A B-ARR-mediated cytokinin transcriptional network directs hormone cross-regulation and shoot development

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Cytokinin fulfills its diverse roles in planta through a series of transcriptional responses. We identify the in vivo DNA binding site profiles for three genetically redundant type-B ARABIDOPSIS RESPONSE REGULATORS (B-ARRs): ARR1, ARR10, and ARR12. The expression and genome-wide DNA binding locations of the three B-ARRs extensively overlap. Constructing a primary cytokinin response transcriptional network reveals a recurring theme of widespread cross-regulation between the components of the cytokinin pathway and other plant hormone pathways. The B-ARRs are found to have similar DNA binding motifs, though sequences flanking the core motif were degenerate. Cytokinin treatments amalgamate the three different B-ARRs motifs to identical DNA binding signatures (AGATHY, H(a/t/c), Y(t/c)) which suggests cytokinin may regulate binding activity of B-ARR family members. Furthermore, we find that WUSCHEL, a key gene required for apical meristem maintenance, is a cytokinin-dependent B-ARR target gene, demonstrating the importance of the cytokinin transcription factor network in shoot development.

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Cytokinin, an N6-substituted adenine derivative, along with other phytohormones orchestrates almost every aspect of plant growth and development, including meristem function, vascular development, biotic and abiotic stresses, and leaf senescence1-5. Cytokinin was first discovered for its ability to promote cell division over fifty years ago6. In the past twenty years, its own biosynthesis and signaling pathways and diverse roles in regulating cellular processes have been revealed by both forward and reverse genetic screens7-12. Cytokinin employs a two-component multi-step phosphorelay for its perception and signaling transduction12-15. In Arabidopsis, there are three cytokinin receptors (ARABIDOPSIS HISTIDINE KINASES; AHK2, 3, and 4) and eleven type-B response regulators (ARABIDOPSIS RESPONSE REGULATORS; B-ARRs)8,13. In cytokinin signaling cascades, the histidine-containing phosphor-transfer proteins (AHPs) act as phosphor-transfer intermediates for various AHK-AHP-B-ARR modules16. Genetic analysis also revealed that only higher order mutants of each family render pronounced developmental phenotypes, indicating redundancy in the cytokinin signaling pathway9.

The cytokinin transcriptional response centrally affects the family of ARRs. Type-B response regulators (B-ARRs) are transcription factors (TFs) with a GARP-like DNA binding domain at their C-termini and a receiver domain at their N-termini. Type-A ARRs (A-ARRs) are similar to the N-termini receiver domain of B-ARRs but do not possess a DNA binding domain. A-ARRs are negative cytokinin regulators but their mechanism of inhibition in cytokinin signaling remains unknown12. The DNA binding domain and protein nuclear localization signal domain at the C-terminus of B-ARRs are responsible for B-ARRs entering the nucleus and binding to their targets while their activation domain is responsible for the activation of cytokinin transcriptional responses. The presence of the receiver domain in B-ARRs is thought to cause inhibition at low cytokinin levels and may block the upstream phosphorelay to B-ARRs. It is postulated that the receiver domain masks the DNA binding domain of B-ARRs until its conformation is altered by cytokinin, which finally results in the activation of B-ARRs15. In previous genetic analyses, five Arabidopsis B-ARRs were shown to act in cytokinin signaling cascades with ARR1, ARR10, and ARR12 playing critical roles in plant growth and development8. The A-ARRs are cytokinin response genes that are the targets of B-ARR TFs12. However, the B-ARRs are not regulated at the transcriptional level by cytokinin but are post transcriptionally controlled11. Recently, B-ARRs were shown to be regulated at the level of protein stability, at least in part, through the ubiquitin-proteasome pathway17.

Previous in vitro studies have identified candidate binding motifs for the B-ARRs18,19 and a “golden list” of cytokinin response genes from microarray expression data and RNA-seq data20. However, the identity of which cytokinin responsive genes may be direct targets of the B-ARRs remains unknown. In addition, most experiments have depended upon treatment with high concentrations of cytokinin, since the targets of B-ARRs are almost impossible to identify at the endogenous levels of cytokinin in transcriptomic studies. Therefore, identification of the genome-wide targets of B-ARRs, with and without cytokinin treatment, would facilitate our understanding of the cytokinin responsive DNA regulatory elements, provide insights into cytokinin primary responsive gene expression, and potentially elucidate the mechanism(s) by which cytokinin ultimately regulates diverse physiological responses. Recently, genome-wide binding sites of ARR10 were identified by chromatin immuno-precipitation sequencing (ChIP-seq) of a tagged, over-expressed ARR10 fusion protein21, demonstrating the utility of in vivo DNA binding studies for cytokinin response pathway analysis.

Cytokinin plays an important but poorly understood role in the maintenance of the stem cell niche and regulation of meristem size22,23. First, inhibition of a subset of A-ARRs by WUSCHEL (WUS) has been demonstrated, although the mechanism of this repression remains unknown24. Since A-ARRs are targets of B-ARRs, it can be postulated that the repression of A-ARRs by WUS involves B-ARRs. Second, the arr1/10/12 triple mutant was shown to produce a smaller size shoot apical meristem2. Third, genetic manipulation of cytokinin levels either by loss-of-function mutants of LONELY GUYS, which are involved in the one step conversion of cytokinin precursors to active cytokinin25, or by over-expression of cytokinin oxidase produces meristem defects26. Finally, plant regeneration requires the proper ratio of cytokinin and auxin27. Therefore, a greater understanding of the targets of B-ARRs may provide a link between the cytokinin transcriptional response and important plant developmental processes such as meristem development.

Our study aims to construct a core cytokinin transcriptional response network with a focus on systematic identification of the binding targets of the B-ARRs, the key players in cytokinin signaling. We take advantage of the power of recombineering28 to generate plants containing epitope-tagged B-ARRs and employ ChIP-seq to identify their genome-wide binding locations. Extensive targeting of multiple type-B ARRs to a common set of genes reveals a conserved core cytokinin transcriptional response network and extensive cross-regulation of the plant hormone pathways. We also demonstrate that the regulation of WUS by B-ARRs is critical for stem cell maintenance in the shoot apical meristem. These findings provide potential avenues to further explore the mechanism operating downstream cytokinin responses that control diverse growth and development processes.

Results

The protein localization of B-ARRs reveals extensive overlap. Previous genetic studies of the arr1/10/12 triple mutant revealed pronounced developmental phenotypes, such as smaller size seedling and adult plants, effects likely due to a smaller shoot apical meristem and insensitivity to cytokinin treatment2,8. Such studies revealed that ARR1, ARR10, ARR12 are critical components of the cytokinin signaling pathway (Fig. 1a). To explore the cellular distribution pattern of these three B-ARRs, Ypet (yellow fluorescent protein)-tagged B-ARRs lines were generated using a recombineering-based gene tagging technique28. The advantages of this strategy are that both the expression pattern and protein location can be monitored. Moreover, because the near-by gene (cis-) regulatory information is maintained, these tagged gene constructs provide a state nearest to the native expression of the endogenous B-ARRs as it is currently technically possible in plants28. ARR1, ARR10, and ARR12 tagged lines were generated in the Col-0 background and were used for ChIP-seq experiments. The functionality of these constructs was also confirmed by successful complementation of the arr1/10/12 triple mutant phenotype (Fig. 1b).

Previous organ-specific expression analysis using reverse transcription PCR and promoter reporter analysis using GUS staining revealed an overlapping expression pattern of B-ARR8,29. We used our ARR-recombineering lines to study the expression pattern of B-ARRs in three-days-old seedlings in the absence and presence of cytokinin (10 µM 6-Benzylaminopurine (6-BA)). We found that all three B-ARRs had similar pattern expressed in roots, hypocotyl, and cotyledons (Fig. 1c; n = 3 for each ARR gene). Consistent with previous findings, all three B-ARRs were localized in the nucleus18,29-31. However, the localization of ARR1 upon 6-BA treatment was more obvious in root tip and cotyledons than in other tissues (Fig. 1c-ARR1 +
BA). In contrast, the 6-BA treatment had less impact on the localization of ARR10 compared to other tested-ARRs (Fig. 1c-ARR10). This was consistent with previous findings identifying ARR10 as the most stable B-ARR. Additionally, the intensity of ARR12 was slightly lower than the mock treatment in the root hair region of roots (Fig. 1c-ARR12-BA). One apparent discrepancy with previous results was the expression of ARR1, ARR10, ARR12 in columella cells of root tips (Fig. 1c, square bracket).

Construction of a cytokinin network using B-ARRs targets. The targets (genes near the DNA binding sites of B-ARRs) of three key B-ARRs (1, 10, 12) were identified by ChIP-seq using long-day (16-h light/8-h dark cycle) conditions at 22 °C and 3-days-old seedlings growing vertically on plates containing Murashige and Skoog (MS) medium (Fig. 2a, and Supplementary Fig. 1, Supplementary Data 1). The binding profiles of all three factors were generated either in the absence of 6-BA (endogenous level of cytokinin) or in the presence of 10 μM 6-BA (cytokinin treatment) for 4 h, or 3 days (only for ARR1). Without 6-BA treatment, 2815 (ARR1_m), 4822 (ARR10_m), and 823 (ARR12_m) targets were identified using a cutoff of p-value 1E-16 (MACS2 peak caller, cutoff: +/-1.5 kb of genes). Using the same standards, the cytokinin treated samples had higher numbers of targets for all three B-ARRs (5128 (ARR1_BA), 6272 (ARR10_BA), and 6240 (ARR12_BA)). An increase in the number of targets upon cytokinin treatment might result from either protein stabilization or modification by phosphorylation or both processes. Interestingly, samples treated with 10 μM 6-BA for three days showed even further increase in the number of targets for ARR1, up to approximately 10,000. B-ARR binding sites detected by ChIP-seq were highly enriched near gene transcription start sites (TSS) (Fig. 2b and Supplementary Fig. 2). They were enriched in regions 1.5 kb upstream and 1 kb downstream of genes, but enrichment dropped dramatically beyond 1 kb downstream of genes.

From comparison of genes near in vivo DNA binding sites for these B-ARRs at endogenous and elevated cytokinin levels, a TF-TF interaction network was constructed to analyze the redundant and diverged role of B-ARR family members (Fig. 2c). In addition, a directed gene regulatory network was constructed using the three cytokinin-treated ChIP-Seq results and publicly available DAP-Seq results as edges and changes in target gene expression (as measured by steady state RNA level) as nodes (Fig. 2d). While the B-ARRs are not themselves transcriptionally cytokinin responsive, 6-BA induced an increase in B-ARR binding of cytokinin “target genes” suggesting they regulate the expression of cytokinin responsive genes. Individual B-ARR shared many targets between mock and 6-BA treatment datasets. For example, 85% (4075 of 4822 mock treated plants) of ARR10 mock targets overlap with 4 h 6-BA targets (Fig. 2c). All 10 A-ARRs (ARRs 3, 4, 5, 6, 7, 8, 9, 15, 16, and 17), well-known cytokinin response genes, were among the targets of B-ARRs (Fig. 3a). At endogenous cytokinin levels, ARR1, ARR10, and ARR12 shared 503 targets (Fig. 3b). In addition, ARR1 and ARR10 shared another 1915 targets. In contrast, ARR12 shared less targets than either ARR1 or ARR10. At the elevated cytokinin levels (4-h 6-BA treatment), a set of 3373 targets were shared by ARR1, ARR10, and ARR12 (”common set of targets”) while 8770 targets were bounded by at least one of the three B-ARRs (”union set of targets”) (Fig. 3c, Supplementary Data 2). Additionally, ARR1 shared more targets with ARR10 than ARR12. The results indicated that many targets under mock treatment (endogenous cytokinin) were also bound by B-ARRs upon 6-BA treatments of 4 h or 3 days (Fig. 3d). However, the number of target genes increased from 2815, 5128, to 10,340 up 6-BA treatments (Figs. 2c and 3d).
differentially expressed in response to cytokinin treatment (Supplementary Data 3 and Data 4). In total, 554 genes were treated for 4 h and using an
were done using RNA-seq data from plants treated with 6-BA

Fig. 2 Cytokinin-dependent transcriptional response network. a Potential target genes near the binding sites of B-ARRs by different treatments (mock treatment (m), treatment with 10 µM 6-BA for 4 h (BA), or treatment for 3 days (BA3d)). Nearby genes were determined by B-ARR binding within 1.5 kb upstream and downstream of the gene’s annotation. The total number of nearby genes for each treatment is printed on each bar. b Distribution of ChIP-seq peaks around a normalized gene (1 kb) for ARR1_m. The base position relative to the transcription start site (TSS). c Heatmap showing number of the shared target genes of the B-ARRs TF-TF interaction network. Conditions are: mock treatment: ARR1_m, ARR10_m, ARR12_m; 4-h 10 µM 6-BA treatment: ARR1_BA, ARR12_BA; 3-day 10 µM 6-BA treatment: ARR1_BA3d. d A sub-network graph of the B-ARRs gene regulatory network. Nodes were either type-A ARRs or transcription factors from the core target genes of B-ARRs with DAP-seq data available. The sizes of nodes are in proportion to the max peak score

The overall correlated profile of peak locations was found (Supplementary Fig. 3a). Compared to dataset 1 of Zubo et al.21 2783 (69.5%) out of 4004 potential targets were also identified in our study (Fisher’s Exact test, p-value < 0.001, Supplementary Fig. 3b). If only the ARR10 "regulated targets" (those with evidence of transcriptional activity) in dataset 2 of Zubo’s study were considered, 87.4% were overlapping with our B-ARR union target set (Supplementary Fig. 3c). Though the recombineering experiment and the over-expressing experiment showed a high degree of agreement, our combined analysis of three B-ARRs experiment and the over-expressing experiment showed a high degree of agreement, our combined analysis of three B-ARRs

whereas the expression of 2323 genes were affected in the triple mutant (q-value <= 0.05 and 1.6-fold changes as cutoff, Supplementary Fig. 4a, b). When previous transcriptomic data and recent CaMV 35S over-expression ARR10 ChIP-seq data21 were compared, the expression of 813 common targets and 1713 union targets were observed to be affected by cytokinin treatment (Supplementary Data 5). These union target genes that showed cytokinin-induced expression changes were designated as “core target genes” of the B-ARRs. The top 50 genes ranked by their maximum peak scores contained eight known cytokinin biosynthesis/degradation or response genes (16%) including type-A ARRs (ARR4, 5, 7,15), cytokinin receptor (WOL/ATHK4), and cytokinin degradation enzyme (CKX5) and showing a 10-fold enrichment (p < 0.001, binomial test, Supplementary Fig. 5a, 5b). Finally, the intersection of 1713 core target genes and a previous large-scale TF binding dataset33 was used to construct a cytokinin
transcriptional gene regulatory network (Fig. 2d, Supplementary Data 6), providing a framework for future studies of cytokinin response genes.

A negative feedback loop in the cytokinin regulatory network. Previous indirect evidences including genetic studies, transcriptional profiling results, and promoter deletion analyses of A-ARRs suggested that these genes are targets of B-ARRs [20,34,35]. Our results show significant increase of B-ARR binding to the promoter of A-ARRs in response to cytokinin (Fig. 3a) and A-ARR genes are among the top-ranking targets of multiple B-ARRs (percentile ranking <5%). Upon exogenous cytokinin treatment (6-BA treatment for 4h to 3 days), B-ARRs show significant increase of binding to A-ARRs (Fig. 3e) and their downstream target genes (Fig. 3f; Wilcoxon rank-sum test \( p < 0.001 \)). In contrast, there was not much change in binding at the promoter of the EIN3 binding factor (EBF2) (Fig. 3e), a negative regulator in the ethylene signaling pathways [36]. Interestingly, a few B-ARRs, including ARR1, ARR10, ARR12, ARR18, and ARR14 were among low-ranking targets (Fig. 3e). Overall these analyses support a scenario in which the promoters of the A-ARR genes are bound by B-ARRs representing an efficient feedback mechanism to fine-tune cytokinin responses in the plant.

**Fig. 3** A-ARRs are direct targets of B-ARR transcription factors. a AnnoJ browser snapshots of A-ARR genes, targets of B-ARR TFs. b Venn diagram describing the number of target genes for each of three key B-ARRs in the mock treatment (m). c Venn diagram describing the number of target genes for each of three key B-ARRs in the 10 \( \mu M \) 6-BA 4-h treatment (BA). d Venn diagram describing the number of target genes increase for ARR1 in a cytokinin time course treatment, 0 h (m), 4 h (BA), 3 days (BA3d). e Peak scores (−Log10 p-value) of A-ARRs in various cytokinin treatments. f Box plot showing the distributions of the binding peak scores of B-ARR targets. A-ARRs were highlighted in red circles. Asterisks (*** *) indicates significant difference using Wilcoxon rank-sum test (\( p < 0.001 \)).
Functional classification of B-ARR targets. A previous study of binding sites for the master transcriptional regulator for the plant hormone ethylene revealed major feedback loops where EIN3 directly targeted almost all essential genes in the ethylene signaling pathway, as well as key regulators of other phytohormone pathways. To find out whether this TF-governed auto-regulation and cross-regulation with other pathways also holds true or not for B-ARRs, we performed gene ontology (GO) analysis of target genes of B-ARRs using the top 3000 genes ranked by IDR score. These analyses revealed enrichment for similar biological function and processes for all tested B-ARRs (Supplementary Fig. 6). GO enrichment analysis was consistent with the fundamental and diverse role of cytokinin (Supplementary Fig. 6). B-ARR DNA binding sites are highly associated with plant hormone responsive genes and cytokinin genes (GOTERMS: response to plant hormone stimulus p-value 1.5E-26, two-component signal transduction p-value 6.3E-15). Among the 3373 common targets of the three B-ARRs (ARR1/ARR10/ARR12) tested, we observed enrichment of hormone-related genes (Fig. 4a, Supplementary Data 7). Additionally, the top 300 genes ranked by ARR10 peak scores were used to refine the GO analysis. The top GO terms are similar in the global analysis for the targets of three B-ARRs. These targets of B-ARRs include the primary cytokinin response genes, A-ARRs and the cytokinin receptor AKH4. Although B-ARRs are not regulated at the transcriptional level by cytokinin, it is interesting that several (ARR1, ARR10, ARR12, ARR14 and ARR18) were found in the B-ARR target gene list as being under control of B-ARRs. In addition, B-ARRs were found to bind at cytokinin biosynthesis and degradation pathway genes (Fig. 4a, Supplementary Data 7). Thus, the transcriptional responses directed by B-ARRs may include nearly every step from cytokinin perception and signaling transductions to the TFs. Like EIN3, the response of B-ARRs to cytokinin may involve cross-regulation with other plant hormone biosynthesis, signaling, and response pathways (Fig. 4a). B-ARRs were found to target the auxin receptor genes TIR1 and AFB2, as well as the auxin transportation efflux carrier genes, the Pin-formed and Pin-formed-like (PIN3/4/7), and the GH3s genes which mediate auxin conjugation. Auxin transcriptional response regulators, including several ARFs and many Aux/IAAs genes, were also among the list of B-ARR targets (Fig. 4a, Supplementary Data 7). The most striking features among the hormone-related targets of B-ARRs are genes encoding the master transcriptional factors such as MYC2, PIFs, BES/BZR, and ERFs (Fig. 4a), each responsible for mediating the transcriptional responses to other plant hormones. B-ARRs also target genes encoding plant hormone receptors such as BRI1/BAX1/BAX2, PYR/PYLs, and TIR1/AFB2 (Fig. 4a). In addition, B-ARRs targeted the plant hormone negative signaling component genes, such as Aux/IAAs for auxin, EBFI/2 for ethylene, BIN2 for brassinosteroid, DELLAs for GAI and RGA for gibberellin.

Next, we inquired into whether the transcription of B-ARR target genes are regulated by cytokinin levels (Supplementary Data 3 and Data 4). The regulated genes were then layered into plant hormone pathways targeted by B-ARRs (Supplementary Data 7). Cytokinin treatment increased gene transcripts for most A-ARRs, AKH4, and cytokinin degradation enzymes in the cytokinin pathways (Supplementary Data 7). Whereas gene transcripts were shown to decrease for important negative regulators of other hormone pathways, including several IAAAs, and GH3s for auxin, SnRK3.14 and NRT for abscisic acid (ABA), as well as modification enzyme for salicylic acid (SA) (Supplementary Data 7). In addition, in the triple mutant background, the auxin receptor AFB2 and the auxin efflux carrier PIN7 were down-regulated (Supplementary Data 7, gene names underscored) whereas TASA3 and the ethylene receptor ETR2 were up-regulated (Supplementary Data 7, gene names in green). These results suggest that cytokinin pathway TFs target important regulators of other plant hormones pathways, potentially leading to diverse outputs for developmental and growth programs, as well as responses to environmental cues.

Numerous studies have reported that TF binding does not necessarily coincide with changes in gene transcription. In the case of EIN3, only 30% of the ET-induced binding events were associated with transcriptional changes. We examined genes in the “golden list” for cytokinin responses which were identified by Bhargava using a meta-analysis of multiple expression datasets, to determine the overlap with genes that we identified as B-ARR targets. In all, 116 (73.4%) of 158 up-regulated genes in the golden list were identified among the 8770 union binding targets of three B-ARRs, suggesting significant association between B-ARR binding and cytokinin-induced transcriptional responses (Fig. 4b; Fisher’s Exact Test p < 0.001). When compared to the 3373 common targets of three B-ARRs, the percentage of overlap dropped to 35.4% which is still highly significant (Fig. 4b; Fisher’s Exact Test p < 0.001). Similarly, 35 (51.4%) of 68 down-regulated genes in the golden list were overlapped among the 8770 union targets of B-ARRs (Fisher’s Exact Test p < 0.001) although only 26.5% of these were among the 3373 common targets of the three key B-ARRs (Fig. 4b; Fisher’s Exact Test p < 0.001). A small portion (74 of 226 or 32.7%) of genes in the golden list were not found in the list of B-ARR targets. They might be either indirect cytokinin response genes or the targets of other B-ARRs that were not tested in this experiment. It is interesting that activators like B-ARRs directly target cytokinin repressed genes which may involve the recruitment of other co-regulators. Only 490 (14.5%) of 3373 common targets of three B-ARRs (ARR1/ARR10/ARR12) were affected transcriptionally either in triple mutant or by cytokinin treatment (Fig. 4c). A subset of 162 common targets that the binding of B-ARRs responded to cytokinin treatment also changed their expression upon cytokinin treatment (Fig. 4d, Supplementary Fig. 4c). When another dataset of cytokinin response genes was used, 82% of overlap between the expressed gene list and our ChiP-seq data were observed (Fisher’s Exact Test, p < 0.001).
affect B-ARR binding through the receiver domain. Although previous in vitro-derived B-ARR binding-motifs were identified by Weirauch et al.51, our study reveals that the subfamily-1 B-ARRs share a similar DNA-binding motif and provides direct in vivo evidence of the DNA-binding signatures of B-ARRs. The similarity between 6-BA-treatment-specific DNA binding signature of B-ARRs and that of the constitutive active ARR1ΔDDK provides additional insight into the in planta mechanism by which cytokinin modulates TF function. The B-ARR-6-BA motif was enriched in the promoter regions (~1.5 k to + 100 bps surrounding the TSS of 83% (3334/4012) of ARR1 bound genes compared to the whole genome background (Fisher’s Exact Test, p < 0.001, Supplementary Fig. 3e). In the Zubo et al. study, 85% (687/804) of the ARR10 “regulated target” genes also showed enrichment of the B-ARR-6-BA motif (Fisher’s Exact Test, p < 0.001, Supplementary Fig. 3e). While the B-ARR-6-BA motif is commonly present in 25,188 (75%) of all promoters in the Arabidopsis genome, only 3334 (13%) of these were bound by ARR1, suggesting that the motif is not sufficient to mediate the transcriptional response to cytokinin. In addition, we compared the ratio of TF binding sites identified in the 5’ and the 3’ (Supplementary Data 8). In the top 5% target genes examined, there was a 2-fold enrichment of (5’/3’) which dropped to 1.8-fold in the top 10% target genes and 1.7-fold for binding sites relative to all genes. There was also preferential binding at 5’ regions of the B-ARRs, which was consistent with the binding profile of other cytokinin regulated genes (Supplementary Data 9). These finding reveal that the top-ranking target genes have slightly preferable binding at their 5’ as opposed to 3’ but there are many ChIP-seq peaks (potential regulatory elements) present at gene 3’ ends as well.

**B-ARRs target to WUS in stem cell maintenance.** Cytokinin is a central player in shoot apical meristem initiation and maintenance.22,23. The fact that the arr1/10/12 triple mutant produces a smaller size shoot apical meristem also implies that the cytokinin transcriptional responses are important for stem cell maintenance.24. It is possible that cytokinin signaling directly targets either WUS or CLV3, genes that control the meristem size, the WUS-CLV3 loop.25,55. Interestingly the WUS gene which plays an important role in shoot apical meristem maintenance, a cytokinin-dependent process,22,55, was a consistent low-ranking target of B-ARRs. We further explored dynamic binding of B-ARRs at the promoter of WUS and identified the B-ARR binding site within the promoter of WUS. After 4 h of cytokinin treatment, clear binding of ARR1 at the WUS promoter could be observed which became even more apparent in the 3-day hormone treatment samples (Fig. 3e). Thus, WUS is likely a, cytokinin-dependent, target of ARR1 (Fig. 3e). Similarly, cytokinin-induced binding at the promoter of WUS was also found for ARR12, showing a significant increase with hormone treatment and ARR10 targeting the promoter of WUS at both mock and
cytokinin treatment conditions, although with less binding (Fig. 3e).

Two lines of evidence point to WUS as a candidate target of cytokinin TFs, such as the B-ARRs. First, cytokinin signaling revealed by two component sensor (TCS) is higher in WUS domain than any of the other domains in shoot apical meristem. Second, cytokinin induces expression of a WUS transcriptional reporter gene. Interestingly, as mentioned above cytokinin (1 mM) concentrations of cytokinin are consistent with a previous report that activation of transcriptional reporter pWUS::GFP in the shoot apical meristem requires high (1 mM) concentrations of cytokinin. Since a high concentration of cytokinin was required to activate pCLV3 expression, a constitutively active transcription factor (GR) was introduced into plants using a Dex inducible system. An enlarged shoot apical meristem was demonstrated the presence of a B-ARR-6-BA motif in the promoter of WUS as a candidate target of B-ARRs. Interestingly, the B-ARR-6-BA motif is adjacent to HD-ZIP response elements, suggesting possible interactions with other TFs that may be required for stem cell maintenance in the shoot apical meristem (Fig. 6a). Indeed, recent reports provide evidence for the interaction between B-ARR and HD-ZIP proteins. Two lines of evidence point to the functional significance of B-ARRs-mediated TF binding and increased WUS gene expression, a constitutively active form of ARR1 was introduced into plants using a Dex inducible system. Deletion of the signal receiver domain (ΔDDK) of ARR1 (a B-ARR) has been shown to constitutively activate cytokinin signaling by unmasking the transactivation function of ARR1. The ARR1 deletion construct was fused to the glucocorticoid-inducible artificial transcription factor (GR) and expressed from the cauliflower mosaic virus (CaMV) 35S coat protein gene promoter to generate dexamethasone (Dex) inducible 35S::ARR1ΔDDK:GR. In the absence of dexamethasone (Dex), transgenic plants carrying the 35S::ARR1ΔDDK:GR construct were phenotypically normal. Upon dexamethasone induction, these plants displayed a phenotype similar to tissue explants propagated on cytokinin culture medium. If B-ARR binding at the B-ARR-6-BA motif in the promoter of WUS activates WUS transcription, then, based on current knowledge of this pathway, transcriptional activation of CLV3 is subsequently expected to lead to increased transcription of A-ARRs by the 35S::ARR1ΔDDK:GR. To test this model, the 35S::ARR1ΔDDK:GR construct was introduced into plants carrying pCLV3:mtGFP-ER (CLV3 promoter driving the expression of endoplasmic reticulum-localized green fluorescent protein), a fluorescent reporter for stem-cells and 35S::YFP-29-1 (ubiquitous promoter driving the plasma membrane-localized yellow fluorescent protein) and marker for cell boundaries which allows visualization of all SAM cells. We observed that constitutive activation of cytokinin signaling led to expansion of stem-cell domain marked by the expression of endoplasmic reticulum-localized green fluorescent protein, a fluorescent reporter for stem-cells and 35S::YFP-29-1 (ubiquitous promoter driving the plasma membrane-localized yellow fluorescent protein) and marker for cell boundaries which allows visualization of all SAM cells. When plants were grown with cytokinin, we identified a single binding site in the WUS promoter with the B-ARR-6-BA motif (AGATAT) located at the peak summit (Fig. 6a) along with increased binding (Fig. 6b black bar). These findings are consistent with a previous report that activation of transcriptional reporter pWUS::GFP in the shoot apical meristem requires high (1 mM) concentrations of cytokinin. Since a high concentration of cytokinin was required to activate WUS, the effect had been previously thought to be indirect. However, our results demonstrate the presence of a B-ARR-6-BA motif in the WUS promoter, implying that WUS may be an in vivo target of B-ARRs. Interestingly, the B-ARR-6-BA motif is adjacent to HD-ZIP response elements, suggesting possible interactions with other TFs that may be required for stem cell maintenance in the shoot apical meristem (Fig. 6a). Indeed, recent reports provide evidence for the interaction between B-ARR and HD-ZIP.
LhG4/pMX6xOPs:ARR1ΔDDK:GR also showed a similar “ball” of SAM phenotype (Supplementary Fig. 7b). RNA-seq of “ball” cells isolated from the SAM clearly showed ARR1ΔDDK was over-expressed compared to the full length of ARR1 (Fig. 6f). Moreover, transcriptional activation of both WUS, and CLV3 was observed (Fig. 6f) although CLV3 was not found to be a target of any of the tested B-ARRs. These results are consistent with the hypothesis that B-ARRs activate WUS expression, which in turn activates CLV3. Therefore, our results provided a link between the cytokinin transcriptional response and the WUS-CLV3 circuit in the shoot apical meristem (Fig. 6g).

Discussion

In this study, we employed recombineering to engineer a Ypet-tag onto three B-ARRs, ARR1, ARR10, and ARR12. This system enabled monitoring of endogenous expression and protein localization patterns for three B-ARR TFs. It also allows real time...
visualization of the cytokinin primary TFs which was previously only done by a GUS fusion. The recombinered B-ARRs were also used for ChIP-seq experiments, allowing the identification of in vivo binding sites and putative target genes. Comparison of binding profiles from both endogenous and elevated cytokinin conditions showed marked differences in both motif sequence and target gene number. Based on these dosage experiments, there may be as many as 10,000 cytokinin response genes in the B-ARR network. Thus, a limiting factor might be the amount of endogenous cytokinin to modify B-ARRs.

The genome-wide identification of the targets of B-ARRs provides a new resource to understand how cytokinin may regulate diverse plant growth and developmental processes, as well as respond to stresses in conjunction with other phytohormones at different regulatory layers, such as biosynthesis, transportation, perception, or signal transduction. The tissue/cell expression patterns of B-ARRs are quite similar. Similarly, in vivo ChIP-seq targets for the three B-ARRs also resemble one another. When cytokinin levels are elevated, B-ARRs target many plant hormone negative regulators, such as A-ARRs, Aux/IAA, and ERFs. Targeting of multiple negative regulators in multiple hormone pathways might provide a quick and effective avenue to abate this hormone imbalance ensuring quick reequilibration of responses. Additionally, a recent ChIP-seq study using tagged, over-expressed ARR10 identified a set of ARR10-bound cytokinin responsive genes. Since over-expression of ARR10 was able to rescue arr1/10/12 triple mutants, it is not surprising that the list of targets (81.5% or 3265 out of 4004 genes) shows a statistically significant overlap with the targets that we identified using recombinered ARR genes (Supplementary Fig. 3b, Fisher’s exact test, p < 0.001). The ARR10 over-expressing ChIP-Seq results shared 2783 gene targets with our ARR10.BA and had 1221 unique targets not identified by ARR10.BA. Interestingly, the 2783 overlapping target genes found by both studies had higher peak scores than unique target genes (Supplementary Fig. 8a), suggesting that these are high confidence ARR target genes. However, expression of the native level of the ARRs using recombinered genes may allow the identification of more authentic, cytokinin-response relevant target genes. In this regard, dataset 1 of Zubo et al. contained 1221 additional targets that did not show enrichment for any meaningful GO terms while 3489 targets unique to the recombinereing ARR10 ChIP-seq data showed significant enrichment for plant hormone GO terms (Supplementary Fig. 3b). Moreover, over-expression of ARR10 identified known target gene for other B-ARRs (Supplementary Fig. 3b and 3d; Fig. 8b). However, the number of unique targets dropped to 739 when we compared these binding sites to ChIP-seq results for other B-ARR 6-BA treated tissues (Supplementary Fig. 3b, 3d). Moreover, the 482 targets shared between other recombinereed B-ARRs and ARR10 over-expressing line had lower peak scores compared to the 2783 shared ARR10 target genes between our study and Zubo et al. (Supplementary Fig. 8b), suggesting potential off-target binding in ChIP-seq experiments using ARR10 over-expressing plants. Combined, these studies provide biochemical evidence confirming genetic redundancy among B-ARR factors (Supplementary Fig. 3d). Interestingly we also uncovered a change of the B-ARR binding motif upon cytokinin treatment which was likely missed in earlier studies since this observation requires comparing B-ARRs at both endogenous conditions and cytokinin treatment (Fig. 5). Finally, gene targets identified by over-expression of ARR10 by Zubo et al., identified a number of the top-ranking B-ARR targets in our study (Supplementary Data 10). Thus, our findings provide unique information not available in previous studies, providing novel insight about full response of the plants to cytokinin.

Although several types of in vitro experiments identified potential DNA binding motifs for B-ARRs, the in vivo binding sites identified here provided a unique opportunity to further analyze DNA binding events. Cytokinin promoted B-ARR motif switching, from a more degenerated motif to a canonical B-ARR-6-BA motif (AGATHY). B-ARRs are not regulated at transcriptional level by cytokinin but are post-transcriptionally modified. One possibility is that without such modification B-ARRs only loosely bind their targets; further studies are necessary to explore the impact of phosphorylation on the motif site selection. ARR1 showed the highest cytokinin-dependent enrichment of binding to its targets. Without cytokinin treatment, only 2815 potential targets were identified but the number increased to 5128 after just four hours of cytokinin treatment, and it further increased to 10,340 targets when treated for 3 days. These targets could be further arranged in a hierarchical manner into early binding (targets found in both mock and 4-hour BA treatment as 1st), short BA treatment binding (targets found in both 4-hour and 3-day BA treatment as 2nd), and longer BA treatment binding (targets found only in 3-day BA treatment as 3rd) (Supplementary Fig. 5). We speculate that longer cytokinin treatment may change chromatin structures such that more binding sites become available. Although the high dose and 3-day treatment may potentially result in false positives peaks, the high overlap between lower level hormone treatments and these highdose experiments confirm the relevant cytokinin responsiveness. This cytokinin-dependent binding of ARR1 to its targets might be explained by hormone-dependent alteration in ARR1 protein stability. Alternatively, a high concentration of cytokinin may trigger the phosphorylation cascade resulting in activation of TF B-ARRs, and these phosphorylated B-ARRs may bind their targets more tightly than those lacking the modification. In this two-component multiple phosphorelay system, it is thought that phospho-activated B-ARRs change the conformation of their receiver domain. The model asserts that B-ARRs constitutively occupy their binding sites, only becoming “active” upon phosphorylation. Our time-series ChIP-seq analysis provides correlative evidence that cytokinin increases both binding of B-ARRs to their targets and the number of targets bound. However, this suggestion must be further addressed by complementary experiments, such as the reduction of endogenous cytokinin levels using a regulated expression of CKX3 to examine the impact of endogenous cytokinin removal on the interaction between B-ARRs and their targeted promoters. Alternatively, using mutations at the conserved phosphorylation sites in B-ARRs may also help to test this model.

Importantly, we identified a B-ARR binding site in the promoter of WUS gene, which encodes a homeodomain TF that was shown to repress a subset of A-ARRs. WUS has been suggested to work in conjunction with the cytokinin pathway to establish a stem cell niche ensuring early embryogenesis and later for maintenance of the shoot apical meristem. The study using the new reporter revealed that the WUS expression domain overlaps with SAM regions where cytokinin activity is the highest. Similarly, the pWUS::GFP-er reporter can be activated in SAM by cytokinin but only at a high concentration of cytokinin. However, subsequent (expected) activation of a pCLV3::GFP-er reporter was not observed. A previous explanation of why WUS does not robustly respond to low cytokinin levels was that the expression of WUS was only in the a specific (WUS) subgroup of the SAM and that the cytokinin was not accessible to the WUS domain. Our results indicate that most of the potential binding motifs of B-ARRs in the promoter region of WUS were unoccupied. However, we identified one B-ARR-6-BA motif, located precisely at the peak summit of the conserved narrow peak, in multiple B-ARRs data sets. The identification of a strong in vivo
B-ARR DNA binding site within the promoter of WUS that requires a high level of cytokinin, provides new direct evidence for this association. In addition, the B-ARR-6-BA motif (−420bp) was found to be within 57bps as two HD ZIP response elements (−540 to −565) that were previously identified as WUS regulatory sites49. It is possible that the coordination or competition among these TFs is a feature of WUS regulation, which is itself controlled by a WUS-CLV3 negative feedback loop, keeping the meristem size constant in each species. Moreover, the activation of WUS by B-ARRs downstream of cytokinin signaling should also be tightly attenuated in the shoot apical meristem. When a constitutive active form of ARR1 was introduced, removing the possibility of feedback inhibition, the activation of WUS led to an expansion of stem cell domain. Interestingly, ectopic expression of ARR1ADDK did not lead to an enlarged SAM, indicating the possible existence of an unknown inhibition mechanism from other domains within the SAM. Expression of ARR1ADDK, under the control of pCLV3 promoter, resulted in an enlarged shoot apical meristem (“ball” of SAM). This phenotype may result from activation of WUS by B-ARRs in the stem cell that cannot be dammed. Since type-A ARRs are highlighted by B-ARRs, increasing the level of type-A ARRs may cause increased expression of CLV3 as ARR7 and ARR15 are required for the expression of CLV322. However, as negative regulators, the inhibition mechanism of type-A ARRs to cytokinin signaling remains unknown51. Similarly, the activation mechanism of type-A ARRs leading to CLV3 expression is also not clear. If CLV3 is the sole interface between B-ARR and A-ARRs leading toactivate of CLV3, then transcription of WUS would be expected to shut down.

The “ball” of the SAM phenotype and high levels of both WUS and CLV3 expression observed in pCLV3:ARR1ADDK system suggests that B-ARRs activate the transcription of WUS, then activate CLV3 leading to expand the shoot apical meristem, resulting in the “ball” of SAM. Previous reports of pCLV3:WUS expression resulted in similar phenotypes, further providing functional relevance to our model61,62. This result is also consistent with the idea that multiple feedback loops exist in the shoot apical meristem, to adjust its size22. The introduction of a mutation in the B-ARR binding site in the promoter of WUS would test the idea that type-A ARRs (ARR1, ARR10, and ARR12) was then examined under Zeiss confocal microscopy 710 (Zeiss) using identical settings (6% laser power; master gain: ch 1 910, ch 2 248; digital gain 1, digital offset 0; pinhole 281 µm; filter ch 521-546).

The active form of ARR1 was constructed using a deletion of the receptor domain of ARR1 that was previously described51. The deletion called ΔARR1 was cloned into pENTR D/TOPO with GR fusion at the C-terminus and then the entire ΔARR1ΔDK-GR gene cassette was moved into the pEG104 transformation vector to be in frame of a N-terminus YFP5. The pENTR D/TOPO:ΔARR1ΔDK-GR was cloned into pmX6xOOp vector and then the transgenic line was combined with the pCLV3:LhG4 line to achieve stem cell-specific expression of ΔARR1ΔDK.

**Observation of phenotypes using the inducible system.** The two-components inducible system was used62. The pCLV3:LhG4 line was in Landsberg erecta (Ler-0) background. The pmX6xOOp:ARR1ΔDKΔGR was transformed into Ler-0 background.

Chromatin preparation and immunoprecipitation. Seedlings were dried by paper towel and transferred in 1% formaldehyde solution. Cross-linking occurred under 5:10-5:15 minute cycles with a quick vacuum release in between each cycle. A final concentration of 125 mM glycine was applied for 5 min to deactivate the remaining formaldehyde. Cross-linking resulted in translucent seedling tissue. Tissue was then liquid nitrogen cooled and either stored in −80°C degree or directly ground and an extraction of chromatin was performed as previously described50. Chromatin immunoprecipitation (ChIP) was performed as previously described56 with modifications, including the use of Bioretor possor (Diana, Belgium). Bioretor settings were used: Low, 10 cycles of 25 s on, 120 s off. Sonication was performed in auto-cooling system with water bath at 4°C. A small amount of ethanol was used as control. All ten lines showed the enlarged apical meristem phenotype. Initial observation of phenotypes were done by germinating seeds directly on Dex plates. The pCLV3:LhG4/mpX6xOOp:WUS-GR line was provided by Dr. Reddy’s lab and the same induction method was used.

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**Methods**

**Plant growth conditions.** Three-days-old seedling tissue was collected for these experiments unless otherwise noted. Seeds were surface-sterilized and sown on agar plates (1.8%) containing Murashige and Skoog salts (pH 5.7) and 1% sucrose. Seedlings were grown vertically for 3 days at 22°C under long day condition using a 16-h light/8-h dark cycle. The seedling was subsequently treated with liquid Murashige and Skoog medium (MS) pH 5.7 with 0.08% Silwet 77 containing 10 μM 6-Ba (in DMSO) or mock treated with an equivalent volume of DMSO. The three-days continuous hormone treatment was orchestrated by germinating the tagged lines on MS with 10 μM 6-Ba or MS with the same amount of DMSO as mock. 10 μM 6-Ba was chosen based on the range from 1 μM to 20 μM cytokinin treatment.

**Gene constructs and generation of tagged B-ARRs.** Based on recombining trends24, a Ypet (yellow fluorescent protein) tag was recombining into a transformable bacteria clone (TAC) clone such that the B-ARR gene was located at the center of the large insert clone. We employed the two-step recombining method using the Flapase-Fret system. Positive Ypet clones were obtained first by selection against ampicillin as the insert contained an ampicillin resistant gene. This ampicillin marker was later removed. While the technique leaves a small scar between Ypet and the B-ARRs the method is easier than the GaK system53 and the scar serve as a linker between the tag and the B-ARRs. The Ypet gene was added at the C-terminus of each B-ARR gene of the whole B-ARR family but only ARR1s 1, 10, 12, 11, 13, and 14 were successful. ARR1 was tagged using isoform ARR1-2 since the previous expression data indicated ARR1-2 as the major splice variant. The tagged TACs were sequenced to confirm sequence fidelity of the B-ARRs, the junction, and the fusion to Ypet within TAC. The tagged TAC clones were transformed into GV3101 strains and transformed wild type Columbia-0 plants using the flowering dipping method60. After screening for Basta resistance, putative tagged lines were identified by PCR using forward recombining test primer and reverse Ypet primers. The PCR products were gel-purified and sequenced to confirm the in planta tagging junction. The expression of the tagged B-ARRs (ARR1, ARR10, and ARR12) was then examined under Zeiss confocal microscopy 710 (Zeiss) using identical settings (6% laser power; master gain: ch 1 910, ch 2 248; digital gain 1, digital offset 0; pinhole 281 µm; filter ch 521-546).

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The commercial anti-GFP antibody (Thermo Fisher Scientific, A11111) was used for the immunoprecipitation reactions. Five microgram antibody and 30 μl of Dynabeads M-280 Sheep anti-Rabbit IgG (Thermo Fisher Scientific, catalog 512040) were used for each reaction. The incubation was performed using previously described buffer at 4°C overnight (16h). The Dynabeads were washed using a low stringent wash buffer followed by a high stringent buffer, a quick rinsing, and 5 min rotating at 4°C. A final wash buffer was applied to clear the detergent from previous buffers followed by another 5 min rotating. During each wash, the tubes were quickly centrifuged before returning to the magnetic stand and trace amounts of buffer were removed to avoid non-specific binding carryover. The resulting ChIP DNA was collected in two elution buffers (100 μl each) at 65°C and combined. The proteinase K digestion occurred at 55°C. The reverse of cross-linking was done at 65°C overnight (16h). The ChIP DNA was then purified by extraction using phenolchloroformisooamyl alcohol (25:24:1) (Sigma, p8023) twice in a phase lock gel system before the ethanol precipitation step using glycogen for the pellet observation. The pellets were then washed with 70% cooled ethanol and dried in a speed-vac. The ChIP DNA was then dissolved in 50 μl water for subsequent library preparation.
gene body TSS. The B-ARR-6-BA motif matrix was used by Homer to identify the locations of the motif sequence on the whole genome. Promoter regions were defined as −1500 to +100 bps relative to the TSS. Gene ontology analysis was conducted using DAVID GO to identify the top 3000 genes ranked by IDR peak score of each experiment were evaluated for GO term overrepresentation (The p-values were corrected for multiple testing). Total 200 bps flanking the binding summits of 1000 bps flanking peaks for each experiment were also used for identification of each DNA binding motif using MEME-ChIP suite. The list of genes associated with cytokinin pathway was downloaded from Gene Ontology Consortium (http://www.geneontology.org). The heatmap of the B-ARRs target genes was hierarchically clustered based on the Euclidean distances, calculated from the number of shared targets between each factor for different conditions. A directed network was constructed based on the relationship between TFs and their binding targets by igraph (http://igraph.org) with nodes representing either type-A or TFs with significant transcriptional changes defined by the meta-analysis (Supplementary Data 5) and edges representing either ChIP-Seq binding of type-B ARR or DAP-Seq binding of TFs. The network was visualized in Cytoscape (v3.4.0). DAP-Seq data was downloaded from the website (http://neomorph.salk.edu/dap_web/pages/index.php) and only samples without "amp" label were used for this analysis.

**RNA-seq and data analysis.** To compare the arr1/10/12 triple mutant with the wild type, three-days-old seedlings were collected without any treatment. For cytokinin quantification, 10 μL 6-BA or an equal volume of ethanol was applied to each sample. RNA was isolated using RNeasy plant kit (Qiagen, cat #74904) and libraries were prepared by NeoPre Library Prep System (illumina). Alignments were done by TopHat (v2.0.8, using TAIR10, Bowtie 2, and default parameters) and differential expression was called by CuffDif (Cufflinks v2.1.1, using TAIR10 and default parameters). The significantly differentially expressed genes used 1.6-fold change and q-value <=0.05 as cutoff.

**Data availability.** Raw and processed data can be found with GEO deposition accession number GSE94486. The authors declare that all other data supporting the findings of this study are available within the manuscript and its supplementary files or are available from the corresponding author upon request.

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Author contributions
J.R.E. and M.X. conceived the project. M.X. generated the materials and all data. M.X., H.C., L.H., R.C.O., M.N.S., and J.R.E. analyzed data. M.X. and J.R.E. wrote the manuscript that also were edited by R.C.O., L.H., M.N.S., and J.R.E. and H.C. C., L.H., R.C.O., M.N.S., and J.R.E. conceived the project. M.X. generated the materials and all data. M.X., H.C., L.H., R.C.O., M.N.S., and J.R.E. analyzed data. M.X. and J.R.E. wrote the manuscript that also were edited by R.C.O., L.H., M.N.S., and H.C.

Additional information
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