Brassinosteroids Counteract Abscisic Acid in Germination and Growth of *Arabidopsis*

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Brassinosteroids (BRs) are involved in multiple plant growth and development processes, such as cell elongation, photomorphogenesis, flowering time control, and stress responses. The phytohormone abscisic acid (ABA) is crucial to plant development and adaptation to stressful environments. The receptors and pathways of BRs and ABA have been deeply studied. But the relationship between them remained largely unknown and there are only few reports about it. Our experiments showed that the BR-deficient and BR-insensitive *Arabidopsis* mutants *det2*, *bri1–5* and *bri1–9* were more sensitive to ABA than the wild type (Ws-2), especially the *det2* and *bri1–9* mutants. Germination, hypocotyl and root elongation, and stomatal apertures of the mutants were more severely inhibited by ABA. All the results suggest that BRs counteract ABA in regulating plant growth, and the interaction may be complicated. The possible mechanisms are discussed.

Key words: Abscisic Acid, Brassinosteroids, Germination, Stomatal Aperture

Introduction

The establishment of seed dormancy or germination in higher plants is influenced by environmental cues, such as moisture, light, and temperature. Many hormones are indispensable to growing and germination, such as gibberellins (GAs), auxin, brassinosteroids (BRs), abscisic acid (ABA), cytokinin (CK), ethylene, salicylic acid (SA) (Yuan and Lin, 2008). More and more attention is paid to interactions among different hormones.

BRs are the only known class of plant steroid hormones with structural similarities to their animal counterparts. They are widely distributed in the plant kingdom and are active at very low concentration. BRs are involved in multiple plant growth and development processes, such as cell elongation, vascular development, senescence, photomorphogenesis, flowering time control, and stress responses (Clouse *et al.,* 1996; Li and Chory 1999; Krishna, 2003; Kagale *et al.*, 2007; Jager *et al.*, 2008). Evidences have proved that BRs play an important role in germination of *Arabidopsis* (Steber and McCourt, 2001).

Much of our understanding of the balancing control by these hormones in determining the developmental state of the seed comes from studies involving hormone biosynthetic and response mutants in *Arabidopsis*. These studies make use of mutants in two BR genes, de-etiolated-2 (*DET2*) and brassinosteroid-insensitive-1 (*BRI1*). *DET2* encodes a steroid 5α-reductase required for BR biosynthesis (Chory *et al.*, 1991; Noguchi *et al.*, 1999a). *BRI1* encodes a Leu-rich repeat receptor kinase which is a receptor of BRs (Li and Chory, 1997; Friedrichsen *et al.*, 2000; He *et al.*, 2000). In the present study, *bri1–5* and *bri1–9* were used. They are all insensitive to BRs and cannot be rescued by exogenous BRs (such as BL, one of the most potent BRs). The only difference was the mutation locus in the BRI1 protein (Noguchi *et al.*, 1999b). The amino acid Cys^{69} of *bri1–5* was changed to Tyr^{69} and the amino acid Ser^{662} of *bri1–9* was changed to Phe^{662}. On the other hand, the co-receptor BAK1 (Li *et al.*, 2002), BRI1's

substrate BSKs (Tang *et al.*, 2008), and the downstream GSK3-like kinase BIN2, which regulates the activity of the nuclear transcription factors, have been identified and studied in detail too. However, no direct interaction has been observed between BRI1 and BIN2, and it remains unclear how BRI1 kinase at the plasma membrane transduces the signal to cytoplasmic components of the BR pathway (Gendron and Wang, 2007).

The phytohormone ABA has a vital function in plant adaptation to stressful environments by regulating stomatal apertures and the expression of stress-responsive genes, and in plant development such as seed maturation, germination and seedling growth (Leung and Giraudat, 1998; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003). ABA-biosynthetic (*aba*) and ABA-insensitive mutants (*abi*), and the mutant enhanced response to ABA (*era*) were widely used in studies of ABA signaling pathways.

In the present study, bioassays were performed with *Arabidopsis* to address the question whether exogenous ABA affects the germination and stomatal movement of BR-deficient and BR-insensitive mutants. The results showed that BRs and ABA are antagonistic to each other.

Material and Methods

Plant material and growth conditions

Arabidopsis ecotypes Wassilewskija-2 (Ws-2) and the mutants *bri1–5, bri1–9* (brassinosteroid-insensitive), and *det2* (de-etiolated) were used in these experiments. Seeds were imbibed for 3 d at 4 °C in water to encourage synchronous germination, and then sown in a mixture of humus and common soil. Plants were watered to saturation with 1/4 strength Hoaglands solution three times a week and grown in a growth chamber with a photoperiod of 16 h light and 8 h dark at 22 ºC.

Germination experiments

ABA (Aldrich, USA) was dissolved in 95% ethanol, diluted to a 10 mM stock solution, and filtered with a sterile dialyzer. ABA was added to the autoclaved 1/2 Murashige and Skoog basal culture medium (pH 5.8) after cooling to approx. 55 °C. Imbibed seeds were sterilized with 0.1% $HgCl_2$ for 5 min, followed by four to six washes with sterile water. Seeds were sown to 1/2 Murashige and Skoog basal culture medium containing the indicated concentration of ABA, then moved to constant fluorescent lighting (50 μmol m^{-2} s^{-1}) at 22 ºC. Seeds with emerging cotyledons were scored as germinated.

Assays of hypocotyl and root elongation inhibition

Seeds were sterilized and planted in 1/2 Murashige and Skoog basal culture medium containing the indicated concentration of ABA as described above. After 10 d the lengths of hypocotyls and roots were determined for each hormone concentration, and an average was calculated. The kinked hypocotyls or roots were pulled straight during measurement using forceps. Inhibition of hypocotyl and root growth was expressed relative to the mean growth of the same genotype on medium without ABA.

Assays of stomatal movement

For stomatal aperture assays (Shen *et al.*, 2006), leaves were floated in the buffer containing 50 mM KCl and 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES, pH 6.15) under a halogen cold-light source at 200 μmol m^{-2} s^{-1} for 2 h followed by addition of different concentrations of ABA. Stomatal apertures were measured on epidermal strips after 2 h of further incubation using a compound microscope and an ocular micrometer to estimate ABA-induced closures. To study the inhibition of opening, leaves were floated on the same buffer in the dark for 2 h before they were transferred to the cold-light source for 2 h in the presence of ABA; then stomatal apertures were determined. Within a single experiment, 20 or 30 apertures were measured per treatment. Microsoft Excel was used to calculate average stomatal apertures and standard errors.

Statistics

Values presented are means ± one standard deviation (SD) of three replicates. Statistical analyses were carried out by analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC, USA). Differences between treatments were separated by the least significant difference (LSD) test at a 0.05 probability level.

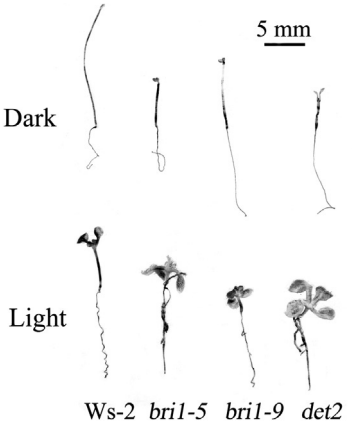

Fig. 1. Phenotypes of the wild type (ecotype Ws-2), *bri1–5, bri1–9,* and *det2* grown in the light (bottom) and in the dark (top). The scale bar is equivalent to 5 mm.

Results

BR mutants are dwarf phenotype

Fig. 1 verifies that BRs play a major role in the growth and development of plants, independent of the seedlings were grown in the light or in the dark. The hypocotyl elongation of Ws-2 was almost two times that of *bri1–5, bri1–9,* and *det2*, suggesting that BR is very important for cell elongation and vascular development. When the plants were 40 d old, the length of the wild type was almost 4–5 times longer than those of the mutants (data not shown).

BR mutants show increased sensitivity to ABA in germination

If BRs play a role in germination, one would expect that BR mutants show a germination phenotype. ABA is a hormone which also plays a key role in regulating seed dormancy and germination. We examined the germination of BR mutants and the wild-type plants in the presence of different concentrations of ABA. After 2 d, the germination of *bri1–5* and *det2* were similar to that of the wild type (Fig. 2), but the germination of *bri1–9* was lower. After 7 d, when the ABA concentration was lower than 0.05 μM, the difference of germination was not significant (Fig. 2). When the ABA concentration was high (0.25 μM), *det2* showed 12% of germination, *bri1–9* 14%, while 45% of the wild-type plant germinated, proving that both *bri1–9* and *det2* had increased sensitivity to ABA in germination. However, an interesting phenomenon was that *bri1–5* was not as sensitive to ABA as the other mutants, and its germination was similar to the wild type.

Different ABA restraint of hypocotyl and root elongation of the mutants

ABA regulates not only the seed germination, but also seedling hypocotyl and root elongation. We determined the hypocotyl and root elongation of 10-d-old seedlings. The result (Fig. 3) showed that at all levels of ABA, *bri1–9* and *det2* were more sensitive to ABA than the wild-type plant.

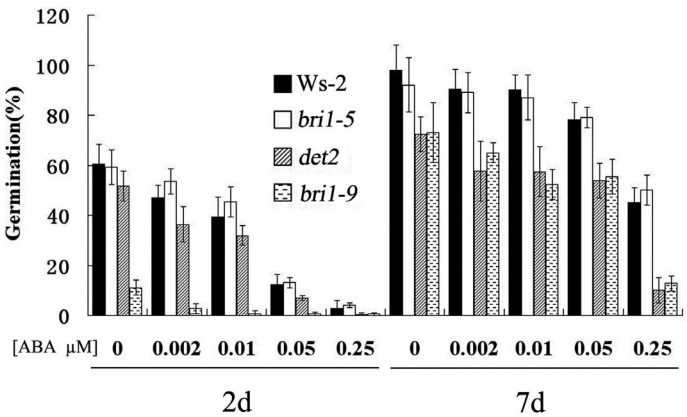

Fig. 2. Effects of ABA on seed germination of BR mutants. Seeds were planted on 1/2 Murashige and Skoog medium and the germination (emergence of radicals) was scored at the indicated times. Error bars show standard deviations (n = 3).

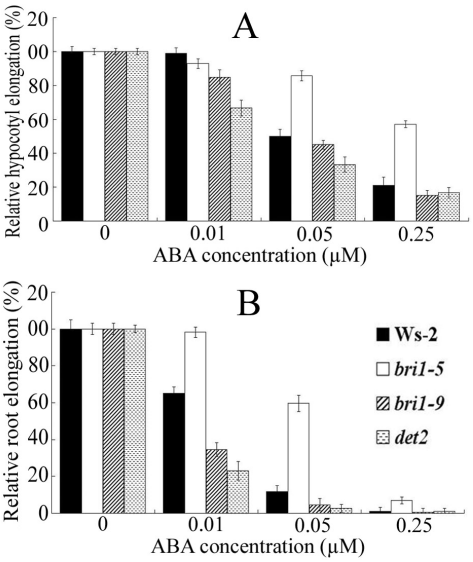

Fig. 3. Effects of ABA on hypocotyl and root inhibition of BR mutants. Inhibition of hypocotyl and root growth is expressed relative to the mean growth of the same genotype on medium without ABA. Error bars show standard deviations (n = 3).

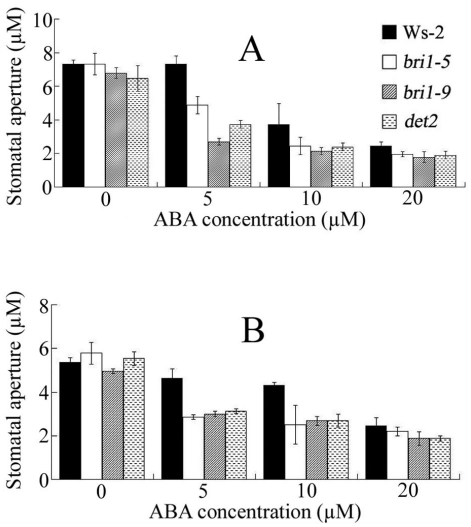

Fig. 4. Effects of ABA on (A) stomatal closure and (B) stomatal opening inhibition of BR mutants. To study the inhibition of the stomatal opening, leaves were incubated in the dark for 2 h for full closure, then transferred to the cold-light source for 2 h in the presence of ABA. Error bars show standard deviations (n = 3).

Contrastively, the elongation of *bri1–5* was less affected by ABA than the wild type.

Different stomatal closure and stomatal opening inhibition of the mutants

ABA is a vital phytohormone that regulates mainly the stomatal aperture. As expected, ABA induced the stomatal closure (Fig. 4A). At 5 μM ABA, compared with untreated plants, the stomatal closure of the mutants was about 50% (the wild type was almost not affected at this concentration), and the stomata closed continually with the ABA concentration increased. Similarly to the stomatal closure, the ABA-induced inhibition of the stomatal opening was also more significant in the mutants than in the wild type (Fig. 4B).

Discussion

The interactions between hormone pathways have become a focus for many laboratories studying hormone signaling. Our results showed that BR-deficient and BR-insensitive mutants were more sensitive to ABA than the wild type. At 0.25 μM of ABA, *det2* showed 12% germination, *bri1–9* showed 14% germination, but the wild type showed 45% germination (Fig. 2). This is consistent with another report (Steber and McCourt, 2001). At a low concentration of ABA (0.01 μM), hypocotyl and root elongation of *bri1–9* and *det2* was inhibited more seriously than of the wild type (Fig. 3).

So far, it is clear that the major receptor of BRs is BRI1, a Leu-rich repeat (LRR) transmembrane receptor kinase, located on the cell surface. BRI1 has an extracellular domain containing 25 LRRs, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain (Wang and He, 2004). It transduces BR signals across the plasma membrane, but how the signal transports mediate genomic effects is still unknown. Possibly there are BR ligands in the cytoplasm connected with ABA signals (He *et al.*, 2007). When the gateway of BR signals is cut down, the ABA signaling would be prompted. Therefore, BR-deficient and BR-insensitive mutants are more sensitive to ABA. The putative ligands are the new working point for future studies.

Stomatal movement is markedly regulated by plant hormones. ABA and methyl jasmonate (MJ) suppress stomatal opening, while CKs and auxin promote stomatal opening (Mansfield *et al.*, 1990; Gehring *et al.*, 1990). It was clear that the stomata of BR mutants closed much sooner and severer (Fig. 4A). Similarly, stomatal opening inhibition in the mutants was much stronger than in the wild type (Fig. 4B). Although some ABA receptors and related components have been identified, many ABA signaling components remain to be discovered. Calcium (Ca^{2+}) plays an essential role in plant cell signaling (Hepler, 2005) and has been shown to be an important second messenger involved in ABA signal transduction (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Fan *et al.*, 2004). Two calcium-dependent protein kinases regulating ABA signal transduction also have been found in *Arabidopsis* (Zhu *et al.*, 2007). Hereby, cytosolic Ca^{2+} may be the common target, which cannot only be induced by ABA but also be regulated by BRs during the restriction of stomatal opening (Suhita *et al.*, 2003; Haubrick *et al.*, 2006). The partially BR-insensitive *Arabidopsis* mutant *det3* shows altered Ca^{2+} responses and thus altered stomatal apertures and guard cell physiology (Allen *et al.*, 2000). However, the detailed mechanism is far from clear.

The results showed an interesting phenomenon that *bri1–5* is not more sensitive to ABA than the other mutants. The mutation locus of *bri1–5* is $Cys^{69}\rightarrow Tyr^{69}$ (domain of paired cysteines) and of *bri1–9* it is $Ser^{662}\rightarrow Phe^{662}$ (domain of leucine-rich repeats, known as LRR). LRR is a so important motif that a single amino acid substitution will passivate the BRI1 protein (Friedrichsen *et al.*, 2000; Noguchi *et al.*, 1999a). $Cys^{69}\rightarrow Tyr^{69}$ mutation in *bri1–5* does not affect the downstream BR signaling components, although it becomes insensitive to BRs. Downstream components to ABA signals should not be affected in the *bri1–5* mutant, therefore *bri1–5* is not sensitive to ABA. Compared with *bri1–5*, the mutation locus of *bri1–9* is on the LRR domain. Downstream signaling components of *bri1–9* may be changed and *bri1–9* loses the ability to rivalize ABA signaling.

In summary, BRs generally counteract ABA on root growth, seed germination, and possibly stomatal movement. BR-related mutants display altered sensitivity to ABA. Further works should pay more attention to the mechanism of ABA-BR cross-talks.

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