Cytokinin Regulates Type-A *Arabidopsis* Response Regulator Activity and Protein Stability via Two-Component Phosphorelay[W]

Jennifer P.C. To,[a,1] Jean Deruère,[a,1] Bridey B. Maxwell,[a] Veronica F. Morris,[a,2] Claire E. Hutchison,[a] Fernando J. Ferreira,[a] G. Eric Schaller,[b] and Joseph J. Kieber[a,3]

[a] Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280
[b] Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

The plant hormone cytokinin regulates many aspects of growth and development. Cytokinin signaling involves His kinase receptors that perceive cytokinin and transmit the signal via a multistep phosphorelay similar to bacterial two-component signaling systems. The final targets of this phosphorelay are a set of *Arabidopsis thaliana* Response Regulator (ARR) proteins containing a receiver domain with a conserved Asp phosphorylation site. One class of these, the type-A ARRs, are negative regulators of cytokinin signaling that are rapidly transcriptionally upregulated in response to cytokinin. In this study, we tested the role of phosphorylation in type-A ARR function. Our results indicate that phosphorylation of the receiver domain is required for type-A ARR function and suggest that negative regulation of cytokinin signaling by the type-A ARRs most likely involves phosphorylation-dependent interactions. Furthermore, we show that a subset of the type-A ARR proteins are stabilized in response to cytokinin in part via phosphorylation. These studies shed light on the mechanism by which type-A ARRs act to negatively regulate cytokinin signaling and reveal a novel mechanism by which cytokinin controls type-A ARR function.

INTRODUCTION

Two-component signaling systems are used by prokaryotic and eukaryotic organisms to sense and respond to changes in the environment (Stock et al., 2000; West and Stock, 2001). In a canonical two-component system, a stimulus is perceived by a sensor kinase, which autophosphorylates on a conserved His residue in the kinase domain. The signal is transmitted by transfer of the phosphoryl group to a conserved Asp residue on the receiver domain of a response regulator. Variations of the simple two-component system involve intermediate elements in the phosphotransfer from the sensor kinase to the response regulator. Receiver domain phosphorylation induces conformational changes, which, in most response regulators, release repression of the output domain to allow the activation of downstream processes, often transcriptional regulation. In some response regulators, these conformational changes allow specific interactions with target proteins.

The cytokinin signaling pathway is the best-characterized system employing two-component elements in plants (Kakimoto,

2003; Ferreira and Kieber, 2005; Maxwell and Kieber, 2005; Muller and Sheen, 2007). Cytokinins were discovered by their ability to promote division in cultured cells (Miller et al., 1955) and have since been implicated in almost every aspect of plant growth and development and in the responses to various biotic and abiotic environmental cues (Mok and Mok, 2001; Sakakibara, 2006).

In *Arabidopsis thaliana*, the three cytokinin receptors (*Arabidopsis* Histidine Kinase2 [AHK2], AHK3, and AHK4) are hybrid His kinases that contain a fused receiver domain in addition to an input (a cytokinin binding CHASE domain) and a His kinase domain (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001a, 2001b; Yamada et al., 2001). In response to cytokinin binding, these receptors autophosphorylate on a conserved His residue and relay this phosphoryl group to *Arabidopsis* Response Regulators (ARRs) via an intermediate set of histidine phosphotransfer (Hpt) proteins called the *Arabidopsis* Hpt proteins (AHPs) (Suzuki et al., 1998; Hutchison et al., 2006). Similar cytokinin signaling components have been characterized in other plant species (Asakura et al., 2003; Ito and Kurata, 2006; Jain et al., 2006; Pareek et al., 2006; Du et al., 2007).

The *Arabidopsis* response regulators fall into four classes based on phylogenetic analysis and domain structure: type-A ARRs, type-B ARRs, type-C ARRs, and the *Arabidopsis* pseudo-response regulators (APRRs) (Schaller et al., 2007). The 10 type-A *ARR*s are primary transcriptional targets of cytokinin signaling and contain short C-terminal extensions beyond the conserved receiver domain (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al., 2000). The 11 type-B ARRs contain C-terminal output domains that have DNA binding

and transactivating activity (Sakai et al., 1998, 2000). Type-B ARRs are positive regulators of cytokinin signaling that control the transcription of a subset of cytokinin-regulated targets, including the type-A *ARR*s (Hwang and Sheen, 2001; Sakai et al., 2001; Tajima et al., 2004; Mason et al., 2005; Taniguchi et al., 2007; Yokoyama et al., 2007). The type-C *ARR*s are more distantly related to type-A and type-B *ARR* receiver domain sequences. They do not contain the output domain of type-B ARRs and are not transcriptionally regulated by cytokinin, although their overexpression results in reduced sensitivity to cytokinin (Kiba et al., 2004). The ARRs all contain the conserved Asp required for receiver domain phosphorylation in bacterial response regulators, and phosphotransfer from an AHP to representative members of all three ARR groups has been demonstrated in vitro (Suzuki et al., 1998; Imamura et al., 2001, 2003; Kiba et al., 2004; Mahonen et al., 2006a). The APRRs lack the conserved Asp phosphorylation site, and some play a role in modulating circadian rhythms (McClung, 2006).

At least 8 of the 10 type-A *ARR*s act as partially redundant negative regulators of cytokinin signaling (Kiba et al., 2003; To et al., 2004; Lee et al., 2007b). ARR4 interacts directly with the red light receptor phytochrome B and, along with other type-A ARRs, modulates the response to red light (Sweere et al., 2001; To et al., 2004). A subset of type-A ARRs are direct targets of the transcription factor WUSCHEL and regulate shoot apical meristem function (Leibfried et al., 2005). ARR3 and ARR4 are involved in controlling the circadian clock, and this function is opposed by ARR8 and ARR9 (Salomé et al., 2005). While it is clear that type-A ARRs play a role in multiple signaling pathways, little is known with regard to their mechanism of action.

There are two general models by which type-A ARRs can act to negatively regulate cytokinin signaling (Figure 1A). In the first, the type-A ARRs may compete with positively acting type-B ARRs for phosphoryl transfer from the upstream AHPs, similar to the chemotaxis system in *Sinorhizobium meliloti* (Schmitt, 2002). A second model is that type-A ARRs regulate the pathway through direct or indirect interactions with pathway components, as observed in *Escherichia coli* chemotaxis (Bourret and Stock, 2002). These two models are not mutually exclusive.

Here, we explore the mechanism by which the type-A ARRs negatively regulate cytokinin signaling and the role of phosphorylation in this process. We show that type-A ARR function requires phosphorylation and that the type-A ARRs likely interact with other components in a phosphorylation-dependent manner to generate negative feedback on the signaling pathway. In addition, we show that a subset of the type-A ARR proteins are stabilized by cytokinin, revealing a novel level of control of these components.

RESULTS

To investigate the role of phosphorylation in type-A ARR function, we generated site-directed mutations in *ARR5* that alter the conserved phosphorylation site in the receiver domain (Figure 1B). The analogous conserved Asp in ARR7 (Asp-85), a closely related type-A ARR, has been shown to be required for receiver domain phosphorylation (Lee et al., 2007a). We mutated the conserved phosphoryl-accepting Asp-87 in ARR5 to Ala (ARR5^{D87A})

to test whether phosphorylation of the type-A ARRs is necessary for their function. An analogous D→A mutation in the bacterial response regulator CheY has been shown to disrupt gene function with negligible changes in protein structure compared with the unphosphorylated wild-type CheY protein (Bourret et al., 1993; Alon et al., 1998; Sola et al., 2000). The conserved Asp-87 residue in ARR5 was also mutated to Glu (ARR5^{D87E}). Analogous D→E changes in some bacterial and yeast response regulators can partially mimic the phosphorylated and active protein form (Klose et al., 1993; Moore et al., 1993; Brown et al., 1994; Gupte et al., 1997; Lan and Igo, 1998). Previously, we had further shown that a similar D→E change in ARR7, another type-A ARR, acts as a gain-of-function mutation (Leibfried et al., 2005). Likewise, a similar D→E change in the receiver domain of a type-B ARR (ARR1) resulted in an activated form of this transcription factor, presumably mimicking phosphorylation (Sakai et al., 2001). All wild-type and mutant ARR5 proteins could interact with AHP2 in a yeast two-hybrid assay (see Supplemental Figure 1 online), indicating that the Asp-87 mutations do not strongly disrupt ARR5 protein folding and that the interaction between ARR5 and AHP2 is not dependent on ARR5 Asp-87 phosphorylation.

ARR5 Function Requires Receiver Domain Phosphorylation

To test whether ARR5^{WT}, ARR5^{D87A}, and ARR5^{D87E} are functional in planta, an *arr3,4,5,6* mutant, which is hypersensitive to cytokinin, was transformed with genomic constructs expressing myc-tagged wild-type and mutant ARR5 from the endogenous *ARR5* promoter (Figure 1). We identified multiple independent transgenic lines, and four lines that represented a range of expression levels of the different transgenes (Figure 1D) were tested for cytokinin sensitivity by a seedling root elongation assay, as described previously (To et al., 2004).

Reintroduction of a wild-type genomic *ARR5* gene was sufficient to restore wild-type–like cytokinin sensitivity to the *arr3,4,5,6* mutant (Figures 1C and 1E). If the *ARR5* transgene were expressed identically to the endogenous *ARR5* gene, then the ARR5^{WT} transgenic lines should closely resemble the *arr3,4,6* mutant. However, in the four lines examined, cytokinin resistance was restored beyond that of *arr3,4,6* to nearly wild-type levels and further increased resistance to higher levels of cytokinin (Figures 1C, 1E, and 1F). One explanation for this is that the roles of *ARR3*, *ARR4*, *ARR5*, and *ARR6* are interchangeable in this cytokinin assay and that the transgenic copy of *ARR5* in these lines is overexpressed. To test this, we isolated RNA from whole seedlings and seedling roots grown under assay conditions and analyzed the level of *ARR5* transcripts by real-time PCR. In wild-type seedlings, no significant increase in the steady state level of *ARR5* transcript was observed in response to the low levels of cytokinin used in this assay (Figure 1G). In three of the four *arr3,4,5,6*+ *genomicARR5^{WT}* lines, the steady state level of *ARR5* transcripts was significantly higher than in the wild type on 10 nM benzyladenine (BA) in both whole seedlings and seedling roots (Figure 1G; data not shown). Consistent with the model that cytokinin resistance correlates with the level of *ARR5*, all four transgenic lines showed increased resistance to cytokinin at 25 to 100 nM BA comparable to an ARR5-overexpressing line (Figures 1F and 2), which correlates with higher *ARR5* expression
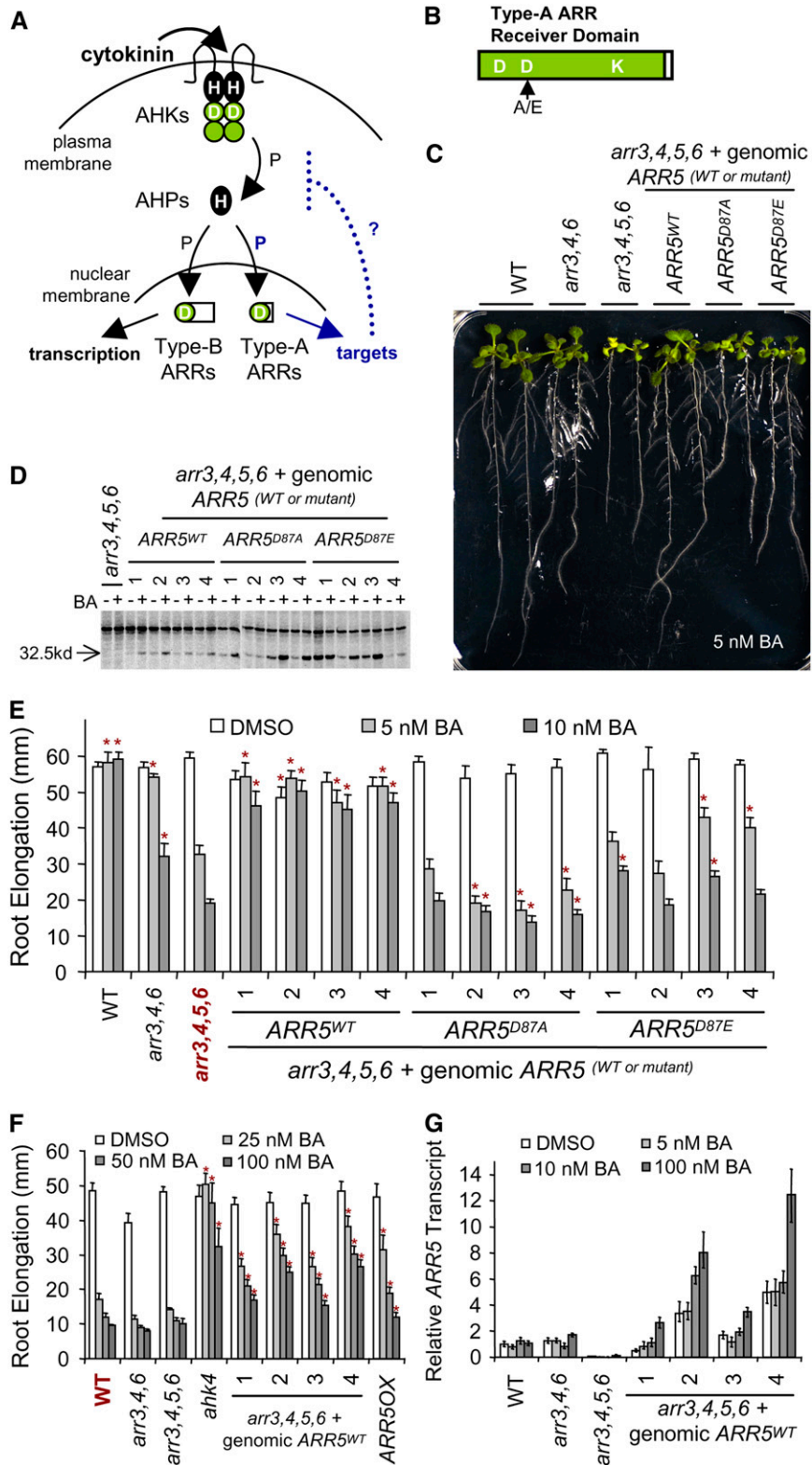
Figure 1. ARR5 Function Is Dependent on the Phosphorylation of Its Receiver Domain.

(A) Model of type-A ARR function in cytokinin signaling. Cytokinin is perceived by AHKs, which autophosphorylate and transmit the signal via AHPs to

at 100 nM BA than in the wild type (Figure 1G). The two lines displaying the highest level of *ARR5* (lines 2 and 4) also showed the strongest cytokinin resistance at 25 to 100 nM BA (Figure 1F). Overexpression of *ARR5* in these lines is most likely due to positional effects of the transgene and/or the insertion of multiple tandem copies of *ARR5*. Surprisingly, one line (line 1) displayed close to wild-type levels of *ARR5*, despite displaying nearly wild-type cytokinin sensitivity in root assays. One explanation could be that this line may overexpress *ARR5* in a specific subset of root cells, which may not be detected in our analysis of RNA from whole seedlings or full-length roots. An alternative explanation is that the addition of the myc epitope tag may increase the translatability of the transgenic *ARR5* transcript, or may increase the stability of the protein relative to endogenous ARR5, thus allowing higher levels of protein accumulation.

If phosphorylation is required for ARR5 function, then introducing an ARR5^{D87A} genomic fragment should not rescue the cytokinin-hypersensitive phenotype of *arr3,4,5,6*. We analyzed four independent transgenic lines that expressed ARR5^{D87A} protein at levels comparable to the four *arr3,4,5,6+ genomicARR5^{WT}* lines (Figure 1D). In all four lines, introduction of the *ARR5^{D87A}* transgene into *arr3,4,5,6* did not decrease the sensitivity to cytokinin, and in three lines, ARR5^{D87A} expression further increased cytokinin sensitivity compared with the parental line (Figures 1C and 1E). Thus, phosphorylation of the receiver domain is required for *ARR5* function. The increased sensitivity in some transgenic lines may be explained by ARR5^{D87A} acting in a dominant negative manner.

ARR5^{D87E} Phosphomimic Is Partially Active

In bacterial systems, altering the Asp phosphorylation target to a Glu can sometimes mimic the phosphorylated form, resulting in a partially activated response regulator (Klose et al., 1993; Moore et al., 1993; Brown et al., 1994; Gupte et al., 1997; Lan and Igo, 1998). This change can also block phosphorylation of the activated response regulator, thus preventing further activation (Klose et al., 1993; Moore et al., 1993). If type-A ARRs negatively regulate cytokinin signaling by acting as phosphate sinks and thus reducing the flow of phosphates to the type-B ARRs, then ARR5^{D87E} should be completely nonfunctional. By contrast, if type-A ARRs act by interacting with other proteins in a phosphorylation-dependent manner, then a phosphomimic mutant may partially complement the *arr5* loss-of-function mutation in the *arr3,4,5,6* parental line. To test this, we introduced a genomic *ARR5^{D87E}* transgene into *arr3,4,5,6*. Four independent transgenic lines showed transgenic protein expression comparable to that of *arr3,4,5,6+ genomicARR5^{WT}* and *arr3,4,5,6+ genomicARR5^{D87A}* (Figure 1D). In three of the four lines examined, ARR5^{D87E} partially restored cytokinin resistance significantly above that of the *arr3,4,5,6* parental line (Figures 1C and 1E). Importantly, in three *arr3,4,5,6+ genomicARR5^{D87E}* lines, cytokinin responsiveness was restored significantly above that of the *arr3,4,5,6+ genomicARR5^{D87A}* lines (Student's *t* test, P < 0.05 at 5 nM BA), indicating that the activation of the ARR5^{D87E} protein is specific to phosphomimickry and is not due to nonspecific phosphorylation at other sites in the absence of the conserved Asp. The results indicate that mimicking the phosphorylated protein form is at least partially sufficient for ARR5 function. The effect of ARR5^{D87E} is weaker than that of ARR5^{WT}, which is consistent with a partial activation of the mutant receiver domain and the inability of ARR5^{D87E} to be fully activated by phosphorylation (Figure 1E) (Moore et al., 1993). This partial complementation by *ARR5^{D87E}*, which is unlikely to receive a phosphoryl group from the AHPs, indicates that ARR5 does not function entirely as a phosphate sink. Furthermore, it suggests that the conformational state of phosphorylated ARR5 is likely to be the active state for interactions with target proteins.

Figure 1. (continued).

ARRs in a His (H)-to-Asp (D) multistep phosphorelay. Type-A ARRs may compete for phosphotransfer with type-B ARRs or interact with targets to negatively regulate the pathway.

(B) Type-A ARR protein is shown with conserved Asp (D) and Lys (K) residues characteristic of receiver domains. The conserved phosphorylation target Asp (D) in the receiver domain is mutated to Ala (A) or Glu (E).

(C) to **(G)** Complementation of *arr3,4,5,6* hypersensitivity to cytokinin inhibition of root elongation. Homozygous T3 seedlings were grown on vertical Murashige and Skoog (MS) plates supplemented with the specified concentrations of BA or 0.1% DMSO control under constant light for 9 d.

(C) Two representative seedlings grown on 5 nM BA per genotype are pictured. Note: line 3 of *arr3,4,5,6+ genomicARR5^{WT}*, line 4 of *arr3,4,5,6+ genomicARR5^{D87A}*, and line 1 of *arr3,4,5,6+ genomicARR5^{D87E}* in **(D)** are shown.

(D) Transgenic seedlings express cytokinin-inducible myc-tagged wild-type and mutant ARR5 proteins. Full-length roots were harvested from 9-d-old light-grown seedlings and were treated with 1 μM BA or 0.1% DMSO control for 2 h. Total proteins were extracted from root tissues and separated by SDS-PAGE. ARR5-myc proteins were detected by protein gel blotting with anti-c-myc antibody. Note that a protein band of ~35 to 40 kD that binds nonspecifically to anti-c-myc can be detected in all lanes, including untransformed *arr3,4,5,6*. The arrowhead indicates the position of a 32.5-kD protein marker.

(E) Root elongation of seedlings from four independent transgenic lines was quantified between days 4 and 9 at the indicated cytokinin concentrations. Error bars represent SE (*n* > 30). Asterisks indicate statistically significant differences from *arr3,4,5,6* (indicated in red) at the given concentrations of BA (Student's *t* test, P < 0.05).

(F) Root elongation of seedlings were analyzed as in **(E)**. Error bars represent SE (*n* > 30). Asterisks indicate statistically significant differences from the wild type (indicated in red) at the given concentrations of BA (Student's *t* test, P < 0.05). *ARR5OX* is as described for Figure 2.

(G) *arr3,4,5,6+ genomicARR5^{WT}* expresses *ARR5* transcript. RNA was extracted from seedlings grown under the same conditions as in **(C)** to **(F)** and used for real-time RT-PCR analysis. *ARR5* relative expression was normalized to β-*tubulin* levels and to the wild-type DMSO control using REST 2005 version 1.9.12. Error bars represent the upper and lower limits of 95% confidence intervals.

Overexpression of Type-A ARRs Confers Cytokinin Resistance

To test whether increasing the levels of other type-A ARRs can confer cytokinin resistance, we expressed ARR4, ARR5, ARR6, ARR7, and ARR9 in wild-type *Arabidopsis* as myc-epitope–tagged fusion proteins from the constitutive cauliflower mosaic virus (CaMV) 35S promoter. One representative line that expressed a detectable level of myc-ARR fusion protein was selected and analyzed for cytokinin responsiveness (Figures 2A and 2B). All transgenic lines tested were significantly more resistant to 25 nM BA than the wild type in root elongation assays (Figure 2C) (Student's *t* test, P < 0.05) but less resistant than the loss-of-function cytokinin receptor mutant *ahk4*.

A Subset of Type-A ARR Proteins Are Stabilized by Cytokinin

The regulation of protein turnover plays an important role in controlling several phytohormone signaling and biosynthetic pathways (reviewed in Dreher and Callis, 2007). We analyzed ARR5 protein turnover using a dexamethasome (DEX)-inducible myc-tagged ARR5 line (DMA5). Continuous growth of DMA5 seedlings on 10 nM DEX results in reduced sensitivity to cytokinin, indicating that the ARR5 myc fusion protein in DMA5 is functional (see Supplemental Figure 2 online).
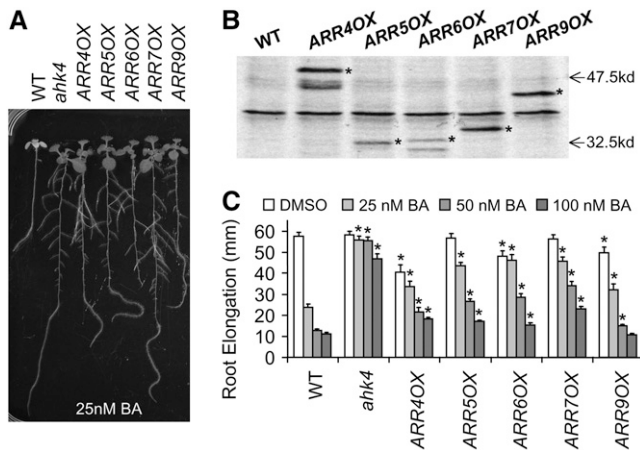

Figure 2. Wild-Type Type-A *ARR* Overexpression Confers Cytokinin Resistance.

Overexpression of *ARR4*, *ARR5*, *ARR6*, *ARR7*, and *ARR9* confers cytokinin resistance.

(A) Seedlings were grown as described for Figure 1E with the specified concentrations of BA or 0.1% DMSO control.

(B) Transgenic seedlings express myc-tagged ARR proteins, detected as in Figure 1E. Bands corresponding to the relevant protein products quantified in Figure 3 are noted with asterisks. Lower molecular mass bands in ARR4 and ARR6 may represent degradation products. Arrows indicate the positions of 47.5- and 32.5-kD protein markers.

(C) Root elongation was measured as described for Figure 1. Error bars represent SE (*n* > 30). Asterisks indicate statistically significant differences from the wild type at the given concentrations of BA (Student's *t* test, P < 0.05).

The myc-ARR5 protein is rapidly degraded following the inhibition of de novo protein synthesis by cycloheximide (CHX). To test whether ARR5 protein turnover is regulated by cytokinin, we compared ARR5 protein steady state levels and degradation rates in the presence and absence of cytokinin. ARR5 protein accumulated to higher steady state levels in the presence of cytokinin, and this is the result of a decreased rate of protein degradation (Figure 3A). Stabilization of ARR5 was effective within 30 min of cytokinin application and was sensitive to concentrations of BA as low as 10 nM (Figures 3A and 3B). Cytokinin increased ARR5 protein stability when added simultaneously with the CHX treatment, indicating that the stabilization of ARR5 protein by cytokinin does not require de novo protein synthesis (Figure 3C).

To test whether other type-A ARR proteins are stabilized by cytokinin, we analyzed the turnover of their respective myc fusion proteins expressed from the CaMV 35S promoter. The five type-A ARR proteins that we examined exhibited different rates of protein turnover (Figures 3D and 3E). The half-lives of the myc-ARR5 and myc-ARR6 fusion proteins were estimated to be 100 and 60 min, respectively. myc-ARR4, myc-ARR7, and myc-ARR9 proteins exhibited longer protein half-lives, ~140, 160, and 180 min, respectively. In the presence of exogenous cytokinin, the myc-ARR5, myc-ARR6, and myc-ARR7 fusion proteins were stabilized, with protein half-lives estimated to be >300 min. The turnover of the myc-ARR4 and myc-ARR9 fusion proteins was not significantly affected by cytokinin (Figures 3D and 3E).

Cytokinin-Mediated Stabilization of ARR5 Involves Two-Component Phosphorelay

To test whether the stabilization of ARR5 by cytokinin is mediated by the two-component signaling pathway, we expressed myc-ARR5 in the background of two-component element mutants. In the *ahk3,4* and *ahp1,2,3,4* mutants, cytokinin treatment failed to stabilize myc-ARR5 (Figures 4A and 4B). These data indicate that an intact AHK-AHP phosphorelay is required for cytokinin to delay the turnover of type-A ARR proteins. Interestingly, cytokinin-mediated stabilization of myc-ARR5 was also reduced in a multiple type-B ARR loss-of-function mutant (*arr1,2,10,12*) (Figure 4C). As de novo protein synthesis is not required for the stabilization of ARR5 by cytokinin, this result suggests that type-B ARRs are required for the transcription of an element involved in the stabilization of ARR5 that is expressed prior to cytokinin application in this assay. However, *arr1,2,10,12* mutants still retain some response to cytokinin stabilization of myc-ARR5, supporting the model that phosphorelay plays a role in regulating myc-ARR5 turnover.

We tested the hypothesis that type-A ARR proteins are stabilized by phosphorylation by analyzing the turnover of ARR proteins mutated in the conserved Asp phosphorylation target. We expressed ARR5^{D87A}, ARR5^{D87E}, ARR7^{D85A}, and ARR7^{D85E} from the CaMV 35S promoter and compared their kinetics of protein turnover with those of their respective wild-type proteins. The myc-ARR5^{D87A} protein was degraded more rapidly than myc-ARR5^{WT} in the absence of exogenous cytokinin; 15 min after CHX treatment, myc-ARR5^{WT} levels decreased 20%,
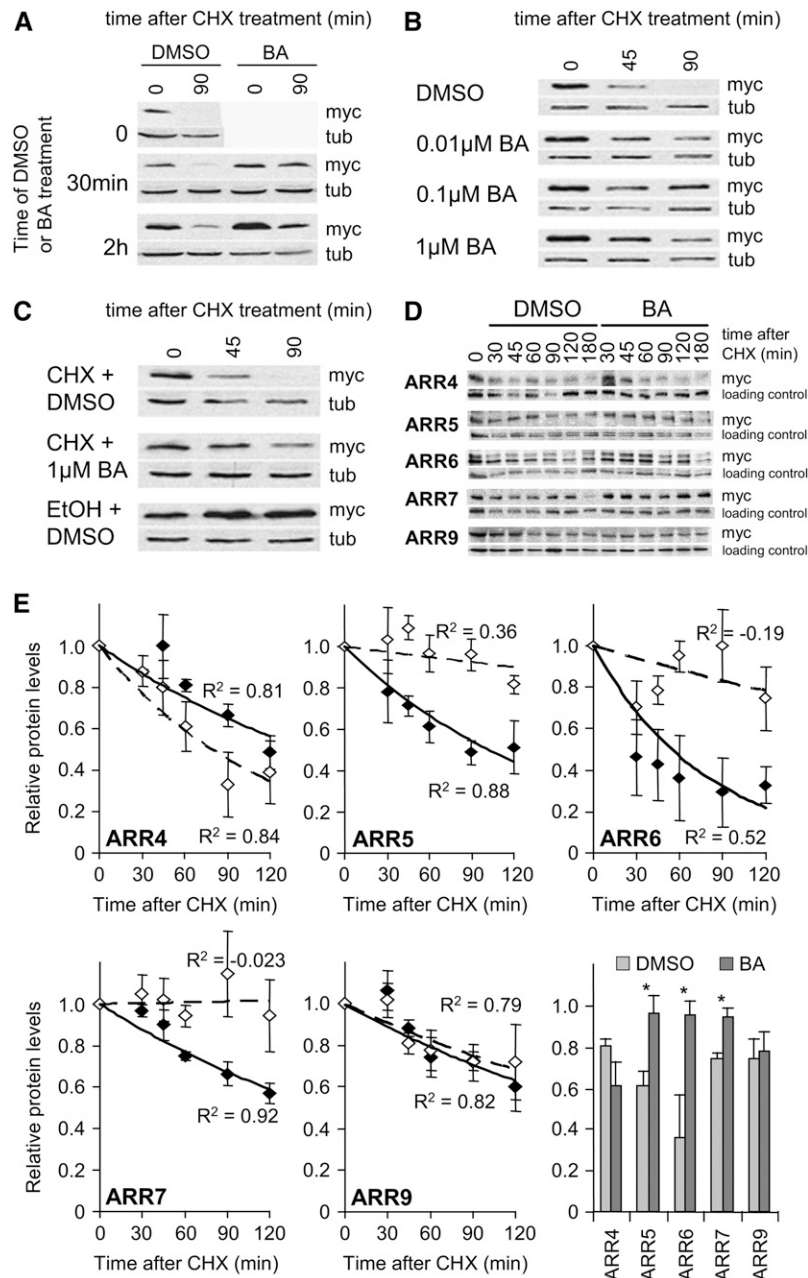
Figure 3. A Subset of Type-A ARR Proteins Are Stabilized by Exogenous Cytokinin Application.

(A) to **(D)** myc-ARR5 protein is stabilized by exogenous cytokinin. myc-ARR5 protein was generated in 7-d-old light-grown seedlings in a DEX-inducible myc-ARR5 (DMA5) line by a 2-h 1 μM DEX treatment.

(A) Cytokinin stabilization of myc-ARR5 protein occurs within 30 min and is effective at 2 h. After DEX induction of myc-ARR5 protein production, 1 μM BA or 0.1% DMSO control was added for the times indicated at left before CHX treatment.

(B) Cytokinin stabilization of ARR5 is sensitive to low concentrations of BA. Seedlings were treated with the indicated concentrations of BA or DMSO control during DEX treatment, followed by CHX application.

(C) Cytokinin stabilization of myc-ARR5 does not require new protein synthesis. After DEX treatment, 200 μM CHX (or ethanol [EtOH] control) and 1 μM BA (or 0.1% DMSO control) were applied simultaneously and myc-ARR5 protein turnover was analyzed as in **(A)**.

(D) A subset of type-A ARRs are stabilized by exogenous cytokinin application. Seven-day-old light-grown *ARR4OX*, *ARR5OX*, *ARR6OX*, *ARR7OX*, and *ARR9OX* seedlings were treated simultaneously with 200 μM CHX and 1 μM BA or 0.1% DMSO control. Three independent experiments were conducted with consistent results, and one representative blot is shown.

(E) Relative myc-ARR protein levels were normalized to loading control and to myc-ARR protein levels at time 0. The results from three independent experiments were averaged and shown with error bars indicating SE. Note that the upper band for ARR6 was quantified. An exponential best-fit curve was fitted through the data points to estimate protein half-life. Correlation coefficient (R^2) values are indicated as a measure of curve fit. Closed symbols and solid lines represent DMSO control. Open symbols and broken lines represent BA treatment. The bottom right panel shows relative protein levels at 60 min after CHX treatment. Asterisks indicate statistical differences between BA treatment and DMSO control within each transgenic line (Student's *t* test, P < 0.05).
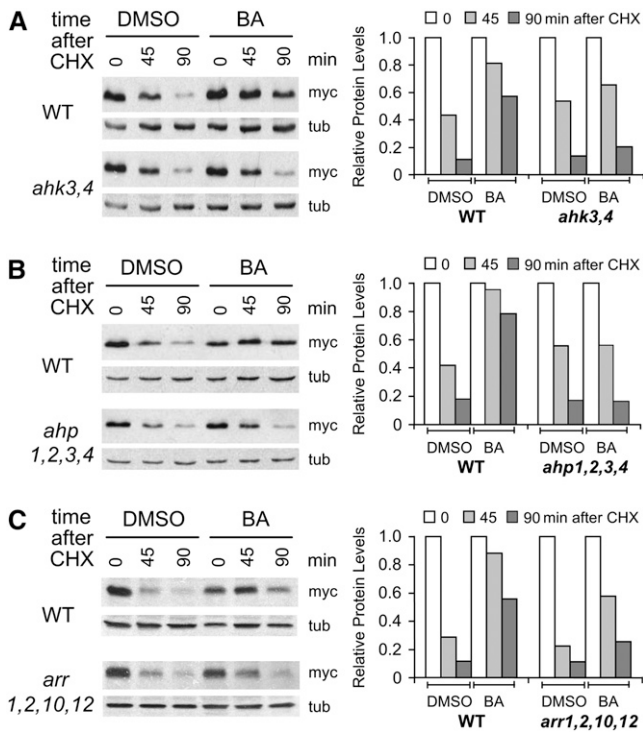
Figure 4. Cytokinin Stabilization of ARR5 Requires Upstream Cytokinin Signaling Genes.

Protein turnover of DEX-inducible myc-ARR5 was examined in the background of the cytokinin signaling mutants indicated. Seedlings were treated and analyzed as described for Figure 3B. Relative protein levels were normalized to tubulin and to myc-ARR5 levels at 0 min after CHX treatment.

whereas myc-ARR5^{D87A} levels decreased $\sim$40%, compared with the initial protein levels (Figures 5A to 5C). Cytokinin treatment resulted in a strong stabilization of myc-ARR5^{WT} protein, but this was not observed with the myc-ARR5^{D87A} protein (Figures 5A to 5C). Consistent results were observed in wild-type and mutant ARR5 proteins expressed from genomic constructs used for the complementation of arr3,4,5,6 (Figure 5D). Similarly, in the absence of cytokinin, myc-ARR7^{D85A} protein was turned over more rapidly than myc-ARR7^{WT} (Figures 5E to 5G), and cytokinin treatment resulted in a stabilization of ARR7^{WT} but not ARR7^{D85A} (Figures 5E to 5G). The rapid turnover of ARR5^{D87A} and ARR7^{D85A} both in the presence and absence of cytokinin suggests that the phosphorylation of Asp-85/87 plays a role in regulating the turnover of these ARR proteins.

To further test the role of phosphorylation in type-A ARR protein stability, we analyzed the protein turnover of myc-ARR5^{D87E} and myc-ARR7^{D85E} phosphomimic mutants. When expressed from the CaMV 35S promoter, basal myc-ARR5^{D87E} protein turnover was slower than that of myc-ARR5^{WT}. At 60 min after CHX addition, myc-ARR5^{WT} proteins decreased by >40%, whereas myc-ARR5^{D87E} proteins only decreased by 10% (Figures 5A to 5C). In the presence of cytokinin, myc-ARR5^{D87E} may be weakly stabilized (Figures 5A to 5C), but the response is

greatly muted relative to that of myc-ARR5^{WT}. Degradation of the myc-ARR7^{D85E} protein was also reduced in the absence of cytokinin and was not altered significantly by cytokinin application (Figures 5E to 5G). The delayed protein turnover of myc-ARR5^{D87E} and myc-ARR7^{D85E} in the absence of cytokinin suggests that the protein conformation induced by phosphorylation of the conserved Asp contributes to protein stability.

DISCUSSION

Type-A ARRs Are Likely to Negatively Regulate Cytokinin Signaling by Phospho-Dependent Interactions

We investigated the mechanism by which type-A ARRs regulate cytokinin signaling. Two distinct, but not mutually exclusive, models for this mechanism are proposed: one invokes phosphocompetition between the type-A and type-B ARRs, and the other involves phospho-dependent interactions of the type-A ARRs with target proteins (Figure 1A). To test these models, we generated two site-directed mutants targeting the Asp-87 residue of ARR5, ARR5^{D87A}, and ARR5^{D87E}. This Asp residue is conserved among response regulator family proteins and has been shown to be the target of bacterial two-component phosphorelay (Bourret et al., 1990; Stock et al., 2000; West and Stock, 2001). Substitutions at this conserved Asp have also been shown to abolish receiver domain phosphorylation in other ARRs (Kim et al., 2006; Lee et al., 2007a). In bacterial response regulators, this invariant Asp resides in the conserved active site of the receiver domain that actively catalyzes phosphotransfer from histidine kinases, Hpts, and small molecular phosphodonors (Lukat et al., 1992; Stock et al., 2000; West and Stock, 2001). Analogous D→A and D→E substitutions in bacterial response regulators have been shown to eliminate receiver domain phosphorylation (Bourret et al., 1990, 1993; Drake et al., 1993; Klose et al., 1993; Moore et al., 1993). The D→A change has been shown to disrupt protein function (Bourret et al., 1993; Klose et al., 1993; Moore et al., 1993; Alon et al., 1998), and this defect is not due to perturbations in protein structure (Sola et al., 2000). In some bacterial response regulators, the D→E change has been shown to result in constitutive but partial activation that is independent of two-component phosphorelay (Klose et al., 1993; Moore et al., 1993; Gupte et al., 1997; Lan and Igo, 1998). Response regulator activation by the D→E mutation has also been demonstrated for Skn7 in yeast and a type-B ARR (Brown et al., 1994; Sakai et al., 2001).

The ARR5 D87A substitution did not disrupt protein interactions with AHP2 in our yeast two-hybrid analysis, indicating that the D→A change does not significantly alter ARR5 protein folding. When we expressed ARR5^{D87A} under the control of the native ARR5 promoter in a multiple loss-of-function type-A arr mutant background, ARR5^{D87A} failed to complement the cytokinin hypersensitivity defect of the arr mutant. In addition, the ARR5^{D87A} protein further increased the cytokinin sensitivity of the arr3,4,5,6 mutant. One explanation for the dominant negative effect of ARR5^{D87A} is that the protein is unable to be activated by phosphorylation but retains its ability to interact with the AHPs, thus reducing the activation of other type-A ARRs. In addition,
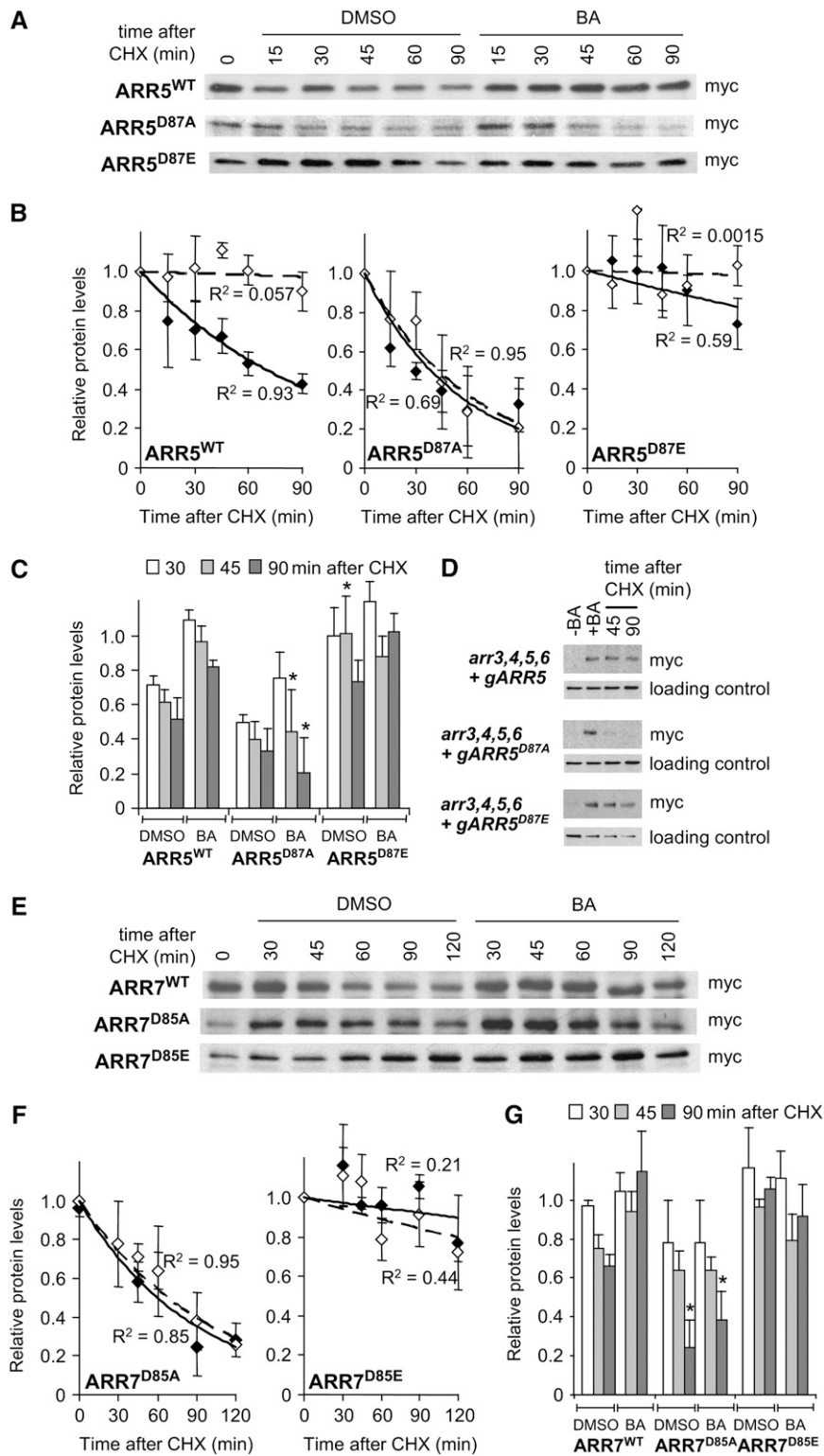
Figure 5. ARR5 and ARR7 Protein Stability Is Dependent on the Conserved Phosphorylation Target Asp.

Seedlings expressing the proteins indicated were grown, treated, and analyzed as described for Figures 3D and 3E. In **(C)** and **(G)**, asterisks indicate significant differences in relative protein levels from ARR5[WT] or ARR7[WT] after the same treatment (Student's *t* test, P < 0.05). The data for triplicate analysis of ARR7[WT] protein degradation is presented in Figure 3E. In **(D)**, genomic versions of myc-ARR5 were first induced by cytokinin to elevate myc-ARR5 protein levels before treatment with CHX.

cytokinin-inducible stabilization of the ARR5 protein, which is dependent on two-component phosphorelay, is abolished in the ARR5[D87A] protein. Together, these results indicate that the ARR5 D87A substitution eliminates phosphoryl Asp-57–dependent activation, which is required for type-A ARR function.

The ARR5[D87E] protein also retained its ability to interact with AHPs in yeast, indicating that the protein folding is relatively conserved. Expression of ARR5[D87E] in a multiple type-A *arr* mutant resulted in the partial rescue of cytokinin hypersensitivity, which is the opposite effect compared with ARR5[D87A]. In addition, basal protein stability of the ARR5[D87E] protein was elevated in both the absence and presence of exogenous cytokinin, consistent with the ARR5[D87E] protein being a phosphomimic. Together, these results indicate that the ARR5 D87E substitution renders a partially activated type-A ARR, which is functional in negatively regulating cytokinin signaling despite its inability to be phosphorylated on Asp-57.

Our results from both the ARR5[D87A] and ARR5[D87E] mutants have demonstrated that type-A ARRs require phosphorylation on the conserved Asp for function in vivo and that a nonphosphorylatable, partially activated form of the type-A ARR protein can partially rescue a loss-of-function mutant. Together, our data provide evidence that the phosphorylated type-A ARR protein can negatively regulate cytokinin response independently of its ability to compete for phosphoryl groups with the type-B ARRs and suggest that this negative regulation may be mediated through phosphospecific interactions with target proteins.

It is interesting that the expression of ARR5 wild type, ARR5[D87A], and ARR5[D87E] produced distinct effects on cytokinin sensitivity in planta and that the three proteins also displayed different levels of basal protein stability. In fact, the population of the unphosphorylated bacterial response regulator NtrC has been reported to consist of a mix of both active and inactive receiver domain conformations, and the population is shifted to predominantly active forms upon phosphorylation (Volkman et al., 2001). This finding is consistent with the residual activity found in the unphosphorylated bacterial response regulator CheY (Barak and Eisenbach, 1992). Our data suggest that the ARR5 D→A and D→E mutations shift the ARR5 protein population toward the inactive and active receiver domain conformations, respectively, which may exhibit distinct properties.

Although our results suggest that type-A ARRs function in cytokinin signaling through phospho-dependent interactions, they do not rule out a role for type-A ARRs in phosphocompetition. A recent study indicates that the cytokinin receptor AHK4 determines phosphate flux through the system by regulating a bidirectional phosphorelay to and from the AHPs (Mahonen et al., 2006b). A bidirectional phosphorelay is also used by the bacterial Arc two-component system to mediate signal decay: the phosphoryl group from the ArcB response regulator is transferred back to the receiver domain of the ArcA tripartite His kinase via its His transmitter domain (Georgellis et al., 1998; Pena-Sandoval et al., 2005). It is possible that type-A ARR function may act by a similar mechanism of reverse phosphotransfer from type-B ARRs to type-A ARRs via AHPs, because phosphotransfer from AHPs to both type-A and type-B ARRs has been demonstrated in vitro (Suzuki et al., 1998; Imamura et al., 2001, 2003) and, in the presence of cytokinins, type-A ARRs, type-B ARRs,

and AHPs mostly localize to the same subcellular compartments (Hwang and Sheen, 2001; Imamura et al., 2001, 2003; Kiba et al., 2003). One argument against ARR5[D87E] acting as a phosphomimic is that the protein may be nonspecifically and less efficiently phosphorylated at an alternative site in the absence of the conserved Asp. However, the catalytic nature of the conserved active site surrounding the Asp phosphorylation target suggests that nonspecific phosphorylation on the receiver domain is unlikely (Stock et al., 2000; West and Stock, 2001). More importantly, the lack of activation of the ARR5[D87A] control indicates that the activation of ARR5[D87E] must be a property specific to the D87E substitution.

A previous study examined shoot formation from cultured *Arabidopsis* roots overexpressing *ARR4* and *ARR8* and reported that overexpression of *ARR4* resulted in cytokinin hypersensitivity, whereas overexpression of *ARR8* resulted in cytokinin insensitivity (Osakabe et al., 2002). While we have not examined the effect of *ARR8* overexpression, our analysis of *ARR4* (as well as *ARR5*, *ARR6*, *ARR7*, and *ARR9*) overexpression in this study, combined with other overexpression reports (Hwang and Sheen, 2001; Kiba et al., 2003; Lee et al., 2007b), as well as loss-of-function mutants from our previous work (To et al., 2004), are consistent with *ARR4*, as well as the other type-A ARRs, acting as negative regulators of cytokinin signaling. One explanation for this discrepancy is that *ARR4* may act as a positive element in a subset of cytokinin responses, such as shoot initiation. Indeed, we have found antagonistic interactions among type-A ARRs in other physiological roles, such as in controlling rosette size, petiole length, and circadian rhythms (To et al., 2004; Salomé et al., 2005).

Previous overexpression studies have also produced conflicting data on the role of phosphorylation on type-A RR function. Cytokinin resistance conferred by overexpression of a rice (*Oryza sativa*) type-A RR was disrupted by mutating the conserved phosphorylation target to either an unphosphorylatable residue or a phosphomimic (Hirose et al., 2007). Similar results have also been reported for overexpression of ARR22, which is a type-C ARR (Kiba et al., 2004). One explanation is that the cytokinin-insensitive phenotype conferred by overexpression of wild-type type-A and type-C RRs may reflect an inappropriate diversion of phosphate flow from the Hpts to the abnormally high levels of type-A and type-C RR proteins, which would decrease the activation of the type-B RRs. Whether this proposed phospho-competition is an artifact of overexpression or accurately reflects the role of endogenous type-A RR proteins is an open question. By contrast, disruption of the conserved phosphorylation site did not significantly alter the ability of type-A ARRs to reduce or enhance a cytokinin-responsive reporter when overexpressed in protoplasts (Hwang and Sheen, 2001). These differences may reflect differences between the assay systems in these overexpression studies. Our study expressing ARR5 under its endogenous promoter in a loss-of-function mutant background clearly shows that phosphorylated type-A ARRs can negatively regulate cytokinin response in vivo, independently of phospho-competition with the type-B ARRs, and that this negative feedback regulation may be mediated via interactions between the type-A ARR in its phosphorylated protein conformation and target proteins.

Cytokinin Regulates Type-A ARR Function in Part by Protein Stabilization

Control of protein stability through the proteasome degradation machinery is a common mechanism for the regulation of plant hormone responses (reviewed in Dreher and Callis, 2007). Indeed, mutants of *RPN12* and *COP9/CIN4/FUS10*, which are subunits of proteasome regulatory structures (reviewed in Dreher and Callis, 2007), are cytokinin-insensitive (Vogel et al., 1998; Smalle et al., 2002), suggesting that cytokinin signaling may also be regulated by the proteasome. One possible explanation is that these mutants have higher levels of type-A ARR protein due to decreased degradation. However, ARR5 protein stability is not altered in *rpn12a-1* or *cin4/cop9/fus10* (see Supplemental Figure 3 online), indicating that cytokinin insensitivity in these mutants is probably due to a distinct mechanism.

In this study, we have shown that cytokinin regulates the turnover of a subset of type-A ARR proteins and that this occurs in the absence of de novo protein synthesis. Cytokinin-mediated stabilization of ARR5 is disrupted in mutants of upstream phosphorelay components, suggesting that phosphorylation of type-A ARRs by two-component elements is required for protein stabilization by cytokinin. In addition, the unphosphorylatable ARR5^{D87A} and ARR7^{D85A} mutant proteins are less stable and their stability is not altered by cytokinin treatment, whereas the partial phosphomimics, ARR5^{D87E} and ARR7^{D85E}, exhibit reduced protein turnover compared with the wild-type proteins, consistent with the idea that type-A ARR protein turnover is determined by the phosphorylation state of the receiver domain. Furthermore, stabilization by cytokinin is compromised in the *arr1,2,10,12* mutant, which is disrupted in cytokinin-activated transcription factors and thus should have no direct effect on the phosphorylation state of the type-A ARRs. These results suggest that the mechanism for the stabilization of ARR5/ARR7 is dependent on type-B ARR basal transcription, because de novo protein synthesis is not required for type-A ARR stabilization.

A model in which ARR5 and ARR7 turnover is regulated by the phosphorylation status of their receiver domains is consistent with the finding that the yeast response regulator, SSK1, is degraded by the 26S proteasome pathway and the degradation of SSK1 is inhibited by the upstream phosphotransfer protein YPD1 (Sato et al., 2003). In our yeast two-hybrid analysis, the steady state protein levels of ARR5^{D87E} prey fusion proteins are higher than those of ARR5 or ARR5^{D87A} fusion proteins, suggesting that the ARR5 protein may also be subject to phosphorylation-dependent proteasome degradation in yeast.

Why Are a Subset of Type-A ARRs Stabilized?

The finding that cytokinin stabilizes a subset of type-A ARRs, apparent negative regulators of cytokinin signaling, appears distinct from other known phytohormone signaling pathways involving proteasome degradation machinery, such as auxin, gibberellin, and ethylene, which generally function to activate or stabilize positively acting transcription factors (reviewed in Moon et al., 2004; Fleet and Sun, 2005; Dreher and Callis, 2007). However, in this study, we have shown that expression of the

phosphomimic ARR5^{D87E} can partially complement a multiple type-A ARR loss-of-function mutant; furthermore, our previous results showed that ARR7^{D85E} overexpression can further induce meristem arrest at a low frequency (Leibfried et al., 2005). Together, these results indicate that these proteins can function without direct phosphotransfer from the AHPs and suggest that phosphorylated, activated, and stabilized type-A ARR proteins may interact with other targets, possibly to regulate output beyond the cytokinin signaling circuitry.

Type-A ARRs, as a group, have been shown to be transcriptionally upregulated by cytokinin and to function as redundant negative regulators of cytokinin signaling (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al., 2000; Hwang and Sheen, 2001; Kiba et al., 2003; To et al., 2004; Lee et al., 2007b). Phenotypic analyses of loss-of-function and gain-of-function mutants have indicated that subsets of type-A ARRs may play distinct physiological roles (Sweere et al., 2001; To et al., 2004; Leibfried et al., 2005; Salomé et al., 2005; Lee et al., 2007b). In this study, we have shown that individual type-A ARRs differ in both their intrinsic protein stabilities and the effect of cytokinin on their protein turnover, which may suggest a mechanistic basis for functional specificity among type-A ARRs. Interestingly, the effect of cytokinin on type-A ARR protein turnover appears to correlate with their phylogenetic and functional relationships. The type-A ARR proteins that are stabilized by cytokinin, ARR5, ARR6, and ARR7, fall into a subset of ARRs that are more similar in receiver domain sequence and contain shorter C-terminal sequences (D'Agostino et al., 2000). ARR5, ARR6, and ARR7 transcription are also highly induced by cytokinin (D'Agostino et al., 2000) and are regulated by WUSCHEL, likely to mediate interaction between cytokinin signaling and meristem activity (Leibfried et al., 2005). The type-A ARR proteins that are not stabilized by cytokinin, ARR4 and ARR9, are less similar to ARR5, ARR6, and ARR7 in receiver domain sequence and contain longer C-terminal regions (D'Agostino et al., 2000). In addition, ARR4 and ARR9 are also less transcriptionally upregulated by cytokinin (D'Agostino et al., 2000) and play a cytokinin-independent role in modulating the circadian clock (Salomé et al., 2005). The C-terminal regions of type-A ARR proteins may impart specificity in protein regulation. Cytokinin regulation of protein turnover of a subset of type-A ARRs may reflect another mechanism for modulating their function in specific processes, such as meristem activity.

In summary, we have shown that cytokinin regulates type-A ARR activity by two-component phosphorelay, in part through the control of protein stability. Targets of phosphorylated and activated type-A ARRs may modulate cytokinin signaling or other functions and remain to be determined.

METHODS

Plasmid Constructs

A genomic ARR5 DNA fragment (from 1.6 kb upstream of ATG through the entire length of cDNA excluding the stop codon) (D'Agostino et al., 2000) was amplified by PCR from genomic DNA isolated from wild-type *Arabidopsis thaliana* ecotype Columbia seedlings and inserted into the

pENTR/D-TOPO vector (Invitrogen) to generate Gateway entry clone pAR5g. Full-length cDNAs of ARR4, ARR5, ARR6, ARR7, ARR9, and AHP2 were amplified by PCR and inserted into the pENTR-D Gateway entry clone vector (Invitrogen) to generate Gateway entry clones pAR4cs, pAR5cs, pAR6cs, pAR7cs, pAR9cs, and pAP2cs. In the coding region for ARR5 in pAR5g and pAR5cs, the 87th codon GAT encoding Asp-87 of ARR5 cDNA, was changed to GCT, encoding Ala, by site-directed mutagenesis to generate pAR5gDA and pAR5DAcs, respectively. The same codon for Asp-87 was changed to GAG, encoding Glu, to generate pAR5DEs and pAR5gDE. In pAR7s, Asp-85 was changed to Ala and Glu by site-directed mutagenesis to generate pAR7DAcs and pAR7DEcs, respectively. All entry clones were sequence-verified.

For ARR5 complementation constructs, a genomic ARR5 fragment was transferred from pAR5g, pAR5gDA, and pAR5gDE into Gateway-compatible binary vector pGWB16 (a gift from Tsuyoshi Nakagawa, Shimane University) to generate pB16-5gw, pB16-5gDA, and pB16-5gDE, respectively. Each of the resulting constructs carried the endogenous *ARR5* promoter driving the expression of wild-type or mutant ARR5 with a 4× C-terminal myc tag.

For ARR overexpression constructs, full-length ARR cDNAs were transferred from Gateway entry vectors pAR4cs, pAR5cs, pAR6cs, pAR7cs, pAR9cs, pAR5DAcs, pAR5DEcs, pAR7DAcs, and pAR7DEcs into the Gateway-compatible binary vector pGWB18 (a gift from Tsuyoshi Nakagawa) by LR recombination (Invitrogen) to generate pB18-4w, pB18-5w, pB18-6w, pB18-7w, pB18-9w, pB18-5DA, pB18-5DE, pB18-7DA, and pB18-7DE, respectively. In each of the resulting constructs, expression of an *ARR* cDNA carrying a 4× N-terminal myc tag was driven by the constitutive CaMV 35S promoter.

To generate a DEX-inducible 6× N-terminal myc-tagged ARR5 construct, a full-length ARR5 cDNA fragment was introduced into a 6× myc vector via *Eco*RI sites and subcloned into pTA7002 (Aoyama and Chua, 1997) to generate pDMA5.

Plant Materials and Transgenic Lines

Arabidopsis plants of the Columbia ecotype were used in all experiments as the wild-type control unless stated otherwise. Mutant lines *arr3,4,5,6* (To et al., 2004), *ahk3,4* (Rashotte et al., 2006), *arr1,2,10,12* (Rashotte et al., 2006), and *ahp1,2,3,4* (Hutchison et al., 2006) have been described previously.

All transgenic plant lines described here were generated in the Columbia ecotype background by introducing binary plasmid constructs via *Agrobacterium tumefaciens*–mediated floral dip (Clough and Bent, 1998). pB16-5gw, pB16-5gDA, and pB16-5gDE were introduced into *arr3,4,5,6* to generate *arr3,4,5,6+ genomicARR5^WT^*, *arr3,4,5,6+ genomicARR5^D87A^*, and *arr3,4,5,6+ genomicARR5^D87E^*, respectively. At least eight independent single-locus insertion lines were analyzed in the T2 generation and taken to T3 homozygosity. Detailed characterization of homozygous T3 progeny from four independent lines is presented in this article.

pB18-4w, pB18-5w, pB18-6w, pB18-7w, pB18-9w, pB18-5DA, pB18-5DE, pB18-7DA, pB18-7DE, and pDMA5 were introduced into wild-type Columbia to generate *ARR4OX*, *ARR5OX*, *ARR6OX*, *ARR7OX*, *ARR9OX*, *ARR5^D87A^OX*, *ARR5^D87E^OX*, *ARR7^D85E^OX*, *ARR7^D85E^OX*, and *DMA5*, respectively. Transgenic T1 seedlings were selected on MS agar plates (see below) supplemented with 30 μg/mL hygromycin and 50 μg/mL carbenicillin. Transgene expression was confirmed in homozygous hygromycin-resistant T3 seedlings by protein gel blotting of whole seedling protein extracts and detection with anti-c-myc POD antibody (Roche Applied Science). For each construct, the results from one representative line are presented.

To generate DEX-inducible myc-ARR5 lines in the various genetic backgrounds, *ahk3,4* was crossed to *DMA5*. pDMA5 was introduced into *ahp1,2,3,4* and *arr1,2,10,12* and selected as described above.

Plant Growth Conditions

Seeds were surface-sterilized and cold-treated at 4°C for 3 d in the dark and grown at 23°C under constant white light (∼100 μE). Seedlings were grown on MS medium containing 1× MS salts, 0.05% MES buffer, and 1% sucrose, pH 5.7. For cytokinin response assays, seedlings were grown on vertical MS plates with 0.6% phytagel (Sigma-Aldrich) supplemented with a dose range of N^6-BA or 0.1% (v/v) DMSO carrier control as described previously (To et al., 2004). For protein assays and transgenic seedling selection, seedlings were grown on horizontal MS plates with 0.8% bactoagar.

Quantitative Real-Time RT-PCR

Ten-day-old light-grown seedlings in cytokinin response assays were harvested, and total RNA was extracted from both whole seedlings and full-length roots using an RNeasy kit according to the manufacturer's instructions (Qiagen). cDNA was generated from the RNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with *Taq* DNA Polymerase Hot-Start Version, buffer, and deoxynucleotide triphosphates according to the manufacturer's instructions (Takara Mirus Bio) supplemented with 0.3× SYBR Green (Molecular Probes) and ARR5 primers ARR5F3 (5′-TCTGAAGAT-TAATTTGATAATGACGG-3′) and ARR5R2 (5′-TCACAGGCTTCAATAAG-AAATCTTCA-3′) or β-tubulin primers TUB4s (5′-AGAGGTTGACGAGCA-AGATGA-3′) and TUB4a (5′-AACAATGAAAGTAGACGCCA-3′). Real-time PCR was performed in an Opticon2 PCR machine (MJ Research) using the following thermocycler program: (1) 2 min at 95°C; (2) 15 s at 95°C; (3) 15s at 60°C; (4) 15 s at 72°C; (5) optical read, repeat 34 cycles of steps 2 through 5, followed by a final analysis of product melting temperature to confirm the PCR product. Each biological sample was analyzed at least twice in triplicate. The relative expression for ARR5 (normalized to β-tubulin as a reference gene and to the wild type grown on DMSO as a control sample) and 95% confidence intervals were determined using REST 2005 version 1.9.12 (Pfaffl et al., 2002; http://rest-2005.gene-quantification.info). Two independent experiments were performed with consistent results. The data from one triplicate analysis are presented.

Analysis of Protein Stability

For DEX-inducible myc-tagged ARR5, myc-ARR5 protein expression was induced by incubating 7-d-old light-grown seedlings in liquid MS medium with 1 μM DEX supplemented with 1 μM BA or 0.1% (v/v) DMSO carrier control for 2 h. Protein synthesis was inhibited by 200 μM CHX. Seedlings were harvested by flash freezing in liquid nitrogen at the time points indicated.

For lines constitutively overexpressing ARRs, 7-d-old light-grown seedlings were incubated in liquid MS medium with 200 μM CHX supplemented with 1 μM BA or DMSO carrier control. Seedlings were harvested at the time points indicated.

Protein extracts were prepared in 250 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 1× Complete protease inhibitors (Roche Applied Science), and 0.5% β-mercaptoethanol. Protein extracts were separated by SDS-PAGE and transferred to Nitropure membranes (GE). myc-tagged proteins were detected with anti-c-myc POD (Roche Applied Science), and tubulin was detected by rabbit polyclonal anti-tubulin and secondary goat anti-rabbit POD antibodies (Chemicon) and visualized by chemiluminescent detection (Perkin-Elmer) by autoradiography. Films were quantified using ImageQuant software (Molecular Dynamics). myc-ARR protein levels were normalized to signal from β-tubulin or from nonspecific anti-c-myc hybridization to a 35- to 40-kD protein. Three independent ARR protein degradation time course experiments were conducted for each line, and the results were averaged. Protein half-lives of myc-ARRs were

estimated by plotting an exponential best-fit curve to the averaged data from three independent experiments.

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for the genes characterized in this study are as follows: *ARR4* (At1g10470), *ARR5* (At3g48100), *ARR6* (At5g62960), *ARR7* (At1g19050), and *ARR9* (At3g57040).

Supplemental Data

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

Supplemental Figure 1. Mutations Targeting the Conserved Phosphorylation Target Asp Do Not Disrupt ARR5 Protein Interaction with AHP2 in the Yeast Two-Hybrid Assay.

Supplemental Figure 2. Exogenous DEX Application Enhances Cytokinin Resistance in DMA5 Seedlings.

Supplemental Figure 3. Mutations in RPN12a and COP9/CIN4/FUS10 Do Not Alter myc-ARR5 Protein Stability.

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