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Cytokinins play critical roles in plant growth and development, with the transcriptional response to cytokinin being mediated by the type-B response regulators. In Arabidopsis (*Arabidopsis thaliana*), type-B response regulators (ARABIDOPSIS RESPONSE REGULATORS [ARRs]) form three subfamilies based on phylogenic analysis, with subfamily 1 having seven members and subfamilies 2 and 3 each having two members. Cytokinin responses are predominantly mediated by subfamily 1 members, with cytokinin-mediated effects on root growth and root meristem size correlating with type-B *ARR* expression levels. To determine which type-B ARRs can functionally substitute for the subfamily 1 members *ARR1* or *ARR12*, we expressed different type-B *ARR*s from the *ARR1* promoter and assayed their ability to rescue *arr1 arr12* double mutant phenotypes. ARR1, as well as a subset of other subfamily 1 type-B ARRs, restore the cytokinin sensitivity to *arr1 arr12*. Expression of *ARR10* from the *ARR1* promoter results in cytokinin hypersensitivity and enhances shoot regeneration from callus tissue, correlating with enhanced stability of the ARR10 protein compared with the ARR1 protein. Examination of transfer DNA insertion mutants in subfamilies 2 and 3 revealed little effect on several well-characterized cytokinin responses. However, a member of subfamily 2, *ARR21*, restores cytokinin sensitivity to *arr1 arr12* roots when expressed from the *ARR1* promoter, indicating functional conservation of this divergent family member. Our results indicate that the type-B ARRs have diverged in function, such that some, but not all, can complement the *arr1 arr12* mutant. In addition, our results indicate that type-B *ARR* expression profiles in the plant, along with posttranscriptional regulation, play significant roles in modulating their contribution to cytokinin signaling.

Cytokinins are phytohormones that play critical roles in plant growth and development, including regulation of cell division and metabolism, stimulation of chloroplast development, modulation of shoot and root development, and delay of leaf senescence (Mok, 1994; Haberer and Kieber, 2002; Kakimoto, 2003). Cytokinin signal transduction is mediated by a multistep phosphorelay that involves cytokinin receptors, phosphotransfer proteins, and type-B response regulators (Kakimoto, 2003; To and Kieber, 2008; Werner and Schmülling, 2009). These relay the cytokinin signal from the membrane to the nucleus, where the type-B response regulators induce the transcription of many genes. In Arabidopsis (*Arabidopsis thaliana*), there are three cytokinin receptors (ARABIDOPSIS HIS KINASE2 [AHK2], AHK3, and AHK4; Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001; Kakimoto, 2003), five phosphotransfer proteins (ARABIDOPSIS HIS-CONTAINING PHOSPHOTRANSFER PROTEINS; Hwang and Sheen, 2001; Hutchison et al., 2006), and 11 type-B response regulators (ARABIDOPSIS RESPONSE REGULATORS [ARRs]; Sakai et al., 2001; Mason et al., 2005). Genetic analysis has demonstrated roles for each of these families in cytokinin-mediated processes (Mähönen et al., 2000, 2006; Higuchi et al., 2004; Nishimura et al., 2004; To et al., 2004; Mason et al., 2005; Hutchison et al., 2006; Yokoyama et al., 2007; Argyros et al., 2008).

According to this model, the type-B ARRs play a pivotal role in the early transcriptional response of plants to cytokinin. The type-B ARRs are structurally related, each possessing a receiver domain that is

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phosphorylated on a conserved Asp residue, as well as a long C-terminal extension with a Myb-like DNA-binding domain (Imamura et al., 1999; Hosoda et al., 2002). The ability of the Myb-like domain of type-B ARRs to bind DNA has been demonstrated in several studies (Sakai et al., 2000; Hosoda K, et al., 2002), and multiple lines of evidence support a role of 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; Liang et al., 2012; Tsai et al., 2012). Elimination of three type-B ARRs, ARR1, ARR10, and ARR12, severely curtails the ability of cytokinin to induce changes in gene expression, demonstrating the importance of the type-B ARRs in the initial cytokinin signal transduction pathway and indicating that the type-B ARRs act at the top of a transcriptional cascade (Argyros et al., 2008; Ishida et al., 2008).

The 11 type-B ARRs of Arabidopsis fall into three subfamilies based on phylogenetic analysis, with subfamily 1 containing seven members and subfamilies 2 and 3 each containing two members (Mason et al., 2004). The members of subfamily 1 have been most extensively characterized. The type-B ARRs of subfamily 1 have the broadest expression pattern in Arabidopsis, and genetic analysis indicates that at least five members, ARR1, ARR2, ARR10, ARR11, and ARR12, of the subfamily mediate cytokinin signaling (Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008). In this study, we describe results obtained from two approaches to characterize the roles of type-B ARRs in cytokinin signaling. First, we assessed the function of all 11 type-B ARRs under the same expression context based on their ability to complement the *arr1 arr12* mutant when driven from the *ARR1* promoter. Second, we examined the effect of disruption of type-B ARRs from subfamilies 2 and 3. Results from these studies indicate that the type-B ARRs have diverged in function, such that some, but not all, complement *arr1 arr12*. In addition, our results indicate that type-B ARR expression profiles in the plant, along with posttranscriptional regulation, may play significant roles in modulating their contribution to cytokinin signaling.

RESULTS

Expression and the Contribution of Type-B ARRs to Root Growth

In Arabidopsis, there are 11 type-B ARRs that are divided into three subfamilies based on sequence homology (Fig. 1A; Mason et al., 2004). Data from microarray studies, semiquantitative reverse transcription (RT)-PCR, and GUS reporter analysis indicate that subfamily 1 members *ARR1*, *ARR2*, *ARR10*, *ARR11*, and *ARR12* are the most highly expressed type-B ARRs in the roots (Fig. 1A; Birnbaum et al., 2003; Imamura et al., 2003; Mason et al., 2004; Tajima et al., 2004; Schmid et al., 2005). Genetic studies suggest that *ARR1*, *ARR10*, and *ARR12* are the primary components of the cytokinin response in the root (Mason et al., 2005; Argyros et al., 2008; Ishida et al., 2008). To gain information about temporal regulation of expression for the five family members we could detect by PCR-based techniques, we performed quantitative RT-PCR on RNA isolated from root tips of seedlings 2, 3, 4, and 5 d after germination (Fig. 1B). The region of the root used for our analysis includes the stem cell niche, the cell division zone, the transition zone, and the initial part of the elongation/differentiation zone (Dello Ioio et al., 2008a). Expression of *ARR12* remained relatively consistent during this time period (Fig. 1B). At the other extreme, *ARR11* exhibited a 5-fold increase in expression between days 2 and 5. *ARR1*, *ARR2*, and *ARR10* all exhibited some increase in expression between days 2 and 4, with *ARR1* expression increasing 2-fold during this time period (Fig. 1B). Overall, based on average threshold cycle (Ct) values obtained from quantitative RT-PCR (Fig. 1A), the expression levels of *ARR2* and *ARR11* are substantially less than those of *ARR1*, *ARR10*, and *ARR12*, even at their time point of maximal expression.

To determine if temporal expression patterns of these type-B ARRs correlated with their role in root development, we examined the effect of single type-B *ARR* mutants on root meristem size (Fig. 1C). Root meristem size was determined by counting the number of meristematic cells at days 2 through 7 after germination. The *arr12-1* mutant exhibited an enlarged meristem throughout this time period, whereas the *arr1-3* mutant did not exhibit a strong effect until day 4 (Fig. 1C), which is consistent with previous reports (Dello Ioio et al., 2008b; Moubayidin et al., 2010). The *arr10-5* mutant behaved similarly to the *arr1-3* mutant, also showing little effect early after germination but a more pronounced effect at day 4 and thereafter. The *arr2-5* and *arr11-3* mutants had only a weak effect on meristem size, with their contribution most apparent later. Thus, overall, the effects of the individual type-B *ARR*s on meristem size are consistent with (1) their absolute expression level and (2) temporal changes in their expression level.

Functional Analysis of Subfamily 1 Type-B ARRs in Arabidopsis

The differing expression patterns of the type-B ARRs raised the question as to whether the function of these proteins is interchangeable. To address this question, we took advantage of the partial cytokinin insensitivity (hyposensitivity) of the *arr1 arr12* double mutant (Mason et al., 2005; Argyros et al., 2008) to determine which type-B ARRs could functionally substitute for activity of *ARR1* (or *ARR12*, as this mutant-based assay is not unequivocal for *ARR1*). We expressed different members of subfamily 1 from the *ARR1* promoter (Fig. 2A), incorporating a Myc epitope tag into the transgene to facilitate detection and comparison of transgene expression. To minimize

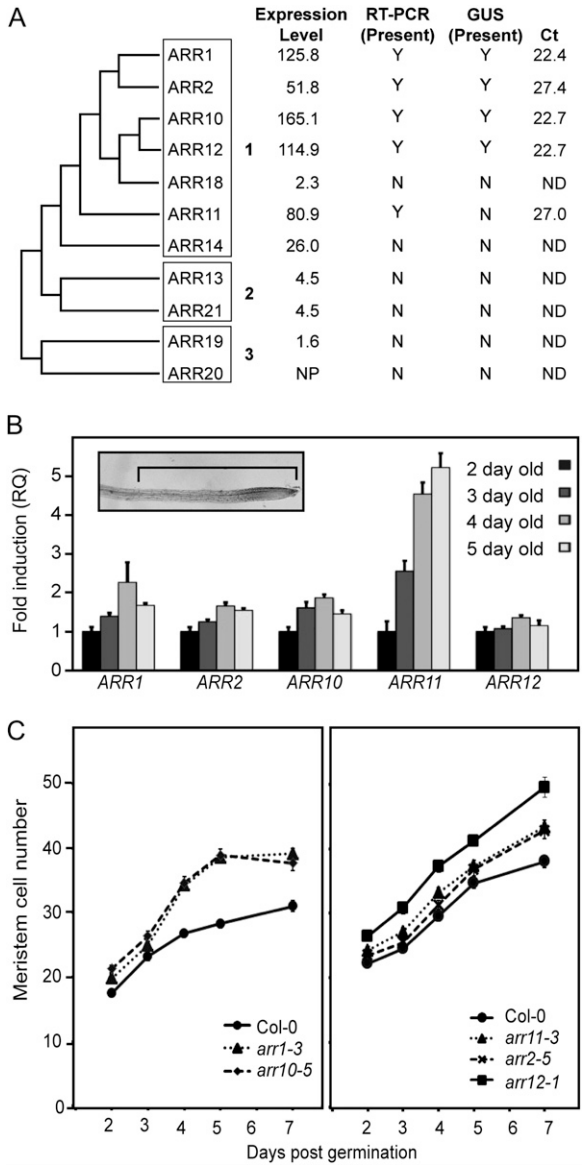

Figure 1. Expression of type-B *ARR*s in Arabidopsis roots varies during early stages of growth and correlates with effects on root meristem size. A, Expression of type-B *ARR*s based on microarray analysis, RT-PCR, GUS fusion analysis, and quantitative RT-PCR. A cladogram based on the receiver domains of subfamily 1, 2, and 3 type-B ARRs was constructed using the phylogeny.fr pipeline (Dereeper et al., 2008). Absolute expression level in 17-d-old roots is derived from the microarray data of Schmid et al. (2005), as accessed through the Arabidopsis eFP Browser (Winter et al., 2007), with the housekeeping gene *β-TUBULIN3* (At5g62700) having an expression level of 1264.4 by way of comparison. The presence (Y) or absence (N) of the type-B ARRs in roots based on RT-PCR and translational GUS fusions is from Mason et al. (2004). Average Ct values are from the point of maximal expression based on quantitative RT-PCR analysis in root tips, with a lower Ct value indicating higher expression. NP, Gene was not represented on the array; ND, not determined. B, *ARR1*, *ARR2*, *ARR10*, *ARR11*, and *ARR12* transcript levels in root tips 2, 3, 4, and 5 d after germination. Transcript levels are expressed relative to day 2. Inset image shows a 5-d-old root tip (tip on right) with the 1-mm region used for isolation of RNA indicated. C, Effect of individual *arr* mutations on meristem size. Meristem size was determined by counting cell number as described (Dello Ioio et al., 2008b). Error bars represent SE. Significant differences from the wild type (Bonferroni-corrected comparison of statistical difference, $P < 0.05$) are found for *arr1* (days 4–7), *arr2* (days 4 and 7), *arr10* (days 2 and 4–7), *arr11* (days 5–7), and *arr12* (days 2–7).

potential adverse effects of a tag on function, only a single 10-amino acid Myc epitope was used, and the tag was incorporated at an analogous position at the amino termini of each encoded protein, proximate to the receiver domain. This type of functional analysis has been used before, notably to examine the function of the ethylene receptor family in plant growth (Wang et al., 2003), and circumvents artifacts that can arise due to ectopic overexpression, such as that driven by the *Cauliflower mosaic virus* 35S promoter.

Multiple independent transgenic lines were assayed for their ability to functionally complement cytokinin hyposensitivity of the *arr1 arr12* mutant. We found that ARR1 (6/6 lines), ARR2 (6/6 lines), ARR10 (9/11 lines), and ARR12 (11/14 lines) but not ARR11 (0/11 lines), ARR14 (0/16 lines), or ARR18 (0/14 lines) could restore cytokinin sensitivity to the *arr1 arr12* mutant in root growth assays. Data for a subset of these lines is shown in Figure 2B, with a line capable of rescue being included if any such was observed. We included the *arr12* mutant in this analysis because it contains wild-type *ARR1* and thus represents the level of response one might anticipate if transgenic *ARR1* were expressed in *arr1 arr12* under completely native conditions. We defined complete complementation of *arr1 arr12* as a recovery to wild-type sensitivity or better, with a response that is significantly different from that of *arr1 arr12* ($P < 0.05$). We defined partial complementation as a recovery to at least 25% of the wild-type sensitivity, with a response that is significantly different from that of *arr1 arr12* ($P < 0.05$). In the absence of cytokinin, wild-type, *arr12*, *arr1 arr12*, and the transgenic lines are all of similar appearance, but significant differences can be observed in their root growth response to 1 μM benzyladenine (BA; Fig. 2B). Transgenic expression of *ARR1*, *ARR2*, *ARR10*, and *ARR12* all reverted the cytokinin insensitivity of *arr1 arr12* to wild-type levels or better (i.e. a complete complementation of the mutant phenotype). By contrast, transgenic expression of *ARR11*, *ARR14*, and *ARR18* failed to complement the mutant phenotype, although a slight but statistically significant increase in cytokinin sensitivity was noted for one line each of *ARR11* (line 1) and *ARR14* (line 7).

This same pattern of complementation was also observed in hypocotyl elongation assays, where cytokinin normally acts to inhibit hypocotyl growth in dark-grown seedlings (Supplemental Fig. S1). Hypocotyl length was similar for all lines in the absence of cytokinin, but in the presence of cytokinin, the transgenic lines of *ARR1*, *ARR2*, *ARR10*, and *ARR12* were all capable of partially or completely reverting the cytokinin insensitivity of *arr1 arr12* to a wild-type level of sensitivity. The same pattern

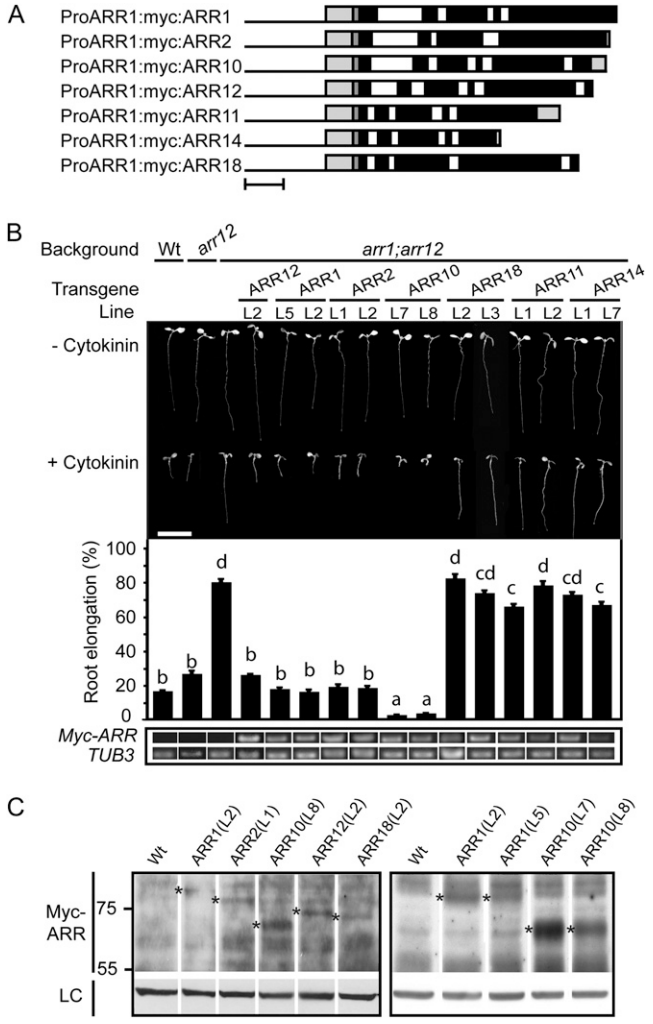

Figure 2. A subset of subfamily 1 ARRs functionally complement the root growth phenotype of the *arr1 arr12* mutant. A, Schematic of the subfamily 1 constructs used in this study. Bar = 500 nucleotides. Black line indicates *ARR1* promoter, light-gray box on left side indicates *ARR1* 5′-UTR, dark-gray box indicates myc sequence, black boxes indicate exons, white boxes indicate introns, and light-gray box on right side indicates 3′-UTR. B, Root growth inhibition by 1 μM BA. The top portion shows representative 7-d-old seedlings grown in presence or absence of 1 μM BA. The middle portion shows the root elongation response of seedlings grown on media containing 1 μM BA expressed as a percentage of root growth of siblings grown on dimethyl sulfoxide (DMSO) control media. Root growth from day 4 through day 7 was measured. Lines were analyzed for significant differences in their responsiveness to cytokinin based on Tukey's multiple range test among the means on the ANOVA ($P < 0.05$). Lines designated with the same letter exhibit no significant difference. The bottom portion shows transcript levels of the *ARR* transgenes in the roots of 7-d-old seedlings, based on RT-PCR from the common sequence involving the *ARR1* 5′-UTR and the c-Myc epitope tag. *β-tubulin3* (At5g62700) was used as a loading control. Amplicons are of the same exposure. Error bars represent SE. Bar = 1 cm. C, Protein levels of selected *ARR* transgenes based on immunoblot analysis using the Myc epitope tag. Asterisks indicate full-length transgenic protein. Hsp70 protein was immunologically detected as a loading control (LC). Predicted molecular masses are 76.4 kD (ARR1), 73.8 kD (ARR2), 62.9 kD (ARR10), 66.8 kD (ARR12), and 69.7 kD (ARR18).

of complementation was also observed in the ability of the transgene to rescue the enlarged seed size phenotype observed in the *arr1 arr12* mutant (Supplemental Fig. S1). The inability of ARR11, ARR14, and ARR18 to rescue the *arr1 arr12* mutant is not due to poor transgene expression, as their expression was similar to or higher than other family members that rescued the *arr1 arr12* phenotypes (Fig. 2B; Supplemental Fig. S2). We were also able to confirm protein accumulation for several of these transgenic proteins (Fig. 2C). We could consistently detect the tagged version of ARR10 (predicted molecular mass of 62.9 kD), and also detected less abundant protein bands corresponding to the tagged versions of ARR1 (76.4 kD), ARR2 (73.8 kD), ARR12 (66.8 kD), and ARR18 (69.7 kD). We could not detect ARR11 (59.8 kD) or ARR14 (44.3 kD), although in many cases, type-B ARR protein levels were below our limits of detection or obscured by nonspecific background bands, even under conditions where rescue was observed. These data support a functional difference among the subfamily 1 type-B ARRs.

In tandem with the physiological response phenotypes, we also examined molecular responses to determine how gene regulation correlates with the ability of the transgenes to functionally complement the *arr1 arr12* mutant (Fig. 3A). For this purpose, we examined the cytokinin-mediated induction of the primary-response genes *ARR15* and *ARR5* (Taniguchi et al., 2007; Argyros et al., 2008) and repression of *HIGH-AFFINITY K^+ TRANSPORTER1* (*HKT1*; Mason et al., 2010). *ARR15* and *ARR5* are induced approximately 11-fold and 7-fold, respectively, in response to 2-h cytokinin treatment in wild-type roots; however, this induction is severely attenuated in the *arr1 arr12* mutant (Fig. 3A). Despite comparable RNA and protein accumulation (Fig. 2, B and C), *ARR1* but not *ARR18* was able to rescue this molecular phenotype for *ARR15* and *ARR5* expression (Fig. 3A). *HKT1*, a gene whose product is responsible for removing sodium ions from the root xylem, is repressed by cytokinin treatment and significantly elevated in *arr1 arr12* (Fig. 3A; Mason et al., 2010). *ARR1* fully rescued and one of the *ARR18* lines (L3) partially rescued the molecular phenotype for *HKT1* (Fig. 3A). As a complement to this molecular study, we also examined the ability of type-B ARRs to stimulate expression of a cytokinin-regulated *LUCIFERASE* (*LUC*) reporter gene in a transient protoplast expression system (Hwang and Sheen, 2001). ARR1, ARR12, and ARR18 all stimulated *pARR6:LUC* expression, demonstrating that all three proteins are functional transcription factors, but only ARR1 and ARR12 activated the reporter gene in a cytokinin-dependent manner (Fig. 3B). Addition of the N-terminal Myc tag appears to decrease activity of ARR18 based on the transient protoplast assay, but ARR18 activity is still comparable to that of ARR1 and is substantially above that of ARR12, both of which rescue the *arr1 arr12* mutant (Fig. 3B). These results point to a fundamental difference in the ability of ARR1 and ARR18 to regulate expression of known cytokinin primary-response genes and

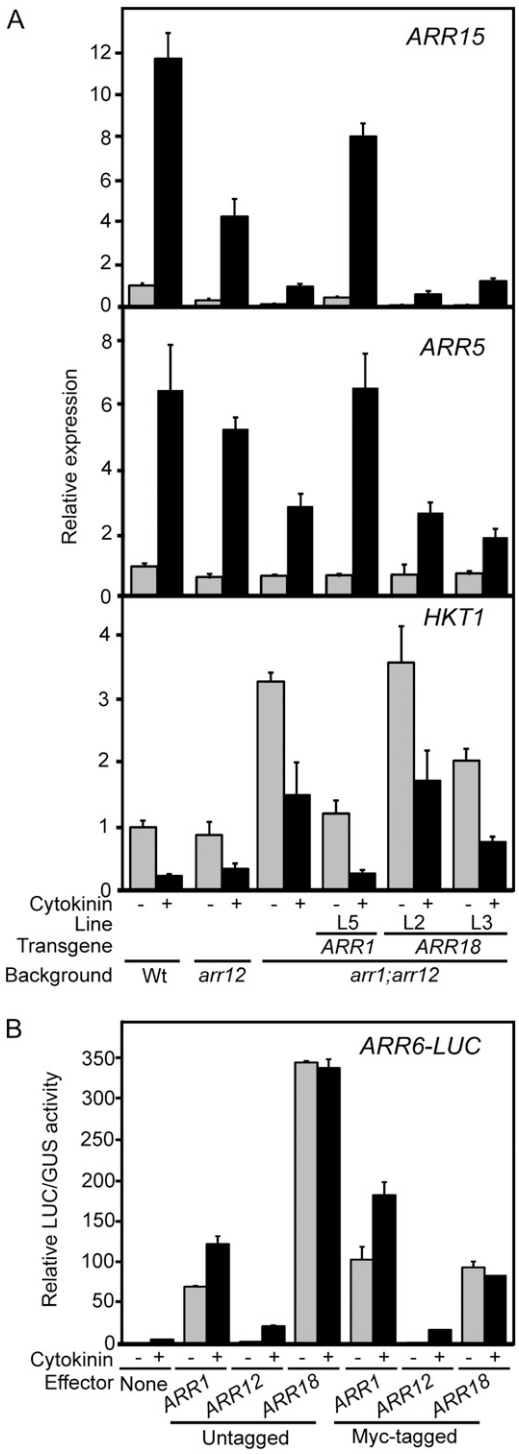

Figure 3. Effect of the *ARR1* and *ARR18* transgenes on cytokinin-regulated expression of cytokinin primary-response genes. A, Transcript levels of *ARR15*, *ARR5*, and *HKT1* were determined in the wild type (Wt), *arr12*, *arr1 arr12*, and transgenic lines of *arr1 arr12* containing either *proARR1*:*myc*:*ARR1* (*ARR1*) or *proARR*:*myc*:*ARR18* (*ARR18*). RNA was isolated from the roots of 14-d-old seedlings treated for 2 h with 10 μM BA or a DMSO vehicle control, and the relative expression levels of *ARR15*, *ARR5*, and *HKT1* were determined based on quantitative RT-PCR. Error bars indicate SE. B, Functional analysis of *ARR1*, *ARR12*, and *ARR18* in the Arabidopsis protoplast transient expression system. Protoplasts were cotransfected with the *ARR6-LUC* reporter and an effector plasmid expressing *ARR1*, *ARR12*, or *ARR18*, using untagged or Myc-tagged versions of the type-B ARRs. Transfection of the reporter gene with empty vector DNA were used as a control. Transfected protoplasts were treated without (–CK) or with (+CK) the cytokinin trans-zeatin (100 nM). The results are shown as the means of relative LUC activities from duplicate samples with error bars that indicate SD.

suggests that their differing abilities to rescue the *arr1 arr12* mutant may be due to such differences in gene regulation.

ARR10 Confers a Hypersensitivity Phenotype When Expressed in the Same Context as *ARR1*

We observed that *ARR10*, when expressed from the *ARR1* promoter, results in a cytokinin hypersensitivity phenotype in the roots, despite accumulating levels of transgene transcript comparable to other lines (Fig. 2B). We found this interesting because *ARR10* transcript is normally present in the wild-type root (Mason et al., 2004; Tajima et al., 2004) and because none of the other type-B ARRs gave a similar hypersensitive phenotype when expressed from the *ARR1* promoter. We therefore examined the hypersensitive phenotype of the *ARR10* transgenic lines in more detail. Based on a dose response analysis for root growth to cytokinin, the *ARR10* lines exhibit hypersensitivity at all cytokinin levels assessed, from 0.001 to 1 μM BA (Fig. 4A). At 1 μM cytokinin, virtually no root growth was observed in the *ARR10* lines (Figs. 2B and 4A), suggesting an almost complete absence of cell division in the root (Argyros et al., 2008).

Cytokinin also plays a key role in shoot regeneration; therefore, we examined how well *ARR10* functionally substitutes for *ARR1* in shoot induction assays. As shown in Figure 4B, wild-type tissue demonstrates increased cell division and greening in response to cytokinin treatment, and this response is substantially decreased in the *arr1 arr12* mutant, similar to previous results (Mason et al., 2005). Transgenic expression of *ARR1* or *ARR12* in the *arr1 arr12* background rescues the shoot induction phenotype to a similar level as that found in the wild type (Fig. 4B). The *proARR1*:*myc*:*ARR10* transgene not only rescues the *arr1 arr12* mutant, but also results in hypersensitivity to cytokinin in this assay based on two criteria. First, the *proARR1*:*myc*:*ARR10* transgenic lines exhibit more substantial greening and shoot development than is evident in any of the other lines. Second, the increased greening and shoot induction from callus occurs at lower levels of cytokinin than with the other lines (Fig. 4B).

In tandem with the physiological response phenotypes, we examined molecular responses of the *proARR1*:*myc*:*ARR10* transgenic line to determine how gene regulation correlates with functional complementation of

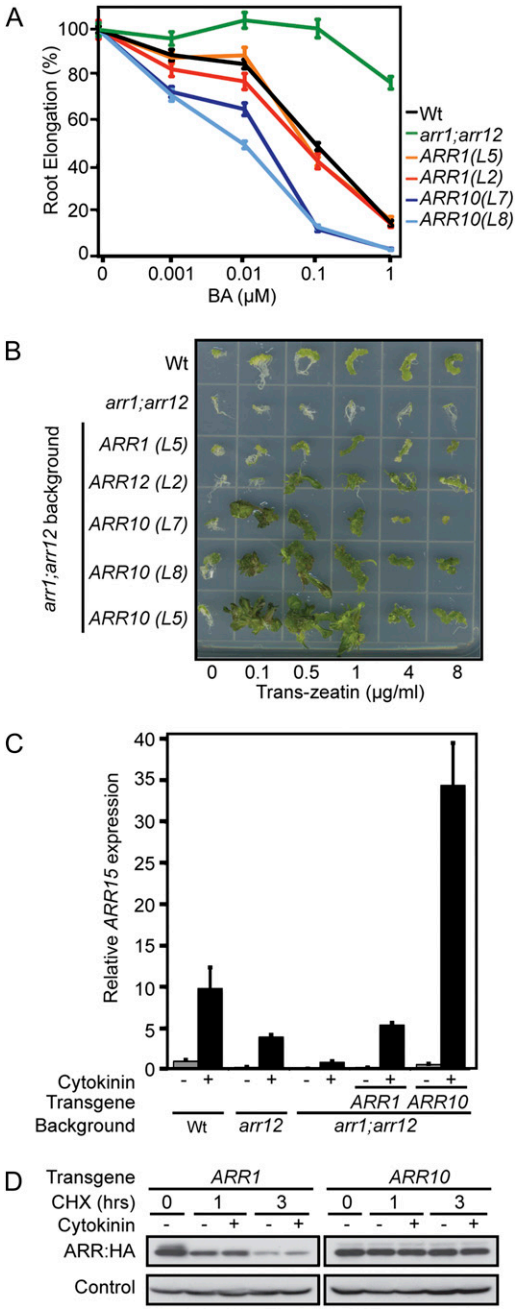

Figure 4. *ARR10* confers cytokinin hypersensitivity when expressed in the same context as *ARR1*. A, The root elongation response of seedlings grown on media containing 0.001, 0.01, 0.1, and 1 μM BA are expressed as a percentage of the root growth of siblings grown on DMSO control media. Root growth was measured from day 4 through day 7. Error bars indicate SE. The mean root growth measurements from untreated lines were 21.1 mm (wild type), 22.4 mm (*arr1 arr12*), 18.6 mm (*tARR1 L2*), 19.5 mm (*tARR1 L5*), 19.2 mm (*tARR10 L7*), and 19.2 mm (*tARR10 L8*). B, Induction of callus formation and greening. Representative hypocotyl segments treated with 0.2 mg L^{-1} indole-3-butyric acid and the indicated concentrations of trans-zeatin are shown after growth for 3 weeks under constant light. C, Relative *ARR15* transcript levels in RNA isolated from roots of 14-d-old seedlings treated for 2 h with 10 μM BA or a DMSO control. *β-tubulin-3* (At5g62700) was used as an internal control. Transgenic lines *tARR1* L5 and *tARR10* L7 were examined. D, Protein levels and degradation of ARR1 and ARR10 proteins in Arabidopsis protoplasts. Equal quantities of *ARR1* and *ARR10* plasmids were transfected into protoplasts. The transfected cells were treated with cycloheximide to inhibit protein biosynthesis, in the absence (–) or presence (+) of trans-zeatin, for the indicated times. ARR1 and ARR10 protein levels were determined by immunoblot analysis with an anti-HA antibody. α-Tubulin protein was immunologically detected as the loading control. Wt, Wild type; CHX, cycloheximide.

the *arr1 arr12* mutant. Consistent with the physiological responses to cytokinin (Fig. 4, A and B), *ARR10* expression resulted in a dramatically increased *ARR15* transcript level following cytokinin treatment (Fig. 4C). However, the basal levels of *ARR15* in the *ARR10* transgenic line were similar to that found in wild-type roots and the *arr1 arr12* line functionally complemented with *ARR1*. This result suggests that the cytokinin hypersensitivity found in the *ARR10* line is due to an enhanced ability to mediate cytokinin-regulated gene expression.

Although the transcript levels were comparable among the lines analyzed (Fig. 2B), protein levels tended to be higher for ARR10 compared with the other detectable type-B ARRs (Fig. 2C). In particular, we consistently observed higher protein levels for ARR10 compared with ARR1, even though both were driven from the *ARR1* promoter. This raised the question as to whether ARR10 protein is more stable than ARR1 protein, which could potentially account for its increased efficacy in functional complementation experiments. Low protein levels of ARR1 in the transgenic lines precluded a direct examination of protein stability in these lines. Therefore, to test this hypothesis, we transiently transfected Arabidopsis protoplasts with epitope-tagged versions of ARR1 and ARR10 and then examined their protein stability following treatment with the protein biosynthesis inhibitor cycloheximide (Fig. 4D). We observed that ARR10 was degraded more slowly than ARR1 following cycloheximide treatment (Fig. 4D). Treatment with cytokinin did not substantially alter the rate of ARR1 and ARR10 degradation. These results indicate that there is an intrinsic difference in the native protein stability of ARR1 compared with ARR10, consistent with the findings of Kim et al. (2012), which may account for the hypersensitivity observed in the *proARR1:myc*:*ARR10* transgenic lines.

Analysis of Subfamily 2 and 3 Family Members Indicates That *ARR21* Can Functionally Complement the *arr1 arr12* Mutant

We previously examined transfer DNA (T-DNA) insertion mutants in members of subfamily 1 and determined roles for *ARR1*, *ARR2*, *ARR10*, *ARR11*, and *ARR12* in several cytokinin-mediated responses, including root growth regulation (Mason et al., 2005;

Argyros et al., 2008). The subfamily 2 and 3 type-B ARRs exhibit more limited expression than members of subfamily 1 (Mason et al., 2004), but this does not necessarily imply a lack of substantive contribution to plant growth and development. To determine the role of subfamily 2 and 3 members in plant growth, we isolated T-DNA insertion mutants in subfamily 2 members *ARR13* and *ARR21* and subfamily 3 members *ARR19* and *ARR20* (Fig. 5A). Because subfamily 2 and 3 members are expressed primarily in the reproductive parts of the plant (Mason et al., 2004), we isolated RNA from these tissues to determine expression levels in the mutants. From RT-PCR analysis of *arr19-1*, *arr20-1*, and *arr21-2*, we determined these mutant lines do not accumulate detectable levels of full-length transcript (Fig. 5B). Lack of transcript is not shown for *arr13-1* because transcript was undetectable in wild-type tissues, as previously reported (Mason et al., 2004). As described below, we were able to express detectable *ARR13* transcript from the *ARR1* promoter, and we were able to use these lines to confirm efficacy of the *ARR13* oligonucleotides for expression analysis (Supplemental Fig. S2). Examination of the single and higher order mutants of subfamilies 2 and 3 revealed no pronounced effects on cytokinin sensitivity in root growth assays (Fig. 5C; Supplemental Fig. S3) or hypocotyl elongation assays (Supplemental Fig. S3). We did not observe any obvious effects on flower development, silique development, or seed size in the mutants.

As an alternative approach to characterize the subfamily 2 and 3 mutations, we generated higher order mutants involving the subfamily 1 mutation *arr1-3*. These higher order mutants were made with *arr1-3*, as it represents a sensitized background for mutant analysis, exhibiting a similar cytokinin sensitivity to the wild type as a single mutant (Fig. 5D) but cytokinin hyposensitivity in combination with other subfamily 1 mutations, such as in the *arr1 arr12* double mutant (Fig. 5D; Mason et al., 2005; Argyros et al., 2008). However, no additive effects were observed when the loss-of-function alleles of subfamily 2 or 3 members were combined with *arr1-3* in a root growth assay (Fig. 5D; Supplemental Fig. S3). A small additive effect was found when *arr19* or *arr21* were combined with *arr1* in a hypocotyl elongation assay, but this was not present in the higher order mutant combinations with *arr1* (Supplemental Fig. S4). Thus, no definitive role for subfamily 2 or 3 members in cytokinin-regulated growth was revealed based on several assays.

The lack of an effect of the subfamily 2 and 3 mutations is likely due in part to their limited expression pattern in the plant (Mason et al., 2004), raising the question as to whether they could play a more prominent role in cytokinin signaling if broadly expressed. We therefore employed the same approach we took with the subfamily 1 type-B ARRs to determine which could functionally substitute for ARR1. Members of subfamilies 2 and 3 were expressed from the *ARR1* promoter and multiple independent transgenic lines assayed for their ability to functionally complement the *arr1 arr12* mutant phenotypes. We found that *ARR19* (0/4 lines) and *ARR20* (0/10 lines) of subfamily 2 and *ARR13* (0/8

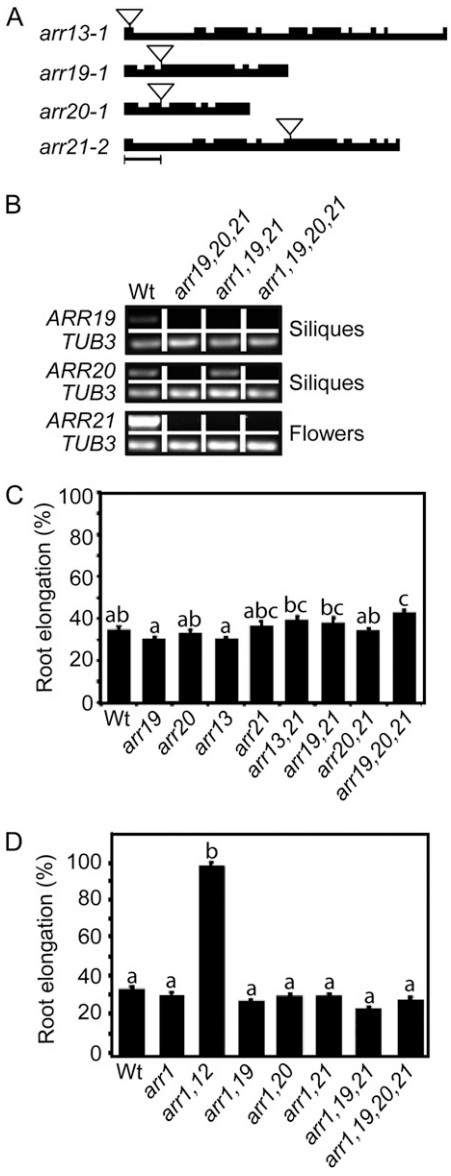

Figure 5. T-DNA insertion mutants of subfamilies 2 and 3 have minimal effect on cytokinin responses. A, Schematic of the T-DNA insertions in type-B ARR subfamily 2 and 3 genes, *arr13-1*, *arr19-1*, *arr20-1*, and *arr21-2*. Bar = 500 bp. B, RT-PCR showing lack of *ARR19*, *ARR20*, and *ARR21* transcript levels in the siliques and flowers (as indicated) of *arr19-1*, *arr20-1*, and *arr21-2* mutant lines. *ARR13* (not shown) was undetectable in the wild type, even after 40 amplification cycles as previously reported (Mason et al., 2004). *β-tubulin3* (At5g62700) was used as a loading control. C, Effect of subfamily 2 and 3 mutants on cytokinin sensitivity of the root. The root growth of seedlings grown on media containing 0.1 μM BA is expressed as a percentage of the growth of siblings grown on DMSO control media. Root growth was measured from day 4 through day 7. Error bars indicate SE. D, Subfamily 2 and 3 mutations exhibit no additive effects on the cytokinin sensitivity of the root when combined with *arr1-3*. Lines were analyzed for significant differences in their responsiveness to cytokinin based on Tukey's multiple range test among the means on the ANOVA ($P < 0.05$). Lines designated with the same letter exhibit no significant difference.

lines) of subfamily 3 were unable to functionally substitute for *ARR1* in root growth assays. Surprisingly, *ARR21* (5/8 lines) could functionally substitute for *ARR1* in the root growth assay, giving either partial or complete restoration of cytokinin sensitivity. Data for a subset of these lines is shown in Figure 6B, with a line capable of rescue being included if any such was observed. A similar trend was also observed in the ability of the subfamily 2 and 3 ARRs to rescue the enlarged seed size phenotype found in *arr1 arr12*, with only a transgenic *ARR21* line (line 8) clearly demonstrating rescue (Supplemental Fig. S5). By contrast, all the subfamily 2 and 3 members demonstrated little or no ability to functionally substitute for *ARR1* in a hypocotyl elongation assay (Supplemental Fig. S5). The inability of *ARR19*, *ARR20*, and *ARR13* to rescue the *arr1 arr12* mutant is not due to poor transgene expression, as they were expressed at comparable levels to the *ARR21* transgene that rescued the mutant (Fig. 6B). From these data, we conclude that *ARR21* can function in cytokinin signal transduction in a similar capacity to the subfamily 1 members *ARR1*, *ARR2*, *ARR10*, and *ARR12* and that the lack of mutant phenotypes in *arr21* is likely due to the restricted expression of *ARR21* and its functional redundancy with other type-B ARRs.

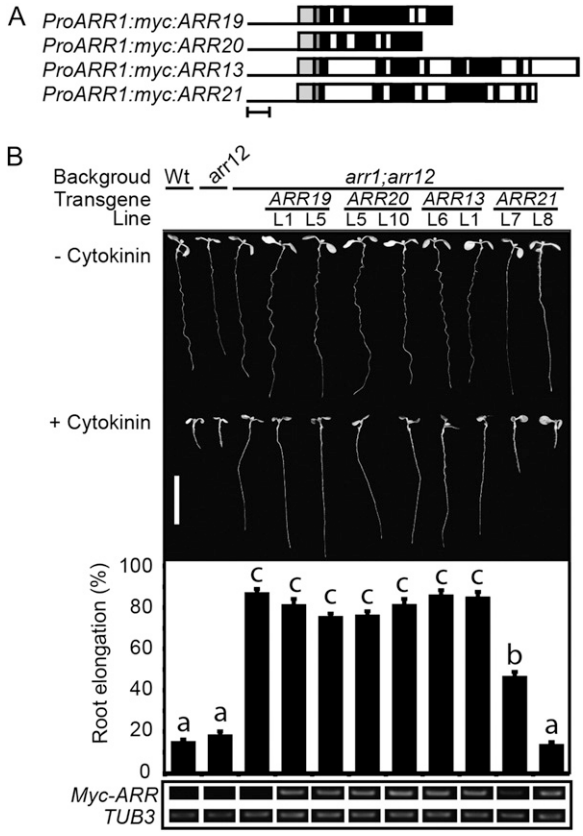

Figure 6. Analysis of subfamily 2 and 3 family members indicates that *ARR21* can functionally complement the root growth phenotype of the *arr1 arr12* mutant. A, Schematic of the subfamily 2 and 3 constructs used in this study. Bar = 500 nucleotides. Black line indicates *ARR1* promoter, light-gray box indicates *ARR1* 5′-UTR, dark-gray box indicates myc sequence, black boxes indicate exons, and white boxes indicate introns. B, The top portion shows representative 7-d-old seedlings grown in presence or absence of 1 μM BA. Bar = 1 cm. The middle portion shows the root elongation response of seedlings grown on media containing 1 μM BA expressed as a percentage of the root growth of siblings grown on DMSO control media. Error bars represent SE. The bottom portion shows transcript levels of the *ARR* transgenes in the roots of 7-d-old seedlings, based on RT-PCR from the sequence encoding the Myc epitope tag. *β-tubulin3* (At5g62700) was used as a loading control. Lines were analyzed for significant differences in their responsiveness to cytokinin based on Tukey's multiple range test among the means on the ANOVA ($P < 0.05$). Lines designated with the same letter exhibit no significant difference in their responsiveness to cytokinin.

DISCUSSION

Previous work to functionally characterize the type-B ARRs has primarily employed a mutant-based approach to assess their contributions to plant growth and development (Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008). However, such an approach is limited because a lack of mutant phenotype may arise due to the genes having low levels of expression, restricted expression patterns, and/or uncharacterized roles in plant development. The type-B ARRs for which functions have been determined are also those that exhibit the broadest expression pattern in Arabidopsis (Mason et al., 2004; Tajima et al., 2004). Subfamily 1 members *ARR1*, *ARR10*, and *ARR12*, in particular, play the most predominant role in regulation of the cytokinin response, consistent with their having a broad expression pattern and also being among the most highly expressed type-B ARRs (Argyros et al., 2008; Ishida et al., 2008). Previous work has also indicated that differences in the temporal expression of *ARR1* and *ARR12* affect their contribution to the regulation of cell division in the root meristem. Based on the analysis of GUS fusions, expression of *ARR1* was noted to increase following germination, while expression of *ARR12* remained constant, and this difference correlated with the effects of *arr1* and *arr12* mutants on root meristem cell number (Dello Ioio et al., 2008b; Moubayidin et al., 2010). We have now extended this analysis by taking a quantitative approach to assess temporal changes in expression at the root tip for the five most abundant type-B *ARR*s and then correlating these data with the effects of the single mutants. Our analysis confirms the prior data on *ARR1* and *ARR12* and also indicates that *ARR10*, which like *ARR1* exhibits a temporal increase in expression following germination, behaves similarly to *ARR1* in control of meristem cell division based on mutant analysis. *ARR2* and *ARR11* play less pronounced roles, consistent with their lower levels of expression in the root. The overlapping role in the control of cell division is likely to be mediated through the common mechanism of transcriptional control of *SHORT HYPOCOTYL2* (*SHY2*), a suppressor of the

auxin response and a transcriptional target of ARR1 and ARR12 (Dello Ioio et al., 2008b; Moubayidin et al., 2010). Thus, overall, the contribution of type-B *ARR*s to the cytokinin response closely correlates with their pattern and levels of expression.

As a means to assess functional similarity within the same developmental context, we expressed all 11 type-B ARRs from the *ARR1* promoter and determined which could rescue the cytokinin insensitivity phenotype observed in the *arr1 arr12* mutant. Results from our studies demonstrate substantial similarity in function among subfamily 1 members ARR1, ARR2, ARR10, and ARR12 and the subfamily 2 member ARR21, all of which can rescue multiple defects found in the *arr1 arr12* mutant. The finding that ARR2 and ARR21 exhibit this level of functional similarity is significant, as these type-B *ARR*s do not display strong mutant phenotypes; thus, their level of contribution to cytokinin signaling is apparently restricted due to their reduced expression profile (Mason et al., 2004; Tajima et al., 2004). Furthermore, a subfamily 1 type-B response regulator from rice (*Oryza sativa*), one phylogenetically related to ARR10 and ARR12, also restores cytokinin sensitivity to *arr1 arr12*, indicating a conserved function for some members of this group between monocots and dicots (Tsai et al., 2012).

The functional similarity of subfamily 1 members ARR1, ARR2, ARR10, and ARR12 is likely related to their ability to regulate a similar set of transcriptional targets, as in vitro studies indicate that the DNA-binding domains of ARR1, ARR2, and ARR10 all bind to a core AGATT sequence (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003; Taniguchi et al., 2007). However, whereas ARR1, ARR2, ARR10, and ARR12 are closely related based on phylogenetic analysis, ARR21 is substantially diverged, raising the question as to why it complements the *arr1 arr12* mutant but not other more closely related type-B ARRs. The complementation we observe for ARR21 is consistent with previous ectopic studies in which activated versions, lacking their inhibitory receiver domains, of both ARR1 and ARR21 resulted in seedlings that displayed severe developmental abnormalities, such as disordered cell division, along with induction of known cytokinin primary-response genes (Sakai et al., 2001; Tajima et al., 2004; Kiba et al., 2005). Sequence analysis of the DNA-binding domains does not suggest any specific residues that correlate with the ability of type-B ARRs to rescue *arr1 arr12* (Tsai et al., 2012). There is, however, a high degree of variation outside of the conserved receiver and DNA-binding domains; thus, more complex interactions not readily identifiable based on sequence homology may play a role in the ability of ARR21 to rescue the mutant phenotype. The finding that *ARR21*, a diverged member of the type-B ARR family, can complement *arr1 arr12* suggests that all members of the family may function as transcription factors, even though this has not been functionally demonstrated for all members.

Six of the type-B ARRs (ARR11, ARR14, and ARR18 of subfamily 1; ARR13 of subfamily 2; and ARR19 and ARR20 of subfamily 3) were unable to rescue the *arr1 arr12* mutant, pointing to functional difference(s) among the 11 type-B ARRs of Arabidopsis. These similarities and differences likely relate in part to the ability of the type-B ARRs to transcriptionally regulate an overlapping set of primary-response genes, based on the known function of type-B ARRs in transcriptional regulation (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; Liang et al., 2012; Tsai et al., 2012). DNA-binding studies indicate that ARR11, unlike ARR1, ARR2, and ARR10, does not bind to the core AGATT sequence (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003; Taniguchi et al., 2007). Similarly, in yeast (*Saccharomyces cerevisiae*) one-hybrid assays, ARR2 but not ARR11 associates with an anther/pollen-specific promoter fragment (Lohrmann et al., 2001). These data are consistent with our finding that ARR11 cannot functionally substitute for ARR1 or ARR12 and suggest that this arises in part from differences in their target specificity. The inability of ARR18 to complement *arr1 arr12* also likely arises in part due to differences from ARR1 in terms of target affinity or specificity based on our transcriptional analysis. Whereas transgenic expression of *ARR1* in *arr1 arr12* facilitated cytokinin-mediated induction of the primary-response genes *ARR5* and *ARR15* and cytokinin-mediated suppression of *HKT1*, transgenic expression of *ARR18* only facilitated the suppression of *HKT1*. In addition, although we observed that ARR18 functioned as a transcription factor in a transient protoplast expression system, it differed from ARR1 and ARR12 in that it did not activate the *ARR6* reporter in a cytokinin-dependent fashion. Differences in the ability of the type-B ARRs to transcriptionally regulate targets need not only arise from individual differences in target affinity or specificity, but could also arise from differences in type-B ARR protein stability (Kim et al., 2012) or their interactions with upstream regulators such as the ARABIDOPSIS HIS-CONTAINING PHOSPHOTRANSFER PROTEINS and/or transcriptional coregulators (Dortay et al., 2006; Kim et al., 2006). For example, ARR2 appears to be specifically activated through an AHK3-dependent phosphorelay in the regulation of leaf senescence (Kim et al., 2006). A potential lack of the relevant regulators would preclude the activation of the type-B ARRs.

The inability of *ARR11*, *ARR14*, *ARR18*, *ARR19*, and *ARR20* to complement the *arr1 arr12* mutant phenotype is not what one would necessarily predict based on previous characterization using transient protoplast assays and overexpression analysis in wild-type plants. In protoplasts, *ARR14* and *ARR20* stimulated a luciferase reporter driven by a concatamerized type-B binding site (*TWO COMPONENT OUTPUT SENSOR*::*LUC*) in a cytokinin-dependent manner, whereas *ARR19* stimulated expression of *TWO COMPONENT OUTPUT SENSOR*::*LUC* in a cytokinin-independent manner, as we saw with *ARR18* using

the *ARR6*::*LUC* reporter (Müller and Sheen, 2008; Zürcher et al., 2013). In a separate protoplast assay, *ARR18* induced an *ARR5*:*LUC* construct by about 50% in the presence of cytokinin (Veerabagu et al., 2012). When ectopically overexpressed in transgenic plants, *ARR18* increased the cytokinin sensitivity, and activated versions of *ARR11*, *ARR18*, and *ARR19* induced a cytokinin-like response (Liang et al., 2012; Veerabagu et al., 2012). Two possibilities, not mutually exclusive, can explain the differences observed between these studies and ours. First, the type-B *ARR*s were overexpressed in these previous studies, rather than being expressed from the *ARR1* promoter, high levels of the type-B ARRs potentially allowing for cross talk with the cytokinin-signaling pathway and/or overcoming a reduced affinity for the target DNA sites of ARR1. Second, these previous studies were performed in a wild-type background, as opposed to the *arr1 arr12* background, raising the possibility that their function is dependent in part on genes regulated through action of ARR1 and/or ARR12. Alternatively, because ARR18 multimerizes (Veerabagu et al., 2012), a physical association with ARR1 and/or ARR12 may allow for indirect transcriptional regulation. Characterization of two-component signaling in plants is likely to be particularly susceptible to artifacts from overexpression based on the known potential for promiscuous interactions in two-component systems (Skerker et al., 2008; Bell et al., 2010; Schaller et al., 2011). This last point is demonstrated by the ability of Arabidopsis cytokinin receptors and response regulators to function within a bacterial two-component system when transgenically expressed in *Escherichia coli* (Imamura et al., 1998; Yamada et al., 2001).

The observed differences in the ability of type-B ARRs to regulate gene expression and restore physiological responses in planta raises the question as to the function of the other type-B ARRs. It is likely that some also participate in cytokinin signaling, but (1) due to lower affinity for their targets, primarily play a role at high cytokinin levels; (2) due to higher rates of turnover, have a proportionately reduced contribution; (3) require additional coregulators to mediate their effects on transcription; and/or (4) regulate expression of different target genes than those regulated by the type-B ARRs implicated in cytokinin signaling. For example, whereas *ARR18* did not functionally complement the *arr1 arr12* mutant, we did find evidence that *ARR18* could regulate a subset of cytokinin-dependent genes in planta. It may also be that some type-B ARRs do not primarily function in cytokinin signaling but regulate the transcriptional response of other plant His kinases, such as AHK1, implicated in the osmotic response (Tran et al., 2007) or CYTOKININ-INDEPENDENT1 implicated in embryogenesis (Pischke et al., 2002; Hejátko et al., 2003). A better understanding of the targets of these type-B ARRs will likely provide key information on how they participate in two-component signaling pathways in plants.

Interestingly, and unlike the case with any of the other type-B ARRs, we found that expression of *ARR10* in the context of *ARR1* results in hypersensitivity to cytokinin. This level of hypersensitivity is greater than that reported from overexpression of *ARR1*, which showed slightly increased sensitivity to low concentrations of cytokinin but was comparable to the wild type at higher concentrations (Sakai et al., 2001). The cytokinin hypersensitivity in the lines expressing *ARR10* likely arises from the change in the zone of *ARR10* expression combined with the enhanced stability of the ARR10 protein. Based on GUS fusions and transcriptional profiling of the primary root tip, *ARR1* is expressed at similar levels throughout the stele, endodermis, cortex, and epidermis, but *ARR10* is expressed at higher levels in the epidermis than in the other tissues (Birnbaum et al., 2003; Mason et al., 2004; Argyros et al., 2008). Thus, when driven from the *ARR1* promoter, the level of *ARR10* within the internal tissues of the root would increase dramatically. In the transgenic plants, ARR10 protein accumulates to higher levels than ARR1 even though transcript levels are similar, apparently due to their differing rates of protein degradation. A higher level of ARR10 protein with a slower rate of degradation would allow for a greater number of ARR10 proteins to become activated in response to cytokinin and consequently increase the level and duration of the transcriptional response to cytokinin, as previous research has demonstrated that increasing the expression levels of type-B ARRs can enhance the cytokinin sensitivity of transgenic lines (Sakai et al., 2001; Liang et al., 2012). Other mechanisms may also contribute to the hypersensitivity conferred by ARR10, but the posttranscriptional difference in degradation rates between ARR10 and ARR1 alone is predicted to result in an enhanced efficacy for mediating the cytokinin signal.

The cytokinin hypersensitivity conferred by ARR10 has potential agronomic benefits, in particular the ability to increase cytokinin sensitivity in tissue culture. There is considerable variability in regenerative potential observed among plant species, even among different lines of Arabidopsis and rice (Abe and Futsuhara, 1986; Candela et al., 2001; Khalequzzaman et al., 2005), and this can pose a significant problem for transformation of crop plants (Birch, 1997). It was previously found that loss of four or more type-A ARRs, which serve to negatively regulate cytokinin signaling, resulted in increased regenerative potential for tissue culture (To et al., 2004). Here, we find that a change in the expression pattern for a single type-B ARR, ARR10, has a profound effect on regenerative capacity, suggesting that either ARR10 itself or orthologs from other plant species may be used to circumvent the recalcitrance of some crop species to tissue culture techniques.

MATERIALS AND METHODS

Plant Growth Conditions and Growth Assays

Wild-type and mutant lines of Arabidopsis (*Arabidopsis thaliana*) are all in the Columbia ecotype and were grown as previously described (Argyros et al., 2008). Root growth, hypocotyl elongation, shoot induction, and seed size assays were performed as previously described (Mason et al., 2005; Argyros et al., 2008).

Constructs and Generation of Transgenic Lines

To express the type-B ARRs from the *ARR1* promoter in planta, we constructed the binary destination vector, pEARLEY-pARR1:myc-Gateway cassette (GW), which contained an *ARR1* promoter, a *c-myc* epitope tag, and a Gateway cloning site. To construct this vector, a 1.2-kb region corresponding to the *ARR1* promoter, 5′-untranslated region (UTR), and ATG start codon was PCR amplified from genomic DNA using the primers 5′-CTAATCATAGTTACACACGACTTG-3′ and 5′-CATACCTCTCTCTATGTAGCTCG-3′ and ligated into the pCR8 entry vector (Invitrogen K2520–02) according to manufacturer's instructions. We then moved the *ARR1* promoter from the entry vector into the destination vector pEarleyGate303 (Earley et al., 2006) by Gateway technology, thus generating the pEARLY-pARR1-myc intermediate. The *Spe*I/*Bgl*II fragment containing a Gateway cloning site was isolated from pEarleyGate203 (Earley et al., 2006) and cloned into the analogous restriction sites of pEARLY-pARR1-myc to generate pEARLY-pARR1:myc-GW.

For expression in plants, the 11 type-B ARR sequences were amplified from Arabidopsis Columbia genomic DNA using oligonucleotides (Supplemental Table S1) that amplified from the translational start codon and included the stop codon, ligated into the pCR8 entry vector, and moved into pEARLY-pARR1:myc-GW according to the manufacturer (Invitrogen). The pEARLEY-pARR1:myc-ARR constructs were confirmed by sequencing and introduced into the *arr1-3 arr12-1* double mutant by the floral dipping method (Bent and Clough, 1998) using the *Agrobacterium tumefaciens* strain GV1301. For the protoplast transactivation assay constructs, the 35S promoter-GW-octapine synthase fragment from pEarleygate100 and the 35S promoter-myc tag-GW fragment from pEarleygate203 (Earley et al., 2006) were amplified (primers 5′-TAGGTACCGAATTCCAATCCCACAAAAATCTG-3′ and 5′-TAAAGCTTGGTCCTGCTGAGCCTCGA-3′) and cloned into the pBluescript II KS+ (pBS) vector (Agilent Technologies) through *Kpn*I and *Hin*dIII restriction sites to generate the Gateway compatible overexpression vectors pBS-35S-GW and pBS-35S-myc-GW. The genomic fragments of *ARR1* (primers 5′-ATGATGAATCCGAGTCACGGAA-3′and 5′-AACCTGCTTAAGAAGTGCGCTC-3′), *ARR12* (primers 5′-CACCTCTGATCCGAACAATGGGAAAGG-3′ and 5′-TCATATGCATGTTCTGAGTGAACTAAAC-3′), and *ARR18* (primers 5′-ATGAGGGTTCTTGCTGTGGAT-3′ and 5′-CTAAGGTGGAGGAAATGAATCAAAGC-3′) were amplified and cloned into the pCR8/GW/TOPO vector (Invitrogen) to generate the entry clones and then recombined into pBS-35S-GW and pBS-35S-myc-GW for protoplast luciferase assays.

For protein stability assays in protoplasts, complementary DNA sequences for *ARR1* (primers 5′-GGATCCATGATGAATCCGAGTCACGGAAGA-3′ and 5′-AGGCCTAACCTGCTTAAGAAGTGCGCTC-3′) and *ARR12* (primers 5′-CCATGGCTATGGAGCAAGAAATTGAAGTC-3′ and 5′-AGGCCTAGCTGACAAAGAAAAGGGAAAATG-3′) were fused to the hemagglutinin (HA) epitope coding sequence and expressed from the *35SC4PPDK* promoter as described (Sheen, 1996).

T-DNA Insertion Lines

The *subfamily-1* mutant alleles have been previously described (Mason et al., 2005; Argyros et al., 2008), except for *arr2-5* (GABI_269G01). The *arr13-1*, *arr19-1*, and *arr20-1* T-DNA mutant alleles were initially identified by PCR-based screening approaches with a T-DNA insertion population as described (Alonso et al., 2003; Mason et al., 2005), with *arr13-1* (SALK_042719) and *arr20-1* (SALK_009734) both available now from the Salk Collection. The mutant allele *arr21-2* (SALK_005772) was obtained from the Salk Collection. Sequence analysis of *arr2-5* identified the T-DNA junction with *ARR2* as (tacaattgaatatatcctg)tcgttgaatactcatTGCGAATCTTCGAGTTCTTGT, with uppercase letters indicating the *ARR2* sequence and parentheses indicating the T-DNA left border sequence, placing the insertion site within the first exon. Sequence analysis of *arr13-1* identified the T-DNA junction with *ARR13* as GTTGTGGACGATAATCGTGTT(gtaaacaaattgacgcttaa), placing the insertion site within the first exon. Sequence analysis of *arr19-1* identified the T-DNA junction as CACAATCTATTTCATATTTGTGa(tgtaaacaaattgacgct), placing the insertion site within the second intron. Sequence analysis of *arr20-1* identified the T-DNA junction as ACCCGTAGTAAGTAAGTATATtggacgt(tattgtggtgtaaacaaattg), placing the insertion site within the second intron. Sequence analysis of *arr21-2* identified the T-DNA junction as (ttgtctaagcgtcaatttgt)TCACATTAAGGAGCCGTACTT, placing the insertion site within the fifth exon.

RNA Expression Analysis

Total RNA was isolated and first-strand complementary DNA synthesis performed as previously described (Argyros et al., 2008). RT-PCR was used to confirm lack of RNA expression in the T-DNA insertion lines, and the primer sequences used for this analysis can be found in Supplemental Table S2. Semiquantitative RT-PCR was used to confirm and compare expression of the transgenes driven by the *ARR1* promoter. For this purpose, we used a primer against the 5′-untranslated region of *ARR1* (5′-GAGATTCACTTCTATCTCCAACAATTTCG-3′) and against the *c-myc* epitope tag (5′-CAAACTTGTGATCAGATCTTCTTCAGAG-3′). In addition, the presence of full-length transcript was verified for the transgenic lines using gene-specific primer pairs (data not shown). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio, RR041A) according to the manufacturer, as previously described using primer pairs specific for the genes of interest (Supplemental Table S3). *β-TUBULIN3* (At5g62700) was used as a loading and normalization control for RT-PCR and quantitative real-time PCR with primers 5′-TGGTGGAGCCTTACAACGCTACTT-3′ and 5′-TTCACAGCAAGCTTACGGAGGTCA-3′.

Transactivation and Protein Stability Assays in Arabidopsis Protoplasts

Arabidopsis protoplasts were isolated and transfected as described (Hwang and Sheen, 2001; Yoo et al., 2007). For transactivation assays, the *ARR6-LUC* reporter gene was transfected alone or cotransfected with *ARR1*, *ARR12*, or *ARR18* effectors into protoplasts isolated from wild-type plants. Transfected protoplasts were incubated with or without 100 nM trans-zeatin for 3 h under dim light (5 μE m^{-2} s^{-1}). The *UBIQUITIN10-GUS* construct was used as an internal control. For protein stability assays, transfected protoplasts were incubated for 4 h to allow for protein expression and then treated with 100 μM of cycloheximide and 1 μM of trans-zeatin for indicated times.

Protein Isolation and Immunoblot Analysis

Seedling samples were ground in liquid nitrogen and the powder resuspended in isolation buffer containing 50 mM TRIS (pH 7.5), 0.1% (v/v) Nonidet P-40, 10 mM EDTA, 150 mM NaCl, and protease inhibitors (Sigma-Aldrich, P9599). Protoplast samples were frozen, resuspended in isolation buffer, and vortexed. Samples were centrifuged at 16,000*g* for 15 min and the supernatant retained for further analysis. Protein concentration was determined by use of the bicinchoninic acid reagent (Pierce) according to the manufacturer after first adding 0.2% (w/v) SDS to the samples and with bovine serum albumin as a standard. Samples were heated above 65°C in gel-loading buffer, and SDS-PAGE and immunoblotting was performed as previously described (Gao et al., 2008). HA-tagged proteins were detected by using a peroxidase-conjugated anti-HA antibody (Roche Applied Science). Myc-tagged proteins were detected with a monoclonal anti-Myc antibody conjugated to horseradish peroxidase (monoclonal 9E-10; Santa Cruz Biotechnology). Monoclonal antibodies against Hsc70 protein (StressGen) and α-tubulin (Sigma-Aldrich), coupled with goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology), were used for detection of these protein-loading controls.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ARR1 (At3g16857), ARR2 (At4g16110), ARR10 (At4g31920), ARR11 (At1g67710), ARR12 (At2g25180), ARR13 (At2g27070), ARR14 (At2g01760), ARR18 (At5g58080), ARR19 (At1g49190), ARR20 (At3g62670), ARR21 (At5g07210), ARR5 (At3g48100), ARR15 (At1g74890), and HKT1 (At4g10310).

Supplemental Data

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

Supplemental Figure S1. A subset of subfamily 1 ARRs can functionally complement the *arr1 arr12* mutant based on cytokinin-modulated hypocotyl elongation and seed size.

Supplemental Figure S2. RT-PCR confirmation of transgene expression in transgenic lines of *arr1 arr12*.

Supplemental Figure S3. T-DNA insertion mutants of subfamilies 2 and 3 have minimal effect on cytokinin response based on a root growth dose response assay.

Supplemental Figure S4. T-DNA insertion mutants of subfamilies 2 and 3 have minimal effect on cytokinin responses in hypocotyl elongation assays.

Supplemental Figure S5. Ability of subfamily 2 and 3 family members to functionally complement the *arr1 arr12* mutant based on cytokinin-modulated hypocotyl elongation and seed size.

Supplemental Table S1 Oligonucleotides used for cloning type-B ARRs.

Supplemental Table S2. Oligonucleotides used to examine expression of subfamily 2 and 3 T-DNA insertion lines.

Supplemental Table S3. Oligonucleotides used for quantitative RT-PCR.

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