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Multiple Type-B Response Regulators Mediate Cytokinin Signal Transduction in *Arabidopsis* [W]

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Type-B *Arabidopsis thaliana* response regulators (ARRs) are transcription factors that function in the final step of two-component signaling systems. To characterize their role in plant growth and development, we isolated T-DNA insertions within six of the genes (*ARR1*, *ARR2*, *ARR10*, *ARR11*, *ARR12*, and *ARR18*) from the largest subfamily of type-B *ARR*s and also constructed various double and triple combinations of these mutations. Higher order mutants revealed progressively decreased sensitivity to cytokinin, including effects on root elongation, lateral root formation, callus induction and greening, and induction of cytokinin primary response genes. The triple mutant *arr1,10,12* showed almost complete insensitivity to cytokinin under many of the assay conditions used. By contrast, no significant change in the sensitivity to ethylene was found among the mutants examined. These results indicate that there is functional overlap among the type-B ARRs and that they act as positive regulators of cytokinin signal transduction.

INTRODUCTION

Plants use two-component phosphorelays for signal transduction, and elements of these two-component systems have been implicated in plant responses to the hormones cytokinin and ethylene, red light, and osmosensing (Schaller, 2000; Hutchison and Kieber, 2002; Hwang et al., 2002; Schaller et al., 2002; Maxwell and Kieber, 2005). Two-component systems were originally identified in bacteria, and in their simplest configuration they involve a His sensor kinase and a response regulator (Mizuno, 1997; Stock et al., 2000). The His kinase autophosphorylates a conserved His residue in response to an environmental stimulus. This phosphate group is then transferred to a conserved Asp residue within the receiver domain of a response regulator. The response regulator, which is often a transcription factor, then modulates downstream signaling in the pathway. Multistep phosphorelays have also been identified that make use of three components: a hybrid receptor kinase that contains both His kinase and receiver domains in one protein, a His-containing phosphotransfer protein (HPt), and a response regulator (Swanson et al., 1994; Schaller, 2000). In a multistep phosphorelay, the phosphate is transferred from amino acid to amino acid between the proteins in the sequence His → Asp → His → Asp.

Analysis of the *Arabidopsis thaliana* genome reveals 8 His kinases, 23 response regulators, and 5 HPt proteins that contain all of the conserved residues required for enzymatic activity (Hutchison and Kieber, 2002; Hwang et al., 2002; Schaller et al., 2002; Maxwell and Kieber, 2005). The *Arabidopsis* response regulators (ARRs) have been classified into type-A and type-B groups based on domain structure and sequence (Imamura et al., 1999). Type-A ARRs contain a central receiver domain along with short N- and C-terminal extensions and are transcriptionally induced by cytokinin. Type-B ARRs contain a receiver domain followed by a DNA binding domain and, based on several lines of evidence, function as transcription factors (Sakai et al., 2000; Lohrmann et al., 2001; Hosoda et al., 2002).

Analysis of His sensor kinases from *Arabidopsis* suggests that two-component signaling pathways may mediate responses to multiple stimuli. Three His sensor kinases, Arabidopsis Histidine Kinase 2 (AHK2), AHK3, and AHK4, are cytokinin receptors (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001; Kakimoto, 2003); AHK4 is also known as Cytokinin Response 1 (CRE1) and Wooden Leg 1 (WOL1). Two His sensor kinases, Ethylene Response 1 (ETR1) and Ethylene Response Sensor 1 (ERS1), are ethylene receptors (Chang and Stadler, 2001; Schaller and Kieber, 2002). Two other His sensor kinases, Cytokinin Insensitive 1 (CKI1) and AHK1, contain putative ligand binding domains with no significant similarity to the ligand binding domains of the cytokinin and ethylene receptors,

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suggesting their involvement in sensing additional stimuli (Schaller et al., 2002). Two-component signaling pathways have been most clearly implicated in the plant's response to cytokinin, which is thought to incorporate all of the elements of a multistep phosphorelay (Hwang and Sheen, 2001; Hutchison and Kieber, 2002; Heyl and Schmülling, 2003; Kakimoto, 2003). According to the proposed model, the cytokinin receptors autophosphorylate in response to cytokinin. The phosphate is then transferred to an HPt protein, which then enters the nucleus and activates the type-B ARRs by transferring the phosphate to them. Among the transcriptional targets of the type-B ARRs are type-A ARRs, which upon induction act as negative regulators of the initial signal transduction pathway (To et al., 2004).

To date, the strongest evidence for the role of type-B ARRs in mediating the cytokinin signal comes from the analysis of ARR1. A null mutation in *ARR1* results in reduced sensitivity to cytokinin in shoot regeneration and root elongation assays (Sakai et al., 2001). Overexpression of either *ARR1* or *ARR2* in *Arabidopsis* plants results in increased sensitivity to cytokinin (Hwang and Sheen, 2001; Sakai et al., 2001). ARR2, however, has also been proposed to play a role in mediating ethylene signal transduction based on the analysis of a transposon-induced mutation in the Landsberg *erecta* background (Hass et al., 2004). The relatively weak phenotypes revealed by the analysis of individual *ARR* mutations may be the result of functional overlap among the type-B ARRs, a hypothesis consistent with the gene expression patterns (Mason et al., 2004; Tajima et al., 2004). Here, we report on the results obtained by taking a reverse genetic approach to discover the function(s) of *Arabidopsis* type-B ARRs. We isolated T-DNA insertions within six of the seven genes that make up the largest subfamily of type-B *ARR*s. We generated different combinations of these mutations and characterized the mutants in terms of their hormone responsiveness. Our results indicate that there is functional overlap among the genes and support a role for five of the genes in mediating cytokinin signal transduction. By contrast, we did not observe a clear effect on ethylene signal transduction among the mutant combinations tested.

RESULTS

Isolation of T-DNA Insertions in Genes Encoding Type-B ARRs

The type-B family of *ARR*s is composed of one major subfamily of seven genes and two minor subfamilies of two genes each (Mason et al., 2004). The major subfamily (subfamily I) contains *ARR1*, a gene previously implicated in cytokinin signal transduction (Hwang and Sheen, 2001; Sakai et al., 2001). To analyze the function of type-B ARRs, we isolated *Arabidopsis* lines homozygous for T-DNA insertions in six of the seven genes that make up subfamily I: *ARR1*, *ARR2*, *ARR10*, *ARR11*, *ARR12*, and *ARR18*. As shown in Figure 1, multiple insertion alleles were identified for five of the genes, and in all cases except *ARR10* at least one allele represented an insertion within an exon. Eleven of these insertion lines were in the Columbia (Col) ecotype and four were in the Wassilewskija (Ws) ecotype. *ARR14* was the only member of subfamily I for which no insertion allele could be identified. Based on the positions of the insertions, the mutant alleles are predicted to be either null or hypomorphic.

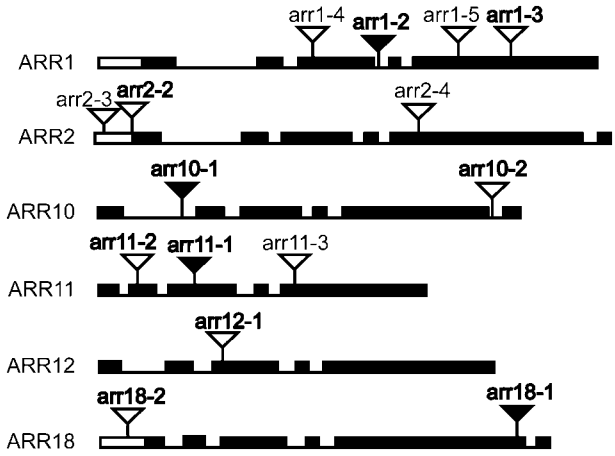

Figure 1. Positions of T-DNA Insertions in Type-B *arr* Mutants.

Boxes represent exons, with black representing coding sequence and white representing the 5′ untranslated region as appropriate. Lines represent introns. Inverted triangles indicate the T-DNA insertions (white triangles represent insertions in the Col background, and black triangles represent insertions in the Ws background). Alleles designated in boldface were used to construct higher order mutants.

To examine genetic interactions among the *arr* mutations, double and triple mutant combinations were generated. All higher order mutant lines were maintained within the Col or Ws ecotypes to avoid complications arising from mixing of the two ecotypes. The alleles used to generate the higher order mutants are indicated in Figure 1.

Root Elongation in *arr* Mutant Seedlings Is Less Sensitive to Cytokinin Inhibition

To assess the role of the type-B ARRs in cytokinin responses, we examined the root lengths of *arr* mutants grown in the presence or absence of cytokinin. As shown in Figures 2, 3, and 4A, root elongation of wild-type seedlings was greatly reduced when treated with 100 nM benzyladenine (BA). Statistical analysis was performed based on Duncan's multiple range test among the means on the analysis of variance, and lines were grouped based on their significant differences at $P < 0.05$ (Duncan, 1975). In the absence of cytokinin, all of the mutant lines had root lengths that were not significantly different from those of the wild type, the only exception being the triple mutant *arr1,11,12*, which had a significantly longer root than the wild type. In the presence of cytokinin, multiple mutant lines (double mutants *arr1,10*, *arr1,12*, *arr10,12*, and *arr12,18* in Col; triple mutants *arr1,2,11*, *arr1,2,12*, *arr1,10,12*, *arr1,10,11*, *arr1,11,12*, and *arr10,11,12* in Col; single, double, and triple mutant combinations containing *arr1* in Ws) showed significant differences from the wild type that were consistent with decreased sensitivity to cytokinin (Figures 2 and 3). Although single mutants in the Col background were not significantly different from the wild type, some higher order mutants

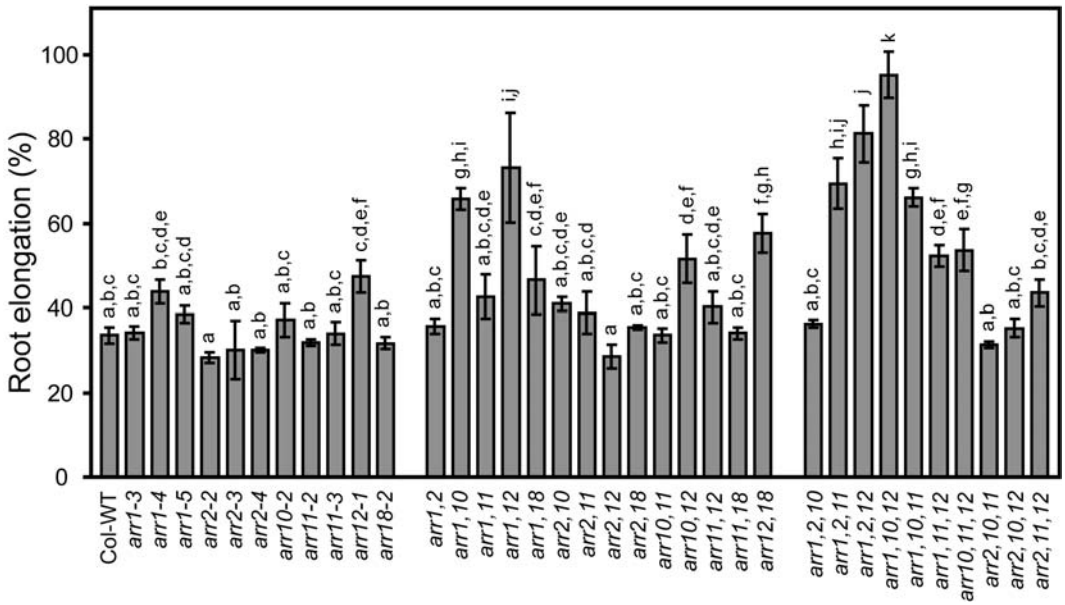

Figure 2. Root Elongation Responsiveness to Cytokinin of T-DNA Insertion Mutants (Col Background).

Seedlings were grown vertically on plates supplemented with 100 nM BA or DMSO vehicle control under constant light conditions at 22°C. Root elongation for each line from days 5 through 8 was measured. The root elongation of each line receiving the cytokinin treatment is expressed as a percentage of its DMSO control. Results shown for each line represent means from at least three independent replicates using at least 14 seedlings each. Error bars represent SE. Lines were analyzed for significant differences in their responsiveness to cytokinin based on Duncan's multiple range test among the means on the analysis of variance ($P < 0.05$). Lines designated with the same letter exhibit no significant difference in their responsiveness to cytokinin.

showed increased resistance to exogenously supplied cytokinin, the triple mutant *arr1,10,12* being almost completely insensitive to 100 nM BA. This finding is consistent with functional overlap within subfamily I of the type-B ARRs. Unlike the *arr1* alleles in Col, the *arr1-2* allele in Ws was significantly reduced in its sensitivity to cytokinin (Figure 3), suggestive of ecotype differences in the contribution of various type-B ARRs to cytokinin sensitivity.

Analysis of the single and higher order mutants in Col provides evidence that multiple subfamily I type-B ARRs contribute to the regulation of root elongation by cytokinin. The *arr1,10,12* triple mutant is significantly less sensitive to cytokinin than are the *arr1*, *arr10*, and *arr12* single mutants or their double mutant combinations, indicating that *ARR1*, *ARR10*, and *ARR12* are all involved in cytokinin regulation of primary root elongation. Similarly, the triple mutant *arr1,2,11* is significantly less sensitive to cytokinin treatment than are the *arr1*, *arr2*, and *arr11* single mutants or their double mutant combinations, indicating that *ARR2* and *ARR11* each also contributes to the cytokinin effect on root elongation. Single and double mutant combinations involving *arr18* also showed a similar additive increase in cytokinin insensitivity based on the analysis of *arr1,18* and *arr12,18*, but these differences were not significant at the $P < 0.05$ level. It should be noted that whereas some of the *arr* alleles, such as *arr1*, *arr10*, and *arr12*, had a generally additive effect in combination, more complex interactions were found with the other *arr* alleles (*arr2*, *arr11*, and *arr18*). This is particularly apparent with the *arr2* allele, which in some instances appeared to exert an antagonistic effect (e.g., the *arr1,2,10* triple mutant was more sensitive to cytokinin than the *arr1,10* double mutant). *ARR1* appeared to make the greatest contribution to the cytokinin response, as mutant combinations containing the *arr1* allele generally showed less sensitivity to exogenous cytokinin than those combinations containing a functional *ARR1* allele.

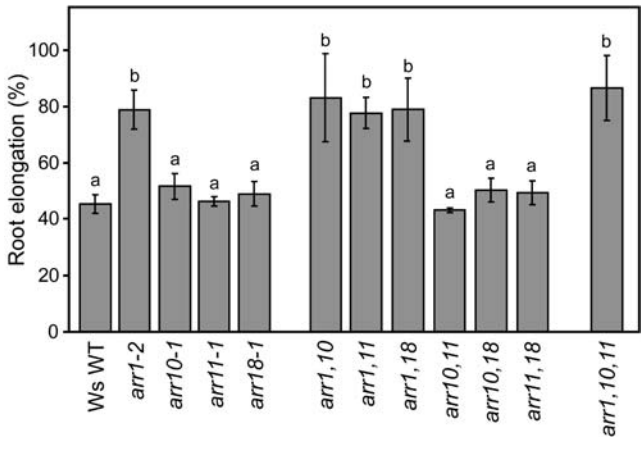

Figure 3. Root Elongation Responsiveness to Cytokinin of T-DNA Insertion Mutants (Ws Background).

Seedlings were grown, root elongation measured, and statistical analysis performed as described for Figure 2. Results shown represent means from at least three independent replicates using at least 14 seedlings each. Error bars represent SE. Lines designated with the same letter exhibit no significant difference in their responsiveness to cytokinin.

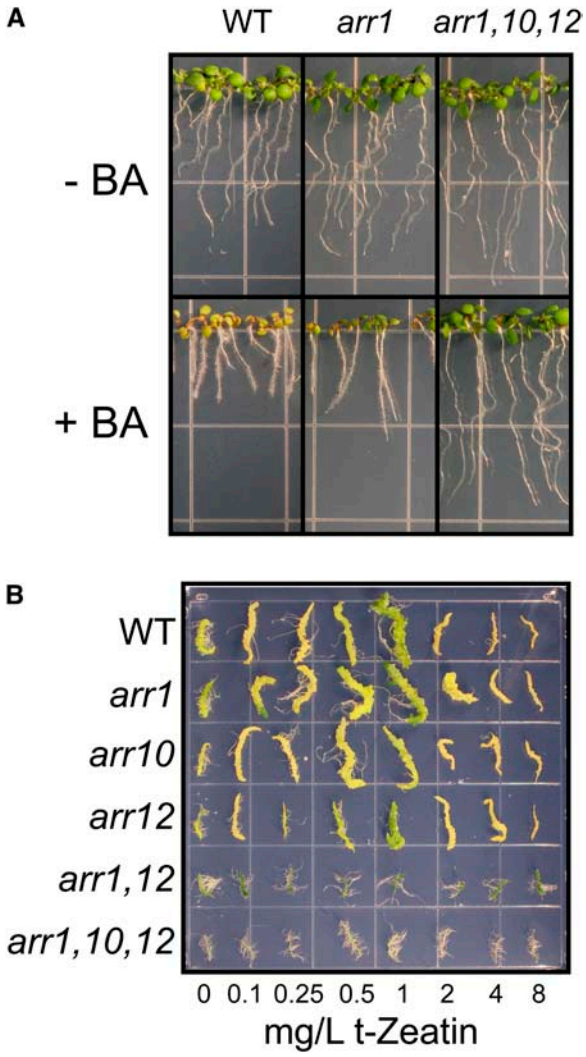

Figure 4. Morphological Phenotypes of *arr* Mutants.

(A) Effect of *arr* mutants on root elongation and shoot greening. The *arr1* and *arr1,10,12* mutants, along with a Col wild-type control, were grown for 8 d in the absence or presence of 100 nM BA. In the absence of BA, the increased root length of the *arr1,10,12* mutant compared with the wild type can be seen. In the presence of BA, the *arr1,10,12* mutant has green cotyledons and lacks a root response to BA.

(B) Induction of callus formation and greening of *arr* mutants. Representative hypocotyl segments treated with 0.2 mg/L indole-3-butyric acid and the indicated concentrations of *t*-zeatin are shown after growth for 3 weeks under constant light.

We compared root elongation in the wild type with several higher order type-B *arr* mutants across a range of cytokinin concentrations between 0.01 and 100 μM BA (Figure 5). A significant difference was found in how the lines responded to the hormone treatment ($F_{4,652} = 11.00$, $P < 0.0001$). Wild-type plants exhibited half-maximal inhibition of root elongation at ~0.03 μM BA. The *arr1,12* double mutant, by contrast, showed half-maximal inhibition at ~20 μM BA, a >600-fold decrease in sensitivity. The *arr1,10,12* triple mutant showed even greater resistance, exhibiting almost complete insensitivity to 10 μM BA but then showing a precipitous decrease in root elongation at 100 μM BA, potentially attributable to toxic effects of this high BA level upon seedlings. The *arr2,10,12* triple mutant was almost as sensitive to exogenous cytokinin as the wild-type plants. This result provides further evidence of the predominant contribution of *ARR1* to the elongation response of primary roots to exogenous cytokinin.

Lateral Root Formation in *arr* Mutant Seedlings Is Less Sensitive to Cytokinin Inhibition

Cytokinin treatment inhibits the formation of lateral roots in *Arabidopsis* (Werner et al., 2001; To et al., 2004). In wild-type *Arabidopsis* seedlings, treatment with 10 nM BA resulted in a significant decrease in the formation of lateral roots; treatment with 100 nM BA virtually eliminated their formation (Figure 6). We examined the effect of BA on lateral root formation in Col plants containing single, double, and triple mutant combinations of *arr1*, *arr10*, and *arr12* and found significant effects of the genotype upon the seedling response to BA ($F_{16,564} = 4.38$, $P < 0.0001$). These *arr* mutations were chosen because they demonstrated a readily discernible additive effect when analyzed in the root elongation assay (Figures 2 and 3). A similar additive effect was observed among these mutants when analyzed for the effects of 100 nM BA on lateral root formation. The single mutants exhibited a nearly wild-type sensitivity to cytokinin, producing virtually no lateral roots per seedling when grown on 100 nM BA. By contrast, the *arr1,12* double mutant produced 3.0 lateral roots per seedling at 100 nM BA, a number that was 36% of that observed in untreated seedlings. The *arr1,10,12* triple mutant exhibited an even stronger phenotype and produced 5.6 lateral roots per seedling, a number that was 78% of that observed in untreated seedlings. Thus, the relative effect of the different *arr* mutants on lateral root formation was similar to that observed in the root elongation response; *arr1,10,12* showed the strongest effect, followed by *arr1,12* and the other mutants.

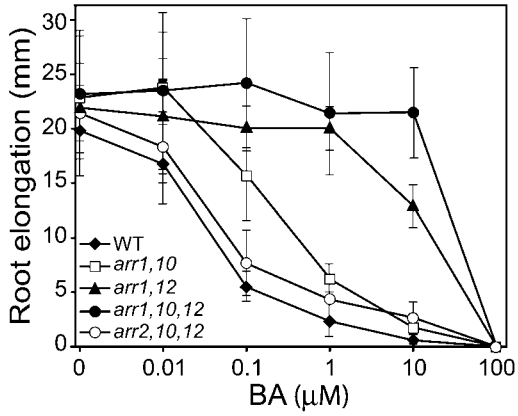

Figure 5. Dose Response for the Effect of Cytokinin on *arr* Mutants.

Seedlings were grown vertically on plates supplemented with the specified concentrations of BA or a DMSO vehicle control. Root elongation between days 4 and 9 was measured. Error bars represent SD ($n > 14$).

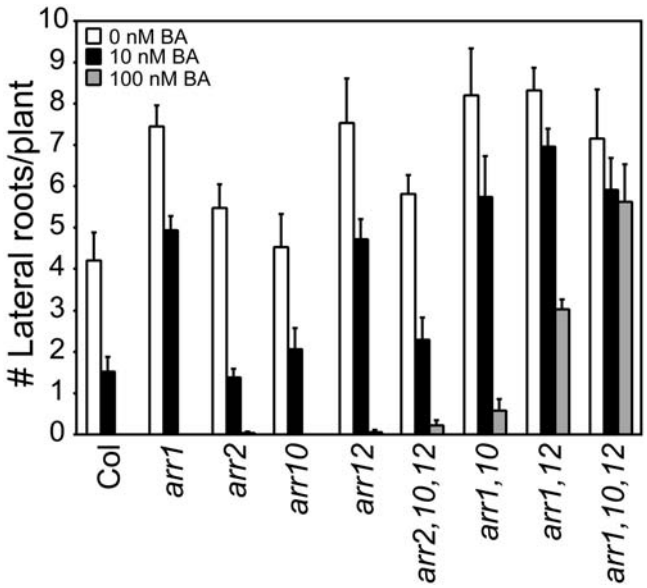

Figure 6. Effect of *arr* Mutants on Lateral Root Formation.

Seedlings were grown vertically on plates supplemented with the specified concentrations of BA or a DMSO vehicle control. The number of lateral roots was determined on day 9. Error bars represent SE ($n > 14$).

Chlorophyll Levels in *arr* Seedlings Are Less Sensitive to Perturbation by Cytokinin Treatment

In the absence of cytokinin, the cotyledons and leaves of the *arr1,10,12* triple mutant appear similar to those of wild-type plants. When grown in 100 nM exogenous BA, the cotyledons and leaves of wild-type rosettes were smaller and paler than those of untreated plants (Figure 4). By contrast, 100 nM BA had no observable effect on the size and color of the *arr1,10,12* mutant. The change from dark to pale rosettes in wild-type plants is attributable to a decrease in chlorophyll levels brought about by cytokinin treatment (To et al., 2004). Therefore, we quantified the chlorophyll levels of wild-type Col and *arr* mutants grown in the absence and presence of 100 nM BA and found a significant difference in how the lines responded to BA ($F_{5,48} = 20.61$, $P < 0.0001$). In the absence of BA, the chlorophyll contents of the wild type and the *arr1,10,12* mutant were not significantly different (2.4 ± 0.33 and 2.27 ± 0.17 μg/mg fresh weight, respectively). The chlorophyll level of the wild type decreased to 0.4 ± 0.16 μg/mg fresh weight in the presence of 100 nM BA (17% of the chlorophyll content found in the absence of BA). By contrast, the chlorophyll level of the *arr1,10,12* mutant demonstrated no significant change in response to treatment with 100 nM BA (2.48 ± 0.33 μg/mg fresh weight). This analysis indicates that the chlorophyll level of the *arr1,10,12* mutants is significantly less sensitive than that of wild-type plants to perturbation by cytokinin treatment; thus, the shoots of the triple mutant are also insensitive to cytokinin.

arr Mutants Have an Altered Response to Cytokinin in Callus/Greening Assays

Cytokinins, in concert with auxin, are capable of promoting cell division, greening, and shoot initiation in cultured plant tissues (Miller et al., 1956; Skoog and Miller, 1957; Mok and Mok, 2001). In addition, cytokinins inhibit root formation in cultured plant tissues (Skoog and Miller, 1957; Mok and Mok, 2001). At 0.2 mg/L indolebutyric acid (auxin), we observed maximal greening and callus induction in Col hypocotyl explants at 1 mg/L concentration of the cytokinin *t*-zeatin (Figure 4B). We observed no shoot formation under these conditions, consistent with the previously observed inefficiency of shoot formation in cultured Col (Valvekens et al., 1988; To et al., 2004). Roots were initiated at *t*-zeatin concentrations of 1 mg/L and below but were observed only occasionally at higher concentrations.

We examined the cytokinin response of excised Col hypocotyls from single, double, and triple mutant combinations of *arr1*, *arr10*, and *arr12* (Figure 4B). The *arr1* and *arr10* single mutants were indistinguishable from wild-type plants. The *arr12* single mutant, however, did exhibit a slight reduction in callus formation. More dramatic phenotypes were observed in the higher order mutants. Although hypocotyl explants of the double mutant *arr1,12* formed green calli, these were substantially reduced in size compared with those formed in the wild type and single mutants. The triple mutant *arr1,10,12* did not green or form visible calli even at the highest *t*-zeatin concentration tested. In addition, both the *arr1,12* and *arr1,10,12* mutants initiated multiple root primordia across the entire range of cytokinin levels tested, including at 8 mg/L *t*-zeatin, at which no root formation occurred in the wild type. These data are consistent with an additive effect of the type-B *arr* mutations on cytokinin sensitivity. They are also consistent with a reduced sensitivity in the type-B *arr* mutants to endogenous as well as exogenous cytokinin because, even in the absence of exogenous cytokinin, the *arr1,12* and *arr1,10,12* mutants exhibited reduced callus formation compared with wild-type plants.

arr Mutations Affect the Cytokinin Primary Response

The assays described above are based on the downstream physiological effects of cytokinin. To determine whether the type-B mutations modulate signaling through the primary cytokinin response pathway, we analyzed the effects of these mutations on the cytokinin-induced transcription of a family of primary response genes: the type-A response regulators (Brandstatter and Kieber, 1998; D'Agostino et al., 2000; Rashotte et al., 2003). The type-B response regulators have been implicated directly in the induction of these primary response genes because of the presence of the ARR1 recognition sequence in their promoters (Rashotte et al., 2003). Therefore, we compared the expression of type-A *ARR*s in the *arr1,10,12* triple mutant and the wild type (Figure 7; see Supplemental Figure 1 online). Dark-grown seedlings were treated for 45 min with either 10 μM BA or a DMSO control. In the absence of cytokinin, *arr1,10,12* showed similar or reduced expression for the type-A *ARR*s compared with the wild type. Thus, in the presence of endogenous levels of cytokinin, the *arr1,10,12* mutant has a reduced level of signaling through the primary pathway.

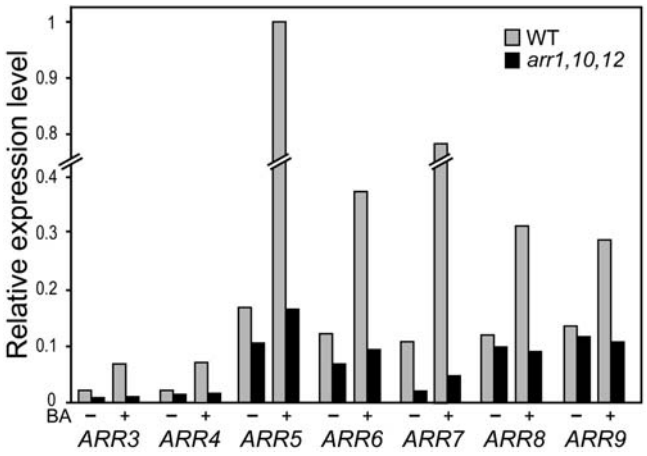

Figure 7. The *arr1,10,12* Mutant Affects the Cytokinin Primary Response.

Total RNA was isolated from 4-d-old wild-type and *arr1,10,12* dark-grown seedlings treated for 45 min with either 10 μM BA or a DMSO vehicle control. The RNA was used to construct a ^{32}P-labeled probe, which was then hybridized to blots containing representatives for each of the type-A *ARR*s. UBQ was included on the blots as an internal control to allow for normalization. Expression of the type-A *ARR*s is reported relative to the maximum signal observed (*ARR5* in the wild-type background treated with BA).

Substantial differences were observed between the *arr1,10,12* mutant and the wild type in response to cytokinin treatment (Figure 7; see Supplemental Figure 1 online). In all cases, the degree of induction for the primary response genes was reduced in the mutant, and in some cases, no induction was observed in response to BA treatment. The effect of the triple mutant upon expression can be clearly seen on the primary response genes *ARR5* and *ARR7*, which of all the type-A *ARR*s examined under our treatment conditions showed the highest levels of expression in the wild type. In the wild type, *ARR5* and *ARR7* were induced 5.9- and 7.2-fold by cytokinin, respectively. In the *arr1,10,12* mutant, the induction of *ARR5* and *ARR7* decreased to 1.6- and 2.3-fold, respectively. The reduction in endogenous levels of type-A *ARR*s in the *arr1,10,12* mutant, coupled with decreases in their induction by cytokinin, resulted in decreased expression levels for all type-A ARRs in the presence of cytokinin. Thus, in the presence of cytokinin, the triple mutant exhibits a lower overall level of signal output from the primary response pathway. The ability of cytokinin to regulate some type-A *ARR* gene expression is likely attributable to the remaining functional ARRs present in the mutant.

Analysis of the Ethylene Response in *arr* Mutants

ARR2 has been implicated previously in modulating the ethylene response in *Arabidopsis* based on the analysis of a transposon-induced mutation in the Landsberg *erecta* background (Hass et al., 2004). Therefore, we examined the effects of *arr2* as well as other type-B *arr* mutations on the triple response of dark-grown seedlings to ethylene. In *Arabidopsis*, the triple response is characterized by an ethylene-induced inhibition of hypocotyl and root elongation, swelling of the hypocotyl, and formation of an exaggerated apical hook (Bleecker et al., 1988; Guzmán and Ecker, 1990).

A quantitative analysis of ethylene responsiveness was performed on the *arr* mutant lines. Seedlings were grown in the dark in ethylene at concentrations ranging from 0 to 100 μL/L, and the hypocotyl lengths were measured after 3.5 d of growth. As shown in Figure 8, no significant difference was observed between the *arr2-1* mutant allele and the wild-type Landsberg *erecta* control ($F_{1,411} = 0.07$, $P > 0.75$), a result differing from that of a previous report using the same mutant allele (Hass et al., 2004). We also observed no significant differences in ethylene responsiveness for any of the mutants in the Col background compared with the wild-type Col control ($F_{3,1108} = 0.92$, $P > 0.4$). The ethylene responsiveness of the *arr2-2* (Figure 8) and *arr2-3* (results not shown) mutant alleles was not significantly different from that of the wild type. The *arr1,10,12* triple mutant, which shows strong cytokinin insensitivity, was also indistinguishable from the wild type in terms of ethylene responsiveness (Figure 8).

We consistently observed a significant reduction in hypocotyl growth in triple mutants that contained the *arr2* allele. In the absence of ethylene, the hypocotyl length of *arr2,10,12* was 8.7 mm compared with 11.7 mm for the wild type and was significantly different from the lengths for the wild type, *arr2-2*, and

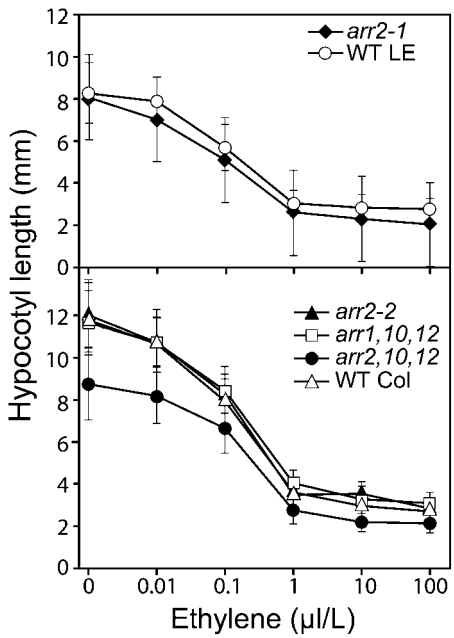

Figure 8. Effect of Type-B *arr* Mutants on the Seedling Growth Response to Ethylene.

The hypocotyl lengths of dark-grown seedlings exposed to the indicated levels of exogenous ethylene were determined after 3.5 d. Analysis was performed using *Arabidopsis* Landsberg *erecta* (LE) and Col lines (top and bottom panels, respectively). The ethylene biosynthesis inhibitor aminoethylvinyl-Gly was included in the medium. Error bars represent SD ($n > 22$).

arr1,10,12 ($F_{3,195} = 46.9$, $P < 0.0001$). Thus, there was a significant effect of this genotype (*arr2,10,12*) on hypocotyl length, even though there was no effect of the genotype on the ethylene responsiveness of the seedling. The reduced hypocotyl length could be attributable to increased ethylene production in the mutant seedlings or to increased sensitivity of the seedlings to endogenous ethylene. The growth medium, however, contained aminoethylvinyl-Gly to inhibit ethylene biosynthesis. In addition, we grew seedlings on silver, which blocks ethylene perception, and observed no reversion of the mutant phenotype (results not shown). This finding suggests that the growth effect occurs either downstream or independently of ethylene perception. The hypocotyls of the *arr2,10,12* mutant were significantly shorter than those of the wild type at all ethylene concentrations tested, even at 100 μL/L ethylene; thus, the growth effect may act independently of the ethylene pathway. Roots of the *arr2,10,12* mutant were not significantly different from those of wild-type plants in the absence of ethylene (2.97 ± 0.58 mm compared with 3.18 ± 0.47 mm, respectively), indicating that the growth effects seen in *arr2,10,12* are not consistent with a general triple response phenotype. A shortened hypocotyl was also observed in the *arr1,2,12* mutant, indicating that other triple mutant combinations containing *arr2* may produce the short hypocotyl phenotype. We conclude from these experiments that *ARR2* acts in concert with other type-B ARRs to regulate hypocotyl growth and that this effect may be ethylene-independent.

DISCUSSION

We characterized six of the seven genes that make up subfamily I, the largest subfamily of type-B response regulators in *Arabidopsis*. Based on their ability to modulate the effect of cytokinin on root elongation, we conclude that five of the genes can serve as positive regulators of cytokinin signaling. Further analysis of a subset of these genes (*ARR1*, *ARR10*, and *ARR12*) indicates that these genes also serve as positive regulators of cytokinin action in lateral root formation, regulation of chlorophyll levels, regeneration from tissue culture, and expression of cytokinin primary response genes. Mutations within the genes of subfamily I typically had additive effects on cytokinin signaling, indicative of functional overlap, but in some instances potentially antagonistic relationships were revealed.

Overlapping Expression and Function of Type-B ARRs

We found a good correlation between the expression and function of the type-B ARRs. First, mutant phenotypes were observed in tissues in which the type-B ARRs were expressed. Second, the functional overlap revealed by mutant analysis correlates with the overlapping expression patterns of the type-B ARRs. Correlation between the expression pattern and overlapping function of the type-B ARRs can be seen in their role in mediating the effect of cytokinin on primary root growth. Overlapping expression of subfamily I members has been found in primary root tips based on ARR:β-glucuronidase (GUS) translational fusions and microarray analysis (Birnbaum et al., 2003; Mason et al., 2004). All members of subfamily I were detected at the primary root tip by microarray analysis, but with differing levels of expression based on relative signal intensity. The *ARR* signal intensities observed by microarray analysis correlate with the level of detection for the ARR:GUS fusions. *ARR10* and *ARR12* had the highest signal intensity; *ARR1* and *ARR2* had the next highest signal intensity; the other members of subfamily I (*ARR11*, *ARR14*, and *ARR18*), which were not detected by GUS fusions, had weaker signal intensity on microarrays.

A gradient of ARR:GUS activity was observed that typically began at the root meristem, reached a peak in the zones of elongation/specialization, and then decreased to below detection limits in the mature root (Mason et al., 2004). Microarray analysis supports the presence of the *ARR*s in the three growth stages examined (meristematic zone, elongation zone, and specialization zone), with different expression levels within each of these zones (Birnbaum et al., 2003). For example, *ARR1*, *ARR2*, and *ARR10* showed their highest levels of expression in the specialization zone. *ARR12* showed a similar expression level in all three zones. As with the GUS fusions, *ARR12* was more abundant in the stele, endodermis, and cortex than in the epidermis, in contrast with *ARR10*, which was more abundant in the epidermis. The expression of subfamily I *ARR*s in the root tip correlated with the effect of *arr* mutations on the cytokinin inhibition of root growth. Whereas single mutants had minimal effects on cytokinin responsiveness, the triple mutant *arr1,10,12* was almost completely insensitive to the effects of exogenous cytokinin on root growth.

The analysis of ARR:GUS translational fusions also revealed overlapping expression of the type-B ARRs at lateral root junctions, in callus tissue, in cells of the apical meristem region, and in young leaves (Mason et al., 2004). *arr* mutants showed reduced sensitivity to cytokinin in lateral root formation, callus induction and greening, and chlorophyll repression assays. These affected tissues correlate with the expression pattern of the type-B ARRs. As found for primary root growth inhibition, the effects of the *arr* mutants were additive in these assays as well. However, there may be differing levels of contribution of the type-B ARRs to various physiological processes based on this mutant analysis. For example, the *arr1* mutation contributes equally to or more than *arr12* to effects on root elongation; but the *arr12* mutation contributes more than *arr1* to effects on callus induction/greening.

Phenotypic Comparison with Other Mutants Affecting Cytokinin Action

Because the type-B *arr* mutants revealed decreased sensitivity to cytokinin based on multiple assays, it is useful to compare the phenotypes conferred by type-B *arr* mutants with those of other mutants with reduced cytokinin action. In previous studies, cytokinin signal transduction has been assessed using two different approaches: (1) by reducing endogenous plant cytokinin levels through overexpression of cytokinin oxidase genes (Werner et al., 2001, 2003) and (2) by isolating mutations in the cytokinin receptors (Inoue et al., 2001; Ueguchi et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004). Transgenic *Arabidopsis* plants, in which the cytokinin levels were reduced by 30 to 50% of their wild-type levels, showed multiple developmental alterations (Werner et al., 2003). These plants had increased root

length, increased number of lateral roots, retarded shoot development, and delayed flowering.

Arabidopsis has a cytokinin receptor family containing three members: AHK2, AHK3, and CRE1 (AHK4/WOL1). Of these, CRE1 appears to play the major role in root responses to cytokinin. Single *cre1* mutants show reduced cytokinin sensitivity in root growth inhibition assays (Inoue et al., 2001; Ueguchi et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004). Double mutants of *cre1* with either *ahk2* or *ahk3* show a greater reduction in cytokinin sensitivity, the *cre1 ahk3* double mutant being almost completely insensitive to cytokinin in a root growth assay (Higuchi et al., 2004). Double mutants also show reduced sensitivity to cytokinin in callus induction assays. The receptor triple mutant displays severe developmental abnormalities, with retarded root and shoot growth, resulting in a stunted and nonviable plant (Higuchi et al., 2004; Nishimura et al., 2004).

These studies indicate that cytokinins can have both positive and negative effects on root growth, depending on the level of transmission through the signal transduction pathway. A partial reduction in signal transmission can result in an apparent increase in root meristem size and activity (Werner et al., 2003; Higuchi et al., 2004), whereas further reduction in transmission results in deceased root meristem size and activity (Higuchi et al., 2004; Nishimura et al., 2004). From these studies, only positive effects of cytokinin were found on shoot growth; reduction in signal transmission results in a smaller and less active shoot apical meristem (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004). The phenotypes we observed in the *arr1,10,12* mutant are very similar to those found in the *cre1 ahk3* double mutant and are consistent with a partial reduction in the level of signal transmission through the cytokinin signal transduction pathway. The other members of subfamily I are likely to mediate the remaining transmission of the cytokinin signal in the *arr1,10,12* mutant, which may explain why we did not observe the reduced activity of root and shoot meristems seen in the triple receptor knockout.

Genetic Interactions between the Type-B ARRs

The effects of the type-B *arr* mutations were generally additive, providing evidence for functional overlap within subfamily I. But the *arr2* mutation displayed more complex interactions with the other *arr* mutations. In root elongation assays, the cytokinin sensitivity of the three *arr2* single insertion alleles was not altered significantly compared with that of the wild type, but in combination with other *arr* alleles both additive and antagonistic effects on root elongation were observed. These effects of *ARR2* are of particular interest given the high degree of similarity between *ARR2* and *ARR1*, which, based on phylogenetic analysis, are likely to have arisen from a recent genome duplication event (Mason et al., 2004). Previous analysis of the type-A *ARR*s has also revealed that some members of gene pairs may have antagonistic relationships (To et al., 2004). The precise mechanism for the apparently antagonist effect is not known, but it could be explained by compensatory changes in the expression of other members of the type-B *ARR* gene family in the mutant backgrounds containing *arr2*.

The effects of *arr2* were not limited to roots. Although single *arr2* mutants exhibited normal hypocotyl growth in dark-grown seedlings, in combination with other *arr* alleles the hypocotyls of dark-grown seedlings were shorter. Of the mutant combinations tested, only those containing *arr2* displayed this phenotype, suggesting a role for type-B ARRs in the regulation of hypocotyl elongation, in which ARR2 may play the predominant role. Hass et al. (2004) previously found that overexpression of an activated form of ARR2 resulted in a decrease in the hypocotyl length of

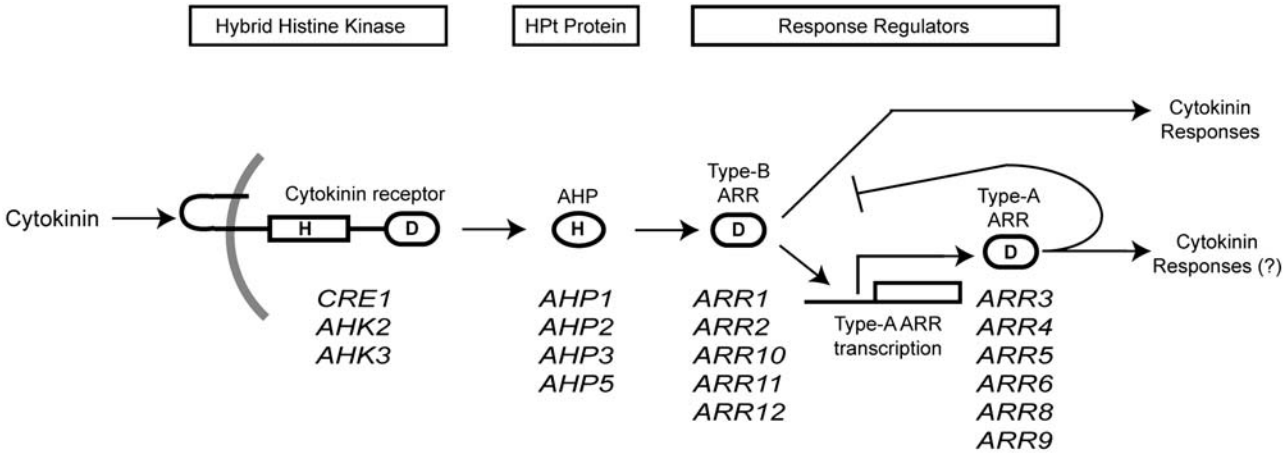

Figure 9. Model for Cytokinin Signal Transduction.

Cytokinin signal transduction is mediated by a multistep phosphorelay that transfers the signal from the plasma membrane to the nucleus. Type-B ARRs act as transcription factors, one target being genes encoding type-A ARRs. The type-A ARRs feed back to inhibit their own transcription and may also mediate other cytokinin responses. The roles of the listed cytokinin receptors (Inoue et al., 2001; Ueguchi et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004), AHPs (C.E. Hutchison, J. Li, G.E. Schaller, J.M. Alonso, J.R. Ecker, and J.J. Kieber, unpublished data), type-B response regulators (Sakai et al., 2001; Hass et al., 2004), and type-A response regulators (To et al., 2004) in cytokinin signal transduction have been confirmed by the analysis of T-DNA insertion mutations.

dark-grown seedlings. This effect is the opposite of what one would predict based on the *arr* mutant phenotype and may be the result of complex interactions with other members of the ARR family. Nevertheless, both studies suggest a role for ARR2 in the regulation of hypocotyl length.

Role in Ethylene Signaling

A subset of the ethylene receptors are His kinases (Schaller et al., 2002), thereby raising the possibility that ethylene receptors participate in a phosphorelay that uses two-component signaling elements such as the ARRs. ARR2 was recently proposed to mediate ethylene signaling based, in part, on the analysis of mutant phenotypes (Hass et al., 2004). Hass et al. (2004) reported a reduction in the ethylene sensitivity of seedlings containing an *arr2* loss-of-function mutation. By contrast, we observed no significant difference from the wild type in the seedling ethylene response when we tested three independent *arr2* insertion mutants, including the same mutant examined by Hass et al. (2004). This difference in results could arise from differences in growth conditions, for, unlike Hass et al. (2004), we used a medium containing Murashige and Skoog (MS) salts and inhibitors of ethylene biosynthesis.

Hass et al. (2004) also report that an activated form of ARR2 induces a constitutive ethylene response–like phenotype in seedlings. Although exhibiting a shortened hypocotyl, the seedlings lack the pronounced apical hook and shortened root that one would expect upon activation of the ethylene signaling pathway (Hass et al., 2004). Our data also support a role for ARR2 in hypocotyl elongation, but given the lack of an effect upon other known ethylene responses, we find no clear evidence supporting a role for ARR2 in mediating ethylene signal transduction.

Role of Type-B ARRs in the Cytokinin Signaling Pathway

The mutant phenotypes observed in the type-B *arr* mutants are likely to result from changes in the normal transcription pattern mediated by the type-B ARRs. The type-B response regulators function in the final step of histidyl-aspartyl phosphorelays and are thought to be transcription factors, based on several lines of evidence. For example, several members of subfamily I (ARR1, ARR2, ARR10, and ARR11) can bind specific DNA sequences and enhance transcription (Sakai et al., 2000, 2001; Hosoda et al., 2002; Imamura et al., 2003). In addition, studies of subcellular localization indicate that members of subfamily I (ARR1, ARR2, ARR10, and ARR12) are localized to the nucleus (Sakai et al., 2000; Hwang and Sheen, 2001; Imamura et al., 2001; Lohrmann et al., 2001; Hosoda et al., 2002; Mason et al., 2004). Among the transcriptional targets for the type-B ARRs are members of the type-A *ARR* family. A previous study of an *arr1* mutant indicated reduced expression of *ARR6* in both the absence and presence of exogenous cytokinin (Sakai et al., 2001). Transient expression assays, using a protoplast-based system, indicated that ARR1, ARR2, and ARR10 were all capable of stimulating the expression of an *ARR6*-reporter construct (Hwang and Sheen, 2001).

Previous mutational analysis has provided evidence that ARR1 and ARR2 contribute to the cytokinin response (Sakai et al., 2001; Hass et al., 2004). The phenotypes observed in these single mutants, however, were weak, involving subtle changes in the response to exogenous cytokinin. Our studies provide evidence that five type-B ARRs of subfamily I participate in the regulation of cytokinin signaling. Evidence for a central role in the establishment of the cytokinin response comes from the study of higher order mutants: the triple mutant *arr1,10,12* exhibits almost complete insensitivity to exogenous cytokinin in several assays. Together, these data indicate that the type-B ARRs function as transcription factors that operate at the last step in the primary cytokinin response pathway. Consistent with this, we find that the basal expression and cytokinin induction of multiple type-A *ARR*s is reduced in the type-B mutant *arr1,10,12*.

In Figure 9, a model for cytokinin signal transduction is shown that incorporates results from this study along with other genetic studies that used loss-of-function analysis. These genetic studies show that the AHPs, the type-B ARRs, and the type-A ARRs likely play a central role in transducing and modulating the cytokinin signal. In fact, to date, no AHP or ARR has been found to function in a pathway independent of cytokinin signal transduction. This raises the intriguing question of the role of these two-component signaling elements in mediating transduction from the other His kinase receptors such as AHK1 and CKI1 and the ethylene receptors. If the type-B ARRs play a role downstream of these other receptors, they may provide a mechanism for integration with the cytokinin signaling pathway or they may, in combination with other transcriptional elements, allow for transcriptional regulation to be tailored to the specific input signal.

METHODS

Isolation of *arr* Mutants

Arabidopsis thaliana T-DNA insertion populations in ecotypes Col and Ws were screened for insertions into type-B response regulators using PCR-based methods as described (Krysan et al., 1996, 1999; Alonso et al., 2003). Additional lines were screened by DNA sequence comparison as T-DNA insertion site information was made available by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/index.html). Gene-specific primers were used in combination with T-DNA–specific primers to identify and confirm T-DNA insertions by PCR (see Supplemental Tables 1 and 2 online). These primer combinations as well as gene-specific primer combinations flanking the sites of T-DNA insertion were used to distinguish heterozygous from homozygous plants. Only lines homozygous for T-DNA insertions were used in subsequent assays.

Double insertion mutants were generated by crossing two single insertion mutants. Triple mutants were generated by crossing a single mutant with a double mutant or by crossing two double mutants with one shared allele. Segregating progeny homozygous for all alleles were identified in the subsequent F2 and F3 generations.

Growth Conditions

Unless stated otherwise, seedlings were grown using the following protocol. Seeds were surface-sterilized in 30% bleach and plated onto medium containing 0.8% (w/v) phytoagar (Research Products International) 1× MS medium containing Gamborg's vitamins (Phytotechnology Laboratories), 1% (w/v) sucrose, and 0.05% (w/v) MES, pH 5.7. The seeds were cold-treated for 3 d in the dark at 4°C before being transferred to continuous white light (~100 μE) at 22°C.

Cytokinin Response Assays

Arabidopsis seedlings were grown on vertical plates containing either 100 nM BA or 0.1% DMSO vehicle control and then photographed. Root lengths were marked on the plates after 4 d of growth, and root growth from days 5 through 8 was measured using ImageJ software (version 1.32; National Institutes of Health). Root growth from days 5 through 8 was used because this eliminates differences caused by variation in the time of germination and because we have observed that the rate of growth accelerates during the first 3 d but is more uniform afterward. Data for the cytokinin dose–response experiments were obtained using the same procedure. The seedlings from the dose–response assays were used for lateral root analysis, in which the number of protruding lateral roots on 8-d-old plants grown on 0, 10, or 100 nM BA was counted with the aid of a Leica MZ16 microscope. Statistical analysis was performed using the SAS system, version 8.2 (SAS Institute). For Figures 2 and 3, Duncan's multiple range test was performed among the means on the analysis of variance (Duncan, 1975).

Callus Induction/Greening Assay

Arabidopsis seedlings were grown vertically in the dark for 3 d and then transferred to dim light (~5 μE) to produce elongated and firm hypocotyls. Hypocotyls were excised and transferred to 1× MS medium with Gamborg's vitamins, 2% (w/v) sucrose, 0.05% (w/v) MES, pH 5.7, and 0.4% (w/v) phytoagar containing 0.5 mg/L 2,4-D and 50 μg/L kinetin. After 4 d, hypocotyl segments were transferred to medium containing 0.2 mg/L indole-3-butyric acid and *t*-zeatin ranging from 0 to 8 mg/L. After 3 weeks, representative callus from each plant line and cytokinin concentration was selected and arranged for a composite photograph.

Expression Analysis of Cytokinin Primary Response Genes

Seedlings were grown in the dark on filter paper overlaid on agar plates with growth medium, and after 4 d of growth, the filters with seedlings were transferred for 45 min to 1× MS medium and 1% (w/v) sucrose containing 10 μM BA or DMSO as a vehicle control. Samples were frozen in liquid nitrogen, and total RNA was extracted using the RNeasy plant mini kit (Qiagen). To prepare the RNA probe, 40 μg of total RNA was treated with DNaseI (Roche Diagnostics) and reverse-transcribed using 1200 units of Superscript III (Invitrogen) with 5 μM gene-specific RT primers and 60 μCi of [α-^{32}P]dCTP (6000 Ci/mmol; Amersham Biosciences) for 80 min at 50°C. A cold chase with 8 μM deoxynucleotide triphosphates was done for 10 min followed by RNaseH treatment.

To prepare type-A slot blots, gene-specific regions (~130 bp each) of the 10 type-A genes were cloned into TOPO pCR2.1 vectors (Invitrogen) and then amplified using BD titanium Taq (BD Biosciences). Approximately 60 ng of DNA per slot was slot-blotted onto a Zeta-Probe membrane (Bio-Rad) according to the manufacturer. Blots were tested for cross-hybridization between the type-A family members by hybridizing a full-length cDNA probe for each of the 10 type-A genes to independent blots and quantifying the percentage hybridization to the closest homolog. Cross-hybridization was no greater than 9%. *UBQ* was included on the blots as an internal control.

The probe was hybridized to the blot for 48 h and washed according to the Zeta-Probe (Bio-Rad 162-0165) oligonucleotide protocol. Blots were then exposed to a phosphor screen (Amersham Biosciences) and quantified using a Storm 840 PhosphorImager and ImageQuant 5.0 software (Molecular Dynamics).

Analysis of Ethylene Response

To examine the triple response of seedlings to ethylene (Chen and Bleecker, 1995; Gamble et al., 2002), seeds were grown on vertical plates containing 0.5× MS basal medium with Gamborg's vitamins (Phytotechnology Laboratories), 0.05% (w/v) MES, pH 5.7, 0.8% (w/v) agar, and 5 μM aminoethylvinyl-Gly to inhibit ethylene biosynthesis by the seedlings. After a 3-d cold treatment at 4°C, plates were brought to 22°C and exposed to light for 15 h. Plates were then placed in 22-liter chambers, and seedlings were grown in the dark in the presence of ethylene at the desired concentration. To examine the growth of seedlings in the absence of ethylene, hydrocarbon-free air was passed through the chamber to remove trace amounts of ethylene synthesized by the seedlings. Seedlings were examined after 3.5 d, time 0 corresponding to when the plates were removed from 4°C and brought to 22°C. To measure hypocotyl length, seedlings on the plates were scanned using an Epson 1670 scanner and Photoshop (Adobe Systems), and measurements were made using ImageJ software (version 1.32; National Institutes of Health). Statistical analysis was performed using the SAS system, version 8.2 (SAS Institute).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the type-B ARRs of subfamily I are as follows: *ARR1* (At3g16857), *ARR2* (At4g16110), *ARR10* (At4g31920), *ARR11* (At1g67710), *ARR12* (At2g25180), *ARR14* (At2g01760), and *ARR18* (At5g58080).

Supplemental Data

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

Supplemental Table 1. T-DNA Insertion Sites and Primers Used.

Supplemental Table 2. Primer Sequences.

Supplemental Figure 1. Slot Blot Analysis for Effect of BA upon Expression of Type-A *ARR*s in the Wild Type and *arr1,10,12*.

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