INTERACTION BETWEEN PHOTORECEPTORS AND BR SIGNALING IN ARABIDOPSIS

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Extensive studies have been performed to elucidate the role of brassinosteroids (BRs), an important class of phytohormone in plant growth, development, and photomorphogenesis. Different wavelengths of light recognized by photoreceptors play a crucial role in plant development. The role of different photoreceptors in BR signaling has not been analyzed. Here we used photoreceptor single mutants, double mutants and even a quadruple mutant to analyze BR-dependent hypocotyl growth and gene regulation. All the photoreceptor mutants differed from the controls in their response to BR, and hypocotyl elongation as well as BR marker gene regulation were inhibited by application of propiconazole (PCZ), a BR biosynthesis inhibitor. In addition, altered Phytochrome and Cryptochrome expression in brassinosteroid insensitive 1 mutant bri1-5 and brassinazole-resistant 1 dominant mutant bzr1-D indicated that BR negatively regulates photoreceptors in transcriptional levels. This is the first study to investigate the connections between BR and photoreceptors in Arabidopsis.

Key words: Arabidopsis, brassinosteroids, morphogenesis, mutants, photoreceptors.

INTRODUCTION

A plant is a sessile organism that is flexible in adapting to diverse environments via morphological changes. Light is the most important environmental cue modulating plant growth and development, perceived by plant sensory photoreceptors such as phytochromes, cryptochromes and phototropins (Casal, 2013). Phytohormones are signal molecules that regulate most cellular processes in plants. Phytochrome A (PhyA) is solely responsible for sensing continuous far-red light, and PhyB is the major receptor for sensing red light (Quail et al., 1995). The Arabidopsis genome encodes two cryptochromes, cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2), which mediate blue-light inhibition of hypocotyl elongation (Ahmad and Cashmore, 1993). The phototropins (phot1 and phot2) are the other blue-light receptors that function in mediating phototropism, chloroplast migration and stomatal opening in Arabidopsis (Briggs and Christie, 2002).

Constitutive photomorphogenic 1 (COP1), a ring-finger-type ubiquitin E3 ligase, moves from the nucleus to the cytoplasm, resulting in disruption of proteasomal degradation and subsequent rapid accumulation of light-related transcription factors to stimulate photomorphogenesis (Ma et al., 2002). Long Hypocotyl 5 (HY5) is a transcription factor which is degraded in a COP1-dependent manner under dark conditions. HY5 is accumulated in the nucleus and required for hypocotyl growth inhibition in light (Oyama et al., 1997).

Phytohormones are plant hormones that regulate plant growth and development. Many reports have described the connections between light and the phytohormone signaling pathway. FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED IMPAIRED RESPONSE1 (FAR1), two key transcription factors in the PhyA signaling pathway, directly bind to the ABA-insensitive (ABI5) promoter to modulate ABA signaling in Arabidopsis (Tang et al., 2013). PHYTOCHROME-INTERACTING FACTOR5
(PIF5) and COP1 have a role in ethylene biosynthesis and ethylene-regulated hypocotyl elongation (Khanna et al., 2007; Liang et al., 2012). Brassinosteroids (BRs) are one of the important groups of plant hormones that have been studied for their crucial role in regulating morphological processes, especially cell elongation (Wang et al., 2012). BRs are perceived by the receptor brassinosteroid insensitive 1 (BRI1), leading to dissociation of BRI1 kinase inhibitor 1 (BKI1) from the plasma membrane, and their co-receptor BRI1-Associated receptor Kinase 1 (BAK1), which interacts with BRI1 and mutually transphosphorylates to form an active BR receptor complex (Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002; Yang et al., 2011). Brassinazole-resistant 1 (BZR1) and BRI1-EMS-Suppressor 1 (BES1) are two well-characterized transcription factors which regulate the expression of thousands of target genes (Sun et al., 2010). Protein stability was highly increased in bes1-D and bZR1-D, gain-of-function mutants of BES1 and BZR1, in which a single proline is substituted by leucine within the PEST domain, and rescued brtl, a BR receptor mutant phenotype (Wang et al., 2002; Yin et al., 2002).

Studies have shown that BR biosynthesis or signaling-deficient mutants fail to maintain morphogenesis in the dark or respond to BR stimuli. Also, BR-deficient or signaling mutants exhibit short hypocotyls and dwarfism in the seedlings and mature stages respectively (Clouse et al., 1996; Li et al., 2013), and that a genetic lesion at the BZR1-dominant mutant bzr1-D which constitutively activates BR signaling. This is the first study of the interactive mechanism of BR and photoreceptors in Arabidopsis.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

Surface-sterilized Arabidopsis seeds were sown on Murashige and Skoog (MS) medium containing 1.2% agar (Sigma, Saint Louis, MO, USA). After two days of stratification at 4°C, seedlings were incubated at 22°C under a 16 h photoperiod. Ten-day-old seedlings grown on 0.5× MS medium were then transferred to soil and grown at 22°C under a 16 h photoperiod.

For 2,4-epibrassinolide (2,4-epiBL) or PCZ-treatment experiments, 30 seedlings were grown on 0.5× MS medium for 7 days with different concentrations of 2,4-epiBL (Sigma, 10, 50, 100, 200 nM) or PCZ (Sigma, 10, 100, 1000, 2000 nM) under continuous light or 24 h dark. Hypocotyl lengths were measured using ImageJ software.

Wassilewskija (WS2) was used as the control line for brtl-5, and Columbia (Col-0) was the control for bzr1-D, all photoreceptor and light-signaling mutants. Information on the mutants tested in this study is given in Table S1.

RNA EXTRACTION AND QUANTITATIVE RT-PCR ANALYSIS

Total cellular RNA was isolated with RNeasy Plant Mini Kits (Qiagen) or TRIzol (Takara, Dalian, Liaoning, China) and subsequently treated with RNase-free DNase (Promega, Madison, WI, USA) to eliminate genomic DNA contamination. The GoScript Reverse Transcription Kit (Reverse Transcription System, Promega) was used to reverse-transcribe 2 μg total RNA, following the manufacturer’s protocol. Real-time PCR was performed, and gene expression was quantified. qRT-PCR products were quantified using Illumina Eco 3.0 software (Illumina, San Diego, California, USA), and the values were normalized against Actin levels from the same samples. The primers used for qRT-PCR are shown in Table S2 and the tested gene information is given in Table S1.

STATISTICAL ANALYSIS

Statistical calculations were performed with Prism 5 (GraphPad, San Diego, CA). All data are expressed as means ± SE. Comparisons employed the t test (*p<0.05; **p<0.01; ***p<0.001).
RESULTS

DIFFERENTIAL HYPOCOTYL GROWTH
OF cop1-4 AND HY5 ox RESPONDS TO BR

Exogenously supplied BR led to significantly elongated hypocotyls (~54%) in light, but excess BR had the opposite effect (~36% shorter than control) under dark conditions (Fig. 1). bzr1-D, a dominant mutant of BZR1, an important BR signaling transcription factor, produced short, curved hypocotyls upon treatment with BR under dark conditions (Fig. 1) (Zhou et al., 2013). These data suggest that BR and light signaling are tightly connected. To test the effects of light on BR-mediated hypocotyl elongation, the Constitutive Photomorphogenic 1 (COP1) mutant cop1-4, Long Hypocotyl 5 (HY5) mutant hy5, Col-0, and HY5 overexpression (HY5 ox) plants were analyzed for their BR-dependent hypocotyl growth in light or dark. cop1-4 showed a normal BR response in hypocotyl elongation regardless of light conditions, while the hy5 mutant was insensitive to BR in light conditions (Fig. 2) (Shi et al., 2011). HY5 ox, however, showed a BR response in the dark similar to that of the wild type and the hy5 mutant (Fig. 2), which differed from that of the cop1-4 mutant.

DIFFERENTIAL HYPOCOTYL GROWTH
OF THE PHOTORECEPTOR MUTANTS
RESPONDS TO BR AND PCZ

To further analyze the light-BR interaction, photoreceptor mutants were tested with different concentrations of 2,4-epiBL supplementation. Wild-type plants showed hypocotyl elongation correlated with BR concentration. Hypocotyl growth of phyA211, phyA211/phyB9 and phot1-2/cry1-2 (quadruple mutant in which phot1, phot2, cry1 and cry2 genes are mutated) was independent of exogenously supplied BR (Fig. 3). phyB9 (~18% weaker than WT) and cry1-2 (cry1 and cry2 double mutant, ~25% weaker than WT) were slightly insensitive to BR, while phot1-2 (phot1 and phot2 double mutant) showed a response to BR similar to that of wild-type plants (Fig. 3).

We tested the hypocotyl growth of photoreceptor mutants treated with BR biosynthesis inhibitor PCZ because the photoreceptor mutants produced longer hypocotyls under normal conditions. phot1-2/cry1-2 (~40% less sensitive to PCZ than WT) and cry1-2 (~30% less sensitive to PCZ than WT) were clearly insensitive to PCZ, while phyB and phyA211/phyB9 (~10% less sensitive to PCZ than WT) were slightly
**Fig. 2.** 2,4-epiBL-mediated hypocotyl elongation in *hy5*, *HY5 ox* and *cop1-4* mutants and Col-0 control plants under light and in darkness. *hy5*, *HY5 ox* and *cop1-4* plants grown under treatment with indicated concentrations of 2,4-epiBL under light and in darkness (a). Hypocotyl elongation patterns under treatment with indicated concentrations of 2,4-epiBL treatment under light (b) and in darkness (c). Error bars are SE of means of three independent experiments.

**Fig. 3.** 2,4-epiBL-mediated hypocotyl elongation in 7-day-old seedlings of photoreceptor mutants and Col-0 control plants. Hypocotyl elongation patterns under treatment with indicated concentrations of 2,4-epiBL under light. Error bars are SE of means of three replicates.
insensitive to PCZ (Fig. 4). The response to PCZ of phyA211 and phot1-2 mutants did not significantly differ from that of wild-type plants (Fig. 4).

We also examined the effect of BR on PCZ inhibition of hypocotyl elongation. Application of 1 μM PCZ drastically shortened hypocotyl length in both Col-0 and phot1-2/cry1-2 plants (Fig. 5). However, 2,4-epiBL treatment rescued hypocotyl growth inhibition by PCZ (Fig. 5).

We compared BR marker gene expression levels between wild-type, photoreceptor mutant and bzr1-D plants. Two BR biosynthetic genes (BR6OX2, DWF4) and signaling genes (SAUR15, ACS5) were analyzed for their expression levels in 7-day-old plants. The results showed that the BR6OX2 level was lower in phot1-2/cry1-2 (~77% lower than in WT) and bzr1-D (~68% lower than in WT), and higher in phyA211 (~2.4-fold) and phyA211/phyB9 (~1.5-fold) than in wild-type plants (Fig. 6a). The DWF4 level was lower in phyB9 (~60% lower than in WT), phot1-2 (~60% lower than in WT), cry1-2 (~57% lower than in WT), phot1-2/cry1-2 (~82% lower than in WT) and bzr1-D (~74% lower than in WT) plants, and higher in phyA211 (~1.5-fold) than in wild-type plants (Fig. 6b). The SAUR15 level was lower in cry1-2 (~56% lower than in WT) and higher in phyA211 (~28% lower than in WT), (~28% lower than in WT), phyB9 (~30% lower than in WT). phyA211/phyB9 (~32%
Photoreceptors interact with BR signaling

**Fig. 5.** PCZ and 2,4-epiBL effects on hypocotyl elongation in *cry1-2/phot1-2* and control Col-0 plants grown on media containing 1 μM PCZ together with indicated concentrations of 2,4-epiBL for 7 days.

**Fig. 6.** Expression levels of BR biosynthetic and signaling genes in shoot tissues of photoreceptor mutants and *bzl1-D* mutants. (a, b) Expression levels of BR biosynthetic genes (*BR6OX2* and *DWF4*). (c, d) Signaling genes (*SAUR15* and *ACS5*) were monitored by qRT-PCR. Error bars are SE of means of three replicates.
lower than in WT), phot1-2/cry1-2 (~3.5-fold) and bzr1-D (~1.9-fold) than in wild-type plants (Fig. 6c). The ACS5 level was higher in phyA211 (~1.7-fold), phyA211/phyB9 (~2.7-fold), phot1-2/cry1-2 (~1.8-fold) and bzr1-D (~2.3-fold) than in wild-type plants (Fig. 6d). These results demonstrate that the photoreceptors are involved in BR signaling.

**BR IS REQUIRED FOR phot1-2/cry1-2 PHENOTYPE AND GENE REGULATION**

The phot1-2/cry1-2 mutant was insensitive to PCZ, and BR marker gene expression in phot1-2/cry1-2 was similar to that in bzr1-D. We further tested the effect of BR activity on phot1-2/cry1-2-mediated marker gene induction. Before testing gene expression, we monitored the effects of a high concentration of PCZ on hypocotyl elongation under dark and light conditions. In the dark, 2 μM PCZ treatment significantly inhibited hypocotyl elongation in de-etched wild-type, cry1-2, phot1-2/cry1-2 and hy5 seedlings but not in the bzr1-D mutant (Fig. 7a). Under light, hypocotyl elongation was completely inhibited by 2 μM PCZ application in all plants tested, but bzr1-D developed larger cotyledons than the other plants (Fig. 7a). Wild-type, bzr1-D and phot1-2/cry1-2 plants were grown on 0.5×MS with or without 2 μM PCZ for 5 days and their SAUR15 and ACS5 levels monitored. SAUR15 and ACS5 expression was higher in bzr1-D (~3- and 1.6-fold respectively) and phot1-2/cry1-2 (~2.7- and 1.5-fold respectively) than in the wild type (Fig. 7b, c). SAUR15 and ACS5 expression levels were higher in bzr1-D (~3- and 1.5-fold respectively) than in wild-type plants; their
levels in wild-type and phot1-2/cry1-2 plants were similar after PCZ treatment (Fig. 7b, c). These data show that hypocotyl elongation under light in phot1-2/cry1-2 requires BR activity.

**BR SIGNALS NEGATIVELY REGULATE PHOTORECEPTORS**

To further test the effects of BR signaling on photoreceptor gene expression, the weak BRI1 mutant bri1-5 and the bzr1-D mutant were used to analyze the expression levels of PhyA, phyB, cry1, cry2, phot1 and phot2. In the bri1-5 mutant we found higher levels of PhyB cry1 and Phot2 (~1.7-, ~1.5- and ~1.8-fold higher respectively), while the levels of PhyA, cry2 and Phot1 in bri1-5 were similar to those in WS2 plants (Fig. 8a). In contrast, versus Col-0 we found lower levels of PhyA (~50% lower in bzr1-D than in Col-0), PhyB (~58% lower in bzr1-D than in Col-0), cry1 (~56% lower in bzr1-D than in Col-0), cry2 (~49% lower in bzr1-D than in Col-0), Phot1 (~83% lower in bzr1-D than in Col-0) and Phot2 (~84% lower in bzr1-D than in Col-0) in bzr1-D (Fig. 8b). These data indicate that BRI1 and BZR1 negatively regulate photoreceptor gene expression.

**DISCUSSION**

Light is a cue for plant growth and development, and is recognized by photoreceptors. BR hormones regulate numerous aspects of plant growth and development, including photomorphogenesis and the interaction between light and FR signaling (Wang et al., 2012). The cop1-4 and HY5 ox mutants differed in their BR responses under dark conditions, suggesting that BR-promoted cop1-4 hypocotyl elongation in the dark is not due to overexpression of HY5. It might be due to the interaction between COP1 and BZR1, recently identified (Kim et al., 2014). The responses of photoreceptor mutants to BR demonstrated that phyA211, phyA211/phyB9 and phot1-2/cry1-2 are significantly insensitive to exogenously supplied BR (Fig. 3). The response of photoreceptor mutants, especially phyB9, phyA211/phyB9, cry1-2 and phot1-2/cry1-2, was similar to that of BR-treated plants growing under light. This suggests that BR treatment is not suitable for examining the relationship between photoreceptors and BR signaling.
To further analyze BR effects on photoreceptor mutants we applied the BR biosynthesis inhibitor PCZ. The cry1-2 and phot1-2/cry1-2 mutants were clearly insensitive to PCZ, while phyB9 and phyA211/phyB9 were slightly insensitive to 100 nM PCZ (Fig. 4). However, a high PCZ concentration (2 μM) completely inhibited cry1-2 and phot1-2/cry1-2 hypocotyl elongation growing under light (Fig. 7a), indicating that hypocotyl growth in cry1-2 and phot1-2/cry1-2 mutants requires BR activity. Analysis of BR marker gene expression in the photoreceptor mutants showed that BR biosynthetic and signaling genes are regulated differently in the photoreceptor mutants (Fig. 6), suggesting that photoreceptors somehow regulate the BR signaling pathway. Without PCZ treatment, BR marker gene expression in phot1-2/cry1-2 was similar to that in the bzr1-D mutant, but with PCZ treatment the SAUR15 and ACS5 expression of phot1-2/cry1-2 was completely suppressed; in bzr1-D, on the other hand, PCZ did not completely inhibit SAUR15 and ACS5 expression (Fig. 7b, c). These data are correlated with the results on PCZ-mediated hypocotyl elongation in phot1-2/cry1-2 and bzr1-D (Fig. 7a). The results also suggest that blue-light receptors negatively regulate BR signaling. In contrast, photoreceptor expression in bri1-5 (BR-insensitive) and bzr1-D (BR-sensitive) mutants indicated that BR signaling negatively regulates photoreceptors at transcription level (Fig. 8).

This is the first time the interaction between BR and light signaling has been tested using photoreceptors and BR mutants. Taken together, our results suggest that light signaling tightly regulates the BR response, which may occur partially via photoreceptors (Fig. 9). Further experiments should elucidate the exact role of each photoreceptor in BR signaling.

CONCLUSIONS

BRs play different roles in hypocotyl elongation under light (promotion) and darkness (inhibition). The key light-signaling gene mutant cop1 exhibited light-independent BR-mediated hypocotyl elongation. The photoreceptor mutants phyA211, phyA211/phyB9 and cry1-2/phot1-2 exhibited insensitive responses to BR, while the blue-light receptor mutants cry1-2 and cry1-2/phot1-2 were more insensitive than other photoreceptor mutants to PCZ, a BR biosynthesis inhibitor. Expression of BR biosynthetic genes was higher in cry1-2/phot1-2. However, supply of PCZ reduced the higher expression of BR biosynthetic genes in cry1-2/phot1-2. BRI1 and BZR1 negatively regulated the transcription of photoreceptor genes.

AUTHORS’ CONTRIBUTIONS

ZZZ, YHX and DNY designed research; ZZZ, XFZ, YTZ and YHX performed research; ZZZ, XFZ, YHX, and DNY analyzed data; ZZZ, YHX and DNY wrote the paper.

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SUPPLEMENTARY MATERIAL

Supplementary material (Tables S1 and S2) for this article can be found in the online version at doi: 10.2478/abcsb-2014-0027

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### TABLE S1. Gene information

| Gene name   | Symbol                  | Mutant   | Phenotype                      | Physiological effect                      | Literature               |
|-------------|-------------------------|----------|-------------------------------|-------------------------------------------|--------------------------|
| BR11        | BR receptor             | bri1-5   | Dwarf                         | Insensitive to BR                        | Bellhadr et al., 2010   |
| BZR1        | BR signaling            | bZR1-1D  | Broad leaves, thick stem      | Insensitive to BR inhibitor PCZ           | Wang et al., 2002       |
| PhytochromeA| Far red light           | phyA211  | No obvious phenotype          | Fails to respond to far red light         | Franklin et al., 2010   |
| PhytochromeB| Red light receptor      | phyB9    | Long petiole                   | Fails to respond to red light             | Franklin et al., 2010   |
| CRYPTOCHROME 1/2| Blue light receptor  | cry1/2   | Long petiole                   | Fails to respond to blue light            | Canamero et al., 2006   |
| Phototropin 1/2| Blue light receptor    | phot1/2  | Phototropism insensitive       | Fails to respond to blue light            | Aihara et al., 2008     |
| BR6OX2      | BR biosynthesis         |          |                               |                                           | Oh et al., 2012         |
| DWF4        | BR biosynthesis         |          |                               |                                           | Oh et al., 2012         |
| SAUR15      | BR inducible gene       |          |                               |                                           | Oh et al., 2012         |
| ACS5        | BR inducible gene       |          |                               |                                           | Oh et al., 2012         |
| COP1        | Light signaling E3      | cop1-4   | Dwarf                         | Light response in the dark                | Ma et al., 2002         |
| HY5         | Light signaling         | hy5      | Long hypocotyl                | Constitutive light response               | Saijo et al., 2003      |

### TABLE S2. Primer sequences

| Primer   | Sequence      |
|----------|---------------|
| BR6OX2 F | ATGGCGGCATGAAATACAAAGGA |
| BR6OX2 R | TGTTCCATCATCCTTCTTCTTC  |
| DWF4 F  | TGGCGGTGTCGGTTAAGAT   |
| DWF4 R  | TGCCGGTGACGGTTAAGAT   |
| SAUR15 F| AAGAGGATCATGCGGCTATATG |
| SAUR15 R| GTAATTGGAACCGCGGCATGGG  |
| Actin F | TTCAGCCTGCTTTCTCTCCTTG |
| Actin R | GAGGGGCTGAAACAGACTTC  |
| ACS5 F  | GCGATGCTTTCTTTTGCTACTC |
| ACS5 R  | TTTCTGGGGCTTTGTGTAAGCTTGT  |
| CRY1 F  | GACCTGAAGAGACGAAG   |
| CRY1 R  | ACTCGGGGACTATGCTTC  |
| CRY2 F  | GCCCTTAAAGGGCTAATAC  |
| CRY2 R  | ATACCTTCCAGATCTTCTC  |
| PHOT1 F | AGTTTCCAGCTAGCATTC   |
| PHOT1 R | TAGCTCAGGATCAACACAC |
| PHOT2 F | CATGTGACACAACAGCGTGT  |
| PHOT2 R | ATTCACAAGCACTCCATC |
| PhyA F  | CTTGCTAATCTAGAGATC   |
| PhyA R  | GTTTGCTGACGGAGTTC   |
| PhyB F  | GCCCTGAAAGGGTTCAGTGTC  |
| PhyB R  | CATCATCAGCATCATGTC   |