Expression Profiling of Cytokinin Action in Arabidopsis[1[w]]

Aaron M. Rashotte, Susan D.B. Carson, Jennifer P.C. To, and Joseph J. Kieber*

University of North Carolina, Biology Department, CB Number 3280, Chapel Hill, North Carolina 27599–3280

The phytohormone cytokinin is an important regulator of plant growth and development; however, relatively few genes that mediate cytokinin responses have been identified. Genome-wide analyses of Arabidopsis seedlings using the approximately 8,300-element Affymetrix Arabidopsis GeneChips (Affymetrix, Santa Clara, CA) to examine cytokinin-responsive genes were conducted, revealing at least 30 genes whose steady-state level of mRNA was elevated and at least 40 that were down-regulated at multiple time points after application of cytokinin. The cytokinin up-regulated genes include the type-A Arabidopsis response regulators (ARRs), which had been shown previously to be cytokinin primary response genes, cytokinin oxidase, which encodes an enzyme that degrades cytokinins, and several transcription factors. Cytokinin down-regulated genes include several peroxidases and kinases and an E3 ubiquitin ligase. We identified a common sequence motif enriched in the upstream regions of the most consistently cytokinin up-regulated genes. This motif is highly similar to the optimal DNA-binding sites for ARR1/ARR2, type-B ARRs that have been implicated in the transcriptional elevation of the type-A ARRs. Additionally, genome-wide analyses of cytokinin receptor mutants (*wol/cre1*) revealed large-scale changes in gene expression, including down-regulation of the type-A ARRs and several meristem and cell cycle genes, such as CycD3. Mutations in CRE1 reduced but did not eliminate the effect of cytokinin on gene expression for a subset of cytokinin-responsive genes and had little or no effect on others, suggesting functional redundancy among the cytokinin receptors.

Cytokinins are a group of adenine derivatives that affect multiple aspects of plant growth and development, including cell division, vascular development, sink/source relationships, apical dominance, and leaf senescence (Binns, 1994; Mok and Mok, 1994, 2001). A pathway for cytokinin biosynthesis and metabolism is emerging from molecular and biochemical studies. This has been highlighted by the recent cloning of several genes encoding enzymes involved in cytokinin biosynthesis or metabolism, including *ipt* (Kakimoto, 2001; Takei et al., 2001), which catalyzes the first committed step in cytokinin biosynthesis; cytokinin oxidase (Houba-Hérin et al., 1999; Morris et al., 1999), which cleaves the N^6 side chain from cytokinins; and several enzymes that catalyze the conjugation of sugar moieties to cytokinins (Martin et al., 1999a, 1999b, 2001). A model for cytokinin perception and signal transduction has emerged that is similar to prokaryotic two-component response pathways (Haberer and Kieber, 2001; Hutchison and Kieber, 2002; Lohrmann and Harter, 2002). A family of genes that are similar to bacterial two-component response regulators, the type-A Arabidopsis response regulators (ARRs), was identified as cytokinin primary response genes (Brandstatter and Kieber, 1998; Sakakibara et al., 1998; Taniguchi et al., 1998; D'Agostino et al., 2000). The cytokinin receptors (CRE1, AHK2, and AHK3) mediate the induction of these type-A ARRs and are similar to bacterial two-component His kinases (Inoue et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). These receptors act through other two-component elements, including Arabidopsis homologs of His phosphotransfer proteins (AHPs) and the type-B ARRs, involved in the up-regulation of type-A ARRs (Hwang and Sheen, 2001; Sakai et al., 2001).

CRE1 (also called WOL and AHK4) is the best characterized of the three cytokinin His kinase receptors. The signal transduction function of this gene in single-cell systems such as *Escherichia coli*, yeast (*Saccharomyces cerevisiae*), and transient Arabidopsis protoplasts has been shown to be dependent on the exogenous application of cytokinin. Furthermore, loss-of-function mutants of CRE1 result in reduced cytokinin sensitivity (Inoue et al., 2001; Ueguchi et al., 2001). Although the interaction between CRE1 and the other cytokinin receptors, AHK2 and AHK3, is unclear, examination of CRE1 mutants has revealed clues regarding its in vivo functions. The *cre1-1* mutation was found to be allelic to the *wol* mutant previously identified in genetic screens for altered root patterning. The severely reduced root growth phenotype of the *wol* mutants is likely to be due to an insufficient number of vascular initial cells, resulting in a lack of phloem tissue (Scheres et al., 1995; Mähönen et al., 2000). CRE1 is also required for the periclinal division that increases the number of cell files in the vasculature of the root and hypocotyl. *CRE1* gene expression is highest in roots and is localized in the vascular precursor cells of

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* Corresponding author; e-mail jkieber@unc.edu; fax 919–962–1625.

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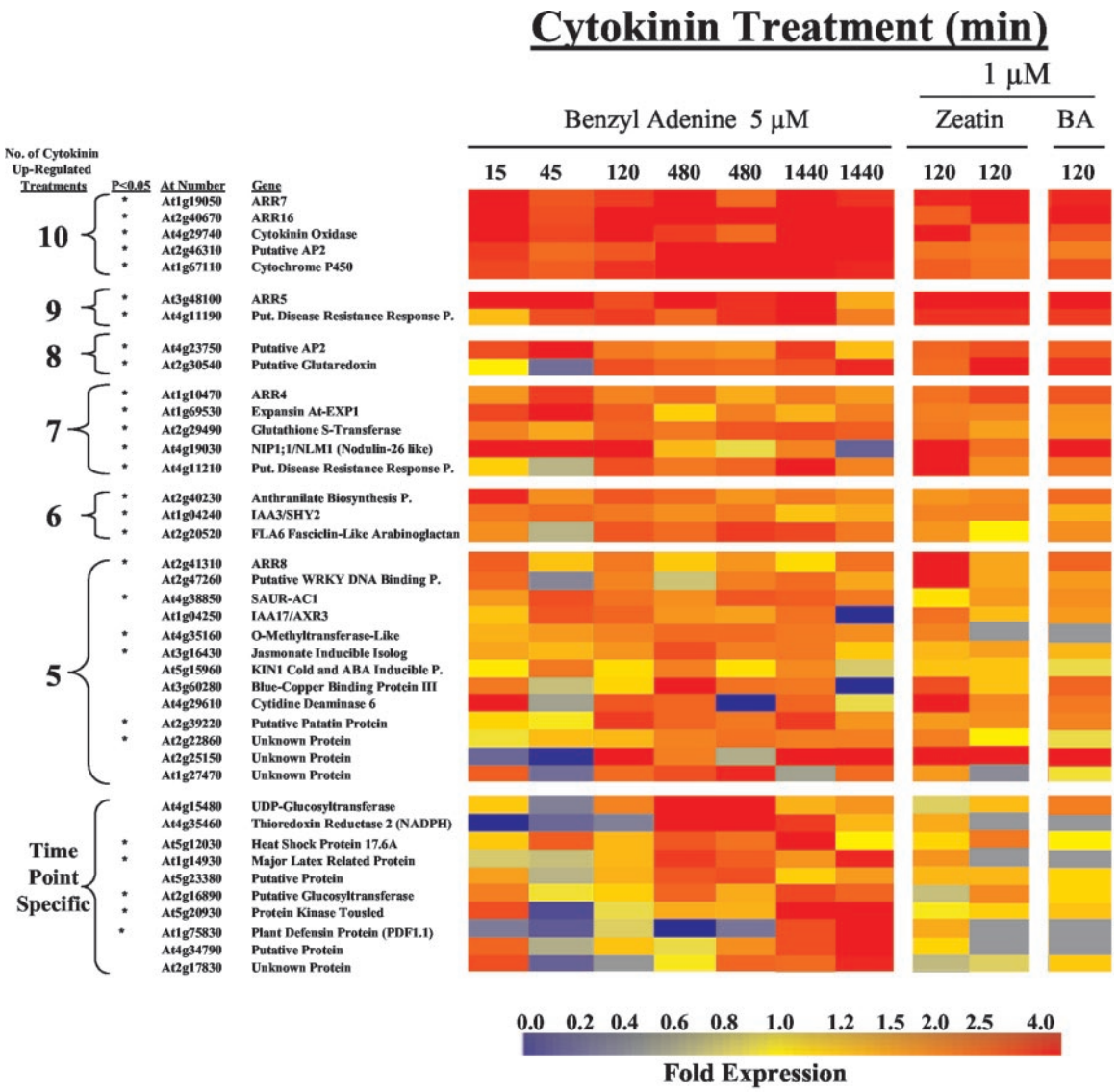

Figure 1. Cytokinin-regulated genes. Expression ratios of genes from wild-type-Arabidopsis seedlings treated under various conditions (compared in all cases with normal media grown seedlings treated with a DMSO control for an identical time) are shown as colored squares. The colors correspond to the relative fold change from the respective controls using the scale shown at the bottom of the figure. Genes with expression levels found to be altered by cytokinin more than 2.0-fold at five or more of the 10 cytokinin time course treatments or at specific time points with a 2.5-fold change and a minimum raw level are shown. The *Arabidopsis thaliana* (At) number and gene description are indicated to the left. The asterisk indicates that a gene is significantly different ($P < 0.05$) due to cytokinin treatment relative to the DMSO control by Welch's *t* test comparisons (see "Materials and Methods"). Treatments shown from right to left are with 5 μM benzyl adenine (BA) for 15, 45, 120, and two replicates of 480 and 1,440 min, 1 μM zeatin for two replicates of 120 min, and 1 μM BA for 120 min. A, Genes whose expression increased more than 2-fold relative to their cognate DMSO control. B, Genes whose expression decreased more than 2-fold relative to their cognate DMSO control. P. refers to Protein.

globular stage embryos, the hypocotyl procambium, the cotyledon shoulders, and the embryonic root (Mähönen et al., 2000). The *wol* mutation is a missense allele that disrupts the cytokinin binding domain of the protein; thus, the phenotype of *wol* appears to be the result of impaired cytokinin perception in tissues where CRE1 is expressed.

Several researchers have examined gene expression in response to application of exogenous cytokinin (Crowell and Amasino, 1994). In one study, 20 genes were identified as induced within 4 h of cytokinin treatment, including two genes encoding ribosomal proteins and a β-expansin (Crowell et al., 1990). Exogenous cytokinin has also been shown to upregulate the cell cycle genes *cdc2* and *CycD3* (Menges et al., 2002). Additionally, both *STM* and *KNAT1* genes, which are involved in meristem function, have elevated expression in Arabidopsis plants overexpressing the bacterial *ipt* gene (Rupp et al., 1999). Recently, a receptor-like kinase gene, *CRK1*, was

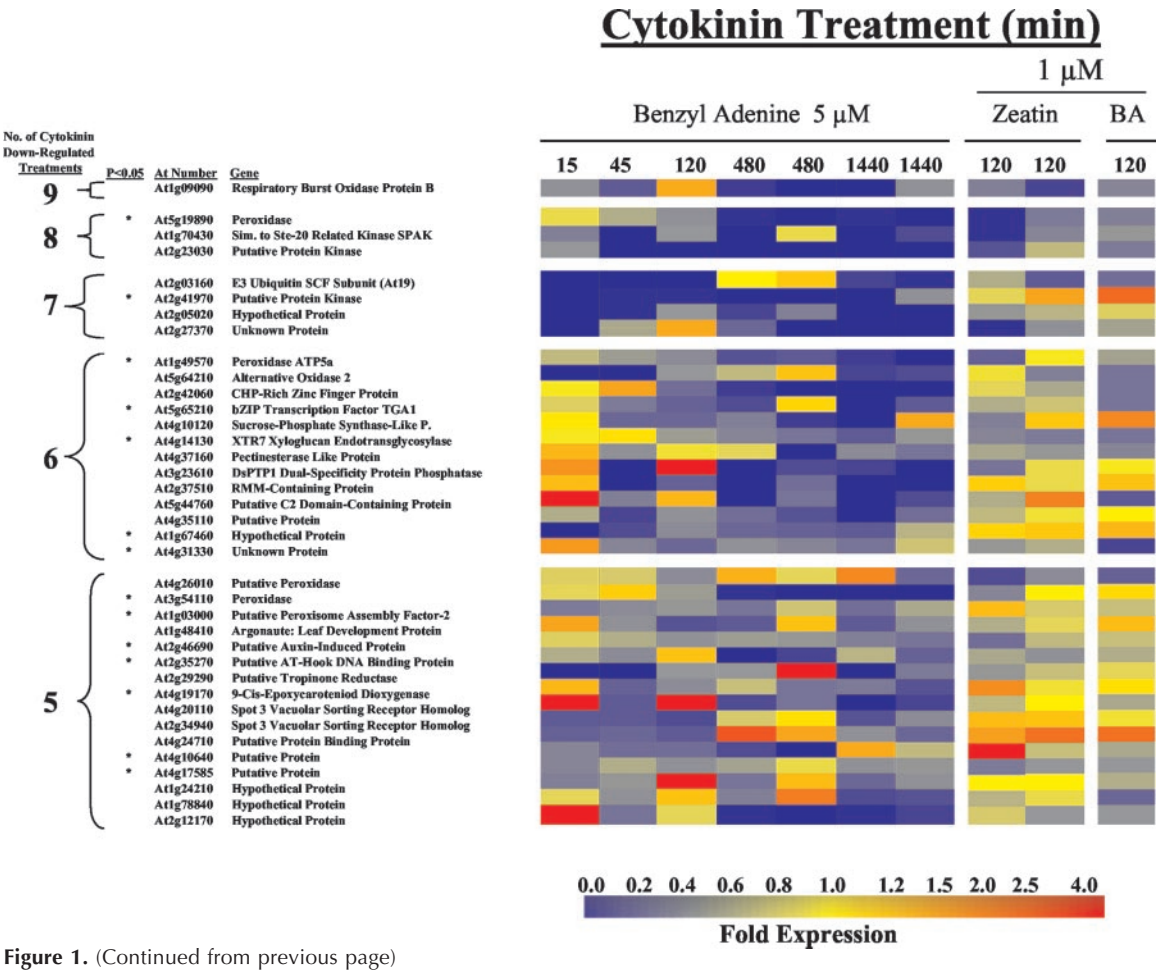

Figure 1. (Continued from previous page)

found to be rapidly down-regulated by cytokinin (Schäfer and Schmülling, 2002). Several studies have also examined cytokinin-regulated gene expression during tissue differentiation including nodulation, senescence, floral development, lateral bud induction, and various aspects of light development (Mok and Mok, 2001). Genes identified in these studies include photosynthetic genes, ribosomal protein genes, nitrate reductase, and many novel genes. However, most of these genes are also induced by other stimuli, most notably light and auxin, and none of these genes are induced with kinetics, suggestive of an immediate early response.

In this study, we sought to identify genes regulated by cytokinin to better understand the mode of action of this hormone. Additionally, we examined the role of the cytokinin receptor CRE1 in mediating the effect of cytokinin on gene expression in Arabidopsis roots.

RESULTS

Regulation of Gene Expression by Cytokinin

Light-grown Arabidopsis seedlings were treated with exogenous cytokinin for various times over a 24-h period, and gene expression was analyzed using the approximately 8,300-element Affymetrix Arabidopsis GeneChips (Affymetrix, Santa Clara, CA). Ten cytokinin treatments were conducted and normalized to control samples prepared and treated identically with dimethyl sulfoxide (DMSO; the solvent used for the cytokinin). For our analyses, we chose a 2-fold change in expression level compared with the control as a minimum for a gene to be called altered in response to cytokinin and a minimum raw value cutoff of 175 to 500, depending on the particular chip hybridization. These raw level cutoffs were assigned based on scatter plot analyses of a treated sample versus its control and used because expression levels of genes below these cutoff thresholds frequently showed variability greater than 2-fold and often had Affymetrix flag calls of Absent (data not shown). In addition, only the cytokinin-treated samples with an Affymetrix flag call of Present and the minimum raw cutoff level were used to identify up-regulated genes; likewise, only the control samples with the same restrictions were used to identify down-regulated genes. By using these restrictions, we sought to distinguish authentic changes in expression from background noise and false positives. Using these param-

eters, the steady-state levels of expression of more than 1,000 genes were altered by cytokinin alone in at least one treatment; however, the majority of these were affected at only a single treatment and generally not in replicate samples (see Supplemental Tables I and II at http://www.plantphysiol.org).

Thirty genes were found to be up-regulated at five or more of the 10 cytokinin time course treatments, and 17 were up-regulated in at least six independent treatments (Fig. 1A). The observation that these genes were reproducibly up-regulated by cytokinin suggests that they are cytokinin responsive. A Welch's *t* test confirmed that all of the top 17 genes showed significant changes in expression levels with cytokinin treatment. We chose to include other genes that fit the criteria described above for cytokinin regulation but that showed a P value ≥ 0.05 because these are still potential cytokinin-regulated genes. These results are further supported by the fact that four of the top 17 genes, *ARR4*, *ARR5*, *ARR7*, and *ARR16*, have been identified previously as cytokinin-responsive type-A ARRs (D'Agostino et al., 2000). Other reproducibly identified cytokinin up-regulated genes include AP2 transcription factors, a P450, and two putative disease resistance response proteins that are novel in their link to cytokinin (Fig. 1A).

Approximately 40 genes were identified that showed greater than a 2-fold decrease in their expression level at five or more of the 10 treatments after cytokinin application (Fig. 1B). One group of genes that showed reductions in their steady-state mRNA levels was a set of genes involved with oxidation, including four peroxidases and two oxidases. Another group of down-regulated genes was three kinases, including an Ste-20 and SNF1-related kinase. Other reproducibly identified cytokinin down-regulated genes include an ubiquitin ligase SCF complex subunit, three transcription factors, and the leaf development protein Argonaute (Fig. 1B). In comparison with the set of induced genes, which contains only two unknowns, the cytokinin-repressed genes include more than one-quarter (11 genes) for which there is no known function (Fig. 1).

A number of distinct induction profiles can be identified from this time course (Fig. 1). There is a set of genes that is elevated or reduced at all time points examined. This includes most of the type-A ARRs, the cytokinin oxidase, the AP2s, and the P450 from the up-regulated list, and the respiratory burst oxidase and the three kinases from the down-regulated list. There is only a small set of genes that are induced rapidly (15, 45, and 120 min), but transiently (return to baseline at 480 and 1,440 min), such as the nodulin-26-like gene NIP1;1, whereas no down-regulated genes appear to fit this pattern. There is a larger set of genes that are up- or down-regulated only upon prolonged cytokinin treatment (>120 min). For the up-regulated genes, these include a glutaredoxin, a disease resistance response protein, and an unknown protein. For the down-regulated genes, these include a zinc finger protein and three of the four down-regulated peroxidases. Another class of genes displays alterations in gene expression at specific time points. Most of these genes are specifically induced or repressed at a single time point, such as a putative peroxidase that is primarily repressed at 120 min by both BA and zeatin cytokinin treatments, as seen in Figure 1B.

Our experimental design using both BA and zeatin treatments allowed us to examine the effect of these different classes of cytokinins on gene expression. At 120 min, seedlings were treated with either BA, a cytokinin with an aromatic-type side chain attached to the N^6 position of the adenine ring, or trans-zeatin, a naturally occurring cytokinin with an isoprenoid-type side chain. Almost every gene found to be up-regulated by BA at both 120-min treatments was also up-regulated in at least one of the two zeatin treatments and vice versa. In contrast, there are many fewer genes down-regulated by zeatin as compared with BA, which suggests that some of these genes may be specifically down-regulated by BA or that they are not actually regulated by cytokinin.

It is important to note that for the genes that have altered expression levels in fewer numbers of cytokinin treatments, there is a decreased confidence that any particular gene is truly regulated by cytokinin. This is reflected in the statistical analysis of the data, which indicates that some but not all of the genes that we identified as cytokinin regulated were found to have significant P values. The genes identified as altered in both replicate treatments for a single time point are potentially transiently cytokinin-regulated genes and are more likely to be truly altered by cytokinin than those affected at only two distinct time points, but not in the replicates. Thus, we have included genes affected in both replicates of any single time point above a 2.5-fold change in expression in Figure 1. Nevertheless, it is possible that some of the genes identified as cytokinin regulated (Fig. 1A) are false positives, and it is likely that we have excluded genes that are weakly induced for a narrow window of time along with genes whose expression levels are relatively low. In summary, the set of genes presented in Figure 1 represents the list of genes found in this study to most likely be regulated by cytokinin, with a decreasing confidence in this conclusion as a gene is identified as altered in fewer cytokinin-treated samples.

Type-B ARR-Binding Sites Are Enriched in the Upstream Regions of Cytokinin-Induced Genes

We used GeneSpring 5.0 software (Silicon Genetics, Redwood City, CA) to search for common motifs enriched within 1 kb upstream of the translational start site of the 17 genes that are consistently up-regulated by cytokinin (those genes induced in six or

more of the cytokinin treatments as in Fig. 1A). The sequence GATCTT was identified as a core sequence motif found significantly enriched in the upstream regions of this set of genes. The relative positions of this motif upstream of the type-A ARRs and the other cytokinin-regulated genes are shown in Figure 2. White boxes represent this common upstream sequence motif GATCTT on the forward strand, and black boxes represent the complement of the motif AAGATC. Upstream regions of the cytokinin-regulated type-A ARRs showed the highest concentration of these motifs, generally clustering in the 500 bp proximal to the coding region. The sequence of this common upstream sequence motif closely matches (G/A)GAT(T/C), the optimal DNA-binding sequence identified in vitro for ARR1 and ARR2, two type-B ARRs (Sakai et al., 2000). Gel shift assays with various derivatives of the optimal type-B ARR-binding site NGAT(T/C) showed a slight preference for A at position 1 and a C at position 5, consistent with part of the common sequence motif we identified (Sakai et al., 2000).

The frequency of this common upstream sequence motif in the upstream regions of the type-A ARRs is positively correlated with the level of their induction by cytokinin. The most highly cytokinin-induced genes, *ARR5*, *ARR7*, and *ARR16*, have multiple copies of this motif. Additionally, genes that are either only weakly induced by cytokinin or not expressed at detectable levels, *ARR3*, *ARR4*, and *ARR17*, have one or no motifs (Fig. 2; data not shown). *ARR6*, which is moderately induced by cytokinin, does not fit this pattern because it has no consensus motif in its upstream region, although two such motifs are found within the fourth intron of this gene. The common upstream sequence motif (GATCTT) should randomly occur approximately 0.32 times per kilobase pair, taking into account the GC content observed in intergenic regions of Arabidopsis (approximately 33%). Our search of all genes present on the Affymetrix GeneChip that are expressed with a raw of at least 500 but not induced by cytokinin (950 genes) revealed that the sequence motif GATCTT is present a total of 358 times in the 1 kb upstream of the translational start site, or approximately 0.38 times per kilobase pair. In contrast, this core sequence motif is present 14 times among the upstream regions of the top 17 cytokinin up-regulated genes and only six times among the upstream regions of the 21 cytokinin down-regulated genes. This enrichment, coupled with the similarity to the previously defined ARR1/ARR2-binding sequence, suggests that this motif may play a role in the transcriptional regulation of these genes by cytokinin.

Effect of Cytokinin on Arabidopsis Two-Component Elements

Previous studies have implicated Arabidopsis homologs of two-component signaling elements in the cytokinin response pathway. We analyzed the expression pattern of these genes under our experimental conditions (Fig. 3; see Supplemental Table III). As noted above, the five type-A ARR genes present on the chip were up-regulated at most of the cytokinin treatments. The three AHP genes on the chip are not affected by cytokinin, nor are the five type-B ARRs, consistent with previous results suggesting that the

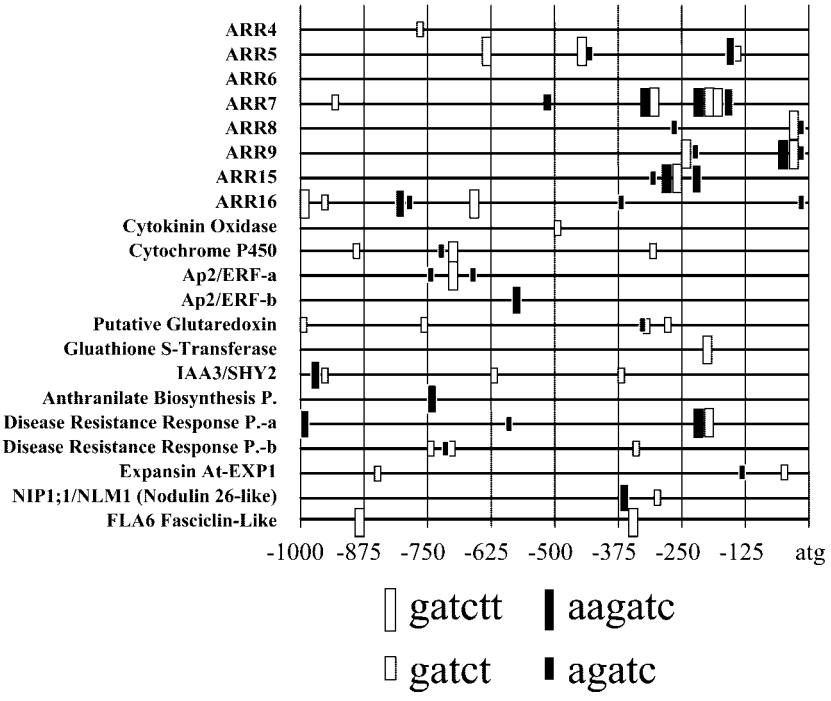

Figure 2. Putative cis-acting motifs in the promoters of cytokinin-regulated genes. The DNA sequences 1,000 bp upstream of the predicted translational start site of the 17 genes identified as cytokinin up-regulated at six or more of the 10 cytokinin time course treatments were analyzed for cis-acting sequence motifs that were significantly enriched in this region, representing potential sites of cytokinin regulation. The positions where the core sequence motif (GATCT) occurs are depicted as white boxes (black boxes represent the complement sequence), and the extended sequence (GATCTT) is depicted as a large white box, with its complement as a large black box.

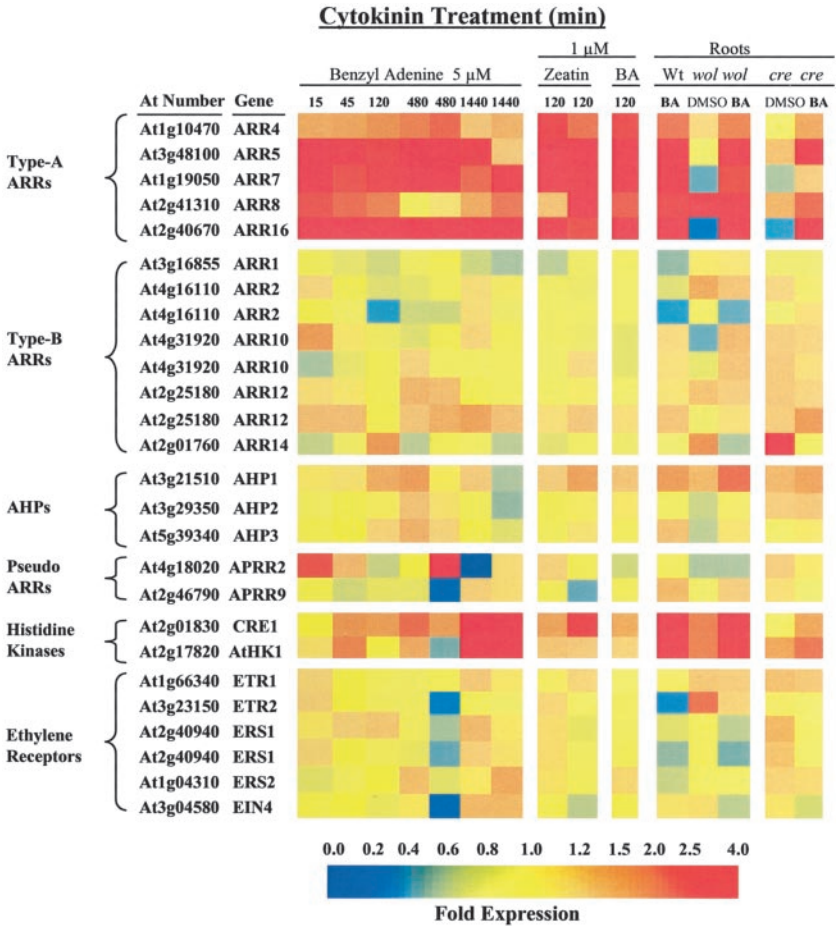

Figure 3. Regulation of two-component response regulator genes by cytokinin and other treatments. The expression ratios of two-component-like genes are depicted for the various samples relative to their cognate controls as described in Figure 1. The colors correspond to the relative fold change from the respective controls using the scale shown at the bottom of the figure. Genes shown are grouped into classes based on their similarity to different two-component elements, with their At number and gene description shown on the left. Cytokinin treatments shown from right to left are with 5 μM BA for 15, 45, 120, and two replicates of 480 and 1,440 min, 1 μM zeatin for two replicates of 120 min, 1 μM BA for 120 min, and root tissue of wild type (Landsburg *erecta* [L*er*]) treated with 5 μM BA for 45 min; *wol* mutant roots and *cre1-1* mutant roots treated with DMSO control or 5 μM BA for 45 min. All root tissue treatments were normalized to the wild-type root with 45 min of DMSO treatment.

steady-state mRNA level of these classes of two-component genes are unaffected by cytokinin treatment (Imamura et al., 1998; Kiba et al., 1999; Lohrmann et al., 1999). The two pseudoresponse regulators, which lack invariant response regulator residues, including the Asp residue that is the target of phosphorylation, and have been implicated in circadian control (Matsushika et al., 2000; Strayer et al., 2000), are also not elevated in response to cytokinin. Interestingly, the cytokinin receptor CRE1 is moderately up-regulated by cytokinin over 24 h, suggesting that application of cytokinin may alter the sensitivity of seedlings to this hormone. The His kinase homolog AHK1, which lacks a CHASE cytokinin-binding domain and has been suggested to play a role in osmosensing (Urao et al., 2000), is also up-regulated by cytokinin. The expression of the ethylene receptor genes was not affected cytokinin.

Effect of CRE1 Mutations on Cytokinin-Regulated Gene Expression

We examined the effects that loss-of-function mutations of the cytokinin receptor CRE1 had on the pattern of gene expression by analyzing RNA isolated from the roots of two different *cre1* mutant alleles, *wol* and *cre1-1*. Root tissue was examined because these mutations have been reported to principally affect root development (Scheres et al., 1995; Mähönen et al., 2000). The *wol* and *cre1* mutations have dramatic effects on gene expression: Approximately 600 genes are elevated and 400 down-regulated in both *wol* treatments (+DMSO and +BA) relative to wild-type roots (Table I; data not shown). The observation that expression levels of these genes are decreased in both treatments of the *wol* background increases the confidence that many are genuine alterations in gene expression caused by this mutation. The large-scale changes in gene expression may largely be a secondary consequence of the *wol* mutation, caused by the lack of phloem tissue in the mutant roots. Consistent with this, several transporter genes, including an aquaporin, and a proline and two sugar transporters, are up-regulated in this mutant, potentially compensating for the reduced ability to uptake nutrients through the phloem (Table I). In addition, the *cre1-1* allele, which has a much weaker root phenotype as compared with the *wol* allele, has a reduced effect on gene expression (approximately 150 genes are elevated and 75 genes down-regulated in both *cre1-1* treatments compared

Table I. *wol- and cre1-regulated genes*

Genes altered at least 2-fold for both *wol* and *cre1* roots treated with DMSO are shown below by At no. and gene description. The expression level of each gene in *wol* and *cre1* treated with 5 μM BA. All values are normalized to wild-type roots treated with DMSO.

*Wol/cre*1 Up-Regulated Genes						*Wol/cre*1 Down-Regulated Genes					
At no.	Gene	*wol*		*cre1*		At no.	Gene	*wol*		*cre1*	
		DMSO	+BA	DMSO	+BA			DMSO	+BA	DMSO	+BA
At2g46670	Transcription factor ATHB-7	4.7	11.8	2.4	2.7	At1g70210	Cyclin Delta-1	0.1	0.2	0.3	0.3
At2g28160	Putative bHLH transcription factor	2.6	0.8	2.3	1.0	At4g34160	Cyclin Delta-3	0.3	0.2	0.5	0.6
At1g43160	RAP2.6 AP2 protein	2.3	1.0	3.2	1.5	At4g20270	CLV1 receptor kinase like	0.1	0.2	0.5	0.7
At2g38470	WRKY DNA-binding protein	4.6	5.7	2.0	1.4	At4g20270	CLV1 receptor kinase like	0.2	0.3	0.5	0.7
At3g46130	R2R3-MYB transcription factor	6.3	5.0	2.3	1.2	At1g75820	Receptor kinase	0.2	0.1	0.4	0.6
At1g67110	Cytochrome P450	5.1	7.2	2.7	3.6	At2g44940	AP2 transcription factor	0.4	0.2	0.4	0.8
At4g40010	Putative Ser/Thr kinase	2.2	3.5	3.0	3.5	At5g38430	Rubisco	0.4	0.3	0.3	0.4
At2g26980	Putative protein kinase	2.5	5.5	2.2	1.8	At4g10340	Lhcb5 protein	0.4	0.5	0.4	0.5
At1g61590	Putative protein kinase	3.1	2.3	2.4	1.1	At1g61520	Chlorophyll *a/b* binding	0.5	0.8	0.3	0.7
At2g16750	Putative protein kinase	4.2	6.0	2.1	1.5	At1g55670	PSI subunit V precursor	0.4	0.7	0.3	0.5
At1g79570	Putative protein kinase	2.0	1.9	2.0	1.6	At2g30570	PSII reaction center protein	0.4	0.8	0.4	0.7
At1g72770	PP2C	2.0	5.4	2.4	2.1	At2g30570	PSII reaction center protein	0.4	0.5	0.4	0.6
At2g13640	Putative nucleotide sugar transporter	2.1	2.1	2.7	1.3	At3g21055	PSII protein	0.3	0.4	0.5	0.6
At1g05030	Putative sugar transporter	2.1	2.3	2.0	1.2	At1g20340	Putative plastocyanin	0.5	0.4	0.3	0.5
At2g16990	Putative tetracycline transporter	2.7	2.8	2.2	1.3	At5g13930	Chalcone synthase	0.5	0.4	0.5	0.9
At2g36830	Putative aquaporin	4.2	4.5	2.0	1.6	At4g16250	Phytochrome D	0.5	3.7	0.1	0.1
At3g47730	ABC transporter	2.0	1.9	2.4	2.1	At4g29080	Phytochrome-associated protein 2	0.2	0.1	0.4	0.5
At2g36590	Putative Pro transporter	2.0	1.2	2.0	1.8	At2g34490	Cytochrome P450	0.1	0.1	0.4	0.5
At2g47160	Putative anion exchange protein	2.6	3.0	2.3	1.6	At4g37430	Cytochrome P450	0.5	0.5	0.4	0.5
At1g30760	Putative berberine bridge enzyme	18.2	16.5	2.1	1.7	At4g39510	Cytochrome P450	0.1	0.6	0.3	0.4
At2g21270	Ubiquitin protein	2.8	2.5	3.3	2.0	At1g22710	SUC2 Suc-proton symporter	0.1	0.1	0.5	0.7
At2g20750	Beta-expansin	13.8	11.7	3.1	1.7	At2g38530	Nonspecific lipid transfer protein	0.3	0.3	0.3	0.3
At2g40100	Lhcb4:3 protein	4.4	9.6	10.1	7.2	At2g38530	Nonspecific lipid transfer protein	0.2	0.2	0.2	0.2
At5g24140	Squalene epoxidase homolog	9.4	7.2	5.4	2.5	At2g38540	Nonspecific lipid transfer protein	0.1	0.1	0.4	0.4
At1g54580	Acyl carrier protein	7.4	5.1	2.4	1.5	At2g36870	Xyloglucan endo-transglycosylase	0.2	0.2	0.4	1.0
At4g13050	Oleoyl hydrolase	2.6	2.2	2.6	1.9	At4g30270	Endo-xyloglucan transferase	0.3	0.2	0.3	0.2
At1g23020	Potential FROHC	5.8	5.8	2.1	1.4	At2g28950	Expansin At-EXP6	0.3	0.2	0.4	0.5
At2g47550	Putative pectinesterase	5.4	3.1	2.5	1.4	At1g20620	Catalase 3	0.3	0.3	0.4	0.3
At3g47400	Pectinesterase-like protein	2.2	1.6	2.5	2.5	At3g12500	Basic endochitinase	0.1	0.1	0.5	0.5
At3g54430	Class IV chitinase	4.1	3.0	2.1	1.1	At4g04840	Putative Met sulfoxide reductase	0.1	0.1	0.3	0.7
At2g04430	mutT domain protein	3.0	2.3	7.3	0.7	At4g27570	UDP Rha	0.1	0.1	0.4	0.3
At2g26930	Putative ripening-associated protein	2.9	3.8	2.1	1.6	At2g20340	Tyr decarboxylase	0.3	0.6	0.4	0.6
At2g39410	Putative phospholipase	2.9	2.5	2.6	1.2	At4g29020	Gly-rich protein	0.0	0.0	0.2	0.2
At2g17420	NADPH thioredoxin reductase	41.3	36.4	3.1	1.9	At3g16380	Pro-rich protein APG isolog	0.1	0.1	0.3	0.1
At2g02990	Ribonuclease	2.4	3.7	2.4	1.5	At4g39330	CAD1	0.2	0.2	0.4	0.5
At2g04150	Subtilisin-like protease	2.0	3.1	2.6	2.2	At1g78830	*S* locus gycoprotein	0.3	0.5	0.5	0.6
At1g64060	Atrboh F	2.2	2.1	2.1	1.5	At2g32990	Putative glucanase	0.0	0.4	0.4	0.6
At2g47490	Putative mitochondrial carrier	2.1	1.7	2.7	1.6	At4g20420	Putative Ser proteinase	0.1	0.2	0.4	0.4
At2g04160	Subtilisin-like protease	2.1	2.6	2.0	2.0	At5g25610	RD22	0.1	0.4	0.3	0.3
At4g25070	Putative protein	2.0	2.2	3.0	1.5	At4g33720	Pathogenesis-related1 precursor	0.1	0.0	0.1	0.1
At4g36640	Putative protein	2.5	3.8	2.3	2.7	At4g33720	Pathogenesis-related1	0.0	0.0	0.1	0.0
At4g26470	Putative protein	2.3	2.8	2.0	1.3	At5g24780	Vegetative storage Protein	0.3	0.1	0.2	0.3
At4g25870	Putative protein	4.0	3.7	2.6	0.9	At2g42840	Protodermal factor 1	0.0	0.0	0.5	0.3
At4g35110	Putative protein	3.5	3.8	2.1	1.6	At4g24360	Putative protein	0.0	0.0	0.1	0.1
At3g47380	Putative protein	4.0	2.3	2.3	2.4	At2g47560	Hypothetical protein	0.1	0.1	0.4	0.4
At2g23890	Hypothetical protein	2.3	1.7	3.0	2.7	At2g33850	Unknown protein	0.0	0.0	0.3	0.4
At2g39690	Hypothetical protein	2.1	1.8	2.9	1.8	At2g39710	Unknown protein	0.4	0.2	0.5	0.7
At4g16350	Hypothetical protein	2.2	2.7	2.7	1.9	At2g16850	Unknown protein	0.2	0.3	0.5	0.6
At1g24310	Hypothetical protein	3.8	3.7	2.6	1.8	At1g67740	Unknown protein	0.4	0.4	0.4	0.5
At2g14530	Hypothetical protein	3.7	2.4	2.5	1.0	At2g44670	Unknown protein	0.1	0.3	0.4	0.4
At2g37210	Hypothetical protein	3.5	7.1	3.3	2.8						
At2g12550	Hypothetical protein	2.6	2.4	4.0	1.9						
At2g48080	Unknown protein	2.6	1.5	2.6	1.2						
At4g10970	Unknown protein	2.6	4.5	2.4	2.7						
At1g05170	Unknown protein	3.9	3.9	2.8	1.6						
At2g20320	Unknown protein	2.1	1.7	2.6	1.3						
At2g01650	Unknown protein	2.1	2.7	2.2	1.9						
At1g30900	Unknown protein	2.7	2.2	2.6	2.1						
At5g49440	Unknown protein	161.5	161.0	2.3	1.0						
At2g33220	Unknown protein	18.3	16.2	6.0	6.9						

with the larger numbers found in *wol* as described above).

Of the genes that are down-regulated in both *wol* and *cre1-1*, one group of particular interest is those involved in regulation of cell division and meristem function, consistent with the primary defect in root development of this mutant. Interestingly, the cell cycle regulators *CycD1* and *CycD3* are down-regulated in both *wol* and *cre1-1* relative to wild-type roots, independent of cytokinin treatment, and the *cdc2* gene is down-regulated specifically in *wol* (see Supplemental Table IV). As in the shoots, we do not observe an induction of either of these genes in response to cytokinin in contrast to previous reports (Riou-Khamlichi et al., 1999; see Supplemental Table IV). The down-regulation of these cell cycle genes is consistent with the reduced meristem function of *wol* and *cre1-1* mutants and could either be a cause (i.e. the lack of expression of the genes due to a reduced cytokinin response leads to reduced cell division in the vasculature precursor cells) or a consequence (the reduced number of dividing cells in the mutant leads to the lower expression of these cell cycle genes). Interestingly, a CLV1 receptor kinase-like gene is also down-regulated in both alleles under all treatments compared with wild type (Table I). *CLV1* plays a role in regulating meristem size in the shoot apical meristem, and, perhaps, this homolog may play a similar role in the root meristem.

The type-A ARR genes downstream of the cytokinin receptor CRE1 were still induced by cytokinin in the *wol* and *cre1-1* mutants, although the fold increase relative to untreated mutant roots was not always to the same level observed in wild-type roots (Fig. 3). Basal expression levels of the type-A ARR and several others of the top cytokinin-inducible genes were also lower in these cytokinin receptor mutants (Table II), as previously observed in the *cre1-1* mutant for two type-A genes, *ARR15* and *ARR16* (Kiba et al., 2002). These CRE1 mutations reduced but did not eliminate the effect of exogenous cytokinin on gene expression on a subset of genes and had little or no effect on the expression of others. This suggests that other cytokinin receptors (e.g. AHK2 and AHK3) can largely compensate for the loss of CRE1 under conditions of high exogenous cytokinin, but the response to endogenous cytokinin levels is partially compromised.

Effect of Inhibition of Protein Synthesis on Cytokinin-Regulated Gene Expression

Generally, genes that are involved in the primary response to a signal are still induced by that signal when protein synthesis is blocked. Furthermore, many primary response genes are elevated upon inhibition of protein synthesis, an effect that has been attributed to the rapid degradation of a negative regulator of transcription or the involvement of a short-lived RNase in the degradation of those transcripts. The effect of inhibition of protein synthesis on cytokinin up-regulated gene expression was examined in seedlings pretreated with cycloheximide, in the presence or absence of cytokinin (Table III).

A large number (236) of genes was found to be elevated greater than 3-fold in response to cycloheximide (data not shown), including many genes previously shown to be up-regulated by cycloheximide treatment such as indole-3-acetic acids (IAAs; Koshiba et al., 1995), type-A ARRs (Brandstatter and Kieber, 1998), and ACS genes (Liang et al., 1992). Approximately one-half of the cytokinin up-regulated genes showed more than a 2-fold alteration in expression levels by cycloheximide treatment alone (Table III), a common feature of primary response and regulatory genes. An additive response to cytokinin and cycloheximide is observed for many of the cytokinin up-regulated genes, including the type-A ARRs that had been reported previously (D'Agostino et al., 2000). This suggests that the majority of the genes that are rapidly induced by exogenous cytokinin are primary response genes.

DISCUSSION

We have analyzed the effect of exogenous cytokinin on gene expression in Arabidopsis seedlings to identify novel cytokinin-regulated genes and to better understand the role of cytokinin in plant developmental processes. Our analysis revealed over 70 genes whose expression is altered by application of exogenous cytokinin in at least one-half of the treatments examined. The genes that are affected by cytokinin and their kinetics of induction provide some clues as to how seedlings respond to elevated cytokinin levels. One reaction to elevated cytokinin at the transcriptional level appears to be a reduction of both the level of active hormone and the sensitivity of the response pathway. The cytokinin signaling response is desensitized by up-regulation of type-A ARRs, which act as negative regulators of the cytokinin response pathway (J. To, G. Haberer, and J. Kieber, unpublished data). The level of cytokinin is potentially reduced by increased expression of a cytokinin-degrading enzyme, cytokinin oxidase, consistent with previous studies demonstrating that cytokinin oxidase enzyme activity was elevated in response to exogenous cytokinin (Terrine and Laloue, 1980; Palmer and Palni, 1987) and by the induction of a gene encoding a potential cytokinin-conjugating enzyme, a putative glucosyltransferase (At2g16890; Fig. 1A). These feedback responses may explain why we do not observe large secondary transcriptional responses after prolonged exogenous cytokinin treatment. In contrast, expression of the cytokinin receptor, CRE1, is up-regulated over time in response to the hormone, which is similar to the induction of ethylene receptors by ethylene. This slight increase

Table II. *Cytokinin-regulated gene Expression in wol and cre1*

The expression levels of cytokinin-regulated genes (from Fig. 1) in either wild type (L*er*) or *wol*- or *cre1*-treated roots are shown below either treated with DMSO or 5 μM BA for 45 min. All values are normalized to wild-type roots treated with DMSO.

Cytokinin Up-Regulated Genes							Cytokinin Down-Regulated Genes						
At no.	Gene	Ler	*wol*	*cre1*			At no.	Gene	Ler	*wol*		*cre1*	
		+BA	DMSO	+BA	DMSO	+BA			+BA	DMSO	+BA	DMSO	+BA
At1g19050	ARR7	5.6	0.5	2.8	0.6	1.4	At1g09090	Respiratory burst oxidase protein	0.7	1.4	1.1	1.1	0.5
At2g40670	ARR16	20.6	0.4	4.4	0.5	3.7	At5g19890	Peroxidase	0.4	1.4	1.1	1.5	0.8
At4g29740	Cytokinin oxidase	8.2	30.5	88.4	0.8	1.8	At1g70430	Ste-20-related kinase SPAK	0.5	1.8	2.6	1.2	1.6
At2g46310	Putative AP2	1.7	0.9	2.0	0.6	2.3	At2g23030	Putative protein kinase	0.9	6.3	16.1	1.7	0.7
At1g67110	Cytochrome P450	6.0	5.1	7.2	2.7	3.6	At2g03160	E3 ubiquitin SCF subunit (At19)	1.1	1.0	1.2	0.7	0.8
At3g48100	ARR5	4.0	0.8	6.0	1.4	5.5	At2g41970	Putative protein kinase	0.7	0.9	0.8	2.1	1.7
At4g11190	Putative disease resistance response P.	2.0	0.1	0.1	0.9	2.3	At2g05020	Hypothetical protein	0.7	0.8	3.5	0.5	0.9
At4g23750	Putative AP2	0.7	1.2	1.2	0.9	0.9	At2g27370	Unknown protein	0.8	0.9	1.0	1.9	1.2
At2g30540	Putative glutaredoxin	5.3	0.1	1.3	0.5	1.0	At1g49570	Peroxidase ATP5a	0.5	5.1	5.4	1.2	0.5
At1g10470	ARR4	2.4	1.2	2.3	1.0	1.8	At5g64210	Alternative oxidase 2	0.3	1.5	2.0	1.8	1.4
At1g69530	Expansin At-EXP1	1.7	1.9	1.4	1.2	1.2	At2g42060	CHP-rich zinc finger protein	0.4	3.5	2.3	1.4	0.5
At2g29490	Glutathione *S*-transferase	3.2	1.9	5.8	1.2	2.5	At5g65210	bZIP transcription factor TGA1	0.7	1.5	1.8	1.3	0.8
At4g19030	NIP1;1/NLM1 (nodulin-26 like)	3.4	1.4	2.5	0.8	1.5	At4g10120	Suc-phosphate synthase like	0.3	0.8	0.4	0.7	0.1
At4g11210	Putative disease resistance response P.	3.8	0.1	0.1	3.0	4.2	At4g14130	Xyloglucan endotransglycosylase	0.6	0.2	0.2	0.5	0.4
At2g40230	Anthranilate biosynthesis P.	1.2	1.2	1.2	0.6	1.0	At4g37160	Pectinesterase-like protein	0.9	1.2	1.0	1.3	1.2
At1g04240	IAA3/SHY2	4.9	0.1	0.5	0.6	1.9	At3g23610	Dual-specificity P. phosphatase	0.3	1.5	2.5	4.0	5.8
At2g20520	FLA6 fasciclin-like arabinoglactan	1.7	0.6	0.4	5.5	4.7	At2g37510	RMM-containing protein	1.0	0.7	0.6	1.4	1.2
At2g41310	ARR8	3.1	5.1	8.8	1.6	2.5	At5g44760	Putative C2 domain-containing protein	4.3	4.6	3.8	1.7	0.2
At2g47260	Putative WRKY DNA binding P.	2.1	0.7	2.1	0.6	0.9	At4g35110	Putative protein	1.6	3.5	3.8	2.1	1.6
At4g38850	SAUR-AC1	1.8	3.0	7.2	0.4	0.2	At1g67460	Hypothetical protein	2.1	3.8	3.5	1.6	0.3
At1g04250	IAA17/AXR3	1.8	0.6	1.0	1.1	1.8	At4g31330	Unknown protein	0.6	0.8	0.6	0.8	0.7
At4g35160	*O*-methyltransferase like	2.5	0.3	0.4	0.9	1.4	At4g26010	Putative peroxidase	0.4	0.4	0.2	1.5	1.4
At3g16430	Jasmonate-inducible isolog	1.3	0.6	0.8	1.2	1.2	At3g54110	Peroxidase	0.6	0.7	0.4	1.8	1.1
At5g15960	KIN1 cold- and abscisic acid-inducible P.	0.8	0.8	1.0	1.7	1.4	At1g03000	Putative peroxisome assembly factor-2	0.9	0.8	0.8	1.7	1.1
At3g60280	Blue copper-binding protein III	1.6	1.1	0.9	2.4	1.9	At1g48410	Argonaute	0.5	2.9	5.7	1.6	1.6
At4g29610	Cytidine deaminase 6	1.2	0.1	0.4	0.3	0.3	At2g46690	Putative auxin-induced protein	0.8	1.0	0.9	0.9	0.8
At2g39220	Putative patatin protein	9.1	0.6	0.4	0.4	0.3	At2g35270	Putative AT-hook DNA-binding protein	0.3	0.8	0.3	1.0	0.5
At2g22860	Unknown protein	1.4	5.0	5.2	1.2	0.8	At2g29290	Putative tropinone reductase	2.8	5.3	1.0	0.9	0.6
At2g25150	Unknown protein	2.2	0.1	1.2	0.7	2.7	At4g19170	9-Cis-epoxycaroteniod dioxygenase	0.6	1.6	4.1	3.6	2.0
At1g27470	Unknown protein	8.5	12.5	21.3	5.7	4.2	At4g20110	Spot 3 vacuolar-sorting receptor	0.6	2.2	2.4	1.5	1.1
At4g15480	UDP-glucosyltransferase	1.5	1.4	1.1	0.6	1.2	At2g34940	Spot 3 vacuolar-sorting receptor	0.9	1.4	1.5	1.3	0.9
At4g35460	Thioredoxin reductase 2 (NADPH)	0.9	1.2	1.7	1.0	1.2	At4g24710	Putative protein-binding protein	1.6	1.2	0.3	2.0	1.0
At5g12030	Heat shock protein 17.6A	0.4	1.4	1.6	1.0	1.5	At4g10640	Putative protein	1.1	1.0	1.6	1.5	1.2
At1g14930	Major latex-related protein	0.7	0.8	1.1	1.1	1.0	At4g17585	Putative protein	1.6	1.2	1.3	0.8	1.3
At5g23380	Putative protein	1.1	0.6	1.3	0.7	0.8	At1g24210	Hypothetical protein	2.4	3.3	2.6	0.4	0.6
At2g16890	Putative glucosyltransferase	1.5	0.3	0.5	0.9	0.9	At1g78840	Hypothetical protein	1.2	0.8	1.0	0.9	0.8
At5g20930	Protein kinase tousled	1.4	1.7	1.9	1.3	1.0	At2g12170	Hypothetical Protein	0.3	0.2	0.2	1.3	0.5
At1g75830	Plant defensin protein (PDF1.1)	0.9	4.0	10.4	0.5	2.6							
At4g34790	Putative protein	0.5	1.6	1.3	0.4	0.2							
At2g17830	Unknown protein	1.3	1.2	2.2	0.4	0.6							

Table III. *Cytokinin-regulated gene expression in the presence of cycloheximide*

The expression levels of cytokinin-regulated genes (from Figure 1) are shown below for seedlings in the presence of 50 μM cycloheximide either treated with DMSO or 5 μM BA for 45 min. All values are normalized to wild-type seedlings treated only with DMSO for 45 min.

Cytokinin Up-Regulated Genes			
At no.	Gene	Cyclohexamide	
		DMSO	+BA
At1g19050	ARR7	16.4	20.8
At2g40670	ARR16	7.2	39.3
At4g29740	Cytokinin oxidase	19.4	47.1
At2g46310	Putative AP2	7.2	10.7
At1g67110	Cytochrome P450	1.4	3.6
At3g48100	ARR5	9.6	14.0
At4g11190	Putative disease resistance response P.	0.8	1.3
At4g23750	Putative AP2	7.1	8.5
At2g30540	Putative glutaredoxin	2.2	7.0
At1g10470	ARR4	2.0	2.4
At1g69530	Expansin At-EXP1	3.0	4.8
At2g29490	Glutathione *S*-Transferase	4.2	5.5
At4g19030	NIP1;1/NLM1 (nodulin-26 like)	0.0	1.8
At4g11210	Putative disease resistance response P.	1.0	2.4
At2g40230	Anthranilate biosynthesis P.	1.8	4.9
At1g04240	IAA3/SHY2	20.8	23.7
At2g20520	FLA6 fasciclin-like arabinoglactan	0.6	0.2
At2g41310	ARR8	7.6	11.7
At2g47260	Putative WRKY DNA-binding P.	8.7	11.1
At4g38850	SAUR-AC1	5.3	8.2
At1g04250	IAA17/AXR3	1.4	2.2
At4g35160	*O*-methyltransferase like	2.0	2.4
At3g16430	Jasmonate-inducible isolog	1.8	1.7
At5g15960	KIN1 cold- and abscisic acid-inducible P.	1.1	1.1
At3g60280	Blue copper-binding protein III	0.8	0.9
At4g29610	Cytidine deaminase 6	19.2	42.2
At2g39220	Putative patatin protein	5.8	8.7
At2g22860	Unknown protein	2.7	3.1
At2g25150	Unknown protein	0.0	0.3
At1g27470	Unknown protein	3.0	1.8
At4g15480	UDP-glucosyltransferase	0.5	0.9
At4g35460	Thioredoxin reductase 2 (NADPH)	1.3	1.7
At5g12030	Heat shock protein 17.6A	0.9	0.9
At1g14930	Major latex-related protein	1.0	1.1
At5g23380	Putative protein	1.5	2.4
At2g16890	Putative glucosyltransferase	4.5	4.5
At5g20930	Protein kinase tousled	1.1	1.8
At1g75830	Plant defensin protein (PDF1.1)	1.0	0.9
At4g34790	Putative protein	1.5	3.2
At2g17830	Unknown Protein	4.4	3.4

may reflect a potential negative regulator role of CRE1 in cytokinin response because His kinases in other two-component systems can act as both His kinases and phosphatases (Stock et al., 2000).

A second potential pattern that emerges from cytokinin-regulated genes is an effect on redox state. Four peroxidases and two oxidases are down-regulated by cytokinin in multiple treatments, and both a glutathione *S*-transferase and a glutaredoxin are up-regulated. The down-regulated peroxidase genes are phylogenetically dispersed within the Arabidopsis peroxidase gene family, which includes 73 members (Tognolli et al., 2002). Peroxidases have been implicated in many physiological processes, including hydrogen peroxide detoxification, lignin biosynthesis, and stress responses (Hiraga et al., 2001; Tognolli et al., 2002). IAA oxidase, which catabolizes auxin, is also a peroxidase (Arnison, 1980), and its down-regulation could lead to an alteration in the levels of IAA. This would provide one mechanism for the synergistic effects of these two hormones. Lee (1974) found that treatment of tobacco (*Nicotiana tabacum*) callus culture with 5 μM cytokinin strikingly reduced the activity of multiple peroxidases, including IAA oxidase activity, although the effect on the IAA oxidase was less pronounced as compared with other peroxidases. Miller (1978, 1979, 1985) noted that cytokinins caused both a rapid promotion and

an inhibition of the apparent peroxidations of several compounds, such as coumarate, the effect being dependent on the concentration and timing of application of the hormone. Potentially, auxin levels could be up-regulated to help balance excess cytokinin levels, if the down-regulated peroxidases have any IAA oxidase activity. Interestingly, several auxin-regulated genes, such as IAA3, IAA17, and SAUR AC-1, were elevated by cytokinin treatment.

A number of transcription factors are rapidly up-regulated by cytokinin, including two AP2/ERF family members and a WRKY DNA-binding protein. The two *AP2* genes cluster within the same subgroup of the large Arabidopsis *ERF/AP2* gene family, and this subgroup as of now has no members with assigned functions (Sakuma et al., 2002). It is possible that they may act in a similar manner to ethylene-responsive *ERF* genes in closely related clades of the *AP2* gene family. In addition, several transcription factors are among the genes that were found to be consistently down-regulated in response to cytokinin treatment (Fig. 1B).

Other genes that we have identified as cytokinin up-regulated reflect previous findings on the effects of cytokinin. Consistent with our identification of a cytokinin up-regulated expansin, cytokinin has been implicated in regulating cell expansion in certain tissues, most notably cotyledons, and a cytokinin up-regulated β-expansin gene has been identified from tobacco (Dowens and Crowell, 1998). Interestingly, there are three protein kinases and a dual-specificity protein phosphatase that are down-regulated by cytokinin and could potentially play a role in cytokinin signal transduction. Three of these genes are more highly down-regulated with extended cytokinin treatment (Fig. 1B), similar to the pattern of another negatively regulated kinase gene identified from tobacco (Schäfer and Schmülling, 2002).

One group of genes (cell cycle and meristem genes) was conspicuously absent from our set of induced genes (Fig. 1A; see Supplemental Table V). The *cdc2* protein kinase, *CycD3* cyclin, and the *STM1* homeobox transcription factor have all been reported previously to be elevated in response to cytokinin (Hemerly et al., 1993; Riou-Khamlichi et al., 1999; Rupp et al., 1999). *CycD3* was reported to be induced by cytokinin, based mostly on analysis of suspension cultures. Induction was also observed in whole seedlings, though this was done using liquid-grown seedlings treated for 24 h with zeatin (Riou-Khamlichi et al., 1999), conditions that are sufficiently different from those used for our experiments to potentially account for these contradictory results. Likewise, increases in *STM1* expression has been observed only in cytokinin-overproducing plants (Rupp et al., 1999), and *cdc2* induction was reported only after 72 h of cytokinin treatment and only in a small subset of root cells (Hemerly et al., 1993). However, we did find several cell cycle genes, including *CycD3*, that are down-regulated in the cytokinin receptor mutants, suggesting cell cycle genes may be responsive to endogenous cytokinin function. Alternatively, the down-regulation of these cell cycle and meristem genes in *wol* and *cre1-1* may simply reflect the altered tissue composition of the mutants.

Our examination of the temporal expression patterns of genes over our time course of treatments revealed two major patterns. The first is a constant pattern of induction or repression from the first 15-min time point to the 24-h time point, including several of the type-A ARRs, cytokinin oxidase and two AP2s that are constantly induced, and a respiratory burst oxidase and several kinases that are constantly repressed. The second major pattern represented by a small set of genes is one of alteration only after prolonged exposure to cytokinin. This occurs both for induced genes such as a putative disease resistance protein and a glutaredoxin, which are up-regulated after 45 min, and for repressed genes, such as the four peroxidases that do not show down-regulation until after 120 min. While other expression patterns can be identified, they are represented by relatively few genes. Although most rapidly induced genes remain altered at later time points, in many cases, a decrease in the magnitude of the response is observed. This suggests that there is a partial desensitization of the response pathway after prolonged exposure to cytokinin.

We were also able to identify a common upstream sequence motif, GATCTT. The similarity of this motif to the previously identified type-B ARR-binding site suggests that type-B ARR genes may mediate induction of genes in addition to type-A ARRs (Sakai et al., 2000). Further analysis is required to determine the functional significance of this motif in cytokinin regulation.

To better understand the role of the cytokinin receptor CRE1 in mediating cytokinin response, we examined expression profiles of cytokinin treatment in the receptor mutant alleles *wol* and *cre1-1*. We found lower baseline expression levels of the type-A ARRs, suggesting that the response to endogenous cytokinin is affected by the loss of this receptor. However, the general trend for gene expression in the presence of cytokinin was a reduction, but not elimination, of the alteration in transcript levels in both receptor mutants. This suggests a redundancy of cytokinin receptor function, probably by the other CHASE domain-containing His kinase homologs, AHK2 and AHK3. There is also another group of genes that show a lack of induction by cytokinin in the receptor mutants or even in some cases a repression in the mutants relative to the wild type. Although some of these genes may be explained by general variation in expression levels, it is possible that for some, the cytokinin signal acts solely through CRE1. Additional analysis of lines disrupted for

CRE1 and other cytokinin receptors may shed further light on this.

In conclusion, we have identified through expression profiling a set of cytokinin-regulated genes. Some of these correspond to previously identified cytokinin-regulated genes, and others are novel cytokinin-regulated genes that provide new insight into the mechanism of cytokinin function. More detailed analyses of the function of these novel genes are in progress to better elucidate their role in cytokinin response. Our examination of CRE1 cytokinin receptor mutants suggests that there is redundancy at the level of cytokinin perception, although levels of downstream genes in the absence of exogenous cytokinin are reduced in these receptor mutants. Further analyses of gene expression with full genome arrays and with additional cytokinin mutants should provide additional insight into the function of this hormone.

MATERIALS AND METHODS

Plant Growth

All plants, except for those in the *wol/cre1-1* experiments, were planted at a density of approximately 500 seeds per plate (150 mm) using 0.8% (w/v) top agar (0.8% [w/v] low-melt agarose in 1× Murashige and Skoog salts) on filter paper (150 mm, Whatman no. 3, Whatman, Clifton, NJ) placed on 1% (w/v) agar containing 1× Murashige and Skoog salts + 1% (w/v) Suc buffered to pH 5.7 with MES (Murashige and Skoog media). Seedlings were stratified at 4°C for 4 d, then grown horizontally under constant light (90 μM m^{-2} s^{-1}) at 22°C for 10 d. The seedlings were then gently scraped off the filter paper into flasks containing 500 mL of liquid Murashige and Skoog media and shaken lightly for 2 h in the light before treatment. At time 0, the indicated concentration of cytokinin (1 or 5 μM BA or 1 μM trans-zeatin) was added, or an equal volume of DMSO was added as a vehicle control to the seedlings, with 0.1% (v/v) DMSO in a final volume of Murashige and Skoog. The treatment of seedlings with cycloheximide was conducted in the same manner as with the cytokinin treatments using either a cytokinin (5 μM BA) or DMSO treatment for 45 min, except that seedlings were treated with 50 μM cycloheximide for 30 min before and during the 45-min cytokinin or DMSO treatment. After the appropriate time in all gene chip treatments, seedlings were harvested and frozen at −80°C until RNA was extracted. For all experiments, RNA from two independent biological replicates was pooled before hybridizing to the chip, except for the *wol/cre1-1*, for which only one replicate was made. For the time course, there was a gene chip for each of the following: 5 μM BA treatment at 15, 45, and 120 min; two treatments at 480 and 1,440 min; a 1 μM BA treatment at 120 min; and two treatments with 1 μM zeatin at 120 min, as shown in Figure 1, along with DMSO control gene chips in parallel with each of the above cytokinin gene chip times. Plants in the *wol/cre1-1* experiments were treated in a similar manner as above except that these seedlings were grown vertically on agar plates without filter paper from a line of seeds such that the roots could be harvested easily. After growth at the above conditions for 10 d, roots were harvested and immediately moved to flasks of liquid Murashige and Skoog, where they were treated in a similar manner as the seedlings above. *wol*- and *cre1*-treated root samples were normalized to two independent biological wild-type root DMSO-treated gene chip samples. Seedlings used in these experiments treated with 5 μM BA and its DMSO control were of the Wassilewskija ecotype, with 1 μM BA and zeatin treatments, and their DMSO control was of the Columbia ecotype. In the *wol/cre1-1* experiment, the L*er* ecotype was used as both the *wol* and the *cre1-1* mutants were in the L*er* background.

RNA Sample and Microarray Preparation

Total RNA was extracted from frozen plant tissue using TRIzol reagent following the manufacturer's instructions (Introgen, Grand Island, NY). Seven micrograms of total RNA was used to synthesize cDNA. A custom cDNA kit (Life Technologies/Gibco-BRL, Gaithersburg, MD) was used with a T7-$(dT)_{24}$ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit (Life Technologies/Gibco-BcL, Gaithersburg, MD). The cRNA was then fragmented in fragmentation buffer (5× fragmentation buffer: 200 mM Tris-acetate [pH 8.1], 500 mM KOAc, and 150 mM MgOAc) at 94°C for 35 min before the chip hybridization. Fifteen micrograms of fragmented cRNA was then added to a hybridization cocktail (0.05 μg μL^{-1} fragmented cRNA; 50 pM control oligonucleotide B2; *BioB*, *BioC*, *BioD*, and *cre1-1* hybridization controls; 0.1 mg mL^{-1} herring sperm DNA; 0.5 mg mL^{-1} acetylated bovine serum albumin; 100 mM MES; 1 M NaCl; 20 mM EDTA; and 0.01% [v/v] Tween 20), and 10 μg of cRNA was used for each hybridization. Arrays were hybridized for 16 h at 45°C in a GeneChip Hybridization Oven 640. The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400. The arrays were subsequently scanned with the Hewlett-Packard GeneArray Scanner (Hewlett-Packard, Palo Alto, CA). Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3′ to 5′ intensity ratios of certain genes.

Data Analysis

Affymetrix gene chip data files were imported into GeneSpring 5.0 software and normalized as recommended by the GeneSpring manual for Affymetrix gene chips. Using 50% median normalization, each treatment chip was specifically normalized to a control chip treated with DMSO for a similar time to the actual treatment. Once the data were normalized within GeneSpring, there were analyzed as a ratio to signal in a variety of ways. Data for all experimental treatments are from a single gene chip compared with its respective DMSO time point control. Data for tables and figures were restricted such that values were considered only if genes had positive expression levels for both treated and control microarrays and if the raw value for a gene on a treated microarray was above a raw expression level of 500 or 175 in the DMSO or treated sample when examining down- and up-regulated genes, respectively. A raw level cutoff of 500 was assigned to all gene chip experiments conducted on seedlings with 5 μM BA or the corresponding DMSO control, whereas a raw cutoff of 175 was assigned to all other gene chips experiments. These raw level cutoffs were assigned because expression levels of genes below these cutoff thresholds frequently showed variability greater than 2-fold and often had Affymetrix flag calls of Absent (data not shown).

Statistical analyses were performed on Affymetrix gene chip treatments normalized as described above and examined as a log ratio to have a normally distributed population for parametric analysis. Welch's *t* tests were performed using the Statistical Group Comparison tool in GeneSpring 5.0 software using the GeneSpring Cross-Gene Error model variances. Comparisons were made between treatments of cytokinin or DMSO either for individual time point treatments or across all time points after a Welch's ANOVA determined that there was no significant effect of the time treatment of samples. A *P* value cutoff of 0.05 was selected for all tests. In addition, the Benjamini and Hochberg False Discovery Rate Multiple Testing Correction option was applied to the examination of the cytokinin up-regulated genes to eliminate against false positives in the larger number of significant genes identified there.

The search for potential cis-acting regulatory sequence motifs in the cytokinin up-regulated genes was conducted using the Find Potential Regulatory Sequence tool in GeneSpring 5.0 software. We searched using Find New Sequences to look for oligonucleotides 5 to 13 bp in length that were enriched in the open reading frames from 0 to 1,000 bp upstream of the top 17 cytokinin up-regulated genes from Figure 1A compared with other genes in the Arabidopsis genome. The sequences GATCTT, GATCTTA, AGATCTTA, AAGATC, and AAGATCTT were identified as having single *P* values below 0.05, observed rates 2 to 3 times the expected rate for those sequences. The only sequences also identified using the above criteria were AAAAAGA and AAAGAAAA, which are likely to simply reflect the A/T-rich value of the non-coding region in the Arabidopsis genome.

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