Cytokinin Antagonizes Abscisic Acid-Mediated Inhibition of Cotyledon Greening by Promoting the Degradation of ABSCISIC ACID INSENSITIVE5 Protein in Arabidopsis[1][C][W]

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In higher plants, seed germination is followed by postgerminative growth. One of the key developmental events during postgerminative growth is cotyledon greening, which enables a seedling to establish photosynthetic capacity. The plant phytohormone abscisic acid (ABA) plays a vital role by inhibiting seed germination and postgerminative growth in response to dynamically changing internal and environmental cues. It has been shown that ABSCISIC ACID INSENSITIVE5 (ABI5), a basic leucine zipper transcription factor, is an important factor in the regulation of the ABA-mediated inhibitory effect on seed germination and postgerminative growth. Conversely, the phytohormone cytokinin has been proposed to promote seed germination by antagonizing the ABA-mediated inhibitory effect. However, the underpinning molecular mechanism of cytokinin-repressed ABA signaling is largely unknown. Here, we show that cytokinin specifically antagonizes ABA-mediated inhibition of cotyledon greening with minimal effects on seed germination in Arabidopsis (*Arabidopsis thaliana*). We found that the cytokinin-antagonized ABA effect is dependent on a functional cytokinin signaling pathway, mainly involved in the cytokinin receptor gene *CYTOKININ RESPONSE1/ARABIDOPSIS HISTIDINE KINASE4*, downstream histidine phosphotransfer protein genes *AHP2*, *AHP3*, and *AHP5*, and a type B response regulator gene, *ARR12*, which genetically acts upstream of *ABI5* to regulate cotyledon greening. Cytokinin has no apparent effect on the transcription of *ABI5*. However, cytokinin efficiently promotes the proteasomal degradation of ABI5 in a cytokinin signaling-dependent manner. These results define a genetic pathway through which cytokinin specifically induces the degradation of ABI5 protein, thereby antagonizing ABA-mediated inhibition of postgerminative growth.

Seed germination and subsequent seedling establishment are key developmental events during plant growth. In Arabidopsis (*Arabidopsis thaliana*), seed germination is morphologically characterized by several distinctive phases, including testa rupture, endosperm rupture, and radicle protrusion (Bewley, 1997; Müller et al., 2006; Piskurewicz et al., 2008). During seed germination, a major physiological event is the degradation and mobilization of the storage compounds that are accumulated during seed maturation and used for the energy supply of a seed during germination. These processes are under the tight control of genetic programs and are regulated by environmental factors, including light, temperature, and osmotic stress (Bewley, 1997; Lopez-Molina et al., 2001, 2002; Borisjuk et al., 2004; Penfield et al., 2005). After germination, postgerminative growth is characterized by cotyledon opening, cotyledon greening, hypocotyl growth, and radicle growth. Cotyledon greening marks the establishment of a seedling to become an autotrophic organism with photosynthetic capacity. In many cases, seed germination and subsequent postgerminative growth are collectively referred to as seed germination.

Seed germination and postgerminative growth are strictly regulated by phytohormones. In particular, abscisic acid (ABA) and GA play predominant and antagonistic roles in the regulation of seed germination (Karssen et al., 1983; Olszewski et al., 2002; Kucera et al., 2005; Nambara and Marion-Poll, 2005). Whereas a high GA-ABA ratio induces, a low GA-ABA ratio inhibits seed germination (Karssen et al., 1983; Kucera et al., 2005). ABA is known to promote seed maturation and seed dormancy but to inhibit seed germination and postgerminative growth. The underpinning molecular mechanism of ABA action during seed germination has been studied extensively. During the past two decades, genetic studies in Arabidopsis have identified many *abscisic acid insensitive* (*abi*) mutants using the germination assay, and several *ABI* genes have been characterized in

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some detail (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994; Leung et al., 1994; Meyer et al., 1994; Finkelstein et al., 1998, 2002; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001). Among these *ABI* genes, *ABI5* is one of the best-characterized genes. *ABI5* encodes a basic Leu zipper transcription factor that acts as a key regulator of seed development and postgerminative growth (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). Whereas the loss-of-function mutations in *ABI5* cause the mutant insensitive to ABA (Koornneef et al., 1984; Finkelstein, 1994), the overexpression of *ABI5* renders hypersensitivity to ABA during germination and postgerminative growth (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). The transcription of *ABI5* and the accumulation of ABI5 protein are highly enriched in developing and germinating seeds and become rapidly decreased to the basal level shortly after seed germination (Lopez-Molina et al., 2001). Moreover, the levels of both *ABI5* mRNA and ABI5 protein are positively regulated by ABA, correlated to the inhibitory effect of the phytohormone on seed germination and postgerminative growth, illustrating *ABI5* as a key regulator in early seedling development (Lopez-Molina et al., 2001, 2002). The stability of ABI5 protein is regulated by the proteasomal degradation pathway, involved in an E3 ubiquitin ligase, KEEP ON GOING, which promotes ABI5 protein degradation (Stone et al., 2006; Liu and Stone, 2010). Moreover, DWA1 and DWA2 (for DWD hypersensitive to ABA1 and ABA2), two substrate receptors of CUL4-DDB1-DWD (for Cullin4-Damaged DNA Binding1-DDB1 binding WD40) E3 ligase complexes, have also been shown to induce ABI5 degradation (Lee et al., 2010). A novel protein, ABI FIVE BINDING PROTEIN (AFP), has also been reported to play a role in the regulation of ABI5 protein degradation (Lopez-Molina et al., 2003). AFP has also been proposed to connect ABI5 with the transcription repressor TOPLESS to repress the ABA response genes (Pauwels et al., 2010).

In addition to ABA and GA, cytokinin has been implied to play an important role in regulating seed germination (Barzilai and Mayer, 1964; Khan, 1971; Black et al., 1974; Thomas et al., 1997). Cytokinin is an essential phytohormone involved in the regulation of various aspects of plant growth and development, including seed germination (Werner and Schmülling, 2009). Cytokinin signaling is mediated by a two-component system-based phosphorelay, through which a phosphoryl group is sequentially transferred from the receptors to downstream components (Kakimoto, 2003; Müller and Sheen, 2007; To and Kieber, 2008; Hwang et al., 2012). In Arabidopsis, three His kinases, CYTOKININ RESPONSE1 (CRE1)/WOODEN LEG (WOL)/ARABIDOPSIS HISTIDINE KINASE4 (AHK4), AHK2, and AHK3, have been characterized as cytokinin receptors (Inoue et al., 2001; Yamada et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Downstream of the receptors, the phosphorelay consists of three additional major components, histidine phosphotransfer proteins (AHPs), type B response regulators (ARRs), and type A ARRs. AHPs accept a phosphoryl group from the cytokinin receptor and then transfer to downstream type B and type A ARRs. Upon phosphorylation, type B ARRs, a group of MYB-type transcription factors, are activated and directly promote the expression of cytokinin response genes, including type A *ARR* genes. The expression of type A *ARR* genes is highly inducible by cytokinin. Upon phosphorylation, type A ARR proteins negatively regulate cytokinin signaling by unknown mechanisms, thereby forming a feedback regulatory loop (Müller and Sheen, 2007; To and Kieber, 2008; Hwang et al., 2012).

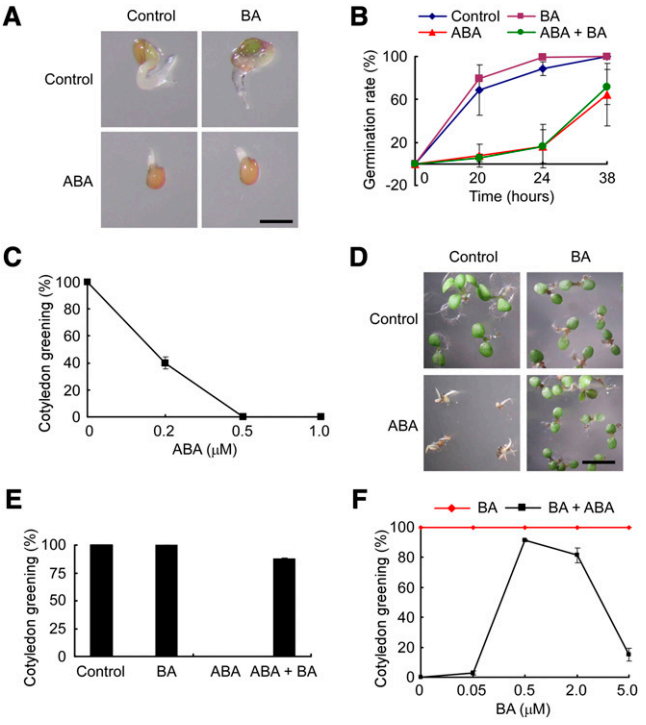

Figure 1. Cytokinin antagonizes ABA to promote early seedling growth. A, Effect of cytokinin on seed germination. Wild-type (Col-0) seeds were germinated on GM medium containing various combinations of 0.5 μM ABA and 0.5 μM benzyladenine (BA) and cultured for 38 h post stratification. The control sample was treated with dimethyl sulfoxide. Bar = 0.5 mm. B, Quantitative analysis of the seed germination rate. Radicle emergence was used as a marker for germinated seeds. C, Inhibition of cotyledon greening by ABA. Wild-type (Col-0) seeds were germinated on GM medium containing various concentrations of ABA for 4 d, and the cotyledon greening rate was scored. D, Cytokinin antagonizes ABA-mediated inhibition of cotyledon greening. Four-day-old Col-0 seeds were germinated and grown on GM medium containing 0.5 μM ABA in the presence or absence of 0.5 μM benzyladenine. Bar = 4 mm. E, Quantitative analysis of the cotyledon greening rate of wild-type (Col-0) seedlings treated with ABA and benzyladenine as in D. F, Dose-dependent effect of cytokinin on antagonizing ABA in cotyledon greening. Wild-type (Col-0) seeds were germinated on GM medium containing 0.5 μM ABA and various concentrations of benzyladenine for 4 d, and the cotyledon greening rate was scored. The data presented in B, C, E, and F are mean values of three independent experiments. More than 50 seeds of each sample were used in each experiment. Error bars indicated the SE (n = 3). [See online article for color version of this figure.]

Recent studies indicate that the active cross talk between ABA and cytokinin plays an important role in the regulation of the abiotic stress response (Ha et al., 2012). The homeostasis of cytokinin is controlled by two key enzymes. Whereas ISOPENTENYL TRANSFERASE (IPT) catalyzes the first rate-limiting step of cytokinin de novo synthesis, CYTOKININ OXIDASE (CKX) irreversibly degrades cytokinin (Sakakibara, 2006). The reduction of cytokinin levels in the *ipt* multiple mutants or transgenic plants overexpressing *CKX* causes a stress-tolerant phenotype (Nishiyama et al., 2011). Consistently, whereas the cytokinin level is decreased under the stress condition, many stress-related genes show an altered expression level in the *ipt* multiple mutants or by external application of cytokinins (Nishiyama et al., 2011, 2012). Mutations in the cytokinin receptor genes or the overexpression of several type A *ARR* genes also cause tolerance to abiotic stresses (Tran et al., 2007; Shi et al., 2012). However, it remains largely unknown how the cytokinin signaling pathway perceives a stress signal (Ha et al., 2012). As an adaptation mechanism, the active cross talk between cytokinin and ABA is also involved in the regulation of lateral root development by modulating the expression of *ABI4* (Shkolnik-Inbar and Bar-Zvi, 2010).

It has long been proposed that cytokinin promotes seed germination, possibly by reverting the inhibitory role imposed by ABA and other factors (Khan, 1971; Black et al., 1974; Mok, 1994; Thomas et al., 1997; Davies, 2004). Note that the term "seed germination" used in these studies referred to both seed germination and postgerminative growth. A recent study identified a gain-of-function mutant, *germination insensitive to ABA mutants* (*gim1*), that shows resistance to ABA during seed germination. *GIM1* encodes an IPT (AtIPT8), also known as PLANT GROWTH ACTIVATOR22 (PGA22), and the *gim1/pga22* mutations cause a remarkably increased level of major cytokinin species (Sun et al., 2003; Wang et al., 2011). The antagonizing effect of cytokinin on ABA-inhibited seed germination was attributed to the cytokinin-repressed expression of *ABI5* (Wang et al., 2011). However, the expression of *ABI5* is only marginally regulated by cytokinin in an ABA-independent manner (Wang et al., 2011), indicating that the cytokinin-regulated *ABI5* transcription is not a major regulatory mechanism. Instead, these observations suggest that an unidentified mechanism, rather than the transcription of *ABI5*, is employed to regulate the interaction between cytokinin and ABA. In this study, we show that cytokinin specifically antagonizes the ABA-mediated inhibition of cotyledon greening, a key developmental event during postgerminative growth. We demonstrate that cytokinin induces the degradation of ABI5 protein, thereby relieving germinating seedlings from the inhibitory effect imposed by ABA.

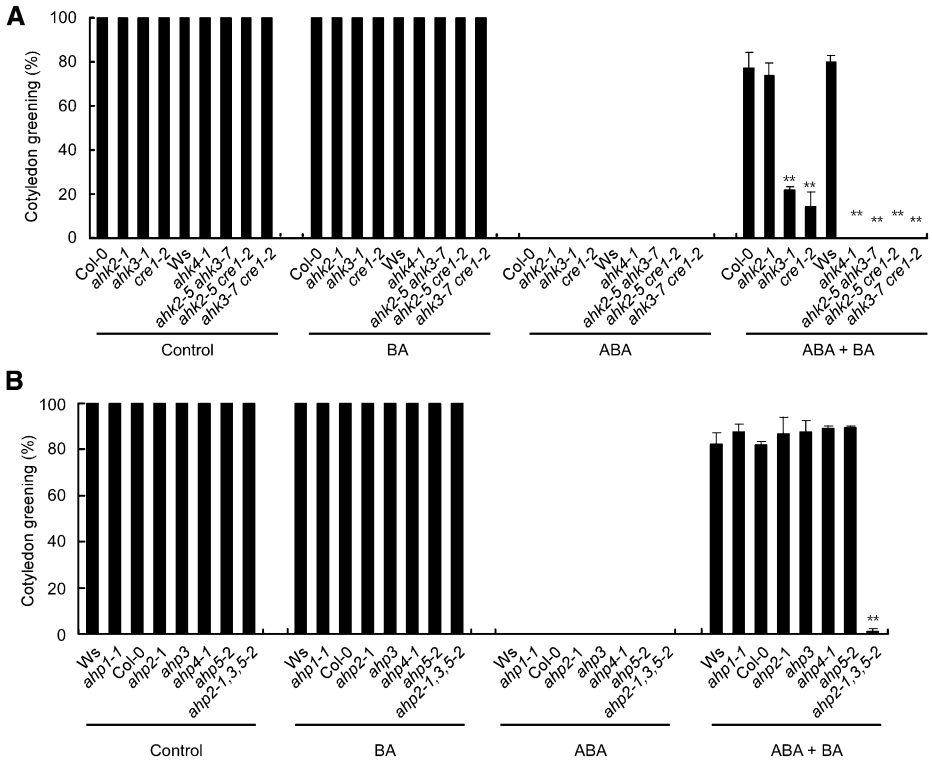

Figure 2. Cytokinin-repressed ABA signaling requires *AHK3*, *AHK4*, and multiple *AHP* genes. Seeds of the wild type (Col-0 and Ws), various cytokinin receptor mutants (A), and *ahp* mutants (B) were germinated and grown on GM medium containing 0.5 μM benzyladenine (BA) and 0.5 μM ABA for 4 d, and the cotyledon greening rate was scored. The control sample was treated with dimethyl sulfoxide. The data presented are means of three independent experiments. More than 50 seeds of each sample were used in each experiment. Error bars indicated the SE ($n = 3$). $^{**}P < 0.01$ (Student's t test).

RESULTS

Cytokinin Promotes Cotyledon Greening by Repressing the Inhibitory Effect of ABA

Seed development and subsequent germination are regulated by several phytohormones, of which ABA promotes seed dormancy and inhibits seed germination. By contrast, cytokinin has been proposed to promote seed germination and postgerminative growth, possibly by antagonizing the ABA-mediated inhibitory effect (Khan, 1971; Wang et al., 2011). Note that many previous studies collectively referred to seed germination and postgerminative growth as seed germination. When wild-type Arabidopsis (Columbia-0 [Col-0]) seeds were germinated in the presence of ABA, the growth of the germinated embryos was arrested (Fig. 1, A and B). As a marker of postgerminative growth and seedling establishment, cotyledon greening was strongly inhibited by ABA, with complete inhibition by 0.5 μM ABA (Fig. 1C). Surprisingly, we found that the cytokinin benzyladenine and 2-isopentenyladenine had no antagonizing effect on the inhibitory effect of ABA on seed germination (Fig. 1, A and B; Supplemental Fig. S1). Instead, cytokinin antagonized the inhibitory effect of ABA on cotyledon greening during postgerminative growth in a dose-dependent manner (Fig. 1, D and E; Supplemental Fig. S2). Notably, cytokinin reverted the ABA effect on cotyledon greening at lower concentrations (approximately 0.5 μM), and the promotive effect of cytokinin was decreased at higher concentrations of cytokinin (Fig. 1F). To further confirm the effect of cytokinin on ABA during cotyledon greening, we analyzed the phenotype of *pga22* that caused estradiol-induced overexpression of *AtIPT8* and an elevated level of cytokinin (Sun et al., 2003). In the seed germination assay, the elevated level of cytokinin produced by the overexpression of *AtIPT8* had no obvious effect on seed germination and no antagonizing effect on the inhibitory effect of ABA on seed germination (Supplemental Fig. S3A). However, the cotyledon greening rates were significantly increased in *pga22* under 0.5 μM ABA and 10 μM estradiol treatments (Supplemental Fig. S3B). These results indicate that cytokinin antagonizes the inhibitory effect of ABA on cotyledon greening with marginal effects on seed germination.

To further test whether cytokinin specifically antagonizes the inhibitory effect of ABA during early seedling establishment, we performed a similar experiment during the postgerminative growth stage. Wild-type seeds were stratified for 24 h, and the germinating seedlings were then transferred onto agar plates supplemented with 0.5 μM ABA in the presence or absence of cytokinin. Whereas ABA strongly inhibited cotyledon greening, cytokinin substantially reverted the inhibitory effect of ABA on cotyledon greening (Supplemental Fig. S4). Similarly, osmotic stress (mimicked by mannitol) and salt stress (NaCl) also inhibited cotyledon greening, which was efficiently relieved by cytokinin (Supplemental Fig. S5). Taken together, these results suggest that cytokinin antagonizes the abiotic stress-mediated early growth arrest.

The Cytokinin-Relieved ABA Inhibitory Effect on Cotyledon Greening Requires a Functional Cytokinin Signaling Pathway

To investigate whether the effect of cytokinin on ABA-inhibited cotyledon greening is dependent on the cytokinin signaling pathway, we first examined the cotyledon greening phenotype of the cytokinin receptor mutants treated with cytokinin and ABA. In the presence of ABA, the *ahk2-1* mutant showed a response to cytokinin similar to the wild type (Fig. 2A), indicating that *AHK2* does not play a major role in cytokinin-promoted cotyledon greening. However, the *ahk3-1* and *cre1-2* (an allele of *ahk4*) mutants displayed significantly reduced sensitivity to cytokinin when treated with both cytokinin and ABA. In particular, whereas nearly 80% of Col-0 cotyledons were turning green, less than 26% and 6% of *ahk3-1* and *cre1-2* mutant cotyledons became green, respectively (Fig. 2A). A similar result was obtained by analyzing *ahk4-1*, an allele of *cre1* in the Wassilewskija (Ws) background (Fig. 2A). We also examined the cotyledon greening phenotype of various combinations of the receptor gene double mutants. Compared with the single receptor gene mutants, all three double mutants displayed remarkably reduced sensitivity to cytokinin in the presence of ABA (Fig. 2A). These results indicate that the cytokinin receptors play redundant roles in antagonizing ABA during cotyledon greening, whereas *AHK3* and *CRE1/AHK4* play a more dominant role in this developmental process. Consistently, the expression of all three receptor genes was detected in germinating seedlings (Supplemental Fig. S6), and the predominantly expressed cytokinin receptor gene in developing embryos is *CRE1/AHK4* (Müller and Sheen, 2008).

We next examined the possible involvement of the *AHP* genes in the cross talk of cytokinin and ABA during the early growth arrest. Single mutations in any of the five *AHP* genes did not show an altered response to cytokinin in antagonizing the inhibitory effect of ABA on cotyledon greening (Fig. 2B), suggesting a high degree of genetic redundancy as revealed in previous studies (Hutchison et al., 2006; Deng et al., 2010). The *ahp2-1 ahp3 ahp5-2* triple mutant is known to cause a severely compromised response to cytokinin (Hutchison et al., 2006). Consistently, cytokinin-induced cotyledon greening was nearly completely lost in the *ahp2-1 ahp3 ahp5-2* triple mutant (Fig. 2B), indicating that the genetically redundant *AHP* genes are functionally required for cytokinin-mediated cotyledon greening. Collectively, the above results suggest that both the cytokinin receptors and AHPs are required for cytokinin-repressed ABA signaling during early seedling growth.

Cytokinin Antagonizes ABA Signaling Mainly via ARR12

Type B ARRs are transcription factors that act downstream of AHPs to positively regulate cytokinin signaling by directly promoting the transcription of type A *ARR* genes. Of the 11 type B *ARR* genes, *ARR1*, *ARR10*, and *ARR12* play essential and redundant roles

in the regulation of cytokinin signaling (Argyros et al., 2008; Ishida et al., 2008). We assumed that cytokinin antagonizes the inhibitory effect of ABA through one or more of these three key type B ARR genes. To test this possibility, we examined the responses of the *arr1-3*, *arr10-5*, *arr12-1*, and *arr12-3* mutants to cytokinin in antagonizing ABA during cotyledon greening. We found that the *arr1-3* and *arr10-5* mutants showed a slightly reduced sensitivity to cytokinin in the cotyledon greening assay (Fig. 3A). However, two allelic mutants, *arr12-1* and *arr12-3*, showed significantly reduced sensitivity to cytokinin in antagonizing the ABA effect (Fig. 3A). Notably, whereas *arr12-1* was a null mutant allele, *arr12-3* contained a transfer DNA insertion in intron 2 and had residual expression of *ARR12* (Supplemental Fig. S7, A and B). Consistently, *arr12-1* showed a stronger phenotype than *arr12-3* in antagonizing the ABA effect in the presence of cytokinin (Fig. 3A). In a double mutant analysis, whereas the *arr1-3 arr10-5* double mutant maintained approximately 35% of the activity, both the *arr1-3 arr12-1* and *arr10-5 arr12-1* double mutants were almost completely insensitive to cytokinin in the cotyledon greening assay (Fig. 3A). These results suggest that

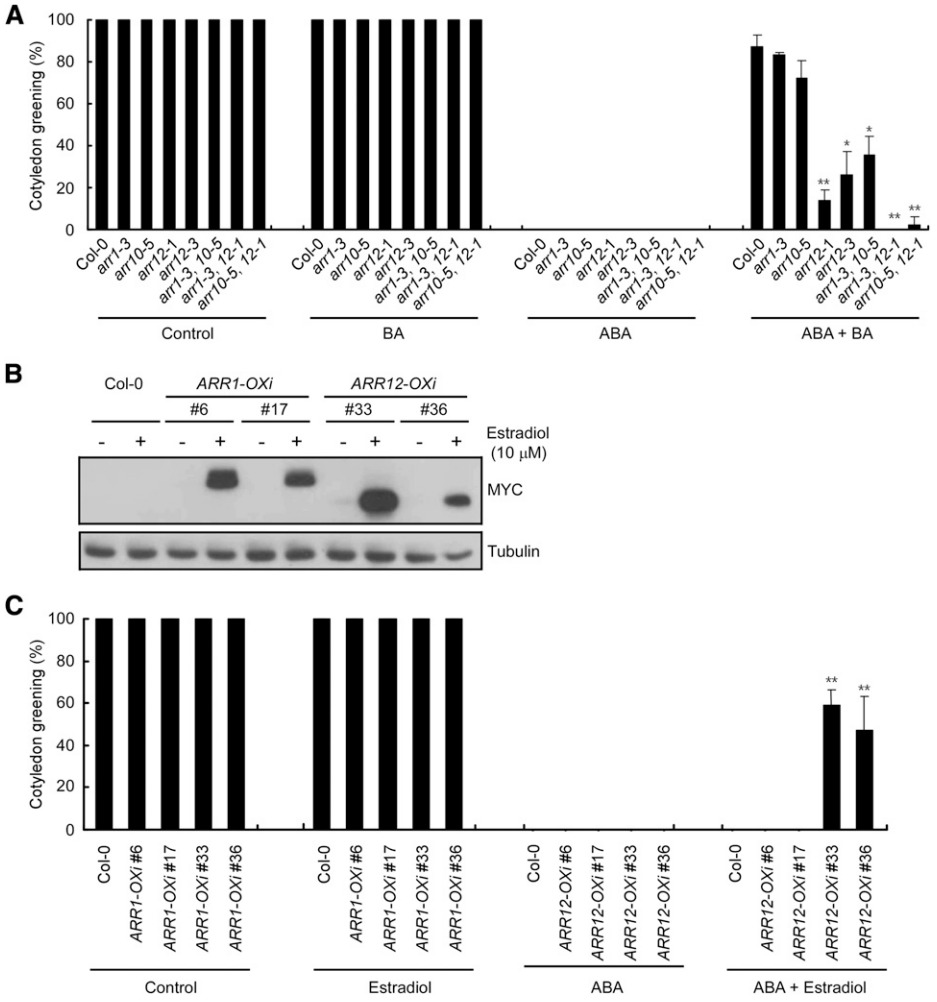

Figure 3. Cytokinin-repressed ABA signaling is dependent on *ARR12*. A, Seeds of the wild type (Col-0) and various mutants of type B *ARR* genes (*ARR1*, *ARR10*, and *ARR12*) were germinated and grown on GM medium containing 0.5 μM benzyladenine (BA) and 0.5 μM ABA for 4 d, and the cotyledon greening rate was scored. The control sample was treated with dimethyl sulfoxide. The data presented are means of three independent experiments. More than 50 seeds of each sample were used in each experiment. Error bars indicated the SE ($n = 3$). *$P < 0.05$, **$P < 0.01$ (Student's *t* test). B, Immunoblot analysis of ARR1ΔDDK-MYC and ARR12ΔDDK-MYC proteins in transgenic seedlings. Seven-day-old wild type (Col-0) and transgenic seedlings (numbers refer to transgenic lines) germinated and grown on GM medium were treated with estradiol for 6 h and then subjected to immunoblotting using an anti-MYC antibody. Equal loading was verified using an anti-tubulin antibody. C, Wild-type (Col-0) and transgenic seeds with the indicated genotypes were germinated and grown on GM medium containing 0.5 μM ABA and 10 μM estradiol for 4 d, and the cotyledon greening rate was analyzed. The data presented are means of three independent experiments. More than 50 seeds of each sample were used in each experiment. Error bars indicated the SE ($n = 3$). **$P < 0.01$ (Student's *t* test).

ARR12 plays an important role in cytokinin-regulated ABA signaling during cotyledon greening.

To further assess the specific role of *ARR12* in antagonizing against ABA, we carried out an overexpression study. We generated two mutated transgenes, *ARR1ΔDDK-MYC* and *ARR12ΔDDK-MYC*, in which the N-terminal region encompassing the receiver domain (the DDK domain) was deleted. The receiver domain in type B ARRs functions as a negative regulatory motif, and the removal of this region causes constitutive transcription activation in the absence of cytokinin (Sakai et al., 2001). These two transgenes were placed under the control of an estradiol-inducible promoter (Zuo et al., 2000) and then transformed into wild-type plants. The accumulation of the transgenic proteins was highly inducible by estradiol as assayed by protein blotting using an anti-MYC antibody (Fig. 3B). The transgenic seeds were germinated and grown on agar plates containing ABA and estradiol, and the cotyledon greening phenotype was scored. Under the assay conditions, overexpression of *ARR1ΔDDK* had no detectable phenotype on antagonizing the inhibitory effect of ABA in cotyledon greening (Fig. 3C). However, overexpression of *ARR12ΔDDK* remarkably increased the cotyledon greening rate in antagonizing the inhibitory effect of ABA, which was comparable to that in wild-type seedlings treated with cytokinin (Fig. 3C). Moreover, the transgenic phenotype of the *ARR12ΔDDK* seedlings was correlated to the level of ARR12ΔDDK protein induced by estradiol (Fig. 3, B and C). These results indicate that overexpression of *ARR12ΔDDK* is sufficient to activate the cytokinin-mediated signaling events in promoting cotyledon greening.

Taken together, these results demonstrate that *ARR12* plays a critical role to specifically repress ABA signaling during cotyledon greening.

Cytokinin Induces Proteasomal Degradation of ABI5 Protein

Given that cytokinin antagonizes ABA during cotyledon greening, we reasoned that cytokinin may target key signaling components of the ABA pathway. To identify the putative targets of cytokinin, we first examined the effect of cytokinin on the expression levels of key ABA signaling components, including two *Sucrose nonfermenting1-related kinase2* (*SnRK2*) genes (*SnRK2.2* and *SnRK2.3*), *ABA-responsive element binding factor/ABA-responsive element binding protein* (*ABF/AREB*) family genes (*ABF1*, *ABF2*, *ABF3*, and *ABF4*), and several *ABI* genes (*ABI1*, *ABI2*, *ABI3*, *ABI4*, *ABI5*, and *ABI8*). We found that cytokinin did not have substantial effects on the expression of these genes (Supplemental Fig. S8). The expression level of most of the ABA-regulated genes remained largely unaltered by cytokinin (Supplemental Fig. S8). The ABA-induced expression of *ABI5* was slightly reduced by cytokinin (Supplemental Fig. S8; reduced approximately 1.3-fold by cytokinin), similar to that observed in a previous study (Wang et al., 2011).

ABI5 is a key regulator involved in ABA-regulated seed germination and postgerminative growth, whereas the accumulation of the *ABI5* transcript and ABI5 protein is positively regulated by ABA (Lopez-Molina et al., 2001; Stone et al., 2006). Since cytokinin had marginal effects on the transcription of *ABI5* (Wang et al., 2011; Supplemental Fig. S8), we examined whether the stability of ABI5 protein is regulated by cytokinin. Because ABI5 protein is rarely detectable following germination and is significantly induced by ABA in a narrow developmental window (Lopez-Molina et al., 2001), we analyzed the accumulation of ABI5 protein in germinating seedlings treated with various combinations of ABA and cytokinin, following an approach similar to that already described (Lopez-Molina et al., 2001). The wild-type seeds stratified in the absence of ABA were transferred to agar plates supplemented with various combinations of ABA and cytokinin and then cultured for different times. Under the assay conditions, ABI5 protein was strongly induced by ABA, as reported previously (Lopez-Molina et al., 2001), but barely detectable in the untreated samples at all the tested time intervals (Fig. 4A). Strikingly, the ABA-induced accumulation of ABI5 protein was efficiently reduced by cytokinin and reached an undetectable level 96 h post

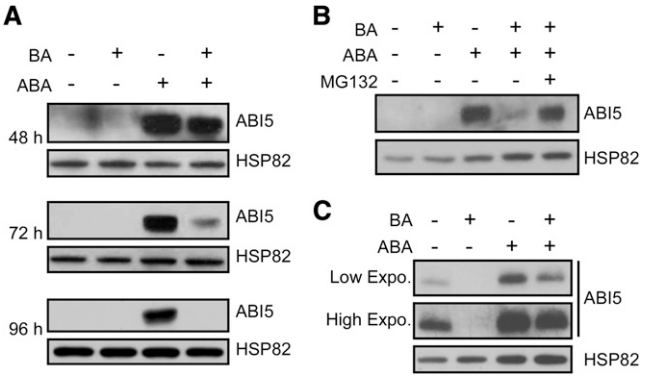

Figure 4. Cytokinin induces the degradation of ABI5 protein. A, Wild-type (Col-0) seeds were germinated and grown on GM medium for 24 h and then transferred to GM medium containing 0.5 μM ABA with or without 0.5 μM benzyladenine (BA) at various time intervals. The control sample was treated with dimethyl sulfoxide. The accumulation of ABI5 protein was analyzed by immunoblotting using an anti-ABI5 antibody. Equal loading was verified by an anti-HSP82 antibody. B, Cytokinin-induced proteasomal degradation of ABI5 protein. Wild-type (Col-0) seeds were germinated on GM medium for 24 h, transferred to GM medium supplemented with various combinations of 0.5 μM benzyladenine, 0.5 μM ABA, and 50 μM MG132, and continued culturing for an additional 72 h. The level of ABI5 protein was analyzed as described in A. C, Eight-day-old *35S:HA-ABI5* transgenic seedlings were incubated in liquid GM medium supplemented with 100 μM cycloheximide for 15 h. After the treatment, the sample was treated with various combinations of 10 μM benzyladenine and 50 μM ABA for an additional 4 h. HA-ABI5 protein was analyzed by immunoblotting using an anti-ABI5 antibody. Images with different exposure times are shown (Low Expo. and High Expo.).

stratification (Fig. 4A). Notably, the cytokinin-induced reduction of ABI5 protein was reversed by MG132 (Fig. 4B), an inhibitor specific to the proteasomal degradation machinery. This result suggests that the cytokinin-regulated reduced accumulation of ABI5 is mediated by the 26S proteasomal pathway, presumably by a mechanism similar to that reported previously (Lopez-Molina et al., 2001, 2003; Stone et al., 2006; Liu and Stone, 2010).

We also analyzed the stability of HEMAGGLUTININ (HA)-ABI5 protein in *35S:HA-ABI5* transgenic plants (Lopez-Molina et al., 2001). Similar to that observed in germinating seeds, the accumulation of HA-ABI5 protein was induced by ABA but was dramatically reduced by cytokinin in 8-d-old seedlings (Fig. 4C), indicating that cytokinin also promotes ABI5 degradation in the established young seedlings. Consistently, overexpression of *ABI5* (*35S:HA-ABI5*) nearly abolished the sensitivity to cytokinin during cotyledon greening (Supplemental Fig. S9), correlated to the accumulation of a higher level of HA-ABI5 protein in the transgenics (Fig. 4C). It should be noted that the stability of other ABA signaling proteins may also be regulated by cytokinin, and it will be interesting to investigate the possible regulatory mechanism on the available specific antibodies recognizing these proteins.

Involvement of the Cytokinin Signaling Components in the Cytokinin-Induced Degradation of ABI5

The data presented above indicate that cytokinin induces the degradation of ABI5, correlated to the cytokinin-promoted cotyledon greening that requires the cytokinin signaling components, mainly including *CRE1/AHK4*, *AHP2*, *AHP3*, *AHP5*, and *ARR12*. We

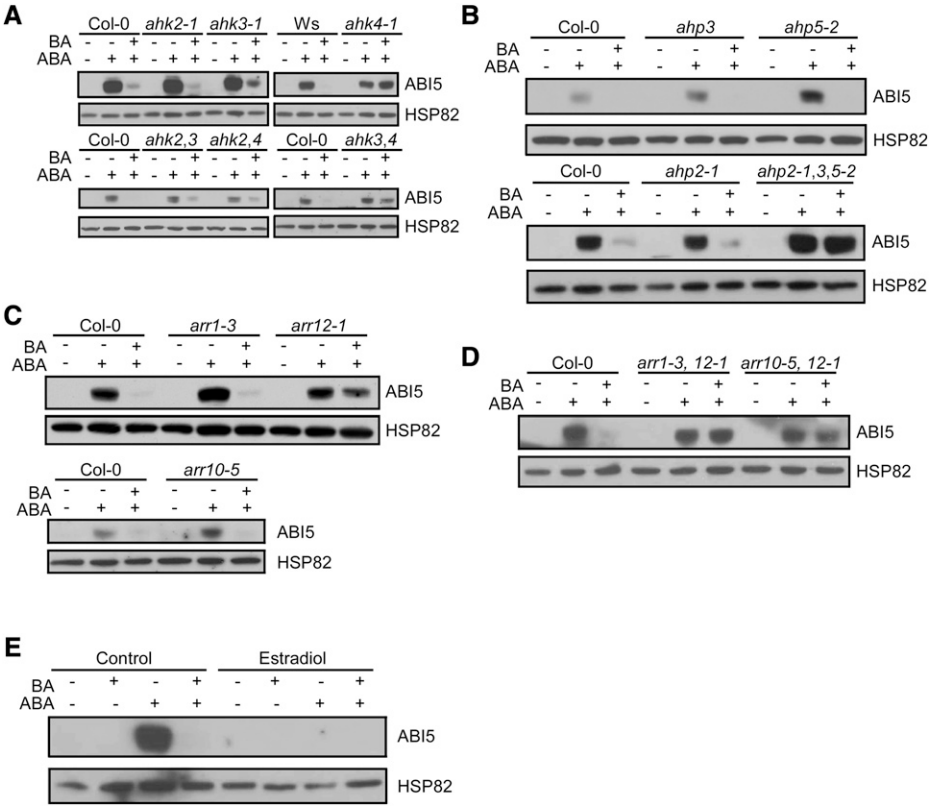

Figure 5. Involvement of cytokinin signaling components in cytokinin-induced degradation of ABI5 protein. A, Requirement of *CRE1/AHK4* in cytokinin-induced degradation of ABI5 protein. Wild-type (Col-0 and Ws) and cytokinin receptor mutant seeds were germinated and grown on GM medium for 24 h, transferred to GM medium containing 0.5 μM ABA with or without 0.5 μM benzyladenine (BA), and continued culturing for an additional 72 h. The control sample was treated with dimethyl sulfoxide. ABI5 protein was assayed by immunoblotting using an anti-ABI5 antibody. Equal loading was verified by an anti-HSP82 antibody. B, Involvement of *AHP2*, *AHP3*, and *AHP5* in cytokinin-induced degradation of ABI5 protein. The assay was performed as described in A. C, *ARR12* is required for cytokinin-induced degradation of ABI5 protein. The assay was performed as described in A. D, Analysis of cytokinin-induced degradation of ABI5 protein in *arr1-3 arr12-1* and *arr10-5 arr12-1* double mutants. Three-day-old seedlings were used for the assay as described in A. E, Degradation of ABI5 protein induced by the overexpression of *ARR12ΔDDK-MYC* under the control by an estradiol-inducible promoter. Transgenic line 33 was used in this experiment. The experiment was performed as described in A with various combinations of treatments as indicated. The samples were treated with or without (control) 10 μM estradiol.

next examined whether these cytokinin signaling components are also involved in the regulation of ABI5 degradation. Whereas the ABA-induced accumulation of ABI5 protein was completely degraded in *ahk2-1* in the presence of cytokinin, a phenotype similar to that in the wild-type seeds, the residual level of ABI5 remained in *ahk3-1* (Fig. 5A). In the *ahk4-1* mutant, however, the cytokinin-induced degradation of ABI5 was nearly abolished (Fig. 5A), indicating that cytokinin-induced ABI5 degradation is mainly dependent on *CRE1/AHK4*, which is predominantly involved in the cytokinin-antagonized effect against ABA during early seedling growth. Similarly, whereas *ahp2-1*, *ahp3*, and *ahp5-2* showed wild-type-like phenotypes in response to cytokinin, the *ahp2-1,3,5-2* triple mutant was insensitive to cytokinin in the regulation of ABI5 degradation (Fig. 5B).

In an analysis of the regulatory roles of *ARR1*, *ARR10*, and *ARR12*, we found that the cytokinin-induced degradation of ABI5 protein in *arr1-3* and *arr10-5* was similar to that in the wild type but was substantially blocked in *arr12-1* (Fig. 5C). Compared with that of the *arr12* single mutants, a stronger phenotype was observed in the *arr1-3 arr12-1* and *arr10-5 arr12-1* double mutants, in which the accumulation of ABI5 protein was nearly not affected by cytokinin (Fig. 5D). Conversely, estradiol-induced overexpression of *ARR12ΔDDK* enhanced the degradation of ABI5 protein (Fig. 5E). Previous studies have shown that *ARR4*, *ARR5*, and *ARR6* are involved in the cytokinin-ABA interaction during seed germination (Wang et al., 2011). Analysis of the accumulation of ABI5 protein in the *arr3 arr4 arr5 arr6* quadruple mutant revealed that the cytokinin-induced ABI5 protein degradation was dramatically reduced in *arr3 arr4 arr5 arr6* (Supplemental Fig. S10), indicating that these type A *ARR* genes also play an important role during cotyledon greening.

Taken together, the above results indicate that key components in the cytokinin pathway are functionally required for the cytokinin-regulated ABI5 protein degradation, correlated to the observation that these components are also predominantly involved in the cytokinin-antagonized effect against ABA during early seedling growth.

ARR12 Functions Upstream of *ABI5* to Regulate Cotyledon Greening

The observation that cytokinin antagonizes the ABA effect on cotyledon greening and promotes the degradation of ABI5 protein implies possible genetic interactions between cytokinin signaling and ABI5. To test this hypothesis, we performed a double mutant analysis by crossing *abi5-8* and *arr12-3*, both of which are in the Col-0 background. The *abi5-8* mutant is a weak allele containing a detectable level of ABI5 protein (Zheng et al., 2012; Supplemental Fig. S11) and showed a partial insensitive phenotype to ABA during cotyledon greening (Fig. 6). In the presence of cytokinin, *abi5-8* showed significantly reduced sensitivity to ABA. Under all assay conditions, the *abi5-8 arr12-3* double mutant showed an *abi5-8*-like phenotype. In particular, the *abi5-8 arr12-3* double mutant showed an *abi5-8*-like phenotype in the presence of both ABA and cytokinin (Fig. 6). These results suggest that *ARR12* genetically acts upstream of *ABI5* to promote cotyledon greening.

DISCUSSION

In this study, we have used multiple approaches to define a genetic pathway that integrates a cytokinin signal into the ABA-mediated early seedling establishment. This pathway mainly consists of the cytokinin receptors *AHK3* and *CRE1/AHK4*, multiple *AHP* genes (*AHP2*, *AHP3*, and *AHP5*), *ARR12*, and *ABI5*. We also demonstrate that *ABI5*, a key transcription factor gene in the ABA signaling pathway, acts downstream of the cytokinin pathway to transduce a cytokinin signal during early seedling growth. Biochemically, we show that cytokinin negatively regulates the stability of ABI5 protein to modulate ABA signaling. These findings reveal an important

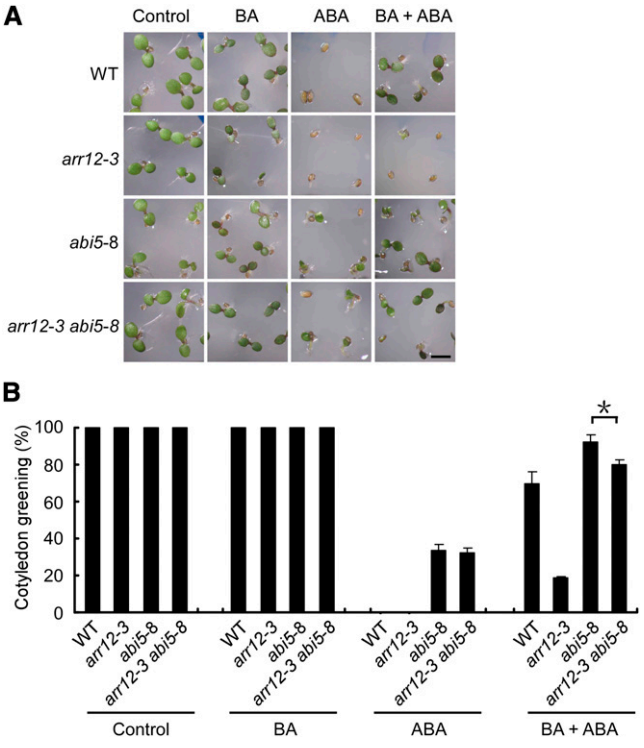

Figure 6. *ARR12* acts upstream of *ABI5* to regulate cotyledon greening. A, Four-day-old seedlings of the wild type (WT; Col-0), *arr12-3*, *abi5-8*, and *arr12-3 abi5-8* mutants were germinated and grown on GM medium supplemented with various combinations of 0.5 μM benzyladenine (BA) and 0.5 μM ABA. The control sample was treated with dimethyl sulfoxide. Bar = 2 mm. B, Quantitative analysis of the cotyledon greening rate in A. The data presented are means of three independent experiments. More than 50 seeds of each sample were used in each experiment. Error bars indicate the SE ($n = 3$). *$P < 0.05$ (Student's t test). [See online article for color version of this figure.]

mechanism that integrates cytokinin signaling and ABA signaling to coordinate plant growth and development.

Seed germination is a complex process initiated with the uptake of water by the quiescent dry seed and terminated with the elongation of the embryonic axis. The key developmental event during postgerminative growth is the establishment of a seedling, involved in the mobilization of the major storage reserves and, morphologically, featuring cotyledon opening, cotyledon greening, and radicle growth (Bewley and Black, 1994). Both seed germination and early seedling establishment are under the tight control of genetic programs and environmental factors. It has been well recognized that ABA plays a vital role by repressing seed germination and subsequent early seedling establishment, and ABI5 is an important regulator of these developmental processes (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001, 2002). In addition, cytokinin has been proposed to play positive roles in regulating seed germination, possibly through antagonizing against the effect imposed by ABA (Khan, 1971; Wang et al., 2011). Unexpectedly, we found that cytokinin is not required for antagonizing the ABA effect during seed germination. Instead, cytokinin plays an important role in early seedling establishment, in particular, cotyledon greening, by antagonizing the inhibitory effect of ABA. Consistently, a major physiological role of cytokinin is to promote plastid differentiation and other photomorphogenesis processes (Chory et al., 1991, 1994; Thomas et al., 1997), which marks the transition from heterotrophic to autotrophic growth of a seedling during early growth. Additionally, both the cytokinin receptor genes and *AHP* genes are involved in far-red light-regulated seed germination (Hutchison et al., 2006; Riefler et al., 2006). Therefore, cytokinin-repressed ABA signaling, particularly during postgerminative growth, represents an important regulatory mechanism to coordinate early seedling establishment.

The basic leucine zipper transcription factor ABI5 has been shown as a key regulator during seed germination and postgerminative growth (Lopez-Molina et al., 2001, 2002). The steady level of *ABI5* mRNA and the accumulation of ABI5 proteins are positively regulated by ABA, thereby forming a positive feedback regulatory loop that acts to monitor environmental changes during seed germination and postgerminative growth (Lopez-Molina et al., 2001). Considering that both the *ABI5* transcript and ABI5 protein are rapidly turned over shortly after seed germination (Lopez-Molina et al., 2001), it is reasonable to assume that the promoting effect of cytokinin on cotyledon greening is likely linked to the cytokinin-regulated ABI5 abundance at the transcriptional or posttranscriptional level. Whereas *ABI5* transcription is not regulated by cytokinin, the ABA-promoted *ABI5* transcription is slightly reduced by cytokinin (Wang et al., 2011; Supplemental Fig. S7). Moreover, we noticed that cytokinin slightly represses the ABA-induced *ABI5* expression (Wang et al., 2011; Supplemental Fig. S7), indicating that the cytokinin-repressed ABA signaling is not executed by the regulation of the transcription of *ABI5*. Instead, an alternative mechanism, rather than the cytokinin-repressed *ABI5* mRNA level, may play a more dominant role in regulating ABI5 activity. In agreement with this notion, we found that cytokinin efficiently induces the degradation of ABI5 protein. Intriguingly, treatment with cytokinin for 72 to 96 h results in nearly complete degradation of ABI5 protein, with a moderately reduced level of *ABI5* mRNA under the assay conditions, suggesting that the stability of ABI5 protein, rather than the steady level of *ABI5* mRNA, is the major regulatory step of cytokinin-mediated ABA signaling. Genetically, cytokinin-induced degradation of ABI5 protein is tightly coupled with the antagonizing effect of cytokinin on ABA signaling, mainly involved in *CRE1/AHK4*, *AHP2*, *AHP3*, *AHP5*, and *ARR12*, further indicating that cytokinin represses ABA signaling by modulating the stability of ABI5 protein during early seedling growth. Given that *ARR12* plays an important role in the regulation of cytokinin-induced ABI5 degradation, an apparent challenge is to fill the gap between *ARR12* and *ABI5*. Notably, ABI5 physically interacts with ARR4, ARR5, and ARR6 (Wang et al., 2011), three type A ARRs that act downstream of type B ARRs. The observation that the cytokinin-induced ABI5 degradation is reduced in the *arr3 arr4 arr5 arr6* quadruple mutant implies that the binding of type A ARR proteins to ABI5 may play an important role in regulating the stability of ABI5 protein. Because both the steady level of type A *ARR* mRNA and the accumulation of type A ARR proteins are positively regulated by cytokinin (Brandstatter and Kieber, 1998; Imamura et al., 1998; To et al., 2007; Ren et al., 2009), it is likely that the type A ARR-ABI5 complex may inhibit ABI5 protein to interact with the proteasomal degradation machinery.

In addition to its regulatory role in seed germination and postgerminative growth, the ABA-cytokinin interaction has also been implicated in the regulation of stress responses (Ha et al., 2012). Whereas abiotic stresses cause reduction of the cytokinin level, the reduced cytokinin level, in turn, results in resistance to salts and drought (Nishiyama et al., 2011). Notably, several key signaling components in the cytokinin pathway have been shown to negatively regulate the stress response (Tran et al., 2007; Shi et al., 2012), indicating that cytokinin-repressed ABA signaling operates not only in seed germination and seedling establishment but also in the stress response. Collectively, these discoveries reveal that cytokinin negatively regulates ABA-mediated stress responses, in particular by promoting ABI5 degradation during early seedling growth, thus illustrating an important mechanism for the adaptation growth of plants in response to various internal and environmental signals.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Col-0, Ws, and Landsberg *erecta* wild-type strains of Arabidopsis (*Arabidopsis thaliana*) were used in this study. The *ahk4-1* and *abi5-4* mutants

were in the Ws background, and all other mutants were in the Col-0 background. The mutants *pga22*, *ahk2-1*, *ahk3-1*, *ahk4-1*, *ahk2-5 ahk3-7*, *ahk2-5 cre1-2*, *ahk3-7 cre1-2*, *ahp1*, *ahp2-1*, *ahp3*, *ahp4-1*, *ahp5-2*, *arr1-3*, *arr10-5*, *arr12-1*, *abi5-4*, *abi5-8*, and *arr3 arr4 arr5 arr6* (Lopez-Molina and Chua, 2000; Inoue et al., 2001; Alonso et al., 2003; Lopez-Molina et al., 2003; Sun et al., 2003; Nishimura et al., 2004; Hutchison et al., 2006; To et al., 2007; Argyros et al., 2008; Zheng et al., 2012) and the *35S:HA-ABI5* transgenic line (in the Landsberg *erecta* background; Lopez-Molina et al., 2001) used in this study have been described previously. The *arr12-3* line (SALK_121983) was obtained from the Arabidopsis Biological Resource Center. The nomenclature of all *ahk* mutants followed Nishimura et al. (2004).

Seeds were surface sterilized with 10% (v/v) bleach and then sown on GM medium (1× Murashige and Skoog salts, 1% Suc, 1× B5 vitamin, 0.05% MES-KOH, and 0.3% Phytagel). Unless otherwise specified, the plates were kept for 2 d in the dark at 4°C (stratification) and then transferred into a tissue culture room under constant white light (100 μmol m^{-2} s^{-1}) at 22°C.

Agrobacterium tumefaciens-mediated transformation of Arabidopsis plants was performed by the floral dip method (Bechtold and Pelletier, 1998). Strain GV3101 was used in all transformation experiments.

Seed Germination Assay and Cotyledon Greening Assay

The same batches of seeds of various genotypes grown under the same conditions were used in all experiments for the seed germination assay and the cotyledon greening assay. After 2 d of stratification at 4°C, seeds were transferred to a tissue culture room. Seed germination was defined as visible radicle protrusion out of the testa, and the germination rate was scored at the indicated times. Unless specified otherwise, green cotyledons were scored at 4 d after transferring from darkness to the tissue culture room. The mean values obtained from three independent batches of seeds (biological repeats) are presented.

Plasmid Construction

All molecular manipulations were performed according to standard methods (Sambrook and Russell, 2001). To construct *ARR1ΔDDK-MYC* and *ARR12ΔDDK-MYC*, 1.7-kb *ARR1* and 1.6-kb *ARR12* genomic DNA fragments were amplified (for the primers used in the PCR, see Supplemental Table S1) and in-frame fused to a 6× *MYC* tag sequence. In these two constructs, the DDK domains were deleted. The resulting fusion genes were cloned between the *Xho*I and *Spe*I sites of a binary vector pER10 (Zuo et al., 2000).

RNA Extraction and Reverse Transcription-PCR

Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. The first strand of complementary DNA (cDNA) was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech) and then used as the template for reverse transcription (RT)-PCR or quantitative RT-PCR as described previously (Deng et al., 2010). The relative expression level of a target gene was normalized with that of *ACTIN7*. All primers used in quantitative RT-PCR are listed in Supplemental Table S1.

Generation of Anti-ABI5 Antibody and Protein Immunoblot Analysis

A cDNA fragment containing the coding sequences of amino acid residues 1 to 300 of ABI5 protein was PCR amplified (Supplemental Table S1) and cloned into the *Bam*HI and *Sal*I sites of pGEX4T-1 (GE Healthcare) to generate a *GLUTATHIONE S-TRANSFERASE* (*GST*)-*ABI5* fusion gene. The expression and purification of GST-ABI5 recombinant was performed according to the manufacturer's instructions. The purified GST-ABI5 recombinant protein was used to immunize mice. The resulting anti-ABI5 antiserum was extensively characterized (Supplemental Fig. S9). Anti-HEAT-SHOCK PROTEIN82 and anti-tubulin antibodies were purchased from Beijing Genomics Institute and Sigma, respectively. Protein immunoblotting was performed as described previously (Ren et al., 2009).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the accession numbers At1g49720 (*ABF1*), At1g45249 (*ABF2*), At4g34000 (*ABF3*), At3g19290 (*ABF4*), At4g26080 (*ABI1*), At5g57050 (*ABI2*), At3g24650 (*ABI3*), At2g40220 (*ABI4*), At2g36270 (*ABI5*), At3g08550 (*ABI8*), At5g09810 (*ACTIN7*), At5g35750 (*AHK2*), At1g27320 (*AHK3*), At2g01830 (*AHK4/CRE1/WOL*), At3g21510 (*AHP1*), At3g29350 (*AHP2*), At5g39340 (*AHP3*), At3g16360 (*AHP4*), At1g03430 (*AHP5*), At3g16857 (*ARR1*), At4g31920 (*ARR10*), At2g25180 (*ARR12*), At3g50550 (*SnRK2.2*), and At5g66880 (*SnRK2.3*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Cytokinin has no antagonizing effect on the inhibitory effect of ABA on seed germination.

Supplemental Figure S2. Cytokinin antagonizes ABA-mediated early growth arrest.

Supplemental Figure S3. Analysis of the seed germination and cotyledon greening phenotypes of *pga22*.

Supplemental Figure S4. Cytokinin antagonizes ABA-mediated postgerminative growth arrest.

Supplemental Figure S5. Cytokinin antagonizes abiotic stress-induced postgerminative growth arrest.

Supplemental Figure S6. Expression of the cytokinin receptor genes in young seedlings.

Supplemental Figure S7. Characterization of the *arr12* mutants.

Supplemental Figure S8. Expression of *ABF/AREB/ABI5* family, *SnRK2*, and *ABI* genes in germinating seeds.

Supplemental Figure S9. Analysis of the cotyledon greening phenotype of *35S:HA-ABI5* transgenic seedlings.

Supplemental Figure S10. Analysis of the cytokinin-induced degradation of ABI5 protein in the *arr3,4,5,6* quadruple mutant.

Supplemental Figure S11. Analysis of ABI5 protein in the wild type and *abi5* mutants.

Supplemental Table S1. Primers used in this study.

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