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ARR1 Directly Activates Cytokinin Response Genes that Encode Proteins with Diverse Regulatory Functions

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Plant cells respond to cytokinins by changing their gene expression patterns. The histidyl–aspartyl (His–Asp) phosphorelay mediates the signal from cytokinin receptors to type-B response regulators including ARR1, which transactivate cytokinin primary response genes. However, the overall architecture of the signal cascade leading to cytokinin-responsive phenomena is still unclear, mainly because it is not known how the His–Asp phosphorelay is connected to downstream phenomena. To reveal events immediately downstream from the phosphorelay-mediated transcriptional activation, we searched for direct-target genes of ARR1 by exploiting ARR1ΔDDK–GR, a chimeric transcription factor that transactivates ARR1 direct-target genes in transgenic plants by glucocorticoid induction. We identified 23 direct-target genes, most of which were found to be cytokinin primary response genes. The *arr1-1* mutation clearly affected the primary response in at least 17 genes, meaning that they respond primarily to cytokinins through the function of ARR1. The 17 genes encode proteins with diverse functions, including type-A response regulators, cytokinin metabolic enzymes and putative disease resistance response proteins. These results provide novel evidence indicating that the His–Asp phosphorelay is connected to diverse regulatory levels of cytokinin-responsive phenomena through ARR1 direct-target genes.

Keywords: *Arabidopsis* — ARR1 — Cytokinin — HiCEP — Phosphorelay — Target gene.

Abbreviations: ARR, *Arabidopsis* response regulator; BA, benzyl adenine; CHX, cycloheximide; DEX, dexamethasone; DRRP, disease resistance response protein; GST, glutathione-*S*-transferase; HiCEP, high coverage expression profiling; HPt, histidine-containing phosphotransfer.

Introduction

Cytokinins play pivotal regulatory roles in plant development, including shoot regeneration from plant tissues, the release of axillary buds from apical dominance, leaf expansion, delay of senescence, vascular cell development, and the differentiation and proliferation of chloroplasts (for reviews, see Mok 1994, Reski 1994). In these processes, plant cells respond to the cytokinin signal by changing their gene expression patterns (Crowell and Amasino 1994, Schmulling et al. 1997). Genome-wide analyses of *Arabidopsis* transcripts have revealed a number of genes responsive to exogenous cytokinins in cultured tissues (Che et al. 2002) and in wild-type and mutant seedlings (Rashotte et al. 2003, Kiba et al. 2004, Brenner et al. 2005, Kiba et al. 2005, Rashotte et al. 2006). Similar profiling analyses have been performed using transgenic lines, including those in which endogenous cytokinin levels are inducibly up-regulated (Hoth et al. 2003) or constitutively down-regulated (Brenner et al. 2005), and those overexpressing regulatory proteins for cytokinin signal transduction (Kiba et al. 2004, Kiba et al. 2005). Although some of the response genes encode proteins with functions closely related to cytokinin-responsive phenomena, the signal transduction pathways from cytokinin perception to their transcriptional regulation have not been clarified in most cases.

Intracellular signal transduction of cytokinin involves a histidyl–aspartyl (His–Asp) phosphorelay, a system also known as a two-component regulatory system because it is typically composed of a sensor histidine kinase and a response regulator in bacteria (for reviews, see Hwang et al. 2002, Aoyama and Oka, 2003, Heyl and Schmulling 2003, Kakimoto 2003, Grefen and Harter 2004, Mizuno 2004, Ferreira and Kieber 2005, Mizuno 2005). An *Arabidopsis* sensor histidine kinase, CRE1/AHK4, has been proven to be a cytokinin receptor by its specific binding to cytokinins in vitro (Yamada et al. 2001), cytokinin-dependent phosphorelay activity in yeast and bacterial cells (Inoue et al. 2001, Suzuki et al. 2001, Ueguchi et al. 2001) and cytokinin-hyposensitive phenotypes of *cre1* mutants (Inoue et al. 2001, Ueguchi et al. 2001). AHK2 and AHK3, which are paralogous proteins to CRE1/AHK4, also act as cytokinin receptors (Higuchi et al. 2004, Nishimura et al. 2004, Kim et al. 2006, Riefler et al. 2006). Because these cytokinin receptors are hybrid-type histidine kinases, which contain a signal receiver domain in the C-terminus, histidine-containing phosphotransfer (HPt) factors are thought to mediate the phosphorelay from the receptors to response

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regulators by analogy with phosphorelays in bacteria and fungi (Suzuki et al. 2002, Hutchison et al. 2006).

The plant response regulators include two subtypes, type-A and type-B (Imamura et al. 1999). Of 23 *Arabidopsis* response regulators (ARRs), 10 and 11 belong to types A and B, respectively (Hutchison and Kieber 2002). Type-A proteins lack known functional structures, except the receiver domains, and constitute one clade in the phylogenic tree of plant response regulators (Hutchison and Kieber 2002). Exogenous cytokinins have up-regulated steady-state levels of all type-A transcripts examined, independent of de novo protein synthesis (Brandstatter and Kieber 1998, D'Agostino et al. 2000), suggesting that all type-A genes are primarily responsive to cytokinins. On the other hand, because type-B proteins consist of an N-terminal receiver domain, a single Myb-repeat-type DNA-binding domain (Riechmann et al. 2000) and a C-terminal extension, they are considered to be transcription factors (Sakai et al. 1998, Sakai et al. 2000, Lohrmann et al. 2001, Hosoda et al. 2002, Imamura et al. 2003). Although their genes are not responsive to cytokinins at the transcript level, some type-B ARRs have been shown to transactivate type-A genes (Hwang and Sheen 2001, Sakai et al. 2001).

Of the type-B ARRs, ARR1 has been studied intensively in terms of both its molecular and biological functions. The DNA-binding domain of ARR1 is bound to DNA with a specific sequence in vitro (Sakai et al. 2000). In a transient expression experiment, a truncated ARR1 protein lacking the receiver domain (ARR1ΔDDK) activates transcription from a promoter containing the ARR1-binding sequence, whereas full-length ARR1 exhibits the transactivation only weakly (Sakai et al. 2000), suggesting that the receiver domain negatively regulates the transactivating function of ARR1. Consistent with this finding, plants expressing ARR1ΔDDK exhibit a phenotype of constitutive cytokinin responses, whereas those overexpressing full-length ARR1 show only a weak phenotype in the absence of exogenous cytokinins (Sakai et al. 2001). Moreover, the phenotype of ARR1ΔDDK plants was induced by glucocorticoid in plants expressing a fusion protein between ARR1ΔDDK and the receptor domain of a mammalian glucocorticoid receptor (ARR1ΔDDK–GR; Sakai et al. 2001). Most importantly, the following two results have demonstrated that ARR1 directly activates the transcription primarily responding to cytokinins. First, in plants expressing ARR1ΔDDK–GR (*35S-ARR1ΔDDK-GR*), glucocorticoid treatment increases transcript levels of the type-A gene *ARR6* independently from de novo protein synthesis, indicating that ARR1 can directly activate the transcription of *ARR6* (Sakai et al. 2001). Secondly, cytokinin-induced transcript levels of *ARR6* are lower in *arr1-1* mutant plants than in wild-type plants, even under conditions inhibiting de novo protein synthesis, indicating that ARR1 is involved in the pathway from cytokinin perception to the primary transcriptional response (Sakai et al. 2001).

To date, genetic and molecular studies have presented a simple feature of the intracellular signal transduction pathway from cytokinin perception to cytokinin primary response genes, where the His–Asp phosphorelay mediates the signal from cytokinin receptors to type-B response regulators. However, it is still difficult to depict the overall architecture of the signal cascade leading to cytokinin-responsive phenomena in plant cells, mainly because the connections between the phosphorelay and downstream phenomena are unclear. Although a number of cytokinin early response genes have been identified by genome-wide expression analyses, it is not known which of them are directly up-regulated by type-B ARRs. To understand cytokinin signal pathways further, it is important to reveal the constituents of direct-target genes of type-B ARRs. Here, we revealed that ARR1 directly transactivates cytokinin primary response genes with diverse regulatory functions, providing novel evidence that the phosphorelay is connected to cytokinin-responsive phenomena at diverse regulatory levels through ARR1 direct-target genes.

Results

ARR1 directly transactivates all type-A ARR genes

To examine candidates of ARR1 direct-target genes for evidence of their direct transactivation by ARR1, we performed a Northern blot analysis in which total RNAs from *35S-ARR1ΔDDK-GR* seedlings treated with dexamethasone (DEX), a glucocorticoid derivative, and/or cycloheximide (CHX), an inhibitor of protein synthesis, were analyzed. Because the transactivating function of ARR1ΔDDK–GR can be induced by DEX independently from de novo protein synthesis (Sakai et al. 2001), direct transactivation by ARR1ΔDDK–GR occurs even in the presence of CHX. However, CHX prevents indirect transcriptional activation that requires the protein functions of the directly transactivated genes. Because ARR1ΔDDK–GR retains the functional structure of ARR1 for specific DNA recognition, ARR1 is thought to recognize the same genes that ARR1ΔDDK–GR recognizes (Sakai et al. 2000, Sakai et al. 2001). Therefore, we considered the genes that are directly transactivated by ARR1ΔDDK–GR to be direct-target genes of ARR1. In addition to the DEX induction experiment, total RNAs from wild-type and *arr1-1* mutant seedlings treated with benzyl adenine (BA) and/or CHX were also subjected to Northern analysis. By comparing the up-regulation levels upon BA treatment in the presence of CHX between the wild type and mutant, we estimated the involvement of ARR1 in the primary cytokinin response of each gene. From the results of these

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Northern analyses, we identified genes that are directly transactivated by ARR1 in their primary responses to cytokinins. As for the induction periods, because the up-regulation of transcript levels was evident at different periods depending on the genes, we used two induction periods for each gene; 1 and 3 h.

First, we examined type-A ARR genes for evidence of their direct transactivation by ARR1. In *35S-ARR1ΔDDK-GR* plants, the transcript levels of all type-A genes were up-regulated by DEX treatment for 3 h in both the absence and presence of CHX, while the up-regulation of the *ARR17* transcript level in the absence of CHX was detected only in the 1 h condition (Fig. 1). These results indicate that ARR1ΔDDK–GR directly transactivated all type-A genes in planta, i.e. they are direct targets of ARR1. Transcript levels of all type-A genes were up-regulated by BA treatment for 3 h in wild-type plants, even in the presence of CHX, whereas the up-regulation of the *ARR17* transcript in the absence of CHX was again detected only in the 1 h condition (Fig. 1). This confirms previous observations that type-A genes are primarily responsive to cytokinins (Brandstatter and Kieber 1998, D'Agostino et al. 2000). The *arr1-1* mutation affected these primary responses relatively strongly for *ARR4*, *ARR6*, *ARR7*, *ARR15*, *ARR16* and *ARR17*, and moderately for *ARR5* and *ARR8* in the 3 h induction (Fig. 1, compare BA-induced levels in the presence of CHX between the wild type and mutant). For *ARR3* and *ARR9*, the mutant effect was modestly but reproducibly detected in the 1 h induction (Fig. 1). These findings indicate that ARR1 is involved in the primary responses of all the type-A genes. From the results of DEX and BA induction experiments (summarized in Table 1), we concluded that all the type-A genes are directly transactivated by ARR1 in their primary responses to cytokinins.

ARR1 directly transactivates cytokinin response genes with various functions

Next, we examined cytokinin response genes other than the type-A genes. As representative cytokinin response genes, we chose genes for which the transcript levels were reported to be up-regulated by >2-fold in at least six of 10 independent time course cytokinin treatments in the study of Rashotte et al. (2003). The genes in this category included four type-A ARR genes, *ARR4*, *ARR5*, *ARR7* and *ARR16*, and 13 other genes with various known or putative functions (Table 1). Using probes specific to these 13 genes, we performed the same Northern analysis as described above.

The cytokinin responses of all the genes were confirmed in the 3 h induction in the absence of CHX, whereas those of an AP2-like gene (At4g23750), a putative glutaredoxin gene and *IAA3/SHY2* were subtle (Fig. 2). In the presence of CHX, BA treatment clearly up-regulated their transcripts in the 1 or 3 h induction, except for two AP2-like protein genes (Fig. 2), indicating that another 11 genes, including the glutaredoxin gene and *IAA3/SHY2*, are primarily responsive to cytokinins. In *35S-ARR1ΔDDK-GR* plants, DEX treatment activated the cytokinin response genes in both the absence and presence of CHX, except for an AP2-like gene (At2g46310), a putative glutathione-*S*-transferase (GST) gene and *NIP1;1/NLM1* (Fig. 2). Although the up-regulation of *IAA3/SHY2* was again subtle in the absence of CHX, it was clearly detected in the presence of CHX (Fig. 2). From these results, 10 genes including *IAA3/SHY2* are considered direct targets of ARR1.

Of the 13 genes examined, nine genes, *CKX4*, *CYP735A2*, two putative disease resistance response protein (DRRP) genes, a putative glutaredoxin gene, *EXP1*, a putative transferase family-protein gene, *IAA3/SHY* and *FLA6*, were found to be both direct targets of ARR1 and primarily responsive to cytokinins. The *arr1-1* mutation clearly affected their cytokinin primary responses in the 3 h induction for *CYP735A2*, *IAA3/SHY*, the transferase family protein gene and the two DRRP genes, and in the 1 h induction for *CKX4*, whereas mutant effects on *EXP1*, *FLA6* and the putative glutaredoxin gene were unclear (Fig. 2). These results (summarized in Table 1) indicate that at least six of the examined cytokinin response genes are directly transactivated by ARR1 in their primary responses to cytokinins.

We also examined some other genes that are closely related to cytokinin-responsive phenomena, i.e. *CDKA;1* and *CYCD3;1* for cell proliferation, *STM* for shoot generation and *CAB1/Lhcb1*3* (At1g29930) for chloroplast development. Some of these genes have been reported to respond to cytokinins at the transcript level (Soni et al. 1995, Rupp et al. 1999, Menges et al. 2002). In our system, however, a modest cytokinin response was seen only for *CYCD3;1* in the absence of CHX (Fig. 3). In *35S-ARR1ΔDDK-GR* plants, none of these genes responded to DEX treatment in either the absence or presence of CHX (Fig. 3).

Profiling analysis of transcripts identified additional direct-target genes of ARR1

To identify novel direct-target genes of ARR1, we performed high coverage expression profiling (HiCEP; Fukumura et al. 2003), a high-resolution quantitative expression analysis in which cDNA fragments with specific sizes are amplified by PCR and fractionated into peaks on sequencing gels (see Materials and Methods for details). Total RNAs from DEX-treated and untreated *35S-ARR1ΔDDK-GR* plants were prepared, and two independent HiCEP analyses were done for each

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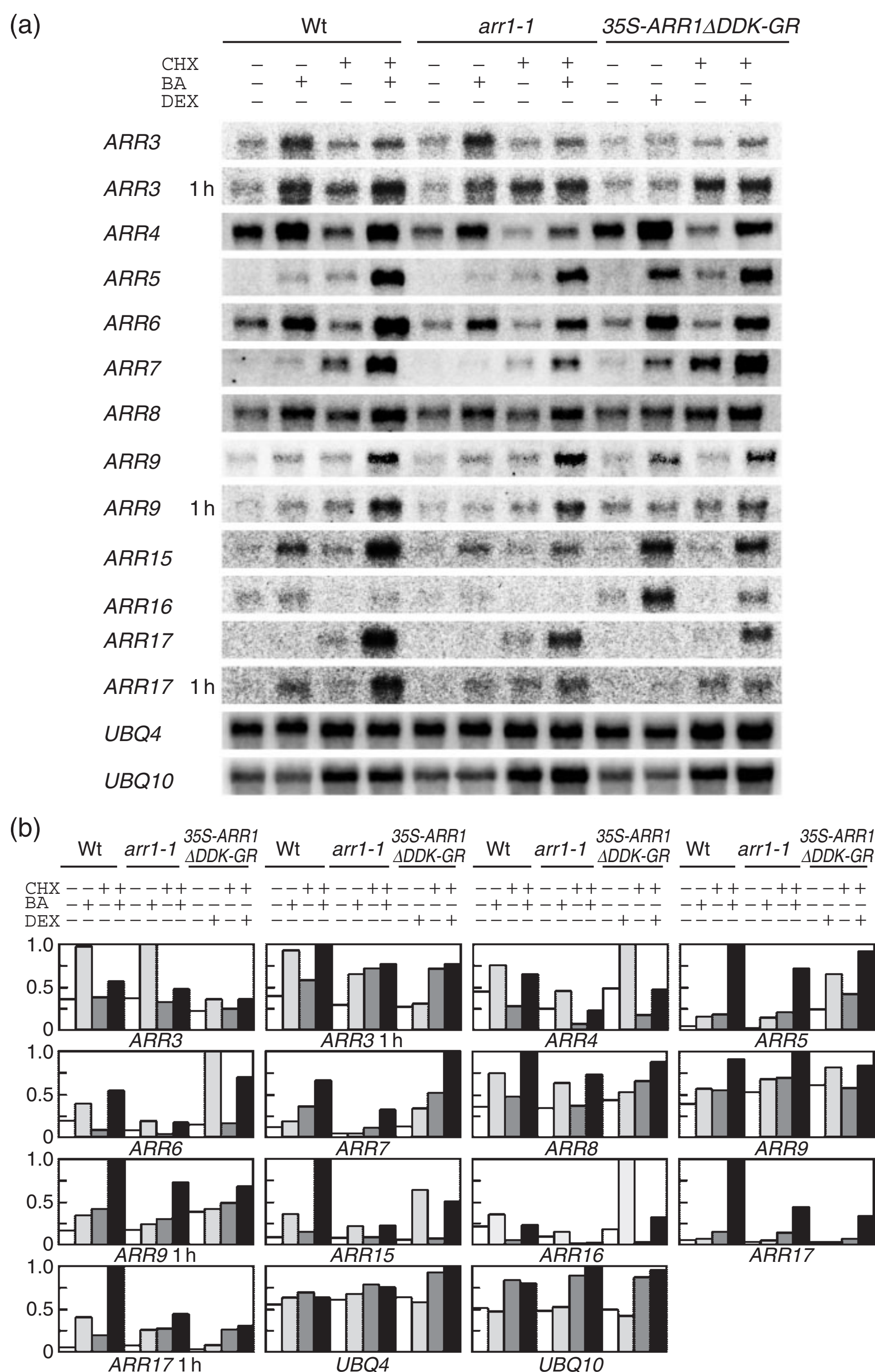

Fig. 1 Northern blot analysis of type-A ARR genes. (a) Type-A ARR genes were examined for the dependence of their cytokinin responses on the ARR1 function and for direct transactivation by ARR1ΔDDK–GR. Northern analysis was performed using total RNAs prepared from wild-type and *arr1-1* seedlings untreated or treated with 5 μM BA and/or 30 μM CHX and *35S-ARR1ΔDDK-GR* seedlings untreated or treated with 30 μM DEX and/or 30 μM CHX for 1 or 3 h, and probes specific to the indicated genes. Results at 3 h are shown. When the effects of chemical treatment or the *arr1-1* mutation were clearer at 1 h than at 3 h, the results at 1 h are also shown. Other results at 1 h and a result of *ARR3* from another independent 3 h induction experiment are shown in Supplementary Fig. S1. Two ubiquitin genes, *UBQ4* and *UBQ10*, were used as negative controls that do not respond to cytokinins. The results of negative control experiments in which wild-type plants were treated with DEX and/or CHX are shown in Supplementary Fig. S4. (b) Relative transcript levels determined by measuring the radioactivity of signal bands are shown graphically for each gene for each induction period. The highest value in each graph was set arbitrarily as 1.

preparation. About 18,000 peaks were detected reproducibly using DEX-treated or untreated preparations (Fig. 4 and Supplementary Table S1). To select a practical number of candidate peaks for further analysis systematically, we set threshold values for two parameters, i.e. a fluorescence level of 500 for the lower DEX-treated value and 1.5-fold for the ratio of the lower DEX-treated value to the higher DEX-untreated value (Fig. 4). We selected 18 peaks satisfying these prerequisites and separating clearly from adjacent peaks (Fig. 4 and Table 2). To assign the peaks to genes, we then determined the sequences of the cDNA fragments that corresponded to the peaks. These genes included *ARR4* and *CKX4*, which were identified as direct targets of ARR1 in previous sections. The other 16 genes were examined for their direct recognition by ARR1 and their responses to cytokinins using Northern analysis.

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Table 1 Summary of results obtained from Northern blot analysis

Gene		Result of the Northern blot analysis[a]				
Code	Product	Induction by BA in the wild type[b]		Effect of *arr1-1* on BA induction in the presence of CHX[c]	Induction by DEX in 35S-ARR1ΔDDK–GR[b]	
		In the absence of CHX	In the presence of CHX		In the absence of CHX	In the presence of CHX
Type-A ARR genes						
At1g59940	ARR3	+	+	+	+	+
At1g10470	ARR4	+	+	+	+	+
At3g48100	ARR5	+	+	+	+	+
At5g62920	ARR6	+	+	+	+	+
At1g19050	ARR7	+	+	+	+	+
At2g41310	ARR8	+	+	+	+	+
At3g57040	ARR9	+	+	+	+	+
At1g74890	ARR15	+	+	+	+	+
At2g40670	ARR16	+	+	+	+	+
At3g56380	ARR17	+	+	+	+	+
Cytokinin-responsive genes						
At4g29740	Cytokinin oxidase CKX4	+	+	+	+	+
At2g46310	AP2-like transcription factor	+	−	n.d.	+	−
At1g67110	Cytokinin hydroxylase CYP735A2	+	+	+	+	+
At4g11190	Putative disease resistance response protein	+	+	+	+	+
At4g23750	AP2-like transcription factor	+/−	−	n.d.	+	+
At2g30540	Putative glutaredoxin	+/−	+	−	+	+
At1g69530	Expansin EXP1	+	+	−	+	+
At2g29490	Putative glutathione *S*-transferase	+	+	−	−	−
At4g19030	Nodulin-like protein NIP1;1/NLM1	+	+	−	−	−
At4g11210	Putative disease resistance response protein	+	+	+	+	+
At2g40230	Putative transferase-family protein	+	+	+	+	+
At1g04240	AUX/IAA-family protein IAA3/SHY2	+/−	+	+	+/−	+

(continued)

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Table 1 Continued

Gene		Result of the Northern blot analysis[a]				
Code	Product	Induction by BA in the wild type[b]		Effect of *arr1-1* on BA induction in the presence of CHX[c]	Induction by DEX in 35S-ARR1ΔDDK–GR[b]	
		In the absence of CHX	In the presence of CHX		In the absence of CHX	In the presence of CHX
At2g20520	Fasciclin-like arabinogalactan protein FLA6	+	+	–	+	+
Genes related to cytokinin-responsive phenomena						
At3g48750	Cyclin-dependent kinase CDKA;1	–	–	n.d.	–	–
At4g34160	D-type cyclin CYCD3;1	+	–	n.d.	–	–
At1g62360	Homeodomain protein STM	–	–	n.d.	–	–
At1g29930	Chlorophyll A-B binding protein CAB1	–	–	n.d.	–	–
Genes selected by HiCEP						
At3g62930	Putative glutaredoxin	+	+	–	+	+
At1g76410	RING finger protein	+	+	+	+	+
At3g44610	Putative protein kinase	+	–	n.d.	+	+

[a] The indicated patterns of transcript levels were confirmed in at least two independent experiments.
[b] '+', '+/–' and '–' indicate that up-regulation of the transcript level by BA in wild-type seedlings or by DEX in *35S-ARR1ΔDDK-GR* seedlings for 1 or 3 h was clear, subtle and not detected, respectively.
[c] '+' and '–' indicate that the effect of the *arr1-1* mutation on the up-regulation by BA in the presence of CHX was detected and not detected, respectively. 'n.d.' indicates that the effect was not determined since the up-regulation by BA in the presence of CHX was not detected in wild-type seedlings.

Northern analysis confirmed that DEX up-regulated the transcript levels of three genes encoding a putative glutaredoxin, a RING-finger protein and a putative protein kinase (Fig. 3). Responses were not clearly observed for the other 13 genes (data not shown). The responses of the three genes were observed even in the presence of CHX in the 1 h induction for the RING-finger protein gene or the 3 h induction for the putative glutaredoxin and protein kinase genes (Fig. 3), indicating that they are direct targets of ARR1. In wild-type plants, the up-regulation of transcript levels by BA was detected for all three genes in the absence of CHX, and for the RING-finger protein gene (1 h) and the putative glutaredoxin gene (3 h) in the presence of CHX (Fig. 3). The *arr1-1* mutation clearly affected the cytokinin responses of the RING-finger protein gene in the 1 h induction (Fig. 3). These results indicate that the RING-finger protein gene is up-regulated by ARR1 in the primary response to cytokinins.

Extended in vitro ARR1-binding sequences appear in the promoter regions of ARR1 direct-target genes at significantly high frequencies

The DNA-binding domain of ARR1 binds specific DNA sequences containing 5′-GAT(T/C)-3′ in vitro (Sakai et al. 2000). However, the core sequence alone is too short to specify the direct-target genes, i.e. it theoretically appears once per 85.3 bp DNA region with a random sequence. To investigate the essential signal structure required for the promoters that are activated directly by ARR1 in planta, we analyzed the promoter sequences of the 23 direct-target genes determined in this study. First, the regions 500 bp upstream from their transcription start sites were surveyed for the core binding sequence.

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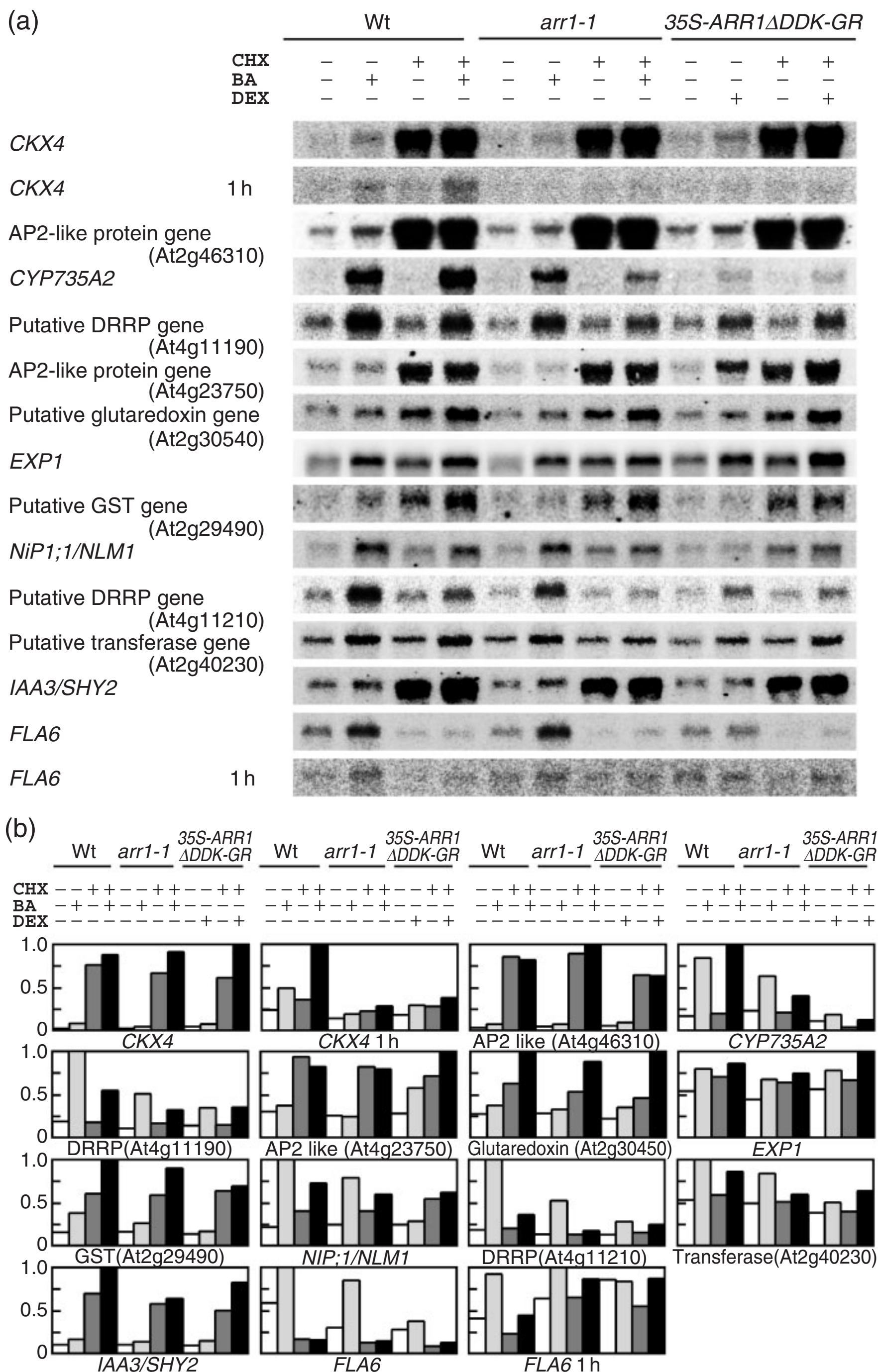

Fig. 2 Northern blot analysis of typical cytokinin response genes other than those for type-A ARRs. (a) Reported typical cytokinin response genes (Rashotte et al. 2003) other than those for type-A ARRs were examined for the dependence of their cytokinin responses on the ARR1 function and for direct transactivation by ARR1ΔDDK–GR. Northern analysis was performed using the same set of RNA preparations as in Fig. 1 (for control, see rows of UBQ4 and UBQ10 in Fig. 1). Results at 3 h are shown. When the effects of chemical treatment or the *arr1-1* mutation were clearer at 1 h than at 3 h, the results at 1 h are also shown. Other results at 1 h and a result of *FLA6* from another independent 3 h induction experiment are shown in Supplementary Fig. S2. The results of negative control experiments in which wild-type plants were treated with DEX and/or CHX are shown in Supplementary Fig. S5. (b) Relative transcript levels determined by measuring the radioactivity of signal bands are shown graphically for each gene for each induction period. The highest value in each graph was set arbitrarily as 1.

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Although the average frequency was slightly higher in the target promoters (9.26 times/promoter) than in 500 randomly selected promoters listed in Supplementary Table S2 (7.55 times/promoter), no characteristics of the distribution pattern were found specifically in the target promoters.

Next, we searched sequences around the core binding sequence for tendencies specific to the target promoters. The 5′-GAT(T/C)-3′ sequence was located in A/T-rich regions and tended to have T at the degenerate position in both the target and the randomly selected promoters (Fig. 5a). Noticeably, both the first and second positions adjacent to the 3′ end were occupied by T at significantly higher frequencies in the target promoters (52.5 and 50.2%, respectively) than in the randomly selected promoters (37.5 and 31.7%, respectively). In addition, at the first

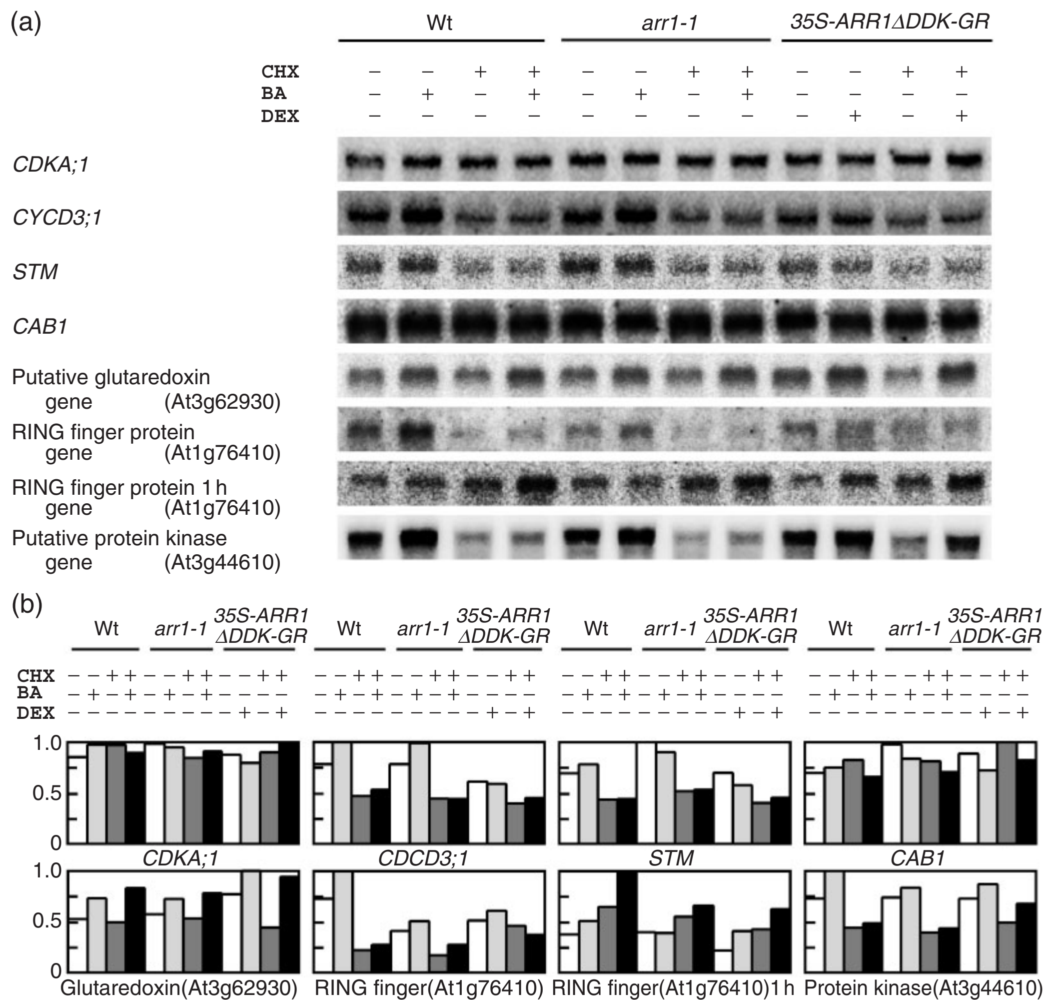

Fig. 3 Northern blot analysis of genes related to cytokinin-responsive phenomena and genes selected by HiCEP. (a) Genes related to cytokinin-responsive phenomena, for cell proliferation (*CDKA;1* and *CYCD3;1*), shoot generation (*STM*) and chloroplast development [*CAB1*], and genes selected by HiCEP analysis were examined for the dependence of their cytokinin responses on the ARR1 function and direct transactivation by ARR1ΔDDK–GR. Northern analysis was performed using the same set of RNA preparations as in Fig. 1 (for control, see rows of UBQ4 and UBQ10 in Fig. 1). Results at 3 h are shown. When the effects of chemical treatment or the *arr1-1* mutation were clearer at 1 h than at 3 h, the results at 1 h are also shown. Other results at 1 h are shown in Supplementary Fig. S3. The results of negative control experiments in which wild-type plants were treated with DEX and/or CHX are shown in Supplementary Fig. S6. (b) Relative transcript levels determined by measuring the radioactivity of signal bands are shown graphically for each gene for each induction period. The highest value in each graph was set arbitrarily as 1.

and second positions adjacent to the 5′ end, A residues appeared more frequently in the target promoters (41.6 and 43.2%, respectively) compared with the randomly selected promoters (30.6 and 29.4%, respectively).

The sequences with either the preferred 5′-AA or 3′-TT extension, 5′-AAGAT(T/C)-3′ or 5′-GAT(T/C)TT-3′, were found at higher frequencies in the 500 bp target promoter regions (3.98 times/promoter) than in those of randomly selected promoters (1.75 times/promoter). Moreover, the sequences containing both extensions, 5′-AAGAT(T/C) TT-3′, appeared much more frequently in the target promoters (0.826 times/promoter) than in the randomly selected promoters (0.094 times/promoter). These sequences were plotted schematically along the 1,000 bp regions upstream from the transcription start sites of the 23 direct-target genes (Fig. 5b).

The DNA-binding domain of ARR1 was preferentially bound to regions containing the extended sequences in the ARR6 promoter

To examine the preferential binding of ARR1 to the extended sequences in a direct-target promoter, gel mobility shift analysis was performed using a recombinant fusion protein (GST–ARR1DBD) between GST and the ARR1

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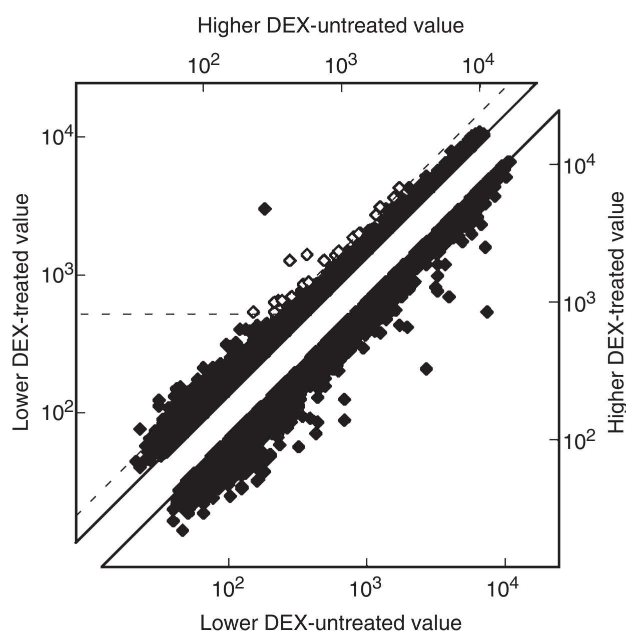

Fig. 4 Scatterplots of HiCEP peaks with DEX-untreated and DEX-treated levels. Two independent HiCEP analyses were performed for each of the DEX-untreated and DEX-treated samples. HiCEP peaks in which the lower DEX-treated levels were higher than the higher DEX-untreated levels and those in which the lower DEX-untreated levels were higher than the higher DEX-treated levels are plotted in the upper left and lower right panels, respectively. Horizontal and diagonal broken lines in the upper left panel indicate thresholds of the lower DEX-treated level (500) and the ratio of the lower DEX-treated level to the higher DEX-untreated level (1.5), respectively. The peaks that were selected for further analysis are indicated as open symbols in the upper left panel.

DNA-binding domain. The fusion protein caused mobility shifts with different signal intensities depending on DNA fragments from the *ARR6* promoter, whereas GST alone did not cause shifts of any DNA fragments (Fig. 6a). As schematically shown in Fig. 6b, The DNA fragment containing three extended sequences (No. 4) conferred an intense signal, and those with one extended sequence (Nos. 3 and 5) conferred less intense signals (Fig. 6b). In contrast, two upstream fragments (Nos. 1 and 2), each of which contained multiple copies of non-extended core sequences only, produced faint signals. Although the preference of ARR1 for each binding sequence in the *ARR6* promoter is unclear, these results suggest that the extended sequences are the primary determinant of ARR1 recognition in the direct-target promoters.

Discussion

We identified a total of 23 genes for which transcript levels were inducibly up-regulated by ARR1ΔDDK–GR, independently of de novo protein synthesis, as direct-target genes of ARR1 (Table 1). Most of them, 21 of 23, were found to be cytokinin primary response genes. Comparison of the wild type and *arr1-1* mutant revealed the involvement of ARR1 in the primary responses of 17 direct-target genes, indicating that ARR1 directly transactivates at least these 17 genes in their primary response to cytokinins. They encode proteins with diverse molecular functions. The cytokinin hydroxylase CYP735A2 and cytokinin oxidase CKX4 catalyze cytokinin synthesis and breakdown, respectively (Werner et al. 2001, Takei et al. 2004). Type-A ARRs are not only thought to be output devices of the phosphorelay, but are also known to be negative regulators of the phosphorelay (Imamura 1999, Kiba et al. 2003, To et al. 2004, Leibfried et al. 2005). IAA3/SHY2 is a transcriptional regulator presumably involved in the cytokinin-directed transcriptional network. DRRPs are probably involved in remodeling of the extracellular matrix because they are considered dirigent proteins mediating lignin and lignan biosynthesis (Gang et al. 1999). Thus the diversity of ARR1 direct-target genes indicates that the phosphorelay signal received by ARR1 is connected to various regulatory levels of cytokinin-responsive phenomena.

Most of the cytokinin primary response genes determined in this study were revealed to be direct targets of ARR1. This suggests that ARR1, and probably paralogous type-B ARRs with similar molecular functions to ARR1 (Sakai et al. 2000, Lohrmann et al. 2001, Hosoda et al. 2002, Imamura et al. 2003), transactivate most of the cytokinin primary response genes. On the other hand, a putative GST gene (At2g29490) and *NIP1;1/NLM1*, both of which are primarily responsive to cytokinins, were not clearly transactivated by ARR1ΔDDK–GR. Although it is still possible that ARR1ΔDDK–GR up-regulated them at levels insufficient for clear detection, effective transactivation of these genes might require factor(s) other than ARR1 (see below). In another case, an AP2-like gene (At4g23750) and a putative protein kinase gene (At3g44610) were activated by ARR1ΔDDK–GR but were not responsive to cytokinins in the presence of CHX. Since they responded to cytokinins in the absence of CHX in a previous study (Rashotte et al. 2003) or this study, it is possible that their cytokinin primary responses existed but could not be detected in the presence of CHX, while their transcript levels strongly up-regulated by ARR1ΔDDK–GR could be detected.

It has been reported that a triple mutation with three type-B genes, *ARR1*, *ARR10* and *ARR12*, strongly affected the cytokinin responses of all examined type-A genes (Mason et al. 2005), including *ARR3*, *ARR5*, *ARR8* and *ARR9*, for which the responses were affected relatively weakly by the *arr1-1* single mutation in our study.

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Table 2 Candidates for ARR1 direct-target genes obtained from HiCEP analysis

Gene[a]		cDNA fragment detected by HiCEP			DEX-treated level of fluorescence[b]	Magnitude of induction[c]
Code	Product	Length of cDNA (bp)	*Msp*I end sequence	*Mse*I end sequence		
At3g62950	Putative glutaredoxin	98	CCGGCG	GCTTAA	1,272	3.16
At4g29740	Cytokinin oxidase CKX4	122	CCGGAC	AATTAA	1,382	2.52
At3g18780	Actin ACT2	70	CCGGTT	AGTTAA	533	2.42
At5g53490	Thylakoid lumenal protein	116	CCGGTC	CCTTAA	628	1.96
At3g44610	Putative protein kinase	41	CCGGCT	GGTTAA	645	1.81
At1g76410	RING finger protein	130	CCGGTA	GATTAA	1,283	1.77
At2g33830	Dormancy/auxin-associated protein	370	CCGGGA	TTTTAA	3,106	1.75
At4g16000	Expressed protein	316	CCGGCC	TGTTAA	534	1.70
At3g59930	Expressed protein	39	CCGGCT	GGTTAA	4,176	1.68
At1g30250	Expressed protein	81	CCGGTG	TTTTAA	685	1.62
At1g10470	Type-A response regulator ARR4	59	CCGGAT	CGTTAA	1,150	1.60
At5g11420	Expressed protein	39	CCGGCT	ACTTAA	1,451	1.60
At5g08690	ATP synthase beta-chain 2	53	CCGGCT	TATTAA	843	1.57
At2g05100	Chlorophyll A-B-binding protein	151	CCGGGG	TCTTAA	860	1.56
At5g42450	Pentatricopeptide repeat protein	34	CCGGCC	TATTAA	1,361	1.54
At3g55970	Putative oxidoreductase	89	CCGGAC	CGTTAA	1,988	1.51
At5g13650	Elongation factor family protein	37	CCGGCC	TGTTAA	2,601	1.51
At3g62930	Putative glutaredoxin	100	CCGGAG	CTTTAA	3,481	1.50

[a] cDNA fragments detected by HiCEP analysis were assigned to genes by determining their sequences.
[b] The lower DEX-treated fluorescence levels in two independent HiCEP analyses are shown.
[c] The ratios of the lower DEX-treated fluorescence levels to the higher DEX-untreated fluorescence levels are shown.

This indicates that ARR1 together with other type-B ARRs regulates its direct-target genes. Moreover, our study revealed that ARR1 contributes to cytokinin responses to different degrees among its direct-target genes. Because some type-B ARRs have slightly different DNA-binding preferences from that of ARR1 (Imamura et al. 2003), direct-target genes of type-B ARRs are also thought to differ from each other in the preference for type-B ARRs. In this context, the genes that were not transactivated by ARR1ΔDDK–GR but were primarily responsive to cytokinins, i.e. the putative GST gene (At2g29490) and *NIP1;1*/*NLM1*, may still respond to cytokinins via activation by type-B ARRs other than ARR1. Recently, a subset of AP2 transcription factors, CRFs, was reported to mediate a large fraction of the early transcriptional response to cytokinins (Rashotte et al. 2006). Cytokinin signaling on these transcription factors might be involved in the effective cytokinin primary responses of some genes examined in our study.

The identified ARR1 direct-target genes, except for a putative protein kinase gene, have been identified as early response genes to cytokinins in previous profiling studies (Hoth et al. 2003, Rashotte et al. 2003, Kiba et al. 2004, Brenner et al. 2005, Kiba et al. 2005). Of the 23 direct-target genes, 11 are up-regulated 15 min after cytokinin treatment (Brenner et al. 2005). On the other hand, *CDKA;1* and *STM*, which respond to cytokinins after relatively long periods (Che et al. 2002, Hoth et al. 2003, Rashotte et al. 2003), were not directly transactivated by ARR1ΔDDK–GR, consistent with our postulate that ARR1 activates the transcription primarily responsive to cytokinins. Transcript levels of the *CYCD3;1* gene have been reported to be up-regulated by cytokinins independently of de novo protein synthesis (Riou-Khamlichi et al. 1999). In our

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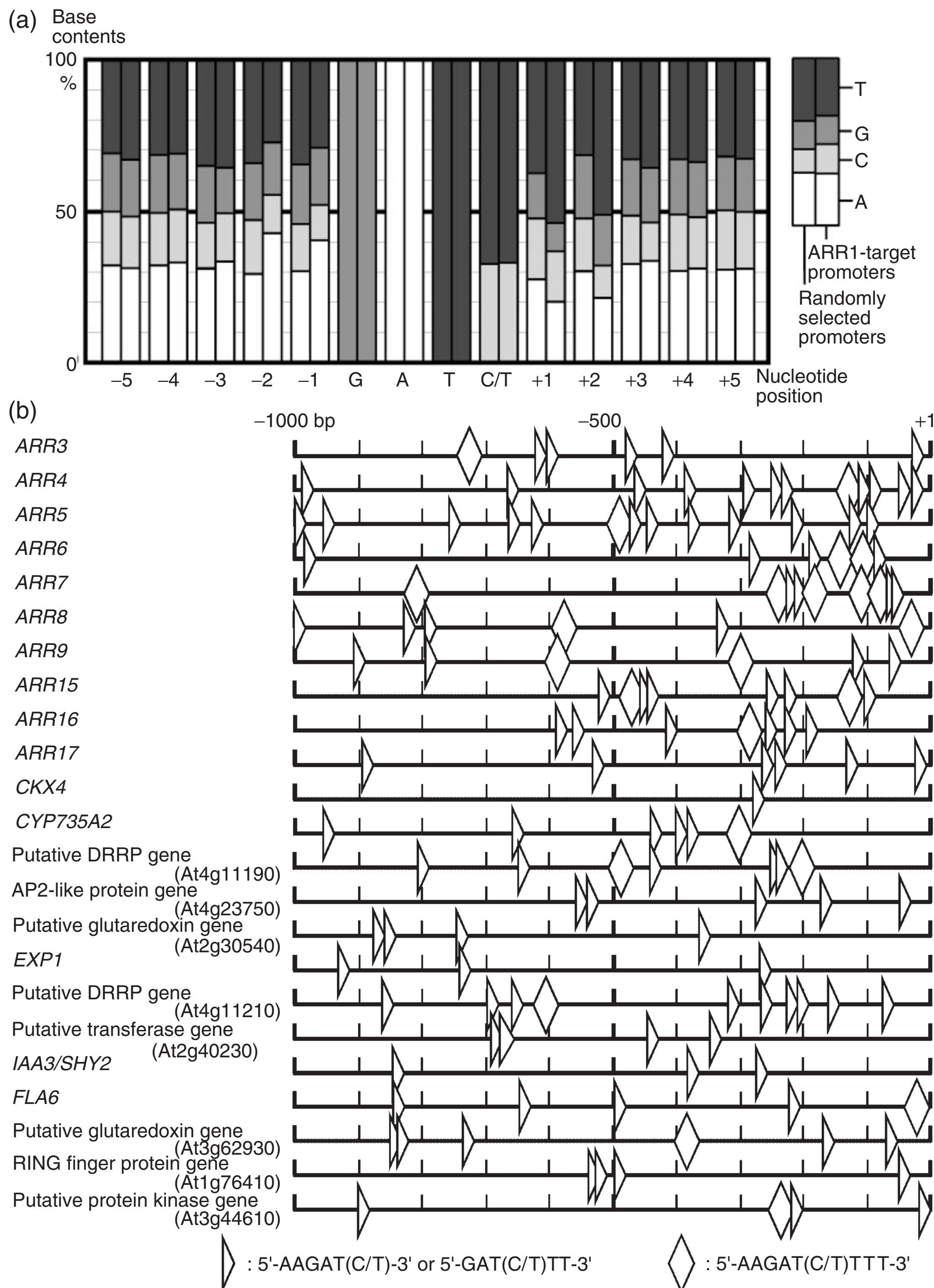

Fig. 5 Sequence analysis of ARR1 target promoters. (a) Analysis of the sequences neighboring the core ARR1-binding sequence in the promoters of ARR1 direct-target genes. Sequences neighboring the 5′-GAT(T/C)-3′ sequence are compared between the 500 bp upstream regions from the transcription start sites of 500 randomly selected genes and 23 ARR1 direct-target genes. The ratios of bases appearing at each upstream or downstream position from the 5′-GAT(T/C)-3′ sequence in randomly selected promoters and ARR1 target promoters are schematically shown in the left and right bars at each position, respectively. (b) Distribution of extended ARR1-binding sequences in ARR1 target promoters. The positions of the sequences, 5′-AAGAT(C/T)-3′ and 5′-GAT(C/T)TT-3′ (triangle), and 5′-AAGAT(C/T)TT-3′ (diamond) are schematically shown in the 1,000 bp regions upstream from the transcription start sites of ARR1 direct-target genes.

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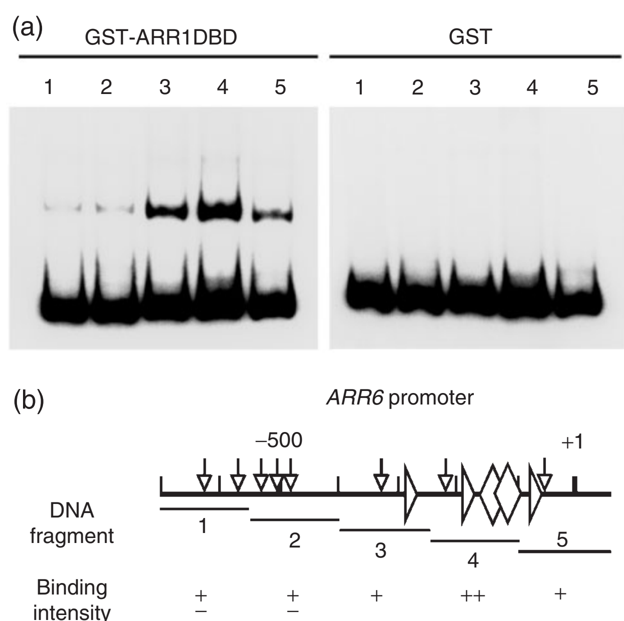

Fig. 6 Gel mobility shift analysis for binding of the ARR1 DNA-binding domain to various regions in the *ARR6* promoter. (a) Labeled DNA fragments (1–5), with the positions indicated schematically in (b), were incubated with *Escherichia coli* lysate containing GST–ARR1DBD or GST, and then subjected to PAGE for detection of their mobility shifts (for details, see Materials and Methods). (b) Intensities of the binding of the DNA fragments to GST–ARR1DBD are shown schematically with the positions of the fragments and the core (vertical arrows) and extended (diamonds and triangles as indicated in Fig. 5) ARR1-binding sequences in the *ARR6* promoter.

study, however, the transcript levels responded to BA only in the absence of CHX, and did not respond to DEX in *35S-ARR1ΔDDK-GR* plants. The cytokinin primary response, and possibly ARR1 transactivation, of *CYCD3;1* might require some factors that were missing from our system.

We analyzed the sequences neighboring the in vitro core binding sequence and found that the sequences accompanied by a 5′ extension, 5′-AA-3′, and/or a 3′ extension, 5′-TT-3′, frequently appear specifically in promoter regions of ARR1 direct-target genes. Consistently, one of these sequences, 5′-GATCTT-3′, has also been found frequently in the upstream regions of cytokinin response genes (Rashotte et al. 2003). The 5′ and 3′ extensions, neither of which is required for the binding to short synthesized DNA in vitro (Sakai et al. 2000), may be required for binding in the context of promoter DNA. This idea is supported by the result of our in vitro binding experiment in which the ARR1 DNA-binding domain was bound tightly to regions containing the extended binding sequences, rather than those containing non-extended core binding sequences, in the *ARR6* promoter. The sequences with both extensions, 5′-AAGAT(T/C)TT-3′, are palindromic or pseudopalindromic, suggesting that ARR1 forms a homodimer or heterodimers with other type-B ARRs.

CCA1, another GARP family transcription factor that regulates circadian rhythms in *Arabidopsis*, interacts with the two sequences, 5′-AAAAATCT-3′ and 5′-AACA ATCT-3′, in the *CAB1/Lhcb1*3* (At1g29930) promoter in vitro (Wang et al. 1997). One of their complementary sequences, 5′-AGATTTTT-3′, partly overlaps with the extended sequences, indicating that a class of GARP family transcription factors recognizes similar DNA sequences. In our analysis, however, the *CAB1/Lhcb1*3* gene was not transactivated by ARR1ΔDDK–GR. This result suggests that the occurrence of the extended sequences is required, but is still not enough to specify the target promoters of ARR1. Detailed analysis of the *cis*-element should be performed to reveal its essential structure, which will enable us to identify ARR1 direct-target genes systematically from the promoter sequence information.

In this study, we screened transcripts for candidates of ARR1 target genes by HiCEP analysis, and then evaluated their transcript levels by Northern analysis. Of the 18 genes selected by HiCEP, the up-regulation of transcript levels was not reproduced by Northern analysis for 13 genes. This may have been caused by the difference in the methodologies used to detect transcripts. Because Northern and HiCEP analyses detect full-length mRNAs and cDNA fragments derived from the 3′ regions of mRNAs, respectively, the determined levels of transcripts may differ between the two methods, especially for unstable transcripts. Interestingly, HiCEP detected down-regulated transcripts by the induced ARR1ΔDDK–GR function (Fig. 4 and Supplementary Table S1), whereas genes corresponding to the transcripts have not yet been determined. Because a number of genes down-regulated by cytokinins have been reported (Che et al. 2002, Hoth et al. 2003, Rashotte et al. 2003, Brenner et al. 2005), the involvement of ARR1 in their down-regulation should also be investigated.

Our results revealed two novel features of cytokinin signal pathways in *Arabidopsis*. First, in most cases, primary response genes to cytokinins were found to be the direct targets of ARR1, suggesting that the His–Asp phosphorelay to type-B ARRs is a major signal pathway from cytokinin perception to primary gene expression. Secondly, more importantly, genes directly transactivated by ARR1 in their primary responses to cytokinins encode proteins with a wide variety of regulatory functions. This means that cytokinin signal pathways expand immediately after the His–Asp phosphorelay and are connected to diverse regulatory levels for cytokinin-responsive phenomena through direct-target

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genes of ARR1. These two features indicate that ARR1 and paralogous type-B ARRs play central regulatory roles in the signal pathways from cytokinin perception to cytokinin-responsive phenomena.

Materials and Methods

Plant materials

Arabidopsis thaliana (ecotype Columbia) was used as the wild-type plant and the parental line for all mutant and transgenic lines. The T-DNA insertional mutant *arr1-1* and the transgenic line containing the *35S-ARR1ΔDDK-GR* gene have been described previously (Sakai et al. 2001). Seeds were germinated on agar medium containing Murashige–Skoog salts, Gamborg-B5 vitamins, 1% sucrose and 0.8% agar, and were grown under constant light at 22°C unless otherwise noted.

Northern blot analysis

Northern analysis was performed as described previously (Sakai et al. 2001). Two-week-old seedlings growing on agar medium were transferred onto sterile filter paper on agar medium, and adapted to open-air conditions at least for 2 d. For induction, the filter paper carrying the seedlings was immersed in an induction solution containing 5 μM BA, 30 μM DEX and/or 30 μM CHX for 1 or 3 h. Complete inhibition of protein synthesis by the CHX treatment was confirmed using transgenic plants carrying a GVG-inducible luciferase gene as described previously (Supplementary Table S3; Ohgishi et al. 2001). Total RNA was isolated from the seedlings and subjected to Northern blot analysis as described previously (Sakai et al. 1998). The DNA fragments used as probes in the Northern analysis were amplified from *Arabidopsis* cDNA by PCR, using the primer sets listed in Supplementary Table S4, and cloned into plasmid vectors. Relative transcript levels were determined for each gene for each induction period by measuring the radioactivity of signal bands on Northern blot filters using a Fujix BAS-2000 Bio-image Analyzer (Fuji Photo Film, Minami-Ashigara, Japan). Responding patterns of transcript levels indicated in Table 1 were confirmed in at least two independent experiments.

HiCEP analysis

HiCEP analysis was performed as described previously (Fukumura et al. 2003). The analysis was repeated twice for each RNA preparation to estimate its reproducibility and experimental error. First-strand cDNA was synthesized using total RNA (10 μg) and 5′-biotinylated oligo(dT) primer, and converted into double-stranded cDNA. The cDNA was digested by the restriction endonuclease *Msp*I and ligated with *Msp*I adaptor (5′-AATGGC TACACGAACTCGGTTCATGACA-3′ and 5′-CGTGTCATGA ACCGAGTTCGTGTAGCCATT-3′). The ligated products bearing biotin at the oligo(dT) end were trapped by magnetic beads coated with streptavidin (Dynabeads M-280 Streptavidin; Dynal Biotech, Oslo, Norway). The cDNA fragments on the beads were digested by the restriction endonuclease *Mse*I. The digested products free from the beads were collected and ligated with *Mse*I adaptor (5′-AAGTATCGTCACGAGGCGTCCTACT GCG-3′ and 5′-TACGCAGTAGGACGCCTCGTGACGATA CTT-3′). This procedure produced a batch of cDNA fragments with the *Msp*I and *Mse*I adaptor sequences at the ends originally distal and proximal to the poly(A) sequence, respectively. The cDNA fragments were amplified using selective PCR composed of 256 separate reactions. Each PCR used one of 16 fluorescence labeled primers with the *Msp*I adaptor sequence and different 3′ dinucleotides (5′-label-ACTCGGTTCATGACACG GNN-3′), and one of 16 primers with the *Mse*I adaptor sequence and different 3′ dinucleotides (5′-AGGCGTCCTACTGCGTA ANN-3′). For labeling, 6-carboxyfluorescein (FAM) was used. The PCR products of each reaction were separated using denaturing gel electrophoresis with an ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA). PCR fragments, each of which corresponded to a particular transcript, were detected as peaks of fluorescence, and the intensity of fluorescence from each peak was thought to correlate with the expression level of the corresponding transcript.

To assign a peak of interest to a gene, fluorescence from the HiCEP peak was detected using Typhoon 9210 (Amersham Biosciences, Uppsala, Sweden), and a slice of gel containing the peak was excised. The DNA was then eluted from the gel, amplified using PCR with appropriate primers, and sequenced.

Promoter sequence analysis

Upstream regions from the transcription start site (the +1 position) were analyzed as promoter regions. The information on transcription start sites was obtained from the RIKEN database of full-length cDNAs and 5′-end expressed sequence tags (http://rarge.gsc.riken.jp/cdna). When no information was available in the database, the 5′-end cDNAs of relevant genes were cloned using a GeneRacer™ Kit (Invitrogen Inc., Carlsbad, CA, USA) and sequenced to determine their transcription start sites. To create a non-biased promoter collection, 500 genes were randomly selected from the RIKEN database, and their upstream regions from the transcription start sites were compiled (Supplementary Table S2).

Gel mobility shift analysis

To prepare the fusion protein GST–ARR1DBD, the cDNA fragment encoding the ARR1 DNA-binding domain (amino acids 236–299) was inserted into the plasmid pGEX-2T (Amersham Pharmacia Biotech) at its multicloning site in-frame with the GST-coding sequence. GST–ARR1DBD and GST were provided as cleared lysates from *Escherichia coli* cells carrying the constructed plasmid and pGEX-2T, respectively. For the binding reaction, cleared lysate containing 50 ng of total protein, 0.2 pmol of a ^{32}P-labeled DNA fragment and 2 μg of poly(dI–dC) were incubated in 20 μl of binding buffer containing 20 mM HEPES (pH 7.4), 200 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2% (v/v) Nonidet P-40 and 10% (v/v) glycerol at 20°C for 30 min. The reaction mixture was then subjected to polyacrylamide (5%) gel electrophoresis as described previously (Sakai et al. 2000).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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