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Type B Response Regulators of *Arabidopsis* Play Key Roles in Cytokinin Signaling and Plant Development [W]

Rebecca D. Argyros,[a,1] Dennis E. Mathews,[b,1] Yi-Hsuan Chiang,[a,1] Christine M. Palmer,[a] Derek M. Thibault,[a] Naomi Etheridge,[a,2] D. Aaron Argyros,[a] Michael G. Mason,[a,3] Joseph J. Kieber,[c] and G. Eric Schaller[a,4]

[a] Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755
[b] Department of Plant Biology, University of New Hampshire, Durham, New Hampshire 03824
[c] Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

The type B *Arabidopsis* Response Regulators (ARRs) of *Arabidopsis thaliana* are transcription factors that act as positive regulators in the two-component cytokinin signaling pathway. We employed a mutant-based approach to perform a detailed characterization of the roles of ARR1, ARR10, and ARR12 in plant growth and development. The most pronounced phenotype was found in the *arr1-3 arr10-5 arr12-1* triple loss-of-function mutant, which showed almost complete insensitivity to high levels of exogenously applied cytokinins. The triple mutant exhibited reduced stature due to decreased cell division in the shoot, enhanced seed size, increased sensitivity to light, altered chlorophyll and anthocyanin concentrations, and an aborted primary root with protoxylem but no metaxylem. Microarray analysis revealed that expression of the majority of cytokinin-regulated genes requires the function of ARR1, ARR10, and ARR12. Characterization of double mutants revealed differing contributions of the type B ARRs to mutant phenotypes. Our results support a model in which cytokinin regulates a wide array of downstream responses through the action of a multistep phosphorelay that culminates in transcriptional regulation by ARR1, ARR10, and ARR12.

INTRODUCTION

Cytokinins are adenine derivatives that play essential roles in regulating plant growth and development (Mok, 1994; Haberer and Kieber, 2002; Kakimoto, 2003). They were initially discovered and named based on their ability to promote cell division of plant cells grown in culture, where they also regulate chloroplast development and shoot initiation (Miller et al., 1956; Skoog and Miller, 1957; Mok, 1994; Haberer and Kieber, 2002; Kakimoto, 2003). In plants, they regulate cell division and metabolism, stimulate chloroplast development, modulate shoot and root development, and delay senescence (Mok, 1994; Haberer and Kieber, 2002; Kakimoto, 2003).

There is now convincing evidence that cytokinin signal transduction is mediated by a two-component system (Hwang and Sheen, 2001; Haberer and Kieber, 2002; Heyl and Schmülling, 2003; Kakimoto, 2003; To and Kieber, 2008). Two-component systems were originally identified in bacteria, and in their simplest form they involve a receptor kinase that autophosphorylates a conserved His residue in response to an environmental stimulus (Mizuno, 1997; Stock et al., 2000). This phosphate is then transferred to a conserved Asp residue within the receiver domain of a response regulator (RR). Phosphorylation of the RR modulates its ability to mediate downstream signaling in the pathway. Of particular relevance to cytokinin signaling is a permutation of the two-component system known as the multistep phosphorelay (Swanson et al., 1994; Schaller, 2000). The multistep phosphorelay makes use of three components: a hybrid receptor kinase that contains both His kinase and receiver domains in one protein, a His-containing phosphotransfer protein, and a RR. In these multistep phosphorelays, the phosphate is transferred in sequence from His to Asp to His to Asp.

Cytokinin signal transduction makes use of all of the elements of a multistep phosphorelay. The cytokinin receptor family of *Arabidopsis thaliana* is composed of the three hybrid His kinases AHK2 (for *ARABIDOPSIS* HISTIDINE KINASE2), AHK3, and AHK4; AHK4 is also known as CRE1 (for CYTOKININ RESPONSE1) or WOODEN LEG1 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001; Kakimoto, 2003). According to the current model, the receptors are activated in response to cytokinins and initiate signaling through a multistep phosphorelay. The phosphorelay incorporates both His-containing phosphotransfer proteins (the five-member *ARABIDOPSIS* HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEIN [AHP] family in *Arabidopsis*) (Hwang and Sheen, 2001; Hutchison et al., 2006) and a specific family of RRs known as the type B *ARABIDOPSIS* RESPONSE REGULATORs (ARRs) (Sakai et al., 2001; Mason et al., 2005). The cytokinin signal is relayed from membrane to nucleus, where the type B ARRs then induce transcription of a second class of RRs known as the type A ARRs

[1] These authors contributed equally to this work.
[2] Current address: School of Molecular and Microbial Sciences, University of Queensland, St. Lucia, Queensland 4072, Australia.
[3] Current address: School of Integrative Biology, University of Queensland, St. Lucia, Queensland 4072, Australia.
[4] Address correspondence to george.e.schaller@dartmouth.edu.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions to Authors (www.plantcell.org) is: G. Eric Schaller (george.e.schaller@dartmouth.edu).
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(Hwang and Sheen, 2001; Sakai et al., 2001). The type A ARRs act as negative regulators of the initial signal transduction pathway and may also regulate downstream responses (To et al., 2004). Mutational analysis supports roles for these signaling elements in many cytokinin-mediated processes (Mahonen et al., 2000; Higuchi et al., 2004; Nishimura et al., 2004; To et al., 2004; Mason et al., 2005; Hutchison et al., 2006; Mähönen et al., 2006; Yokoyama et al., 2007).

According to this model, the type B ARRs play a pivotal role in the early response of plants to cytokinin. The type B ARRs differ from the type A ARRs in that the type B ARRs contain long C-terminal extensions with a Myb-like DNA binding domain referred to as the GARP domain (Imamura et al., 1999; Hosoda et al., 2002). Multiple lines of evidence support the role of the type B ARRs as transcription factors (Sakai et al., 2000, 2001; Imamura et al., 2001, 2003; Lohrmann et al., 2001; Hosoda et al., 2002; Mason et al., 2004, 2005; Rashotte et al., 2006). The 11 type B ARRs of *Arabidopsis* fall into three subfamilies based on phylogenetic analysis: subfamily 1 contains seven members and subfamilies 2 and 3 each contain two members (Mason et al., 2004). Mutational analysis indicates that at least five subfamily 1 members mediate cytokinin signaling (Mason et al., 2005; Yokoyama et al., 2007; Ishida et al., 2008).

Here, through further mutational analysis, we define roles for type B ARRs in the regulation of vascular development, light sensitivity, chlorophyll production, hypocotyl elongation, and cell division in the root and shoot. Microarray analysis of a null mutant for subfamily 1 members *ARR1*, *ARR10*, and *ARR12* demonstrates that these type B mutations affect the majority of the genes under cytokinin regulation of expression. These studies complement similar studies performed on the cytokinin receptors and AHPs (Higuchi et al., 2004; Nishimura et al., 2004; Hutchison et al., 2006; Riefler et al., 2006), and demonstrate that cytokinin regulates a wide array of downstream responses through the action of a multistep phosphorelay that culminates in transcriptional regulation by the type B ARRs.

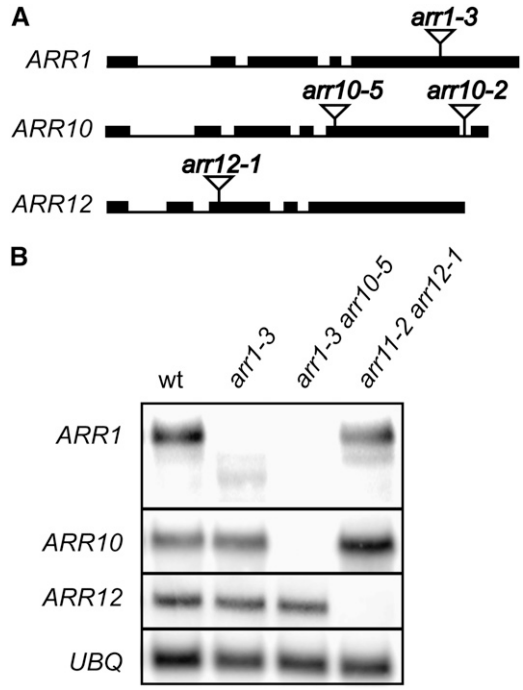

Figure 1. Characterization of the *arr1-3*, *arr10-5*, and *arr12-1* T-DNA Insertion Alleles.

(A) Positions of T-DNA insertions in genes encoding *ARR1*, *ARR10*, and *ARR12*. Black boxes represent exons of the coding sequence, lines represent introns, and triangles indicate positions of T-DNA insertions.
(B) RNA gel blot analysis. Poly(A) RNA from the wild type, *arr1-3*, an *arr1-3 arr10-5* double mutant, and an *arr11-2 arr12-1* double mutant was analyzed by RNA gel blot analysis using *ARR1*, *ARR10*, *ARR12*, or a control ubiquitin (*UBQ*) probe as indicated.

RESULTS

T-DNA Insertion Alleles of *ARR1*, *ARR10*, and *ARR12*

We previously identified T-DNA insertion alleles for six of the seven type B ARR genes that compose subfamily I, including alleles for *ARR1*, *ARR10*, and *ARR12* (Mason et al., 2005). Although the *arr10-2* allele affected cytokinin responsiveness in combination with other *arr* mutant alleles, we hypothesized that it was not a null allele based on the position of the insertion in the last intron (Figure 1A). Therefore, we identified and isolated the *arr10-5* insertion allele from the Salk population, the insertion site of which suggested that it could represent a null allele. To examine genetic interactions among the *arr* mutations, double and triple mutant combinations were generated of the *arr10-5* allele with the *arr1-3* and *arr12-1* alleles.

No full-length transcripts were detected for the mutant alleles based on RNA gel blot analysis (Figure 1B). Also, no partial transcripts were detected for the *arr10-5* and *arr12-1* mutant alleles. A weakly expressed partial transcript for *arr1-3* was detected in the *arr1-3* background but not in the *arr1-3 arr10-5* background. These results suggest that any truncated messages produced from the region upstream of the insertion sites have reduced stability. The lack of full-length transcripts, coupled with reduced expression, is consistent with *arr1-3*, *arr10-5*, and *arr12-1* being strong hypomorphic or null alleles.

Effects of Type B *ARR* Mutants on Root Elongation

β-Glucuronidase (GUS) fusion analyses (Mason et al., 2004) and transcriptional profiling (see Supplemental Figure 1 online) (Birnbaum et al., 2003) support overlapping expression of *ARR1*, *ARR10*, and *ARR12* in the primary root tip. To assess the roles of *ARR1*, *ARR10*, and *ARR12* in cytokinin signaling of the root, we examined the primary root growth of *arr* mutants in the presence and absence of cytokinin (Figures 2A and 2B). Single mutants are similar to wild-type seedlings in their responsiveness to the cytokinin 6-benzylaminopurine (BA), although some slight differences are sometimes observed (Sakai et al., 2001; Mason et al., 2005). Double mutants show varying levels of reduced sensitivity to BA. The *arr1-3 arr12-1* double mutant is the least sensitive to BA, followed by *arr10-5 arr12-1* and then *arr1-3 arr10-5*. None of the double mutants display the level of reduced sensitivity

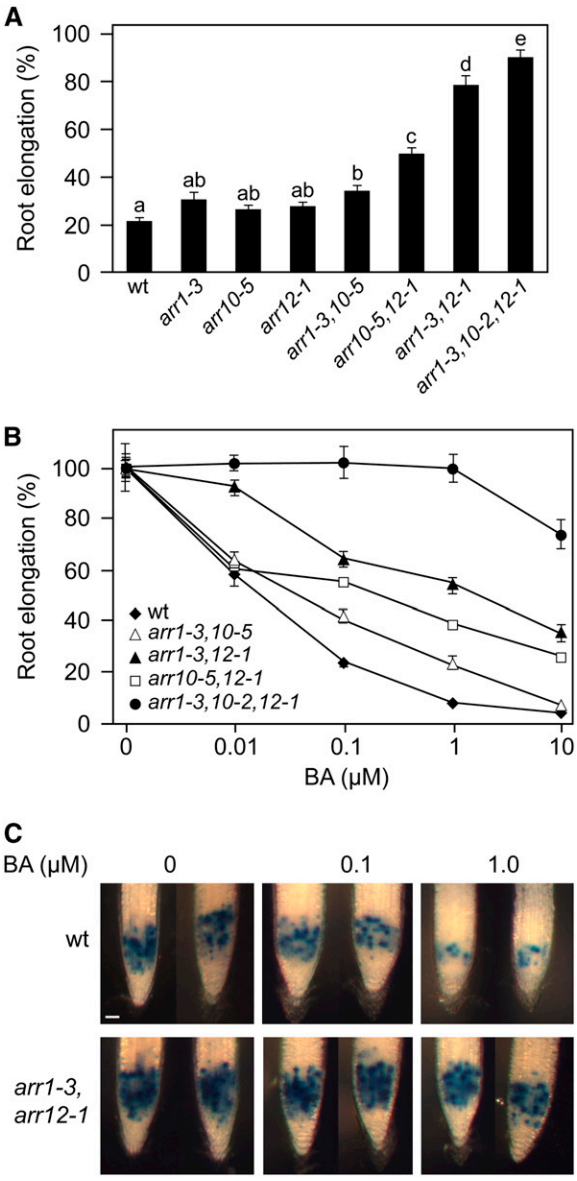

Figure 2. Primary Root Characteristics of T-DNA Insertion Mutants.

(A) Root elongation responsiveness to cytokinin of *arr* mutants. Seedlings were grown on vertical plates supplemented with 1 μM BA or a DMSO vehicle control under constant light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 22°C. Increase in root length from day 4 through day 7 was measured. The root elongation of each line receiving cytokinin treatment is expressed as a percentage of its DMSO control. Error bars represent SE ($n > 10$). The triple mutant *arr1-3 arr10-2 arr12-1* is abbreviated *arr1-3,10-2,12-1*; double mutants are similarly abbreviated. Genotypes 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 ($P < 0.05$). Those genotypes having the same letter exhibit no significant difference in their responsiveness to cytokinin.

(B) Dose response for the effect of BA on *arr* mutants grown under constant low light (25 $\mu E \cdot m^{-2} \cdot s^{-1}$). Error bars represent SE ($n > 18$).

(C) CycB1;1:GUS expression in wild-type and *arr1-3 arr12-1* primary roots grown for 8 d on the indicated concentrations of BA. Bar = 0.1 mm.

observed in the *arr1-3 arr10-2 arr12-1* triple mutant that contains the hypomorphic *arr10* allele (Figures 2A and 2B) (Mason et al., 2005). As described below, the *arr1-3 arr10-5 arr12-1* triple mutant with the *arr10-5* allele results in severe primary root abnormalities and is not directly comparable to the other mutants in this assay. These results are consistent with a functional overlap of *ARR1*, *ARR10*, and *ARR12* in mediating the cytokinin root growth response, but they also indicate that they have varying contributions to this response potentially related to differences in their root expression patterns.

To gain insight into the basis for the difference in primary root growth, we examined the expression of a *CycB1;1:GUS* reporter in wild-type and *arr1-3 arr12-1* backgrounds (Figure 2C). This reporter produces a labile cyclin-GUS fusion protein that is destroyed at the end of mitosis, thereby serving as a marker for cell division (Colon-Carmona et al., 1999). In wild-type plants, GUS staining is observed in the meristematic region of the primary root, but staining is substantially reduced when plants are grown on BA (Figure 2C). Thus, the cytokinin-induced reduction in primary root growth of wild-type seedlings correlates with a reduction in cell division. In contrast with the wild type, cytokinin has substantially less effect on GUS staining in the *arr1-3 arr12-1* line, which remains at higher levels than the wild type at both 0.1 and 1.0 μM BA. Thus, in the *arr1-3 arr12-1* line, cytokinin is less able to inhibit cell division, resulting in increased root growth compared with the wild type.

Altered Primary Root Development in the *arr1-3 arr10-5 arr12-1* Triple Mutant

The *arr1-3 arr10-5 arr12-1* triple mutant produces a short narrow primary root that prematurely ceases growth (Figures 3A to 3C). This contrasts with the single and double mutant combinations of *arr1-3*, *arr10-5*, and *arr12-1* as well as with the *arr1-3 arr10-2 arr12-1* triple mutant (Mason et al., 2005), all of which produce primary roots similar in length to the wild type (Figure 3C). Analysis of root elongation over time indicates that the primary root of the *arr1-3 arr10-5 arr12-1* mutant elongates normally for the first 2 d following imbibition, although growth almost ceases by day 4 (Figure 3C). Even though the primary root of *arr1-3 arr10-5 arr12-1* prematurely terminates, it demonstrates resistance to the BA-mediated inhibition of root growth (Figure 3A). Growth and development of the mutant are initially slowed compared with the wild type, but the mutant is able to continue growth upon production of adventitious roots, which do not exhibit the premature termination observed in the primary root. Use of the *CycB1;1:GUS* reporter in the *arr1-3 arr10-5 arr12-1* line confirms that the meristematic region of adventitious roots contains actively dividing cells, although this activity is lost in the primary root tip (Figure 3D).

Premature termination of primary root growth is also observed in mutant lines of cytokinin receptors and AHPs (Higuchi et al., 2004; Nishimura et al., 2004; Hutchison et al., 2006). Because these lines also exhibit altered vascular development (Hutchison et al., 2006; Kuroha et al., 2006; Mähönen et al., 2006; Yokoyama et al., 2007), we characterized the vasculature of type B *ARR* mutants (Figures 3E and 3F). In wild-type *Arabidopsis* roots, xylem vessels are found as more centrally located metaxylem

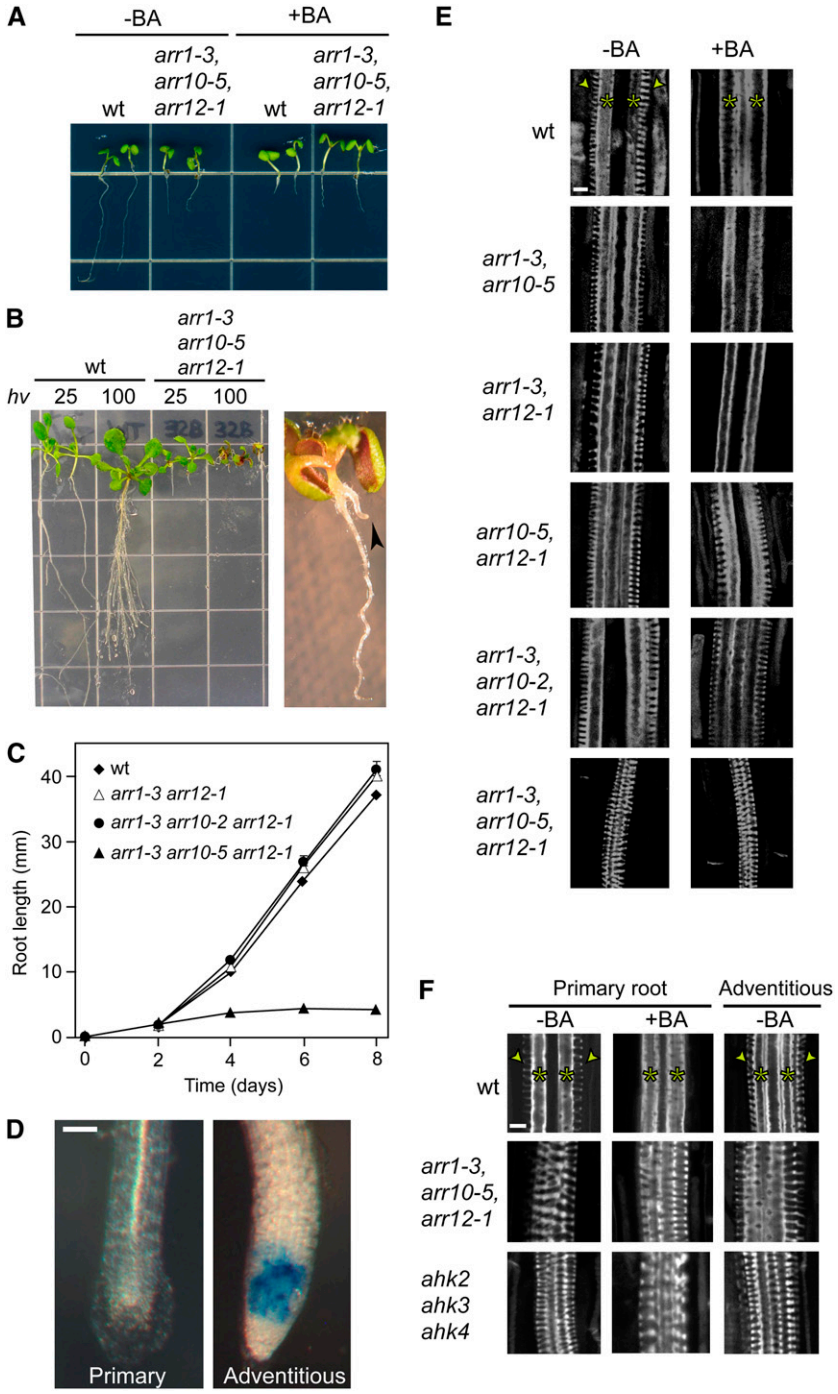

Figure 3. The *arr1-3 arr10-5 arr12-1* Triple Mutant Exhibits a Short Primary Root with Altered Vascular Development.

(A) Wild-type and *arr1-3 arr10-5 arr12-1* seedlings grown for 8 d in the absence (–) and presence (+) of 10 μM at 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light. Squares on the plate are 13.3 mm per side.

(B) Wild-type and *arr1-3 arr10-5 arr12-1* seedlings grown for 18 d at 25 and 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light (hv). A closeup of an *arr1-3 arr10-5 arr12-1* seedling grown under 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ light is shown at right; note the adventitious root (arrowhead) and the purple curled cotyledons. Squares on the plate are 13.3 mm per side.

(C) Time course for primary root growth of wild-type and *ARR* mutant seedlings (100 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light; similar results were obtained for seedlings grown under 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light).

(D) CycB1;1:GUS expression in primary and adventitious *arr1-3 arr10-5 arr12-1* roots from a 29-d-old seedling. Bar = 0.1 mm.

cells surrounded by protoxylem cells. Protoxylem vessels have annular wall thickening, which results in a striated appearance when examined with the microscope. Primary roots of the *arr* double mutants are similar to wild-type roots in that they have both metaxylem and protoxylem. By contrast, all of the vascular cell files in the primary root of the *arr1-3 arr10-5 arr12-1* triple mutant are specified as protoxylem, such that the vasculature is similar to that found in the *ahk2 ahk3 ahk4* triple mutant, which lacks all three members of the cytokinin receptor family (Mähönen et al., 2006; Yokoyama et al., 2007). Vasculature in the adventitious roots of the *arr1-3 arr10-5 arr12-1* triple mutant contains both protoxylem and metaxylem, unlike the adventitious roots of the *ahk2 ahk3 ahk4* triple mutant, which contain predominantly protoxylem.

We also examined the effects of cytokinin on vascular morphogenesis of the *arr* double and triple mutants (Figures 3E and 3F). Treatment of wild-type plants with cytokinin alters vascular morphogenesis, so that the primary and adventitious roots lack protoxylem and contain only metaxylem (Figures 3E and 3F) (Mähönen et al., 2006). The *arr* double mutants vary in how BA affects vascular morphogenesis. The primary root of the *arr10-5 arr12-1* double mutant is resistant to cytokinin and still contains protoxylem when treated with BA (Yokoyama et al., 2007). By contrast, the primary roots of both *arr1-3 arr10-5* and *arr1-3 arr12-1* double mutants are responsive to cytokinin, containing only metaxylem when treated with BA. Thus, the double mutants vary in their resistance to BA depending on what physiological response is being assayed: *arr10-5 arr12-1* shows greater resistance when evaluated based on vascular morphogenesis (Figure 3E), but *arr1-3 arr10-5* shows greater resistance when evaluated based on root elongation (Figures 2A and 2B). The primary root of the *arr1-3 arr10-5 arr12-1* triple mutant consists only of protoxylem regardless of BA treatment, as does the primary root of the *ahk2 ahk3 ahk4* triple mutant (Figures 3E and 3F).

Effects of *ARR* Mutations on Hypocotyl Elongation and on the Deetiolation Response to Cytokinin

Cytokinin treatment of wild-type seedlings results in a reduction in hypocotyl elongation (Su and Howell, 1995). We examined this response in seedlings grown under conditions of reduced light (25 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Figure 4A) or in darkness (Figure 4B). Higher order *arr* mutants are resistant to cytokinin to varying degrees in the hypocotyl growth response assay. The *arr1-3 arr10-5 arr12-1* triple mutant exhibits the greatest cytokinin resistance and is virtually insensitive to all of the BA concentrations tested up to 10 μM BA. The *arr1-3 arr12-1* double and *arr1-3 arr10-2 arr12-1* triple mutants also demonstrate substantially reduced sensitivity to cytokinin in the hypocotyl growth response assay. Similar results were found under both light conditions examined.

When dark-grown seedlings are treated with cytokinin for extended periods of time, the seedlings acquire some of the same developmental characteristics induced by exposure to light (Chory et al., 1994). These include the inhibition of hypocotyl elongation, the promotion of cotyledon expansion, and the induction of leaf development. This deetiolation response was examined in 14-d-old wild-type and mutant seedlings (Figure 4C). Single mutants show a response similar to that observed in the wild type. Double mutants have longer hypocotyls and roots than the wild type but still exhibit effects of cytokinin on cotyledon expansion and leaf development. The *arr1-3 arr10-5 arr12-1* triple mutant lacks the general deetiolation response, exhibiting a longer hypocotyl than the wild type or the lower order mutants; it also shows decreases in cotyledon expansion and leaf development.

Shoot Phenotypes of Type B *arr* Mutants

The rosette diameter of the *arr1-3 arr10-5 arr12-1* triple mutant is smaller than that of the wild type but larger than that of the *ahk2 ahk3 ahk4* cytokinin receptor mutant (Figure 5A). Initial growth of the *arr1-3 arr10-5 arr12-1* triple mutant is more sensitive to light intensity than is wild-type growth (described in the section below), but the difference in rosette size between the mutant and the wild type is observed even under optimized growth conditions. Thus, ARR1, ARR10, and ARR12 are necessary but not sufficient for normal shoot growth, as their loss results in reduced growth compared with the wild type but fails to fully mimic the cytokinin receptor triple mutant.

Leaves of the *arr1-3 arr10-5 arr12-1* triple mutant are reduced in length and width compared with wild-type leaves (Figure 5A). To determine the basis for the reduced leaf size, we examined the epidermal cell size on the adaxial surface of the fifth true leaf after full expansion (Figure 5B). The wild type and the mutant have a similar cell size, but the mutant has substantially fewer cells per leaf. This is consistent with a role for the type B ARRs in regulating cell division in the shoot meristematic region. A D-type cyclin (CycD3;1) was implicated previously in the regulation of cell division by cytokinin in *Arabidopsis* (Riou-Khamlichi et al., 1999; Dewitte et al., 2007), so we examined the expression of *CycD3;1* in wild-type and mutant plants (Figure 5C). Consistent with the decreased cell division in the *arr1-3 arr10-5 arr12-1* mutant, real-time RT-PCR revealed a significant difference ($P < 0.001$, *t* test) in the basal levels of *CycD3;1* expression, which is 43% lower in the mutant compared with the wild type.

Inflorescences and seeds of *arr1-3 arr10-5 arr12-1* also show abnormalities (Figures 5D to 5F). The inflorescence stems of the mutant are reduced in width compared with the wild type (Figure 5D). Flowering of individual inflorescences typically terminates earlier in the triple mutant than in the wild type, and the siliques

Figure 3. (continued).

(E) and **(F)** Fuschin-stained roots showing vasculature. The protoxylem (arrowheads) and metaxylem (asterisks) are indicated on the wild-type root images. Seedlings were grown under 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light for 7 d in the absence (−) or presence (+) of 0.1 μM BA **(E)** or under 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light for 21 d in the absence or presence of 10 μM BA to allow time for adventitious roots to develop **(F)**. The *ahk2 ahk3 ahk4* cytokinin receptor triple mutant is included for comparison. Bars = 5 μm.

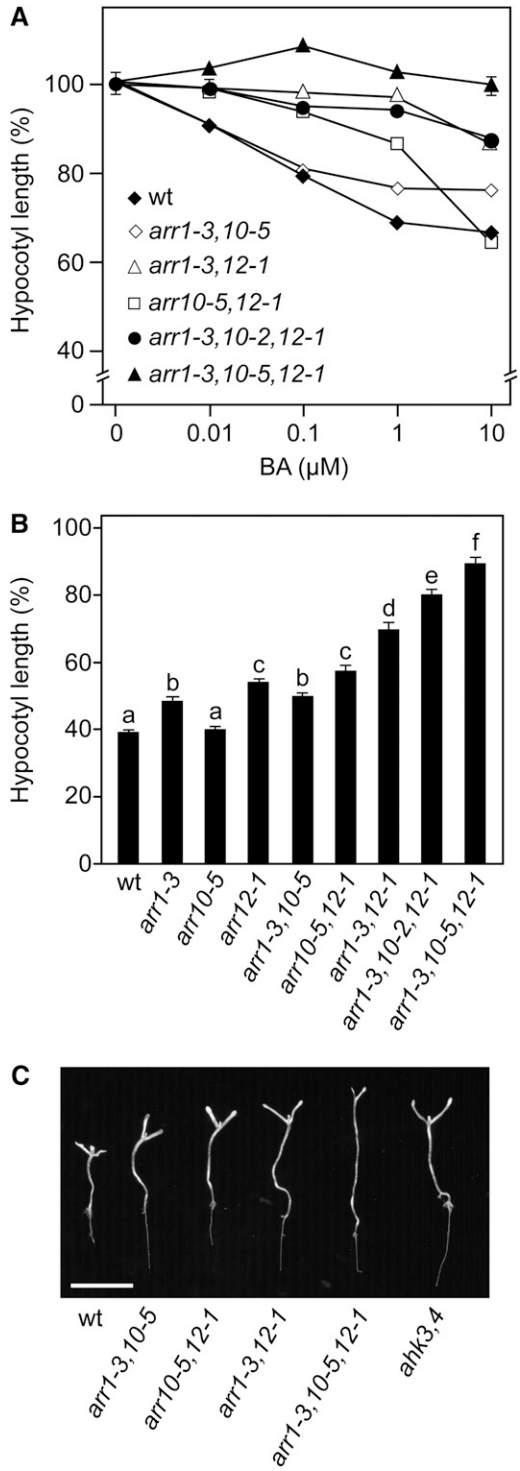

Figure 4. Hypocotyl Elongation and Deetiolation Response of *arr* Mutants to Cytokinin.

(A) Dose response for the effect of BA on hypocotyl elongation of 5-d-old seedlings grown under 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light. The hypocotyl length of each line receiving cytokinin treatment is expressed as a percentage of its DMSO control. The triple mutant *arr1-3 arr10-5 arr12-1* is abbreviated *arr1-3*,*10-5*,*12-1*; other higher order mutants are similarly abbreviated. Error bars indicate SE ($n > 15$).

(B) Effect of 10 μM BA on hypocotyl elongation in 4-d-old dark-grown seedlings. The hypocotyl length of each line receiving cytokinin treatment is expressed as a percentage of its DMSO control. Error bars indicate SE ($n > 16$). Those genotypes having the same letter exhibit no significant difference in their responsiveness to cytokinin.

(C) Deetiolation response in 14-d-old dark-grown seedlings grown on 30 μM BA. Bar = 10 mm.

are shorter than those in the wild type. The seeds produced by the triple mutant are larger than those produced by the wild type, with an ~20% increase in seed length (Figure 5F). The seed size of the single *arr* mutants along with *arr1-3 arr10-5* and *arr10-5 arr12-1* double mutants is similar to that of the wild type, but the *arr1-3 arr12-1* double mutant shows a 14% increase in seed length compared with the wild type. In mutant plants that are grown in short days for >30 d, then moved to long days to induce flowering, floral meristems frequently initiated along the inflorescence in close proximity to each other (Figure 5E).

To examine the shoot responsiveness of *arr1-3 arr10-5 arr12-1* to cytokinin, we grew plants in the absence or presence of 0.1 μM BA. This BA concentration causes the rosettes of wild-type plants to become smaller and paler than those of untreated plants, the paleness resulting from a decrease in chlorophyll levels (Figure 5G) (Mason et al., 2005). In the absence of BA, the triple mutant is smaller and has reduced chlorophyll levels compared with the wild type. Unlike the wild type, there is no decrease in fresh weight or chlorophyll levels upon BA treatment of the triple mutant (Figure 5G). In fact, we observe a slight increase in both fresh weight and chlorophyll levels when the triple mutant is grown on BA, suggesting that the application of exogenous BA may partially compensate for the decreased cytokinin responsiveness of the mutant.

Altered Chlorophyll and Anthocyanin Production in Cytokinin Receptor and Type B ARR Mutants

The *arr1-3 arr10-5 arr12-1* triple mutant shows increased light sensitivity compared with the wild type, the mutant's growth being inhibited by 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ light (Figure 3B). A reduction in light intensity to 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ results in substantially improved growth for the mutant compared with its growth under the higher light intensity. The level of anthocyanin production in the mutant is sharply affected by the light intensity, with the mutant showing an ~20-fold increase in anthocyanin levels compared with the wild type at 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ light (Figures 3B and 6). Growth of the *arr1-3 arr10-5 arr12-1* mutant at 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ results in anthocyanin levels similar to those in the wild type. Chlorophyll levels are reduced in the *arr1-3 arr10-5 arr12-1* mutant, suggesting that a photosynthetic problem underlies the increased light sensitivity. Double *arr* mutants were similar to the wild type in terms of both anthocyanin and chlorophyll contents when grown under 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ light.

To determine if the effect on photopigment production is specific to the type-B mutant or is a more general feature of an impaired cytokinin signaling pathway, we examined

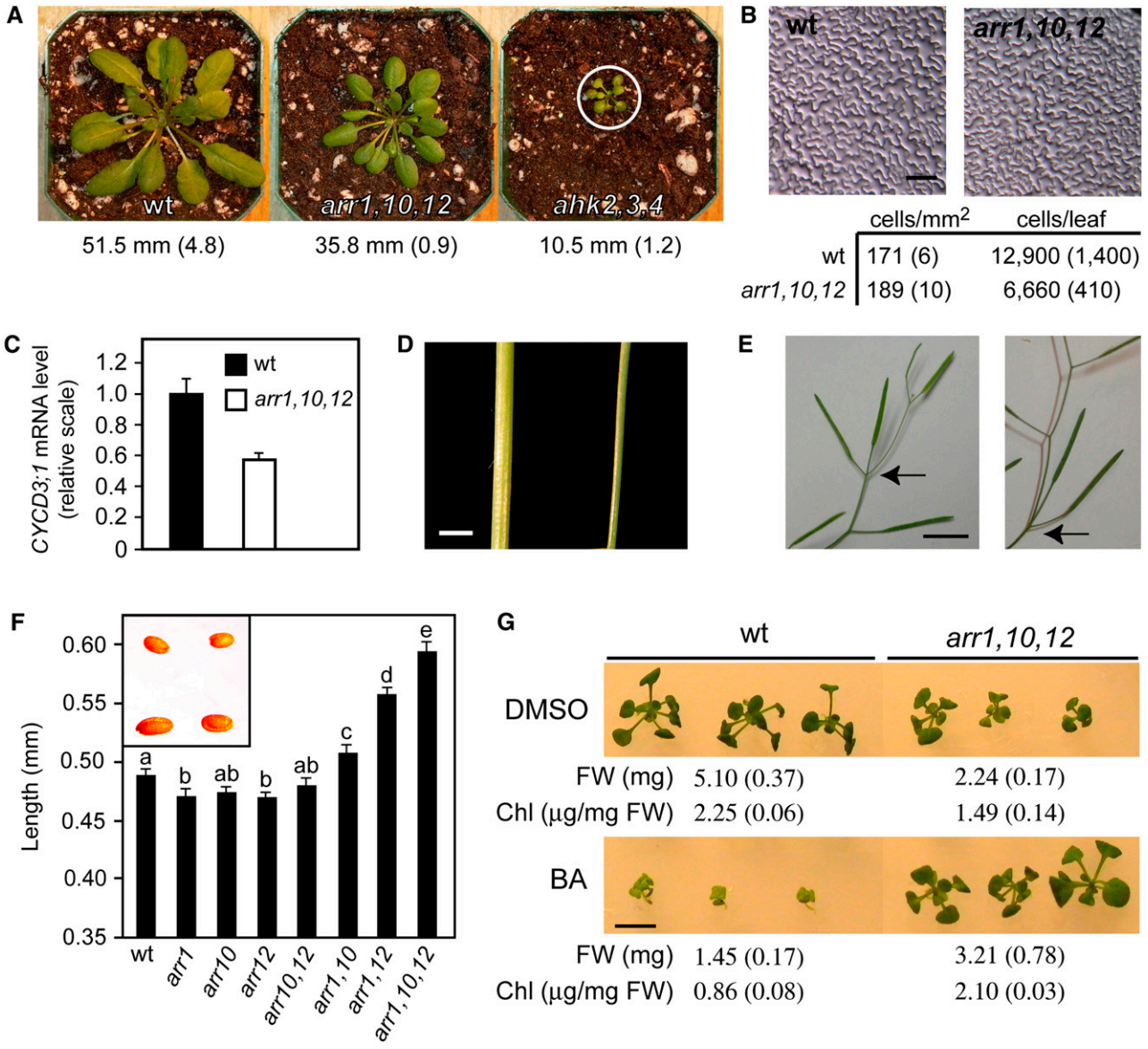

Figure 5. Shoot Phenotypes of *arr1-3 arr10-5 arr12-1* Triple Mutants.

(A) Rosettes of wild type, *arr1-3 arr10-5 arr12-1* (*arr1,10,12*), and *ahk2 ahk3 ahk4* (*ahk2,3,4*) plants grown under short days (8-h daylength) at 60 d old. The *ahk2 ahk3 ahk4* plant is circled. Mean rosette diameters are indicated, with SE in parentheses ($n > 3$). Pot diameter is 62 mm.

(B) Adaxial epidermal cells from fully expanded leaf 5 of wild-type and *arr1-3 arr10-5 arr12-1* plants shown in **(A)**. Mean cells/mm^2 and cells/leaf are indicated, with SE in parentheses ($n = 3$). Bar = 0.1 mm.

(C) Real-time RT-PCR analysis of *CYCD3;1* expression in wild-type and *arr1-3 arr10-5 arr12-1* 14-d-old shoots. Expression level of the wild type is set to 1.

(D) Primary inflorescence stems of wild-type (left) and *arr1-3 arr10-5 arr12-1* (right) plants. Bar = 2 mm.

(E) Altered spacing of siliques in *arr1-3 arr10-5 arr12-1*. Two examples of the mutant phenotype are shown, with arrows indicating positions with altered silique spacing. Bar = 10 mm.

(F) Mean length of seeds from the wild type and *arr* mutants. Error bars indicate SE ($n = 60$). The triple mutant *arr1-3 arr10-5 arr12-1* is abbreviated *arr1,10,12*; other mutants are similarly abbreviated. Those genotypes having the same letter exhibit no significant difference in their seed length. The inset shows wild-type (top) and *arr1-3 arr10-5 arr12-1* (bottom) seeds.

(G) Effect of cytokinin treatment on the growth of the wild type and *arr1-3 arr10-5 arr12-1* triple mutants. Images show 26-d-old plants grown under constant light (25 μE·m^{-2}·s^{-1}) treated with a DMSO vehicle control or with 0.1 μM BA. Fresh weight (FW) and total chlorophyll (Chl) levels are indicated, with SD in parentheses, based on three replicates of five seedlings each. Bar = 5 mm.

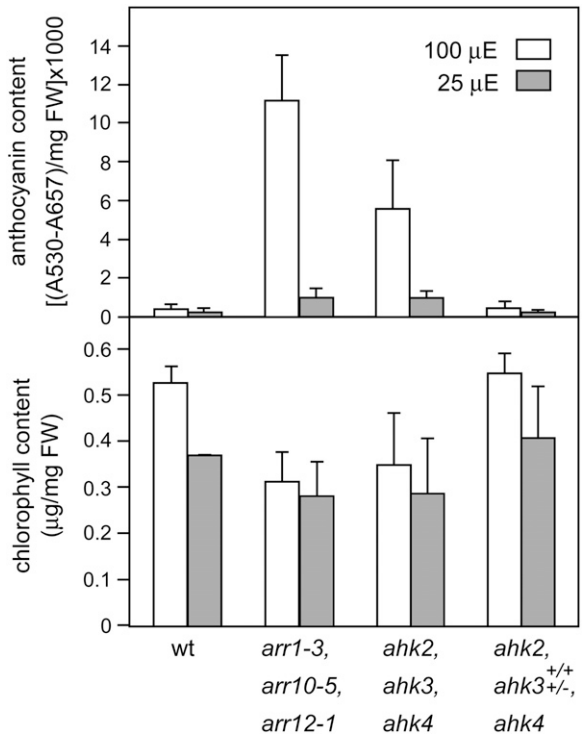

Figure 6. Altered Levels of Anthocyanin and Chlorophyll in *arr1-3 arr10-5 arr12-1* and *ahk2 ahk3 ahk4* Compared with the Wild Type.

Fourteen-day-old seedlings were grown under either 25 or 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light. Seedlings of the wild type, the *arr1-3 arr10-5 arr12-1* triple mutant, the *ahk2 ahk3 ahk4* triple receptor mutant, and a mixed population of *ahk2 ahk4* and *ahk2 ahk3(+/−) ahk4* seedlings were examined for both anthocyanin and chlorophyll levels. Each sample represents the mean of two replicates of 10 seedlings each.

photopigment levels in the triple receptor mutant *ahk2 ahk3 ahk4*. The *ahk2 ahk3 ahk4* mutant also exhibits increased light sensitivity compared with the wild type based on its anthocyanin production, indicating that the alteration in light sensitivity is a phenotype associated with a lack of cytokinin response (Figure 6). Within the same segregating population from which we obtained the *ahk2 ahk3 ahk4* triple mutant, we also examined a pool of the other segregants [representing a mixture of *ahk2 ahk4* and *ahk2 ahk3(+/−) ahk4*] and found these to exhibit wild-type levels of anthocyanin and chlorophyll. Thus, the pronounced light sensitivity is dependent on the loss of all three cytokinin receptors.

Effect of the *arr1-3 arr10-5 arr12-1* Mutant on Cytokinin-Regulated Gene Expression

We performed a microarray analysis to determine the extent to which *arr1-3 arr10-5 arr12-1* affected cytokinin-regulated gene expression. For this purpose, 14-d-old seedlings were treated with 10 μM BA or a DMSO vehicle control for 1 h, and replicate RNA samples were then prepared from the shoots for analysis. Shoots were examined because of the developmental differences existing between the roots of the wild-type and mutant plants (Figure 3). In addition, seedlings were grown under conditions of reduced light to avoid the light-induced stress responses described previously.

From the microarray analysis, we identified a group of 71 genes induced by threefold or more in BA-treated wild-type shoots (Figures 7A and 7B; see Supplemental Data Set 1 online). This set of induced genes includes type A *ARR*s, the cytokinin receptor *AHK4*, and the cytokinin oxidase *CKX4*, all of which have been identified previously as cytokinin-regulated genes (Hoth et al., 2003; Rashotte et al., 2003; Taniguchi et al., 2007; Yokoyama et al., 2007). The *arr1-3 arr10-5 arr12-1* mutant exhibited a severely abrogated response to BA, with only one of these 71 genes (*ARR5*) exhibiting a greater than threefold induction upon treatment with BA (Figures 7A and 7B). The final level of BA-regulated gene expression will depend on the initial expression level coupled with the degree to which cytokinin induces an increase in expression. Therefore, we also compared the expression of these genes in BA-treated wild-type plants to that in the BA-treated mutant. This analysis indicates that 62 of the 71 genes (87%) are expressed at least twofold lower in the mutant than in the wild type, with 46 of the genes being expressed at least fourfold lower. The mutant is also abrogated in its ability to respond to BA in terms of gene repression, based on the analysis of 95 genes repressed threefold or more in BA-treated wild-type plants (Figure 7A; see Supplemental Data Set 1 online). It should be noted that this set of repressed genes exhibits a lower basal expression level in the mutant compared with the wild type in the absence of cytokinin treatment, suggesting a complex role for the type B ARRs in their regulation.

As an alternative approach to characterize cytokinin-regulated expression from the microarray analysis, we examined a set of 15 cytokinin primary response genes previously determined to be direct targets of the type B response regulator ARR1 (Figure 7C) (Taniguchi et al., 2007). Based on our microarray analysis, 13 of these genes are induced in wild-type shoots by 1 h of BA treatment. For 12 of these 13 genes, the level of induction (fold change) by BA in the *arr1-3 arr10-5 arr12-1* mutant was less than that observed in the wild type. In addition, in all 13 cases, the expression level in BA-treated *arr1-3 arr10-5 arr12-1* mutants was reduced compared with that in BA-treated wild-type plants, due to the lower basal expression level coupled with the reduced level of BA induction.

We used real-time RT-PCR to validate the altered expression of the type A *ARR* genes *ARR5*, *ARR7*, and *ARR15*, the cytokinin receptor gene *AHK4*, and the cytokinin oxidase gene *CKX4* in the *arr1-3 arr10-5 arr12-1* mutant (Figure 7D). For all five genes, the basal expression level was reduced compared with that in the wild type, indicating a reduced response to endogenous cytokinin in the mutant. Substantial differences were observed between the *arr1-3 arr10-5 arr12-1* mutant and the wild type in their responses to cytokinin treatment. In all cases, the degree of induction of the primary response genes was reduced in the mutant, with some genes, such as *ARR15*, displaying no induction at all. The decrease in endogenous levels of the primary response gene products, coupled with reduced induction by cytokinin, results in substantially decreased expression levels for all of these primary response genes in the presence of cytokinin.

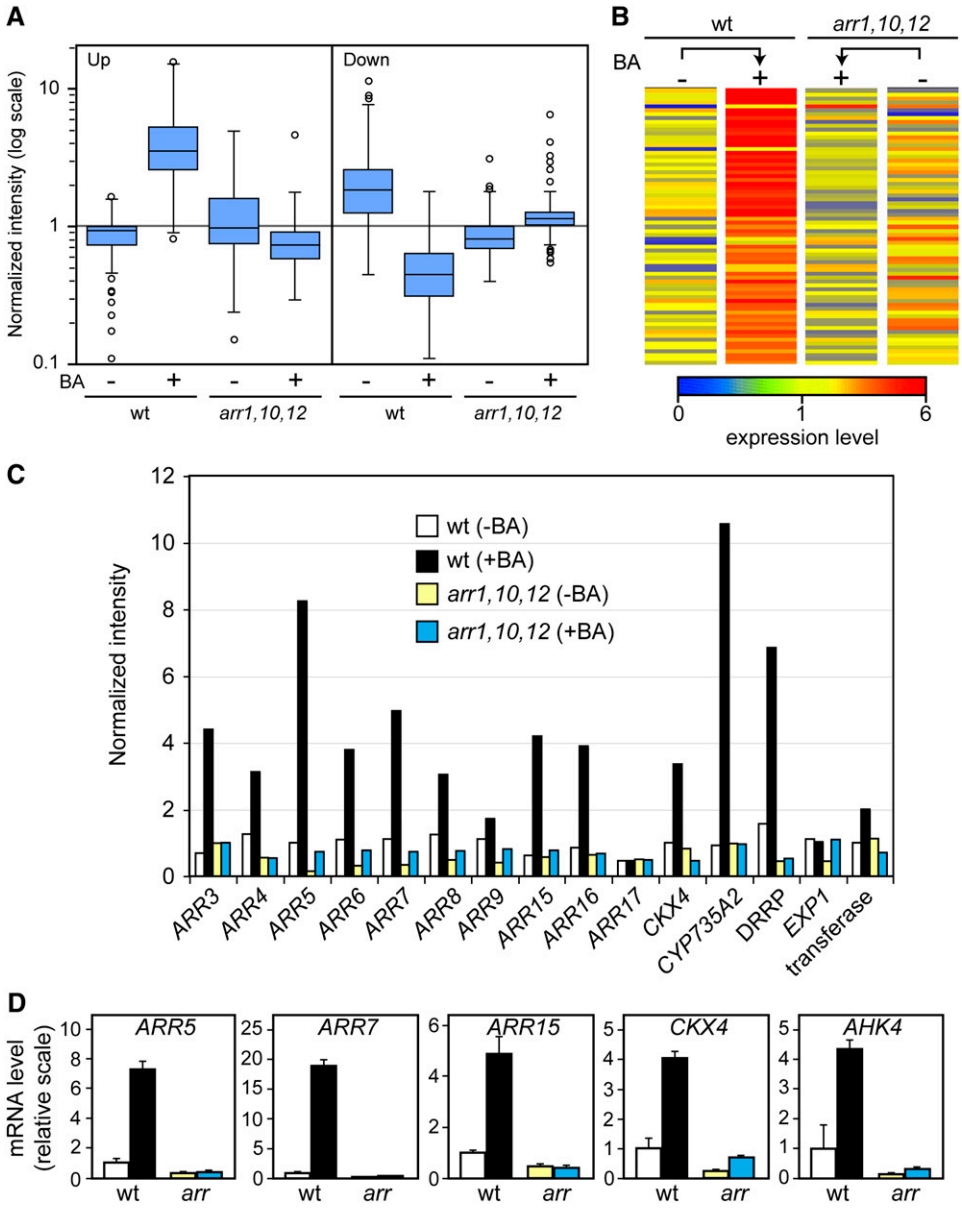

Figure 7. Reduced Transcriptional Response of Cytokinin-Regulated Genes in Shoots of *arr1-3 arr10-5 arr12-1* Triple Mutants Compared with the Wild Type.

Fourteen-day-old wild-type and *arr1-3 arr10-5 arr12-1* (*arr1,10,12*) seedlings were treated with either 10 μM BA or a DMSO control for 1 h, and the gene expression was analyzed in shoots by either microarray (**[A]** to **[C]**) or real-time RT-PCR **(D)**. For microarray analysis, absolute expression levels are given relative to the normalized expression value of 1 based on all genes represented on the array.

(A) Box-plot analysis for the expression levels of 71 genes induced (Up) and 95 genes repressed (Down) threefold or more based on microarray analysis. Whiskers are drawn to 1.5× the interquartile distance, with genes whose expression lies outside this range indicated by open circles.

(B) Expression levels of 71 genes induced threefold or more by BA treatment in the wild type compared with the *arr1-3 arr10-5 arr12-1* mutant. Expression levels are indicated by color according to the scale.

(C) Expression of known cytokinin primary response genes based on microarray analysis. Target genes are as described (Taniguchi et al., 2007) and include type A *ARR*s, the cytokinin oxidase *CKX4*, the cytokinin hydroxylase *CYP735A2*, a putative disease resistance response protein (DRRP), the expansin *EXP1*, and a transferase family protein.

(D) Real-time RT-PCR analysis of the cytokinin-regulated genes *ARR5*, *ARR7*, *ARR15*, *CKX4*, and *AHK4* in wild-type and *arr1-3 arr10-5 arr12-1* (*arr*) mutant shoots. The key is the same as that used in **(C)**.

DISCUSSION

We adopted a mutant-based approach to characterize the roles of the type B RR genes *ARR1*, *ARR10*, and *ARR12* in plant growth and development. As described below, our analyses lend insight to the functional overlap among the type B *ARR*s, their roles in regulating cell division in the root and the shoot, in mediating light sensitivity, and in mediating the transcriptional response to cytokinin. The phenotypes of the type B *ARR* mutants are consistent with those of cytokinin receptor mutants and reveal the importance of *ARR1*, *ARR10*, and *ARR12* in mediating cytokinin responses throughout the plant.

Overlapping Functions of Type B *ARR*s in Cytokinin Signaling

ARR1, *ARR10*, and *ARR12* have all been demonstrated previously to contribute to the cytokinin response in *Arabidopsis* (Mason et al., 2005; Yokoyama et al., 2007; Ishida et al., 2008). Based on the analysis of single, double, and triple mutant combinations, there appears to be extensive functional overlap among the three response regulators, a finding consistent with their overlapping expression patterns (Mason et al., 2004). Single mutants are similar to the wild type in phenotype and their responses to cytokinin (Figures 2B, 4B, and 5F) (Mason et al., 2005). Examination of the double mutants reveals varying contributions for *ARR1*, *ARR10*, and *ARR12* to cytokinin responses. In most cases, the *arr1-3 arr12-1* double mutant has a greater impact than either the *arr1-3 arr10-5* or *arr10-5 arr12-1* double mutant. This was the case for root and hypocotyl growth responses to cytokinin as well as for the mutants' effect on seed size (Figures 2B, 2C, 4, and 5F) . However, the *arr10-5 arr12-1* double mutant is less sensitive to cytokinin's effect on xylem differentiation than the *arr1-3 arr12-1* double mutant (Figure 3E), indicating that the closely related *ARR10* and *ARR12* genes have a substantial contribution to this developmental phenotype.

The triple mutant *arr1-3 arr10-5 arr12-1* exhibits stronger phenotypes than the single or double mutants and results in a plant similar to the *ahk2 ahk3 ahk4* cytokinin receptor triple mutant for a subset of phenotypes. For example, the defects observed in the *arr1-3 arr10-5 arr12-1* primary root, which include premature growth termination, no metaxylem formation, and a lack of cytokinin responsiveness, are indistinguishable from those found in the cytokinin receptor triple mutants (Figure 3). In addition, both triple mutants display a similar reduction in chlorophyll levels and demonstrate light sensitivity characterized by increased anthocyanin production (Figure 6). There is, however, a clear difference between the *arr* and *ahk* triple mutants in their shoot growth. Although the rosettes of both mutants are smaller than that of the wild type, the *ahk2 ahk3 ahk4* mutant has a substantially smaller rosette than the *arr1-3 arr10-5 arr12-1* mutant (Figure 5A). Our results in this respect differ from those of Ishida et al. (2008), in which an *arr1-4 arr10-5 arr12-1* triple mutant was shown to be similar to the *ahk2 ahk3 ahk4* cytokinin receptor mutant in the adult rosette leaf size. These differences in shoot growth between the two studies are likely due to the sensitivity of the *arr* triple mutants to stress, which we found resulted in retarded growth of the mutant (Figure 3B). The *arr1-3 arr10-5 arr12-1* triple mutant also does not display the defects in reproductive growth, characterized by decreased pollen production and almost complete infertility (Nishimura et al., 2004; Riefler et al., 2006), or the defects in adventitious root development found in the cytokinin receptor triple mutant. These developmental processes involving the shoot may involve regulation by other members of the type B ARR family. The residual transcriptional response to cytokinin we detected in shoots of *arr1-3 arr10-5 arr12-1* (Figure 7) may be mediated by these additional *ARR* family members and may account for the partial rescue of the triple mutant when grown on BA (Figure 5G).

Role of *ARR*s in Regulating Cell Division

Our results indicate that the type B ARRs regulate cell division, with differing effects on cell division in the shoot and the root that parallel the known effects of cytokinin. Cytokinin acts as a positive regulator of cell division in the shoot (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Consistent with the type-B ARRs mediating the positive effect of cytokinin in shoots, we found that the leaves of the *arr1-3 arr10-5 arr12-1* triple mutant had approximately half the number of cells found in wild-type leaves (Figure 5B). We also found that shoots of the *arr1-3 arr10-5 arr12-1* triple mutant had reduced expression of *CYCD3;1* (Figure 5C), a D-type cyclin previously implicated in regulating cytokinin effects on cell division (Riou-Khamlichi et al., 1999; Dewitte et al., 2007). The decrease in *CYCD3;1* levels could be due to a decrease in the gene expression per cell and/or a decrease in the percentage of cells expressing this gene. The first possibility would be consistent with the cytokinin induction of *CYCD3;1* reported previously (Riou-Khamlichi et al., 1999), as we found that many cytokinin-regulated genes had lower basal levels of expression in the *arr1-3 arr10-5 arr12-1* mutant (Figure 7). However, *CYCD3;1* is not strongly induced by cytokinin under our growth conditions, showing no induction at 1 h and only 1.8-fold induction after 24 h of treatment with 10 μM BA when analyzed by real-time RT-PCR (Y.-H. Chiang and G.E. Schaller, unpublished data). Thus, although the decrease in CYCD3;1 expression is consistent with a reduction in cell division, transcriptional regulation of CYCD3;1 may not play a role in initiating this cytokinin-mediated response.

Cytokinin generally acts as a negative regulator of cell division in the root (Werner et al., 2003; Ioio et al., 2007). However, plants with substantially reduced cytokinin signaling produce a primary root that terminates prematurely, resulting in what has been referred to as the wooden leg phenotype, characterized by aberrant vascular development and a lack of cell division activity (Mahonen et al., 2000; Higuchi et al., 2004; Nishimura et al., 2004; Hutchison et al., 2006). Our results using a labile cyclin-GUS reporter in the *arr1-3 arr12-1* double mutant indicate that type B ARRs are required to mediate the negative effect of cytokinin on cell division in roots (Figure 2C). Recent studies suggest that cytokinins control the level of root cell division by regulating where the transition zone occurs that separates the elongation zone from the meristematic region (Ioio et al., 2007). Thus, based on this model, the loss of *ARR1* and *ARR12* would result in increased cell division because the meristematic region would remain larger even in the presence of cytokinin. The *arr1-3*

arr10-5 arr12-1 triple mutant exhibits the wooden leg phenotype and, based on the cyclin-GUS reporter, exhibits a loss of cell division in the primary root meristem. The lack of cell division could imply that cytokinin also acts as a positive regulator of root cell division as it does in the shoot meristem, with a low level of cytokinin signal transmission being required for root cell division but higher levels being inhibitory. However, the *arr1-3 arr10-5 arr12-1* mutant, like the cytokinin receptor triple mutant and the *ahp* quintuple mutant, also produces adventitious roots that are still active for cell division (Figure 3D), indicating that the primary root mutant phenotype cannot be generalized to other roots. Thus, the loss of cell division capacity of the primary root may be a secondary consequence of the other defects in primary root development. For example, the primary roots of cytokinin receptor mutants with the wooden leg phenotype have fewer vascular cell files due to a lack of embryonic cell division (Mahonen et al., 2000; Nishimura et al., 2004).

Role of ARRs in Light-Sensitive Growth Responses

The *arr1-3 arr10-5 arr12-1* triple mutant is light-sensitive (Figures 3B and 6). Light stress occurs in plants due to inefficient use of the light energy absorbed by chlorophylls in the antenna and reaction centers, resulting in the production of damaging reactive oxygen species (Gould, 2004; Krieger-Liszkay, 2005). The anthocyanins produced by the *arr* triple mutant in response to light stress would serve as photoprotectants because they can absorb light and act as an optical screen in the leaf epidermis (Gould, 2004; Jung, 2004). In addition, anthocyanins can function as protectants by scavenging for free radicals (Gould, 2004; Vanderauwera et al., 2005).

The light sensitivity of the *arr* triple mutant arises from a defective cytokinin signaling pathway, based on our finding that the *ahk2 ahk3 ahk4* triple mutant was also light-sensitive (Figure 6). Decreased cytokinin signaling in the *arr* and *ahk* triple mutants may contribute to their light sensitivity in several ways. First, cytokinin has a well-documented role in chloroplast biogenesis, including the transcriptional regulation of genes involved in photosynthesis (Chory et al., 1994; Brenner et al., 2005); thus, the mutants may produce an inefficient photosynthetic system less able to use the available light energy. The finding that the mutants have reduced chlorophyll levels is symptomatic of aberrant regulation in the photosynthetic system. Second, several studies have implicated cytokinin in the oxidative stress response (Bianchi et al., 2002; Zavaleta-Mancera et al., 2007); thus, the cytokinin-insensitive mutants may have a decreased ability to respond to and eliminate the reactive oxygen species produced even under normal conditions by the photosynthetic apparatus.

Role of ARRs in Regulating the Transcriptional Response to Cytokinin

Our analysis of the *arr1-3 arr10-5 arr12-1* triple mutant reveals a broader effect of type B ARRs on the global transcriptional response to cytokinin than that uncovered in previous analyses, which made use of *arr* double mutants (Rashotte et al., 2006; Yokoyama et al., 2007). None of the genes identified as cytokinin-induced in the wild type exhibited the same level of induction in the *arr* triple mutant, with 87% of the genes showing a twofold or greater decrease in their responsiveness to cytokinin (Figure 7). Analysis of known cytokinin primary response genes revealed that many have a lower basal level of expression in the mutant (Figures 7C and 7D), indicating that the mutant has a reduced response to endogenous cytokinin. In addition to affecting gene induction, the *arr1-3 arr10-5 arr12-1* triple mutant also had broad effects on the cohort of genes whose expression is repressed by cytokinin application. The type B ARRs do not contain known repressor motifs; thus, this effect is likely to involve additional transcriptional repressors, either that modulate the action of the type B ARRs or that act downstream of the type B ARRs in a transcriptional cascade. We also noted a lower basal level of expression of the repressed genes in the *arr1-3 arr10-5 arr12-1* mutant compared with the wild type (Figure 7A); thus, the type-B ARRs may play a role in inducing the expression of some genes in the absence of cytokinin, and this effect may be reversed in the presence of cytokinin.

Although our results support a requirement of the type B ARRs in general, and ARR1, ARR10, and ARR12 specifically, in mediating the transcriptional response to cytokinin, other families of transcription factors also appear to play significant roles. In the primary response pathway, additional transcription factors are required in combination with the type B ARRs for transcriptional regulation of some genes. For example, mutational analysis indicates that the six-member CYTOKININ RESPONSE FACTOR family of AP2 transcription factors regulates about half of the cytokinin-regulated genes, with substantial overlap among the set regulated by the type B ARRs (Rashotte et al., 2006). In addition, our microarray analysis reveals that cytokinin induces the expression of many transcription factors in a manner that is dependent on ARR1, ARR10, and ARR12. This is consistent with the type B ARRs acting at the head of a transcriptional cascade to regulate the cytokinin response.

In summary, we have shown here that a broad array of phenotypes associated with the *arr1-3 arr10-5 arr12-1* mutant match what have been found in *ahk* and *ahp* mutant combinations. In addition, we have shown that the *arr1-3 arr10-5 arr12-1* mutant severely attenuates the expression of the vast majority of cytokinin-regulated genes. These results thus support a model in which cytokinin regulates downstream responses through the action of a multistep phosphorelay that culminates in transcriptional regulation by the type B ARRs, with ARR1, ARR10, and ARR12 playing a central role in regulating this transcriptional response.

METHODS

Plant Material and Growth Conditions

The wild-type and mutant lines of *Arabidopsis thaliana* were all in the Columbia ecotype. Isolation of the *arr1-3*, *arr10-2*, and *arr12-1* (SALK_054752) mutant alleles, genotyping, and generation of their double and triple mutant combinations were as described previously (Mason et al., 2005). The *arr10-5* mutant allele was obtained from the Salk Collection (SALK_098604). Sequence analysis of *arr10-5* identified the T-DNA junction with *ARR10* as 5′-(gttattaagttgtctaagcg)TCAGAAAGGACTTGGTGGCT-3′,

with uppercase letters indicating *ARR10* sequence and parentheses indicating the T-DNA left border sequence, placing the insertion site within the fifth exon. Double insertion mutants with *arr10-5* were generated by crossing two single insertion mutants. The *arr1-3 arr10-5 arr12-1* triple mutant was generated by crossing the *arr10-5* single mutant with the *arr1-3 arr12-1* double mutant. Segregating progeny homozygous for all alleles were identified in the subsequent F2 and F3 generations. Genotyping of *arr10-5* was performed using primers 5′-GCCACCTTCAGGTGAGAGTTAGACTATGAT-3′ and 5′-AGCTGACAAAGAAAAGGGAAAATGGAGTTT-3′. The cytokinin receptor mutant *ahk2-7 ahk3-3 cre1-12* (referred to here as *ahk2 ahk3 ahk4*) was generated from the T-DNA insertion lines Wisc Dslox 504C06, SALK069269, and SALK048970. Wild-type plants containing the *CycB1;1:GUS* reporter were kindly provided by Peter Doerner (Colon-Carmona et al., 1999) and were crossed with the *arr1-3 arr10-5 arr12-1* triple mutant to generate both double and triple mutants containing the *CycB1;1:GUS* reporter.

Unless stated otherwise, seeds were surface-sterilized and cold-treated for 3 d in the dark at 4°C before being moved into the light. Seedlings for molecular and physiological assays were grown on medium containing 0.8% (w/v) phytoagar (Research Products International), 1× Murashige and Skoog (MS) salts containing Gamborg's vitamins (Phytotechnology Laboratories), 1% (w/v) sucrose, and 0.05% (w/v) MES (pH 5.7). Seeds were grown under continuous white light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 22°C, with white light being generated by standard fluorescent bulbs augmented with 18,000 K fluorescent bulbs (Aqua-Glo; Rolf C. Hagen). Layers of black nylon mesh were used to reduce light levels as needed. For long-term growth, seedlings were initially grown on Petri dishes and then transferred to pots. Short-day growth was performed with an 8-h-light (35 $\mu E \cdot m^{-2} \cdot s^{-1}$)/16-h-dark cycle.

For RNA gel blot analysis, seedlings were grown in liquid MS medium for 14 d under constant light on a platform shaker with gentle agitation (50 rpm). For microarray analysis, seedlings were grown on filter paper on agar medium for 14 d under constant light (25 $\mu E \cdot m^{-2} \cdot s^{-1}$), then transferred to liquid MS medium containing either 10 μM BA or a DMSO vehicle control for 1 h. The roots were removed and discarded, and the shoots were frozen at −80°C until the RNA was extracted.

Root Analysis

Root elongation was measured as described (Mason et al., 2005) using *Arabidopsis* seedlings grown on vertical plates containing either BA at the indicated concentration or 0.1% DMSO as a vehicle control. Root lengths were marked on the plates and measured using ImageJ software (version 1.32; National Institutes of Health). Root elongation was typically measured after at least 3 d of initial growth (e.g., from the beginning of day 4 through day 7) because this eliminates differences due to variation in the time of germination. Statistical analysis was performed using the SAS System, version 8.2 (SAS Institute). For Figures 2, 4, and 5, Duncan's multiple range test was performed among the means on the analysis of variance (Duncan, 1975).

Root xylem was visualized after staining with 0.01% basic fuchsin as described (Mahonen et al., 2000) in both 7- and 21-d-old seedlings, grown in the presence or absence of 10 μM BA, using a Nikon D-Eclipse C1/80-1 confocal microscope with a HeNe laser and a Chroma HQ590/50-nm filter (543 nm excitation, 565 to 615 nm emission). Z sections were collected and projected to give a composite image of the xylem vessels.

Hypocotyl Elongation and Deetiolation Assays

Hypocotyl elongation in the light was measured after 5 d of growth under 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ constant light on plates containing MS medium with 1% (w/v) sucrose using the indicated concentrations of BA. Dark-grown seedlings were grown for 4 d in the presence or absence of 10 μM BA. The deetiolation response to cytokinin was analyzed by growing seedlings on plates containing MS medium with 1% (w/v) sucrose and 30 μM BA for 14 d in the dark, after an initial light treatment for 8 h. In all cases, seedlings were grown on vertical plates and scanned, and their lengths were measured using ImageJ software.

Shoot Analysis

To determine leaf cell size and number, clear nail polish replicas were made from the adaxial surface of fully expanded leaves. Counts were made in 0.6- × 0.6-mm square sections midway between the midvein and the leaf periphery. The total number of cells in the leaf was then calculated by extrapolating this value to the total leaf area. Rosette diameter was determined based on the mean of two measurements taken with a ruler on the same rosette. Seed size was determined by scanning seeds at 6000 dpi on an Epson flatbed scanner, and their lengths were measured using ImageJ software (National Institutes of Health).

Chlorophyll and Anthocyanin Measurements

Chlorophyll and anthocyanin measurements were each performed on two groups of 10 seedlings. Chlorophyll levels were determined by extraction with 100% ethanol for 16 h followed by spectrophotometry, as described previously (Lichtenthaler, 1987). Relative anthocyanin levels were determined by incubating tissue in 350 μL of ethanol and 1% (v/v) HCl overnight. The volume was then brought up to 500 μL with distilled water and extracted with an equal volume of chloroform to separate anthocyanins from chlorophyll. A total of 500 μL of 70% (v/v) ethanol and 1% (v/v) HCl was added to the aqueous phase, and the relative amount of total anthocyanin was determined by first subtracting A_{657} from A_{530} and then dividing by the milligrams fresh weight.

Histochemical Analysis of GUS Activity

Histochemical analysis of GUS activity in stably transformed lines of *Arabidopsis* was performed as described (Jefferson et al., 1987) with a few alterations. Briefly, tissues were immersed in GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide; Gold BioTechnology), 0.1% (v/v) Triton X-100, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 10 mM EDTA, and 100 mM sodium phosphate buffer (pH 7), and left overnight in the dark at 22°C before being cleared of chlorophyll by incubation in 70% (v/v) ethanol for several hours. The GUS-stained tissues were visualized and photographed using an Olympus SZX9 stereomicroscope with a dark field base.

Gene Expression Analysis

Total RNA was isolated using the RNeasy plant kit according to the manufacturer's instructions (Qiagen). A DNase treatment was incorporated into the procedure when real-time PCR analysis was to be performed. The nucleic acid integrity was assessed by evaluating rRNA bands on agarose gels, and its quality was determined by calculating the 260:280 and 260:230 spectrophotometric ratios. When necessary, the RNA was cleaned further by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, followed by precipitation with 0.15 M sodium acetate and 50% (v/v) isopropanol.

RNA gel blot analysis was performed as described (Etheridge et al., 1999; Zhao et al., 2002) using the PolyATract mRNA isolation system (Promega) for mRNA isolation, the NorthernMax-Gly kit (Ambion) for gel electrophoresis, and the Strip-EZ PCR kit (Ambion) for synthesis of single-stranded DNA antisense probes. Radioactivity was analyzed by

phosphorimaging with a Typhoon 8600 Variable Mode Imager (Molecular Dynamics) and the accompanying software.

Microarray analysis was performed using GeneChip ATH1 *Arabidopsis* genome arrays (Affymetrix) on 10 μg of total RNA isolated from shoots. Two independent biological replicates were performed for each experimental treatment. Samples were independently labeled (Genechip IVT labeling kit), hybridized, washed (Fluidics Station 450), and scanned by the Dartmouth Genomics and Microarray Laboratory (http://dms.dartmouth.edu/dgml/) according to the manufacturers' recommended procedures. Affymetrix gene-chip data files were imported into GeneSpring GX 7.3 (Agilent Technologies) for data analysis. The data were normalized per chip to the 50th percentile and per gene to the median. The Genespring Cross Gene Error Model, which combines measurement variation and between-sample variation information in replicate samples to estimate measurement precision, was used to filter data for minimum expression level. For interpretation of data, the Genespring fold change mode was used and putative cytokinin-regulated genes were identified based on their exhibiting a threefold change in the BA-treated sample compared with the control sample. Genes were eliminated from further analysis if the SD of the normalized expression for the replicate samples exceeded 50% of the mean. The data set is deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) with accession number E-MEXP-1573.

For real-time RT-PCR, total RNA was treated with RNase-free DNase I, and RNA (1 μg) pooled from three biological replicates was used as template for first-strand cDNA synthesis using SuperScript III (Invitrogen). Real-time RT-PCR was performed using a mix containing 10 μL of 2× SYBR Premix Ex Taq (TaKaRa Bio), 0.8 μL of forward and reverse primer mix (0.2 μM final concentration), 0.4 μL of 50× ROX reference dye II, 5 μL of cDNA, and 3.8 μL of deionized water. PCR was run on an ABI 7500 fast real-time PCR system (Applied Biosystems) using a 10-min initial denaturation at 95°C, followed by 40 cycles of 95°C (15 s) and 56°C (1 min). Three experimental replicates of each reaction were performed using primer pairs specific for the genes of interest: *ARR5* (5′-GCCTCGTATCGATAGATGTCTTGAAGAAGG-3′ and 5′-TCTGATAAACTCAGATCTTTGCGCGT-3′), *ARR7* (5′-AGAGTGGAACTAGGGCTTTGCAGT-3′ and 5′-CTCCTTCTTTGAGACATTCTTGTATACGAGG-3′), *ARR15* (5′-CTGCTTGTAAAGTGACGACTGTTG-3′ and 5′-AGTTCATATCCTGTTAGTCCCGGC-3′), *CKX4* (5′-CACCCACAAGGGTGAAATGGTCTC-3′ and 5′-TGCGACTCTTGTTTGATCGGAGAG-3′), *AHK4* (5′-ACCGTTGCTAAGTGGAGTGGCTTA-3′ and 5′-ATATTCTCACGATCCTCCTCGCCT-3′), and *CYCD3;1* (5′-CGAAGAAACCCAAGTCCCTCTTCTTC-3′ and 5′-ACCCGACAAATCTTGAATCGGAGA-3′). β-Tubulin (At5G44340) (5′-TTCCGTACCCTCAAGCTCGCTAAT-3′ and 5′-ATCCTCTCGATGTCAATGGTGCGA-3′) was used as a control. Average threshold cycle (Ct) values were generated and analyzed by SDS software version 1.4, which uses the comparative Ct method (Livak and Schmittgen, 2001). Fold change compared with the untreated wild-type control was calculated after normalization to the β-tubulin Ct values. All primer sets generated a single distinct peak during melting curve analysis.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: *ARR1* (At3g16857), *ARR3* (At1g59940), *ARR4* (At1g10470), *ARR5* (At3g48100), *ARR6* (At5g62920), *ARR7* (At1g19050), *ARR8* (At2g41310), *ARR9* (At3g57040), *ARR10* (At4g31920), *ARR12* (At2g25180), *ARR15* (At1g74890), *ARR16* (At2g40670), *ARR17* (At3g56380), *CKX4* (At4g29740), *AHK2* (At5g35750), *AHK3* (At1g27320), *AHK4* (At2g01830), *CYCD3;1* (At4g34160), *β-tubulin* (At5g44340), *CKX4* (At4g29740), *CYP735A2* (At1g67110), *DRRP* (At4g11190), *EXP1* (At1g69530), and a transferase (At2g40230).

Supplemental Data

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

Supplemental Figure 1. Expression Patterns of *ARR1*, *ARR10*, and *ARR12* Based on Transcriptional Profiling of the Primary Root Tip.

Supplemental Data Set 1. Microarray Analysis of Cytokinin-Regulated Genes of Wild-Type and *arr1-3 arr10-5 arr12-1* Shoots.

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