Cryptochrome 2 negatively regulates ABA-dependent seed germination in *Arabidopsis*

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Abstract: Cryptochromes (CRYs) are a group of flavin-type blue light photoreceptors found in plants, animals, and humans. These proteins play diverse functional roles in growth and development. In plants, the role of CRYs in light responses involving the maintenance of circadian rhythms and photomorphogenesis is well known. Upon seed imbibition, abscisic acid (ABA) levels decrease, which allows embryos to germinate and develop into seedlings. However, the molecular mechanism behind the effect of CRYs on hormone-regulated seed germination remains unclear. Here, we found that the *cry2* mutant germinated vigorously in the presence of different concentrations of ABA under both light and dark conditions. The expression of ABA- and GA-responsive genes encoding germination inhibitors was downregulated in the *cry2* mutant compared to wild type even after ABA treatment. Taken together, our results indicate that CRY2 plays an important role in *Arabidopsis* seed germination.
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Abbreviations: ABA, abscisic acid; ABI3, ABSCISIC ACID–INSENSITIVE3; ABI5, ABSCISIC ACID–INSENSITIVE5; CCT1, CRY1 C-terminus; COP1, constitutive photomorphogenic 1; CRY, cryptochrome; GA, gibberellins; GAI, GA-insensitive; GID1A, GA INSENSITIVE DWARF1A; RGA, Repressor of ga1-3; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SPA1, suppressor of phytochrome A 1; UV, ultraviolet

Introduction

Light is perceived by plants via a diverse array of photoreceptors including phytochromes, which detect light in red and far-red regions of the spectrum, and cryptochromes (CRYs), which detect blue, UV (ultraviolet)-A, and UV-B light. CRYs are flavoproteins that regulate plant photomorphogenic development and the circadian clock in both plants and animals. *Arabidopsis* CRY1 and CRY2 mediate the light-dependent stimulation of de-etiolation (affecting hypocotyl elongation), leaf and cotyledon expansion, pigment biosynthesis, stem growth and internode elongation, and photoperiodic control of floral initiation. CRYs were first discovered in *Arabidopsis* and have been identified in a wide range of organisms from bacteria to humans (Li and Yang, 2007; Chaves et al., 2011).

The accumulated data show that blue light affects the expression of up to 20% of the genes in the *Arabidopsis* genome, and CRY1 and CRY2 are the major photoreceptors mediating the blue light regulation of gene expression. CRY1 and CRY2 share sequence similarities with the conserved N-terminal region of DNA photolyase (a DNA-repair enzyme dependent on blue light), but they show no detectable photolyase activity. The poorly conserved C-terminal region of DNA photolyase has less than 15% sequence similarity with CRY1 and CRY2; this region is involved in blue light-dependent ubiquitination and protein turnover (Ahmad et al., 1998; Lin et al., 1998). Transgenic *Arabidopsis* plants overexpressing the C-terminal regions of CRY1 and CRY2 exhibit early flowering. CRY signalling is primarily mediated by the C-terminal regions of these proteins (Li and Yang, 2007; Yang et al., 2000).

Biochemical studies show that the stability and activity of many proteins are regulated by E3 ubiquitin ligase activity. COP1 ubiquitin ligase interacts with the CRY1 C-terminus (CCT1) in response to blue light, whereas COP1 (Constitutive photomorphogenic 1) and its interacting protein SUPPRESSOR OF PHYTOCHROME A 1 (SPA1) interact with CRY2 in response to blue light (Wang et al., 2001; Zuo et al., 2011). These data indicate that CRY-mediated light signalling and development are regulated by COP1.

Seed germination is regulated by diverse biotic

and abiotic factors. Photoreceptors (phytochromes and CRYs) receive light and regulate seed germination, which involves rupture of the testa followed by unified endosperm rupture and radicle protrusion. Environmental factors determine the relative levels of crucial phytohormones such as gibberellins (GAs) and abscisic acid (ABA), which have antagonistic functions in the control of seed germination (Olszewski et al., 2002). However, the regulation of CRYs by ABA during seed germination is not fully understood. Here, we investigated the dark and light responses of CRY1 and CRY2 during *Arabidopsis* seed germination using *cry1* and *cry2* mutants treated with different levels of ABA.

Materials and Methods

Seeds of *Arabidopsis thaliana* of the Columbia (Col) Landsberg erecta (Ler) ecotype were surface-sterilized in 5% sodium hypochlorite and 0.1% Triton X-100 solution for 10 minutes, washed five times in sterilized water, and plated on Petri dishes containing germination medium (MS medium containing 1% sucrose and 0.8% agar) with different concentrations of ABA (0, 0.2, 1, and 5 μM). The plates were stored at 4°C for 2 days to break dormancy and subsequently incubated at 22°C under a 16 h light/8 h dark or continuous dark regime for 2 days. T-DNA knockout mutants of *Arabidopsis cry1* and *cry2* were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). The experiment was repeated three times with similar results.

Estimation of transcript levels of ABA- and GA-responsive genes

Wild-type and *cry2* mutant seeds were treated with 5 μM ABA during imbibition. After 12 hours, total RNA was isolated from the seeds. First-strand cDNA was synthesized from 1 μg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). An equal volume of cDNA was amplified by quantitative real-time qRT-PCR (MyiQ, Bio-Rad) according to the manufacturer's protocol. Specifically, 10 nM of specific primers and template cDNA were combined with 25 μl iQ SYBR Green Super Mix (Bio-Rad), and the reactions were performed under the following thermal conditions: 95°C for 10 min; 45 cycles of 95°C for 10 sec; 60°C for 10 sec; and 72°C for 10 sec. The CT values of target genes were normalized to the CT value of the *actin1* gene and analyzed with iCycler IQ software (Bio-Rad). The experiments were repeated three times. PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi), and their specificity was verified by cloning into the pGEM T-Easy vector (Promega) and sequencing with an ABI 3730xl DNA Analyzer (Applied Biosystems).

Results and Discussion

In *Arabidopsis*, CRY1 and CRY2 are involved in developmental responses such as the inhibition of hypocotyl elongation, stem growth and internode elongation, leaf and cotyledon expansion, UV-B-dependent gene expression, and anthocyanin accumulation. These proteins share common functions because of their highly conserved N-termini, but they have divergent C-terminal domains, which subsequently undergo ubiquitination via light activation (Chaves et al., 2011). Previous studies showed that blue light-dependent CRYs were involved in diverse aspects of plant development and growth. However, the effects of CRYs on ABA-dependent seed germination have not been fully elucidated. We therefore examined the role of CRY1 and CRY2 in ABA-mediated seed germination using *Arabidopsis cry1* and *cry2* mutant seeds. For this experiment, we treated *cry1* and *cry2* mutant seeds with different concentrations of ABA during imbibition under light and dark conditions and then examined their germination. We found that seeds of *Arabidopsis* CRY mutants *cry1* and *cry2* had different sensitivities to ABA compared to wild-type seeds (Fig. 1 and 2). During germination, cotyledon development was significantly delayed in the presence of 1 μM ABA in both *cry1* and wild-type (Col 0) seeds (Fig. 1). Radicles just began to protrude in both wild-type and *cry1* seeds under similar light conditions (Fig. 1). However, in *cry2* seeds, cotyledons clearly developed on 1 μM ABA under the same light conditions, although only protruded radicles were observed on 5 μM ABA under these light conditions (Fig. 1). Under dark conditions, root development was clearly observed in the *cry2* mutant but not in the wild type or *cry1* mutant on 0.2 μM ABA (Fig. 2), while both *cry1* and *cry2* displayed only radical emergence in the presence of 1 and 5 μM ABA. These results indicate that the *cry2* mutant has resistance to ABA.

In barley embryos, blue light promotes the expression of an ABA biosynthetic gene and affects genes involved in GA and ABA metabolism (Gubler et al., 2008), suggesting that CRYs regulate the expression of genes involved in hormone synthesis and metabolism. ABA affects radical emergence but not testa rupture (Schopfer and Plachy, 1984), implying that CRYs can participate in the ABA-mediated germination pathway. Therefore, we speculate that the strong germination of *cry2* seeds in the presence of ABA may disrupt the signalling networks between CRYs and other ABA- or GA-related proteins, or it may impair the expression of signalling-related genes during radical and cotyledon emergence.

We thus examined the expression levels of ABA- and GA-responsive genes in the seeds of the *cry2*

mutant. For this experiment, we treated wild-type and *cry2* seeds with 5 μM ABA during imbibition. After 12 hours of treatment, total RNA was isolated from the samples. We investigated the transcript levels of two ABA-responsive genes, *ABI3* (*ABSCISIC ACID−INSENSITIVE3*) and *ABI5* (*ABSCISIC ACID−INSENSITIVE5*), and two GA-responsive DELLA protein genes, *RGA* (*Repressor of ga1-3*) and *GAI* (*GA-insensitive*), by real-time qRT-PCR with gene-specific primers. We found that the transcript levels of *ABI3*, *ABI5*, and *GAI* were similar in both untreated wild-type and *cry2* samples, while the transcript levels of *RGA* were significantly lower in the untreated *cry2* mutant than in the untreated wild type (Fig. 3). As expected, the expression of *ABI3*, *ABI5*, and *GAI* was induced in both ABA-treated wild-type and *cry2* seeds (Fig. 3). However, the transcript levels of these genes were still lower in the *cry2* mutant than in the wild type. By contrast, the *RGA* transcript levels were not affected by ABA treatment in the wild type or *cry2* mutant (Fig. 3). Finally we examined the transcript level of *GID1A* (*GA INSENSITIVE DWARF1A*) gene encoding a GA receptor. As a result, we found that the transcript levels of *GID1A* were also significantly lower in the untreated *cry2* mutant than in the untreated wild type, and its transcript level was not affected by ABA treatment in the wild type or *cry2* mutant (Fig. 3). These data indicate that the relatively low sensitivity of *cry2* mutant seeds to ABA is due to the low expression of germination inhibitors ABI3, ABI5, GAI, and RGA, and also due to the low expression of GA receptor GID1A. Together, these results suggest that CRY2 plays a critical role in seed germination in a light-independent manner.

Recently, Xu et al. (Xu et al., 2009) reported that transgenic *Arabidopsis* lines overexpressing wheat CRYs exhibit a susceptible phenotype when treated with ABA. In the current study, we showed that *cry2* was resistant to ABA at 1 μM under light conditions and at 0.2 μM in the dark, but *cry1* was not resistant to ABA under either condition. This result indicates that CRY2 is more sensitive to ABA than CRY1 during seed germination.

Considering both previous and current results, we conclude that CRY2 regulates seed germination in a light-independent manner in *Arabidopsis*, and CRY2 is sensitive to ABA during germination. It is possible that ABA disrupts the signalling networks between CRYs and ABA- or GA-related proteins. Therefore, further studies investigating the mechanisms underlying the response of *cry1* and *cry2* to ABA and GA will provide more information about the roles of CRYs during seed germination.

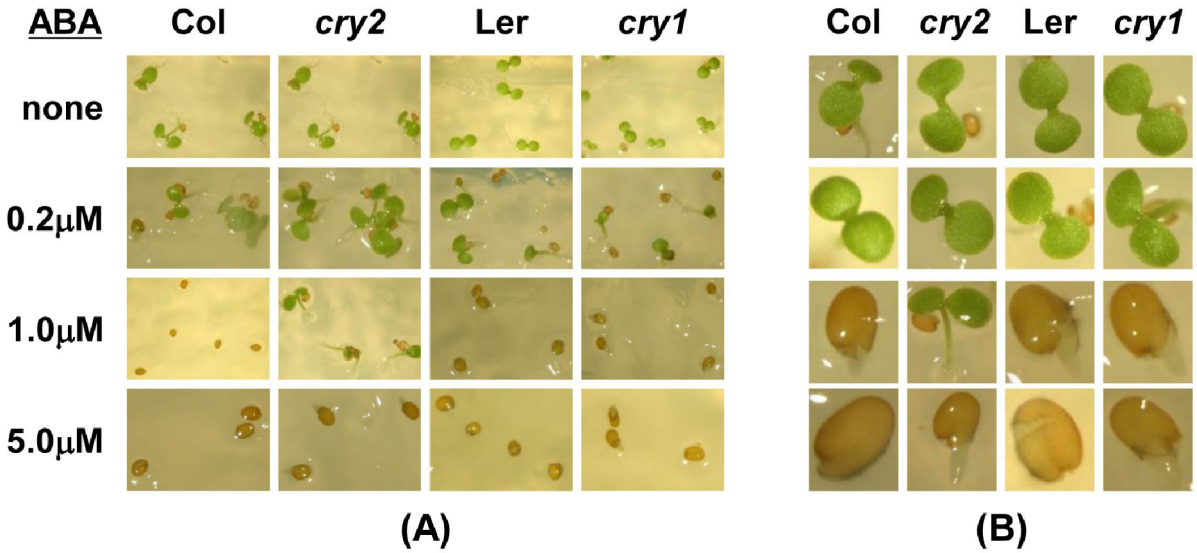

Fig. 1 Effect of ABA on germination of cryptochrome mutants under long-day conditions. (A) Representative photographs of seedlings taken 4 days after planting. (B) Enlarged view of testa rupture and cotyledon and radicle emergence.

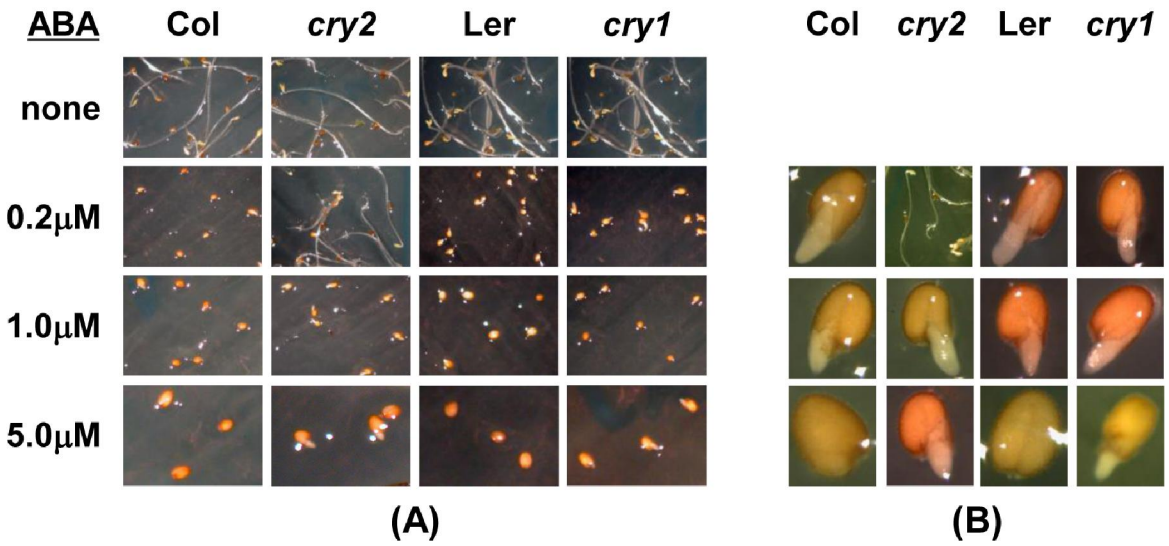

Fig. 2 Effect of ABA on germination of cryptochrome mutants in the dark. (A) Representative photographs of seedlings taken 4 days after planting. (B) Enlarged view of testa rupture and radicle and root emergence.

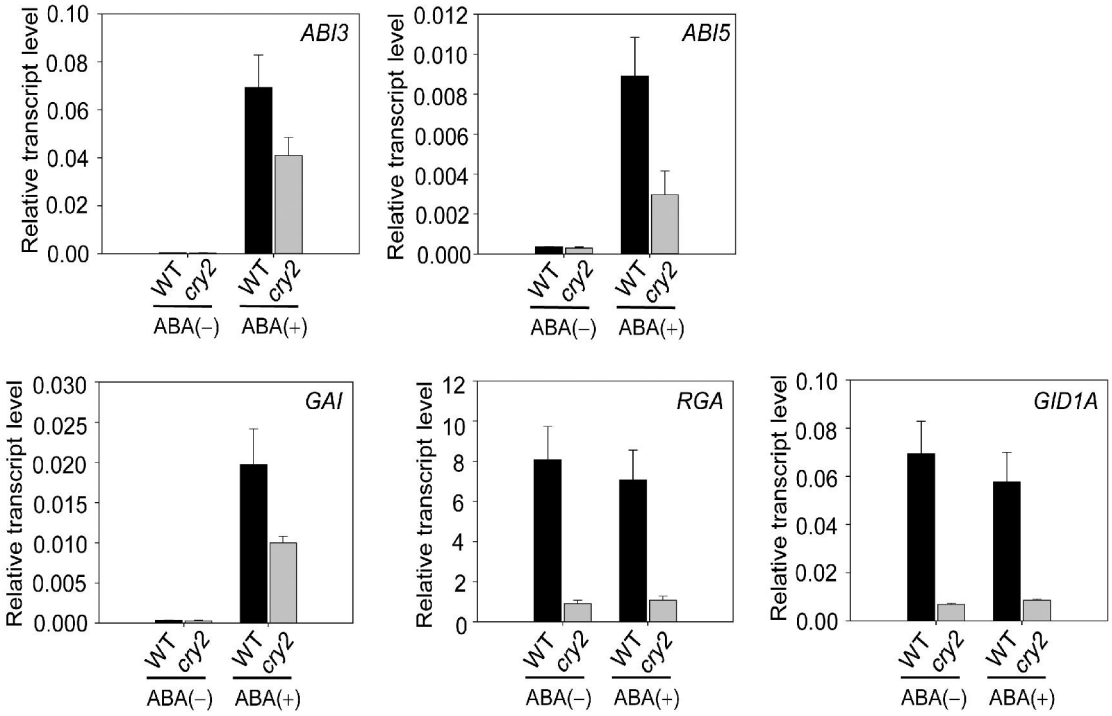

Fig. 3 Transcript levels of ABA- and GA-responsive genes during seed imbibition. Seeds of wild-type and *cry2* plants were imbibed for 12 hours in 5 μM ABA solution. The seeds were used for total RNA extraction, and the expression levels of *ABI3*, *ABI5, RGA*, *GAI* and *GID1A* were then examined by real-time qRT-PCR with gene-specific primers. *ABI3*, *ABSCISIC ACID–INSENSITIVE3; ABI5, ABSCISIC ACID–INSENSITIVE3; RGA*, *Repressor of ga1-3*; *GAI*, *GA-insensitive*; *GID1A*, *GA INSENSITIVE DWARF1A*.

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Running title: Cry2 regulates ABA-dependent seed germination

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