the root’s meristematic activity [31]. A BR receptor-mediated increase of the cytosolic concentration of calcium ions (Ca$^{2+}$) regulates ROS production, thereby reducing the length of the hypocotyl in dark-grown seedlings [32, 33]. Though BRs have been reported to regulate many plant biotic and abiotic stresses through the regulation of ROS homeostasis [27, 34], the role of the cross-regulation between BRs and ROS in root growth is largely unknown.

Here, the participation of BR in root growth and the extent of its cross-regulation with both ethylene and ROS signaling were investigated by characterizing a novel Arabidopsis det2 mutant allele (det2-9) selected on the basis of its short-root phenotype, which proved to be defective with respect to BR synthesis. A key observation was that the det2-9 mutant accumulated more ethylene and ROS than the wild type. The increased accumulations of both ethylene and ROS caused the short root phenotype in det2-9. This study reveals a mechanism about how BRs regulate root growth through a cross-regulation with ethylene and ROS signaling.

### Results

#### Isolation and characterization of a short-root mutant

To identify novel determinants involved in the control of root growth, an ethyl methane sulfonate (EMS)-mutagenized Arabidopsis population was screened by monitoring root length and elongation. One mutant was subsequently named as short root 5 (sr5) (Fig 1A and 1B). The length of the mutant root was only 23% of the one of a wild type (WT) seedling at 7–8 days post germination. A longitudinal zonation pattern analysis showed that the size of its root apical meristem (RAM) was significantly smaller than the WT control (Fig 1C). Both meristem zone (MZ) and transition zone (TZ) in the RAM were substantially reduced in size. Cortical cells in the mutant mature zone were significantly shorter than those in WT, and cell number in the MZ was strongly reduced (Fig 1D and 1E and Table 1). The number of cells formed by the RAM in sr5 was 1.8 fold fewer than that in the WT control. The length of the mutant’s RAM was 67% of WT’s, and both its MZ and TZ were reduced in size (Table 1). The compromised RAM in the mutant was accompanied by an increased cell cycle time which displayed 1.4 times longer than that in the WT control (Table 1). The signal obtained from the mitotic cyclin B1;1 G/M transition marker pCYCB1;1::GUS was much weaker in the mutant than the WT control (Fig 1F), an indication that cell proliferation was inhibited in sr5. The conclusion
was that the mutant’s short root derived from both a reduced MZ cell number and a smaller cell size in the mature zone.

**The mutated sr5 gene encodes DET2, a steroid 5α-reductase in the BR synthesis pathway**

When positional cloning was employed to identify the site of the sr5 mutation, a position on chromosome 2 flanked by the markers W20 and W22 was identified (S1A Fig). Sequencing of

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Table 1. WT and sr5 root growth and comparative analysis of their RAM activity.

| Genotype | RAM length (μm) | MZ length (μm) | MZ no. of cells | TZ length (μm) | Elongated cell length (μm) | Rate of root growth (μm/h) | Cell production rate (cell h⁻¹) | Cell cycle duration (h) |
|----------|----------------|----------------|-----------------|----------------|---------------------------|---------------------------|-------------------------------|-----------------------|
| WT       | 335±33A        | 209±28A        | 35±3A           | 126±17A        | 199±26A                   | 327.8±48.6A               | 1.6±0.3A                     | 14.6±3.2a               |
| sr5      | 222±18B        | 149±7B         | 26±3B           | 73±7B          | 85±11B                    | 74.4±16.8B                | 0.9±0.2B                     | 20.4±5.9b               |

Different letters associated with values indicate a significant (upper-case letter: *P*<0.01, lower-case letter: *P*<0.05) difference between the WT and sr5 means, based on Duncan’s multiple range test.

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the genes present in the critical genomic region revealed that the mutant had a point mutation causing a G-to-A transition at nucleotide position 107 after ATG in At2g38050 (DET2). The root growth and seedling morphology of the reported det2-1 mutant were indistinguishable from those of sr5 (S1B and S1E Fig). Since the F1 hybrid sr5 x det2-1 retained the short-root phenotype (S1C Fig), it was concluded that the sr5 mutation likely involved a lesion in DET2. Moreover the DET2 promoter driving DET2 cDNA fused to GFP-GUS (pDET2::DET2-GFP-GUS) complemented the short-root phenotype in sr5 (S1D Fig), suggesting that the G107A mutation in DET2 led to the short-root phenotype in the sr5 mutant. In seedlings carrying the transgene, GUS activity was detected both in the shoot and the RAM (S2 Fig). DET2 encodes a steroid 5α-reductase acting in the BR synthesis pathway. The phenotype of sr5, which was similar to that observed in det2-1 grown in darkness, was rescuable when the plants were treated with exogenous BR (eBL) (S1B Fig). Since there are eight alleles of det2 mutant have been reported, we renamed the sr5 mutant as the det2-9 which was used for most of the analysis in this study. We compared the expression levels of some BR induced genes between det2-9 and det2-1 through Q-PCR analysis. The results showed that, though both mutants displayed reduced expression levels of TCH4, BAS1, IAA17 and IAA19, the det2-9 mutant has a higher expression level of these BR-induced genes than the det2-1 mutant (S3 Fig). Consistently, the det2-9 mutant had a weaker phenotype compared with the det2-1 mutant (S1 Fig), indicating that the point mutation at position 107 might not be a null allele.

Both ROS-responsive and ethylene-related genes were affected in det2-9

A RNA-seq approach was applied to compare the det2-9 root transcriptome with that of the WT, a total of 1,480 and 1,116 genes were found to be, respectively, up- and down-regulated (Fig 2A). Among the differentially expressed genes, based on the GO analysis we found that there is a statistically significant enrichment in genes annotated as being linked to secondary metabolic process and response to stimulus (P<0.01). It is not surprising for this enrichment considering the dwarf phenotype of the mutant. Though the previous research has shown that exogenously supplied BR can enhance the production of ethylene [26], the ethylene biosynthesis and ethylene response factors were up-regulated in det2-9 according to our RNA-seq analysis (Fig 2B). These genes included 1-aminocyclopropane-1-carboxylate synthase encoding genes, 1-aminocyclopropane-1-carboxylate oxidase encoding genes and ethylene response factor encoding genes (S1 Dataset). According to our GO analysis, we also found that many of genes belong to GO:0000302 (response to reactive oxygen species) were up-regulated significantly in det2-9 (Fig 2C), which was in contrast with the previous reports showing that BR could induce the generation of H2O2 [27, 34]. To confirm the RNA-seq results, we performed a quantitative real time-PCR (qRT-PCR) assay on a selection of 20 ethylene related genes which were differentially transcribed in WT and det2-9 seedlings grown in light and dark growth conditions (Fig 2D and S4 Fig). Though the expression changes of most of ethylene related genes were confirmed, we also found that some genes, for example ACS6, ERF6 and ERF17, had little agreement between the transcript abundance by RNA-seq and qRT-PCR analysis. Considering three independent repeats were done for the confirmations, the results of qRT-PCR analysis are more reliable. These results suggest that both ROS and ethylene signaling were enhanced in the det2-9 mutant.

BRs either positively or negatively regulate ethylene biosynthesis in a concentration-dependent manner

Ethylene signaling in the det2-9 mutant was monitored by the expression of the pEBS::GUS ethylene signaling reporter. The strength of the GUS signal was considerably higher in the
mutant than that in the WT (Fig 3A), suggesting that an enhanced level of ethylene signaling occurred in det2-9. The increased ethylene response in det2-9 was abolished by the presence of 10 nM eBL during seedling growth (Fig 3A). The ethylene content was considerably higher in the det2-9 mutant than that in WT seedlings (Fig 3B and 3C). The transgene line pDET2::DET2-GFP-GUS/det2-9 complemented the higher level of ethylene observed in det2-9 (Fig 3C). Treatment with the BR synthesis inhibitor propiconazole (PPZ) also resulted in higher ethylene content in light-grown WT seedlings, while eBL (10 nM, a concentration which partially rescued the short-root phenotype in det2-9) treated WT or bes1-D (a mutant which displays an enhanced BR signaling response) light-grown seedlings both showed a reduction in ethylene content (Fig 3B). A similar profile of ethylene content was also observed when seedlings were grown in darkness (S5 Fig). All these above chemical treatment experiments and mutant analysis suggest that both exogenous applied low levels of BR and native BR signaling negatively regulated ethylene biosynthesis.
In addition, we also observed that root growth was inhibited gradually by eBL at concentrations ranging from 10 to 5000 nM (Fig 4A and 4B). While the hypocotyl length was unchanged when treated with low concentration of eBL (<500 nM) but reduced sharply when the concentration of eBL greater than 500 nM (Fig 4A). Furthermore, dark-grown seedling hypocotyls treated with higher concentration of eBL (≥500 nM) displayed a typical “triple response”, indicating the enhanced ethylene response (Fig 4A). Therefore, we further examined the effects of BR on ethylene production using different concentrations of eBL. The results showed that ethylene content was greatly reduced in seedlings treated with low concentration of eBL (10 or 100 nM) while it was strongly increased when the concentration of eBL greater than 500 nM (Fig 4D). Consistently, both GUS staining analysis with the pEBS::GUS transgene line and an examination of ethylene response factors (ERFs) expression using qRT-PCR analysis show that low concentrations (10–100 nM) of BR inhibits ERF expression while high concentrations (≥500 nM) of BR enhanced expression (Fig 4C and 4E), consistent with the change ethylene.

Fig 3. The det2-9 mutant accumulates more ethylene than WT. (A) Ethylene-induced GUS activity (pEBS::GUS) in det2-9 and WT. Seedlings of det2-9 and WT were grown for five days either in the presence or absence of 10 nM eBL, and were stained for GUS activity analysis. Each treatment involved 20–30 seedlings; here, representative samples are presented. Bar = 1 cm. (B) Ethylene content in indicated BR-related transgenic and WT nine-day old seedlings exposed to either eBL (10 nM) or propiconazole (2 μM) under light conditions. Data shown are mean±SE (n = 5). **: means of det2-9, bes1-D, WT+eBL, WT+PPZ differ significantly from mean of WT (P<0.01). (C) Ethylene content in five-day old WT, det2-9 and pDET2::DET2-GFP-GUS/det2-9 seedlings in light conditions. Data shown are mean±SE (n = 5). **: means of det2-9 and WT differ significantly (P<0.01).

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levels. In summary, BR either positively or negatively regulate ethylene biosynthesis depends on the levels of BRs.

**The enhanced ethylene response contributes to the short-root phenotype in det2-9**

When det2-9 mutant seedlings were grown on a medium containing either silver nitrate (AgNO₃, an antagonist of ethylene signaling) or 2-aminoethoxyvinyl glycine (AVG) (an inhibitor of ethylene synthesis), the root growth of det2-9 mutant was partially rescued, producing
root lengths almost double than those developed by the non-treated mutant seedlings. However, both treatments inhibited the root growth of WT seedlings (Fig 5A and 5B). In addition inhibition of ethylene signaling by AgNO$_3$ rescued the cortical cell length in det2-9 (S6 Fig). On the other hand, the root cell elongation and root growth of the mutant seedlings was found to be more sensitive to ACC (a precursor of ethylene synthesis) (Fig 5A and 5B and S6A Fig). In addition, both WT and det2-9 mutant seedlings displayed similar cell numbers in root meristem under the same treatment with either AgNO$_3$ or ACC (S6B Fig). This result suggests that the BR deficiency caused short-root phenotypes in det2-9 was mediated by the effect of ethylene signaling on root cell elongation. Consistently, the octuple acs mutant CS16651
(acs2-1/acs4-1/acs5-2/acs6-1/acs7-1/amiRacs8acs11), ein2-5 and the ein3/eil1-1 double mutant, which have defects in either ethylene biosynthesis or ethylene signaling, were less affected by the PPZ treatment than WT (Fig 5C). The short-root phenotype of det2-9 was partially recovered in the det2-9/acs9 double mutant and det2-9/ein3/eil1-1 triple mutant (Fig 5D–5G). These results indicate that the short-root phenotype in det2-9 partly result from enhanced ethylene biosynthesis and ethylene signaling.

BR regulates ethylene biosynthesis via BZR1/BES1-mediated repression of ACS

A promoter analysis showed that promoters of ACS6, 7, 9, 11, along with ACO1 and 3 (all these genes were strongly up-regulated in det2-9, Fig 2) contained a BRRE and/or an E-box, the binding sites for BES1 and BZR1 (Fig 6A). The direct interaction of the ACSs by BES1 or BZR1 was confirmed by a chromatin immunoprecipitation (ChIP)/qPCR analysis in FLAG-tagged BES1 or YFP-tagged BZR1 transgenic lines (Fig 6A). A series of yeast one-hybrid assays were conducted to further verify whether any of these promoters was regulated directly by either BES1 or BZR1. The outcome was that in yeast BES1 interacted with the promoters of ACS7

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Fig 6. Interaction of BZR1/BES1 transcription factors with various ACS promoters. (A) ChIP/qPCR assay. A scheme of the promoters of ACS6, 7, 9 and 11 are shown with the position of BRRE-box (black) and E-box (gray). Graphs show the ratio of bound promoter fragments (P1–P5) versus total input detected by qPCR after immuno-precipitation in p35S:BZR1-YFP and pNP:BES1-FLAG seedlings by YFP or FLAG antibodies. Data shown are mean±SE (n = 9). (B) Yeast one-hybrid binding assay involving BZR1/BES1 and ACS6, 7, 9 and 11 promoters. (C) Transient expression in A. thaliana protoplasts. BZR1 or BES1 transcription factors were co-transfected with either ACS7, 9 or 11 promoters. The LUC to REN ratio is shown and indicated the activity of the transcription factors on the expression level of the promoters. LUC: firefly luciferase activity, REN: renilla luciferase activity. Data shown are mean±SE (n = 9); **: means significant difference compared to control (P<0.01).

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The accumulation of \(\text{O}_2^\cdot\) was unaffected in both det2-9 and det2-9/rbohF double mutant (S10C Fig). And \(\text{O}_2^\cdot\) hyper-accumulation in det2-9/robohD/F is also similar to the one of WT (S10B Fig). NBT staining showed that det2-9 mutant roots identified genes responding to ROS as BR-signaling mutants compared with the WT control (Fig 7A), while the same treatment inhibited root growth in det2-9 mutant than that in the WT control (Fig 7A), while there was no clear difference when 3,3’-diaminobenzidine (DAB) staining was used to visualize the level of \(\text{H}_2\text{O}_2\) present [37] (S9 Fig). This suggests that det2-9 accumulated \(\text{O}_2^\cdot\) but not \(\text{H}_2\text{O}_2\). Treatment with eBL substantially reduced the extent of the \(\text{O}_2^\cdot\) hyper-accumulation in det2-9 (Fig 7A). Meanwhile, the BR-signaling defective mutant bri1-116 hyper-accumulated \(\text{O}_2^\cdot\), while BR-signaling enhanced plants (p35S::BRI1-GFP or bes1-D) accumulated less superoxide anion in their roots compared with WT plants (Fig 7B), indicating that BR signaling suppresses the accumulation of \(\text{O}_2^\cdot\). Therefore, root growth analysis was done using det2-9 mutant seedlings were exposed to two different \(\text{O}_2^\cdot\) scavengers, namely superoxide dismutase (SOD) [38] and 1,3-dimethyl-2-thiourea (DMTU) [39]. The root length in det2-9 was significantly increased in the presence of 0.65U/ml SOD, while the same treatment inhibited root growth in WT seedlings (Fig 7C). Similarly, a concentration of 0.1 to 2 mM DMTU treatment, which has no effect on root growth in WT seedlings, could significantly increase root lengths in det2-9 (Fig 7D).

**The short-root phenotype in det2-9 was partly attributed to the hyper-accumulation of \(\text{O}_2^\cdot\)**

The transcriptomic analysis in det2-9 mutant roots identified genes responding to ROS as BR-targets (Fig 2C). Therefore, we further analyzed det2-9 mutant for defects in ROS using the nitroblue tetrazolium (NBT) staining method to detect the presence of \(\text{O}_2^\cdot\) in vivo [36]. The NBT signal was higher in the det2-9 mutant than that in the WT control (Fig 7A), while there was no clear difference when 3,3’-diaminobenzidine (DAB) staining was used to visualize the level of \(\text{H}_2\text{O}_2\) present [37] (S9 Fig). This suggests that det2-9 accumulated \(\text{O}_2^\cdot\) but not \(\text{H}_2\text{O}_2\). Treatment with eBL substantially reduced the extent of the \(\text{O}_2^\cdot\) hyper-accumulation in det2-9 (Fig 7A). Meanwhile, the BR-signaling defective mutant bri1-116 hyper-accumulated \(\text{O}_2^\cdot\), while BR-signaling enhanced plants (p35S::BRI1-GFP or bes1-D) accumulated less superoxide anion in their roots compared with WT plants (Fig 7B), indicating that BR signaling suppresses the accumulation of \(\text{O}_2^\cdot\). Therefore, root growth analysis was done using det2-9 mutant seedlings were exposed to two different \(\text{O}_2^\cdot\) scavengers, namely superoxide dismutase (SOD) [38] and 1,3-dimethyl-2-thiourea (DMTU) [39]. The root length in det2-9 was significantly increased in the presence of 0.65U/ml SOD, while the same treatment inhibited root growth in WT seedlings (Fig 7C). Similarly, a concentration of 0.1 to 2 mM DMTU treatment, which has no effect on root growth in WT seedlings, could significantly increase root lengths in det2-9 (Fig 7D).

**The peroxidase pathway, but not the NADPH oxidase pathway, is required for the hyper-accumulation of \(\text{O}_2^\cdot\) in det2-9**

The accumulation of \(\text{O}_2^\cdot\) in det2-9 can be the result of the activation of two signaling pathways: peroxidase or NADPH oxidase. When the NADPH oxidase pathway was blocked by the presence of either diphenylene iodonium (DPI) [40] or ZnCl\(_2\) [41], det2-9 mutant roots were insensitive to any treatment (Fig 8A and 8B). Consistent with this result, the abundance of transcripts of the four NADPH oxidase genes (\(\text{RBOHC, D, F and G}\)) was identical in det2-9 and WT (S10A Fig). The root growth response to PPZ treatment of three mutants robohD, rbohf and rbohd/F was also similar to the one of WT (S10B Fig). NBT staining showed that the BR deficiency-induced \(\text{O}_2^\cdot\) hyper-accumulation by PPZ treatment was unaffected in both plants harboring p35S::NADPHD-GFP and the rbohd/F double mutant (S10C Fig). And \(\text{O}_2^\cdot\) hyper-accumulates in det2-9/robohD and det2-9/robohD/F mutants similarly to det2-9, compared
with WT (S10D Fig). These experiments allow us to conclude that the hyper-accumulation of \( \text{O}_2^- \) in det2-9 did not involve the NADPH oxidase pathway. So attention was focused on the peroxidase pathway [29], by treating seedlings with either salicylhydroxamic acid (SHAM) [42] or 1,10-phenanthroline (1,10-Phe) [43], inhibitors of peroxidase activity. The root length of det2-9 was significantly increased by both treatments, whereas root growth of WT was slightly inhibited (S11A and S11B Fig). NBT staining showed that the levels of \( \text{O}_2^- \) in det2-9 reduced sharply when treated with SHAM or 1,10-Phe but no obvious changes were observed when treated with DPI or ZnCl\(_2\) (Fig 8C), which was consistent with the NBT staining observed in det2-9/rbohD and det2-9/rbohD/F mutants compared with WT and det2-9 (S10D Fig). When the transcription of genes encoding peroxidase was investigated, no clear-cut differences were visible between the mutant and WT (S12 Fig), but peroxidase activity was much stronger in the det2-9 mutant and was reduced when seedlings were treated with exogenous BR (Fig 8D). Thus the hyper-accumulation of \( \text{O}_2^- \) in det2-9 was likely the effects of an increased peroxidase activity.

**Relationship between ethylene and \( \text{O}_2^- \)**

Given that the level of both ethylene and \( \text{O}_2^- \) was enhanced in det2-9, the question arose as to whether ethylene and ROS interacted with one another. \( \text{O}_2^- \) accumulation was initially assayed in WT and det2-9 plants treated with either AVG or ACC (Fig 9A). NBT staining showed that the ACC treatment had a positive and AVG had a negative effect on superoxide anion accumulation in WT roots (Fig 9A). This indicates that ethylene induces an accumulation of \( \text{O}_2^- \) in

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*Fig 7. BR represses the accumulation of superoxide anions.* (A) Five-day old det2-9 and WT seedlings grown in the presence or absence of eBL (10 nM), then assayed for the superoxide anion using NBT. Bar = 1 cm. (B) Superoxide anion accumulation in the root tips of BR-signaling enhanced plants (p35S::BRRI1-GFP and bes1-D) and BR signaling deficient plants (bri1-116). Bar = 50 μm. (C, D) Elongation of the primary root of WT and det2-9 seedlings exposed to either (C) superoxide dismutase (SOD) or (D) 1,3-dimethyl-2-thiourea (DMTU). Data shown are mean±SE (n = 30); Asterisks means significant difference from the control-treated plants (*P<0.01).

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Arabidopsis. NBT staining also showed that p35S::EIN3-GFP accumulated more O$_2^-$ than WT, but when treated with PPZ, the extent of O$_2^-$ accumulation was similar among p35S::EIN3-GFP, ein3/eil1-1 and WT (S13 Fig), indicating that there was another pathway independent from ethylene participating in O$_2^-$ accumulation when BR synthesis was blocked with PPZ treatment. In the det2-9 mutant, there was no clear increase in ACC-induced superoxide anion accumulation, but the AVG treatment reduced it, which is also an indication that the increase in superoxide anion accumulation was at least partially dependent on ethylene production in det2-9. Since peroxidase activity in det2-9 was higher than that in WT (Fig 8D), an experiment was conducted to compare peroxidase activity in plants carrying p35S::EIN3-GFP, the ein3/eil1-1 double mutant and WT. The result showed that the peroxidase activity was not clearly affected in p35S::EIN3-GFP, ein3/eil1-1 compared with the wild-type control (Fig 9B), indicating that ethylene signaling pathway is unlikely to activate the POD pathway for BR-regulated accumulation of O$_2^-$ in det2-9 mutant.

To investigate whether the O$_2^-$ accumulation can alter normal ethylene signaling, we compared the expression level of pEBS::GUS when treated or not with methyl viologen (MV, a superoxide anion propagator) treatment. As shown in Fig 9C, the expression level of pEBS::GUS reporter was considerably induced when treated with MV, suggesting that an O$_2^-$...
accumulation can increase ethylene content. We then measured the primary root growth of 
\textit{ein2-5}, \textit{ein3/eil1-1} and wild type when treated or not with MV. Mutants in ethylene signaling 
were more resistant than WT to the negative effects of MV on root growth (Fig 9D). The 
expression of genes encoding ACS and ACO, analyzed by qRT-PCR, increased when treated 
with MV (S14 Fig). These results further indicated that O$_2^-$ accumulation can alter normal eth- 
ylene production.

\section*{Discussion}

The BRs are well recognized as promoters of cell elongation, also in addition to their involve- 
ment in the de-etiolation response, where the opening of the apical hook is thought to require
a decrease in the level of ethylene synthesis [17, 44, 45]. It was found that BR enhances ethylene production through the synergistic interaction with eto1 and eto3 [46]. Another study from the same lab showed that supplying BR exogenously promotes ethylene synthesis in the A. thaliana seedlings via stabilizing ACS5 and ACS9 protein [26]. This BR-induced ethylene production was also observed in mung bean and maize [47, 48]. However, in jujube fruit, 5 μM BR-treated fruits caused a significantly lower level of ethylene during storage and the inhibition fruit ripening [49]. These contradictory results indicate the complicated effects of BR on ethylene synthesis. However, all these observations are based on the chemical treatment with BR.

In this study, a new mutant allele of DET2, det2-9, was identified based on the short-root phenotype (Fig 1 and S1 Fig). DET2 encodes a steroid 5α-reductase involved in BR biosynthesis, catalyzing the formation of campestanol with campesterol as substrates. Since another allele det2-1 and other mutants such as cpd and dwf4 which have defects in different steps of BR biosynthesis also displayed short-root phenotype [50–52], it is unlikely that campesterol accumulation caused the short-root phenotype. The short-root phenotype is most likely a result of the reduced BRs synthesis in det2-9 since externally applied BRs could largely rescued the mutant phenotypes (S1 Fig). Through genetic analysis and chemical treatment, we found that the short-root phenotype in det2-9 was partly the result of over accumulation of ethylene leading to enhanced ethylene signaling (Figs 3 and 5). The ethylene content was considerably higher in the det2-9 mutant than that in WT seedlings (Fig 3B). Treatment with the BR synthesis inhibitor propiconazole (PPZ) also resulted in higher ethylene content in light-grown WT seedlings, while eBL (10 nM, a concentration which enhances the growth of root in det2-9) treated WT or bes1-D (a mutant which displays an enhanced BR signaling response) light-grown seedlings both showed a reduction in ethylene content (Fig 3B). A similar profile of ethylene content was also observed when seedlings were grown in darkness (S5 Fig). Transcriptional profiling showed that a number of ACS genes were up-regulated in the det2-9 mutant both in light and dark growth conditions, consistent with its increased level of ethylene (Figs 2B, 2D and 3 and S4 and S5 Figs). Since the BR signaling transcription factors BZR1 or BES1 bind to ACS promoters to repress their expression (Fig 6), we analyzed BR signaling pathway by using bri1-116 or bin2-1 mutants and found that the BR-mediated down-regulation of ACS genes was greatly reduced in these two mutants compared with det2-9 (S8 Fig), further indicating BZR1 or BES1 mediated BR signaling negatively regulates the expression ACS transcription factors. This result indicates that native physiological levels of BRs negatively regulate ethylene production through BZR1 or BES1 mediated transcriptional regulation of ACSs.

Since our results and Zhu et al.’s observations [49] are in contrast to other reports which showed that BRs enhanced ethylene biosynthesis [26, 46–48], we further did dosage-dependent assay to test the effects of BR on ethylene productions and root growth. Not surprisingly, root growth was inhibited gradually by eBL at concentrations ranging from 10 to 5000 nM (Fig 4A and 4B). However, ethylene content was greatly reduced in seedlings treated with low concentration of eBL (10 or 100 nM) while it was strongly increased when the concentration of eBL greater than 500 nM (Fig 4D). Root growth analysis under both treatment suggests that both high and low levels of ethylene cause a short-root phenotype (Fig 4D), which is consistent with the previous reports. In the octuple acs mutant (CS16651), which has only 10% ethylene level compared with WT, a reduced root growth phenotype was observed [53]. The acs9 mutant also displays a short root phenotype (Fig 5E). The high levels of BR (500 nM or 1000 nM BR) induced ethylene production is also consistent with the previous reports in Arabidopsis [26, 46]. This study together with previous reports clearly showed that BRs either positively or negatively regulate ethylene biosynthesis in a concentration-dependent manner to control root growth through the regulation of ethylene synthesis and superoxide anions accumulation.
growth. Certainly, since BR can also interact with other plant hormones such as auxin, ABA, cytokinin and jasmonic acid to regulate myriad aspects of plant growth and developmental processes in plants [54, 55], externally applied BR treatment caused root-growth phenotype might be also result from the interaction between BR and other plant hormones.

ROS represent not only a by-product of stress response, but also influence growth and development in response to both internal developmental signals and external environmental cues [28]. The contrasting ROS status in the cell proliferation and the cell differentiation zones has recently been shown to be an important driver of root growth [29]. A mitochondria localized P-loop NTPase was also reported to regulate quiescent center cell division and distal stem cell identity through the regulation of ROS homeostasis in *Arabidopsis* root [56]. It has been pointed out that ABA-promoted ROS regulates root meristem activity [31]. In cucumber plants exposed to exogenous BR, H$_2$O$_2$ accumulates as a result of an increased activity of NADPH oxidase [27], while in tomato, the same result is achieved by the up-regulation of *RBOH1* [57]. BR has been documented as inducing a receptor-dependent increase in cytosolic Ca$^{2+}$, which stimulates NADPH oxidase-dependent ROS production [32, 58]. Thus, although the participation of BR in root growth and development is accepted, its interaction with ROS signaling has not been systematically explored to date. Here, a key finding was that the *det2-9* mutant hyper-accumulated O$_2^-$, which in itself likely contributed to the short root phenotype (Fig 7). BR inhibited the synthesis of O$_2^-$ via the peroxidase (Fig 8C and 8D and S11 Fig) rather than via the NADPH oxidase (Fig 8A and 8B and S10 Fig) pathway. These results suggest that H$_2$O$_2$ and superoxide anion respond dissimilarly to BR in *A. thaliana* seedlings. While the level of H$_2$O$_2$ rises rapidly upon exposure to exogenous BR, the one of the superoxide anion is repressed. In addition, the hyper-accumulation of ethylene displayed by *det2-9* contributed to a rise in the superoxide anion content in a peroxidase-independent manner (Fig 9A and 9B).

In summary, according to this study together with the previous reports, a proposed model was given in Fig 10. We suggest that BR inhibits ethylene synthesis by activating the transcription factors BZR1 and BES1 under low levels. These transcription factors bind directly to the ACS promoters, thereby suppressing ACS expression and damping the level of ethylene synthesis under normal growth conditions. While high levels of BR induce ethylene biosynthesis either through increasing the stability of ACSs or influencing auxin signaling regulated ethylene production [47, 59, 60]. The possible regulation mechanism of BES1/BZR1’s activity under different levels of BR maybe refer to the regulation mechanism of ARF3 under different levels of auxin. Recent study has found that ARF3 acts as a repressor or activator depends on auxin concentration [61]. At the same time, BR inhibits the synthesis of O$_2^-$ via the peroxidase pathway, but not NADPH oxidase pathway, which serves to regulate the growth of the *A. thaliana* seedling root. The accumulation of the O$_2^-$ is also partially controlled by ethylene signaling in a peroxidase-independent manner and the O$_2^-$ accumulation can enhance ethylene signaling by increasing the expression of ACSs and ACOs. Understanding how ethylene mediates BR signaling to control the accumulation of the O$_2^-$ represents a logical follow-up research target.

**Materials and methods**

**Plant materials and growing conditions**

All of the *A. thaliana* mutants and/or transgenic lines utilized are in a Col-0 background; the following have been described elsewhere: *det2-1* [62], *bes1-D* [13], *bri1-116* [63], *bin2-1* [64], p35S::BRI1-GFP [65], *ein2-5* [66], *ein3/eil1-1* [67], *acs9* [68], p35S::BZR1-YFP [69], pNP::BES1-FLAG [70], p35S::EIN3-GFP [71], and p35S::NADPH-GFP [72]. And *rbohD*, *rbohF*, *rbohD/F* all described in Torres’ paper [73]. The octuple *acs* mutant (CS16651, *acs2-1/acs4-1/acs5-2/acs6-1/acs7-1/acs9-1/amiRacs8acs11) [53] was obtained from the Arabidopsis Biological Resource Center.
Center (ABRC, Columbus, OH, USA), and the marker lines pCYCB1;1::GUS [74] and pEBS::GUS [75] from early research. The 1501-bp upstream region from the DET2 start cordon and the cDNA of DET2 were amplified and linked to the GFP-GUS reported in gateway vector PKGWFS7.1 [76] to obtain pDET2::DET2-GFP-GUS reporter construct. Prior to germination, the seed was surface-sterilized by fumigation in chlorine gas, held for two days at 4˚C on solidified half strength Murashige and Skoog (MS) medium, then transferred to a growth room providing a 16 h photoperiod and a constant temperature of 20˚C.

Microscopy, growth measurement and histochemical GUS staining

Root tips were imaged by laser-scanning confocal microscopy. The number (obtained from a count of cells in the cortex file extending from the quiescent center to the TZ) and length of cortical and mature epidermal cells were obtained from microscope images using ImageJ software. The criteria for defining the MZ and TZ were those described by Napsucialy-Mendivil et al. [77]. The cell production rate was based on the rate of root growth and the length of fully elongated cells, and the cell cycle time on cell production and the number of cells present in the MZ, as described by Napsucialy-Mendivil et al. [77]. The number of cells displaced from the cell proliferation domain (N_{transit}) during a 24 h period was estimated from the equation

![Diagram](https://doi.org/10.1371/journal.pgen.1007144.g010)
\[ N_{\text{transit}} = \frac{24 \ln 2 N_{\text{MZ}}}{T}, \] where \( N_{\text{MZ}} \) represents the number of cells in the RAM MZ and \( T \) means cell cycle time in hours. Histochemical GUS staining was performed according to the method described by Gonzalez-Garcia et al. [78].

**Map-based cloning of the gene underlying the sr5 mutation**

The mapping population was the F\(_2\) generation of the cross sr5 x Landsberg erecta. Genomic DNA was extracted from each F\(_2\) seedlings showing the sr5 phenotype. Simple sequence length polymorphism markers were used for the initial genome-wide linkage analysis, following Lukowicz et al. [79]. To enable fine mapping, 22 PCR-based markers were designed to target the relevant region of the *Arabidopsis thaliana* genome sequence.

**RNA-Seq**

RNA was isolated from the roots of six day-old det2-9 and WT seedlings using the TRIZol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I to remove contaminating genomic DNA. The preparation was enriched for mRNA by introducing magnetic beads coated with oligo (dT). The resulting mRNA was fragmented into fragments of about 200 nt, and the cDNA first strand was then synthesized via random hexamer priming. After synthesizing the second strand with DNA polymerase I, the ds cDNA was purified using magnetic beads coated with oligo (dT) and End repairation is then performed. Adaptors were then ligated to each end of the fragments, and the products were size-selected by gel electrophoresis. Finally, the fragments were amplified based on the adaptor sequences, purified using magnetic beads coated with oligo (dT) and dissolved in the appropriate amount of Epstein-Barr solution. The concentration and integrity of the ds cDNA was monitored using a 2100 Bioanalyzer device (Agilent Technologies Japan Ltd.). The cDNA was then sequenced using an Ion Proton platform (www.thermofisher.com). Low quality and adaptor sequences were removed and the remaining sequences were then aligned to the *Arabidopsis thaliana* genome sequence using SOAP2 software. Individual transcript abundances were expressed in the form of the number of reads per kilobase per million reads (RPKM), and differentially transcribed genes were identified using the thresholds FDR \( \leq 0.001 \) and \( |\log_2| \geq 1 \) [80].

**qRT-PCR**

The RNA template required for qRT-PCR was isolated using an RNeasy PlantMini kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. After treating with DNase I to remove contaminating genomic DNA, a 2 \( \mu \)g aliquot was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland), following the manufacturer’s protocol. The subsequent qRT-PCRs were run on a MyiQTM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using FastStart Universal SYBR Green Master mix (Roche, Basel, Switzerland). Each sample was represented by three biological replicates, and each biological replicate by three technical replicates. The reference sequence was *AtACTIN2* (*At3g18780*). Primer sequences are given in S2 Dataset.

**Ethylene quantification**

Ten seedlings were placed in a 100 mL vial containing 50 mL solidified half strength MS either with or without eBL or PPZ, and immediately capped. The vials were held under a 16 h photoperiod and a constant temperature of 20˚C. After seven days, a 10 \( \mu \)L sample of the headspace was subjected to gas chromatography using a GC-6850 device equipped with a flame ionization detector (Agilent Technologies Japan Ltd.).
Yeast one-hybrid assay
The coding sequences of BES1 and BZR1 were inserted separately into the EcoRI-XhoI cloning site of pGADT7 (Takara, USA), while the promoter sequences of ACS6, 7, 9, 11, ACO1 and 3 were inserted into the cloning site of pAbAi. The primer sequences used in the construction of the various constructs are given in S2 Dataset. Each of the constructs (including an empty vector for control purposes) was transferred separately into yeast Y1HGold using the PEG/LiAc method. The yeast cells were plated onto SD/-Ura/-Leu medium containing various concentrations of Aureobosidin A to allow for a highly stringent screening of interactions. The procedure followed the manufacturer’s protocol given for the Matchmaker Gold Yeast One-Hybrid Library Screening System (www.clontech.com).

Chromatin immunoprecipitation (ChIP)
Ten day old transgenic plants were used for the ChIP assay following Gendrel et al. [81]. The quantity of precipitated DNA and input DNA was detected by qPCR. For each ACS promoter, primers were designed to amplify a fragment of length ~70–150 bp lying within the 2 kbp of sequence upstream of the transcription start site. The relevant primers are given in S2 Dataset. Enrichment was calculated from the ratio of bound sequence to input.

Transient expression
The BES1 or BZR1 coding sequences were amplified and the resulting sequences introduced into pBI221 to place them under the control of the CaMV 35S promoter. The ACS promoter sequences were amplified and introduced into the pGreenII0800-LUC reporter vector. Both recombinant plasmids were then transferred into A. thaliana protoplasts. Firefly luciferase (LUC) and renilla luciferase (REN) activities were measured using the Dual-Luciferase Reporter Assay System (www.promega.com). LUC activity was normalized against REN activity [82]. Details of all primers used are given in S2 Dataset.

NBT assay for the superoxide anion
The roots of five day-old seedlings were immersed for 15 min in 2 mM NBT in 20 mM phosphate buffer (pH 6.1). The reaction was stopped by transferring the seedlings into distilled water. The material was then imaged under a light stereomicroscope.

Peroxidase activity measurement
Tissue peroxidase activity was measured by a spectrophotometric analysis (420 nm) of the formation of purpurogallin from pyrogallol in the presence of H2O2. The roots of nine day-old seedlings were harvest and weighted. Tissue homogenate was prepared using 9 times phosphate buffer and then centrifuged for 10 min in 3500 rpm. The supernatant was used for peroxidase activity measurement. A single unit of enzyme was defined as the amount catalyzed and generated 1 μg pyrogallol by 1.0 mg fresh tissues in the reaction system at 37˚C. Peroxidase activity was calculated from the formula provided with the peroxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

Accession numbers
Sequence data for genes used in this study can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers:

- DET2 (At2g38050), BES1 (At1g19350), BZR1 (At1g75080), ACS1 (At3g61510), ACS2 (At1g01480), ACS4 (At2g22810), ACS5 (At5g65800), ACS6 (At4g11280), ACS7 (At4g26200),
ACS9 (At3g49700), ACS11 (At4g08040), WOX5 (At3g11260), ARF10 (At2g28350), ARF16 (At4g30080), ARR1 (At3g16857), SHY2 (At1g04240), BRI1 (At4g39400), CYCB1;1 (At4g37490), ACO1 (At2g19590), ACO2 (At1g62380), ACO3 (At2g05710), ACO4 (At1g05010), ACO5 (At1g77330), EBS (At1g22140), EIN3 (At3g20770), EIL1 (At2g27050), ERF6 (At4g17490), ERF13 (At2g44840), ERF17 (At1g19210), ERF104 (At5g61600), ERF105 (At5g51190), TCH4 (At4g57560), BAS1 (At2g26710), IAA17 (At1g04250), IAA19 (At3g15540), ACTIN2 (At3g18780).

Supporting information

S1 Fig. Positional cloning of the gene underlying the sr5 mutation. (A) The mutated gene maps to chromosome 2. The sr5 allele sequence differs from the WT allele of At2g38050 by a point mutation causing a shift from G to A at position 107. (B) Phenotype of five day-old sr5 and det2-1 seedlings exposed to eBL (10 nM) either under lit or non-lit conditions. Bar = 1 cm. (C) Root phenotype of five day-old seedlings of the F1 hybrid sr5 x det2-1 and its reciprocal. Bar = 1 cm. (D) Root phenotype of a five day-old sr5 seedling carrying the transgene pDET2::DET2-GFP-GUS. Bar = 1 cm. (E) Phenotype of WT, sr5 and det2-1 17 day-old seedlings. Bar = 1 cm.

(TIF)

S2 Fig. GUS expression in five-day old sr5 seedling carrying the transgene pDET2::DET2-GFP-GUS. Bar = 50 μm.

(TIF)

S3 Fig. Relative transcript abundance of BR induced genes in seedlings of WT, det2-9 and det2-1.

(TIF)

S4 Fig. Relative transcript abundance of ACC synthase genes (ACS2, 4, 6, 7, 8, 9, 11) in dark-grown seedlings of WT and det2-9. **: means significant difference compared to control (P<0.01).

(TIF)

S5 Fig. The det2-9 mutant accumulates more ethylene than WT when grown in darkness. Ethylene production by five day-old seedlings of various BR-related transgenic and WT seedlings exposed to either eBL(10 nM) or propiconazole (2 μM) in dark conditions. Data shown are mean±SE (n = 5). **: means significant difference compared to control (P<0.01).

(TIF)

S6 Fig. Effect of ethylene on cell length and cell number in RAM. (A) Cortical cell length in the maturation zone of five day-old WT and det2-9 seedlings when treated with AgNO3 or ACC. Data shown are mean±SE (n = 25), Different letters associated with values indicate a significant difference (P<0.01). (B) Cell number in the proliferation domain of five day-old WT and det2-9 seedlings when treated with AgNO3 or ACC. Data shown are mean±SE (n = 25), Different letters associated with values indicate a significant difference (P<0.01).

(TIF)

S7 Fig. Neither BES1 nor BZR1 interact directly with the ACO1 or ACO3 promoters, as indicated by a yeast one-hybrid binding assay.

(TIF)
S8 Fig. A qRT-PCR analysis of genes involved in ethylene production in BR mutants. Relative transcript abundance of ACC synthase genes (ACS6, 7, 9, 11) in WT, det2-9, bri1-116 and bin2-1 when treated with or without eBL (10 nM).

(TIF)

S9 Fig. The production of H₂O₂ in the det2-9 mutant and WT five-day-old seedlings. WT and det2-9 roots are stained by DAB to quantify H₂O₂ levels. Bar = 50 μm.

(TIF)

S10 Fig. The BR-mediated inhibition of superoxide anion synthesis does not operate through the NADPH oxidase pathway. (A) Transcription of RBOH genes, assayed by qRT-PCR in WT and det2-9 seedlings. (B) Relative root length in the mutants rbohD, rbohF and rbohD/F in the presence or absence of propiconazole (2 μM). Data shown are mean±SE (n = 30). (C) NBT staining of root of WT, 35S::NADPHD-GFP and rbohD/F plants exposed to propiconazole (2 μM). Bar = 50 μm. (D) NBT staining of root of WT, p35S::EIN3-GFP and ein3/eil1-1 plants exposed to eBL (10 nM) or propiconazole (2 μM). Bar = 50 μm. (E) NBT staining of root of WT, det2-9, rbohD, rbohD/F, det2-9/rbohD and det2-9/rbohD/F plants. Bar = 50 μm.

(TIF)

S11 Fig. Primary root length of WT and det2-9 seedlings when exposed to inhibitors of peroxidase.

(TIF)

S12 Fig. Transcription of genes encoding peroxidase in det2-9 and WT, assayed by qRT-PCR.

(TIF)

S13 Fig. NBT staining of root of WT, p35S::EIN3-GFP and ein3/eil1-1 plants exposed to eBL(10 nM) or propiconazole (2 μM). Bar = 50 μm.

(TIF)

S14 Fig. Transcription of genes encoding ACC synthase (ACS) and ACC oxidase (ACO) when treated or not with MV, assayed by qRT-PCR. **: means in treated seedling significantly differ from untreated samples (P<0.01).

(TIF)

S1 Dataset. Different expression genes related to ethylene in det2-9.

(XLSX)

S2 Dataset. List of primer sequences used in this paper.

(XLSX)

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Author Contributions

Conceptualization: Zhaojun Ding.

Data curation: Bingsheng Lv, Songchong Lu.
Formal analysis: Bingsheng Lv, Huiyu Tian, Zhaojun Ding.
Funding acquisition: Bingsheng Lv, Zhaojun Ding.
Investigation: Bingsheng Lv, Huiyu Tian, Feng Zhang, Jiajia Liu.
Methodology: Bingsheng Lv, Huiyu Tian.
Supervision: Zhaojun Ding.
Writing – original draft: Bingsheng Lv, Huiyu Tian, Mingyi Bai, Chuanyou Li, Zhaojun Ding.

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