Monitoring genome-wide changes in gene expression in response to endogenous cytokinin reveals targets in *Arabidopsis thaliana*☆

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Abstract Cytokinins have been implicated in developmental and growth processes in plants including cell division, chloroplast biogenesis, shoot meristem initiation and senescence. The regulation of these processes requires changes in cytokinin-responsive gene expression. Here, we induced the expression of a bacterial isopentenyl transferase gene, *IPT*, in transgenic *Arabidopsis thaliana* seedlings to study the regulation of genome-wide gene expression in response to endogenous cytokinin. Using MPSS (massively parallel signature sequencing) we identified 823 and 917 genes that were up- and downregulated, respectively, following 24 h of *IPT* induction. When comparing the response to cytokinin after 6 and 24 h, we identified different clusters of genes showing a similar course of regulation. Our study provides researchers with the opportunity to rapidly assess whether genes of interest are regulated by cytokinins.

Key words: Cytokinin; Gene expression; Isopentenyl transferase; Massively parallel signature sequencing

1. Introduction

Plant growth and developmental processes as well as environmental responses require the action and cross talk of phytohormones including auxin, ABA, brassinolide, cytokinins, ethylene and gibberellin. The molecular mechanisms of many hormone signal transduction pathways have been studied in detail [1–4]. Recent molecular genetic and biochemical approaches suggested that the cytokinin signal is transduced by a phosphorelay pathway similar to those used by bacterial two-component systems [5,6]. Most likely, in *Arabidopsis thaliana* a sensor histidine kinase (AHK) serves as a cytokinin receptor. The AHKs in *A. thaliana* comprise a heterogenous group of receptors implicated in ethylene and cytokinin responses [7]. Genetic screens, complementation studies in His kinase-deficient yeast, and in vitro cytokinin binding assays have demonstrated that *AHK2*, *AHK3*, and *AHK4* may act as cytokinin receptors [8–11]. Overexpression of *AHK4*, but not *AHK2* and *AHK3* increased cytokinin-responsive gene expression in an *Arabidopsis* mesophyll protoplast system [12]. In addition, histidine to aspartate phosphorelays often involve His-containing phosphotransfer factors that transduce the signal by transferring a phosphoryl group from the transmitting domain of an activated histidine kinase to the receiver domain of a response regulator [13]. Indeed, the completion of the *Arabidopsis* genome uncovered a highly homologous family of five genes encoding putative His-containing phosphotransfer proteins (AHPs, [14–16]). These AHPs were capable of undergoing phosphorylation at an essential histidine residue and transferring a phosphoryl group to the receiver domain of response regulators [9,15,17,18]. However, in a protoplast cytokinin response assay, different AHPs had little effect on cytokinin-responsive gene expression [12].

A large number of response regulators (ARRs) have been identified as putative signaling elements downstream of the two-component sensor kinases [5]. Whereas all ARRs harbor a phospho-accepting receiver domain, only a subgroup of ARRs (Type-B in contrast to Type-A) contains an additional conserved C-terminal domain that resembles features of transcriptional activators and indeed is capable of DNA binding [19]. Overexpression of Type-B ARRs (*ARR1* or *ARR2*) increased the transcript abundance of the cytokinin-responsive gene *ARR6*, while the loss-of-function *arr1* mutant resulted in decreased *ARR6* transcript levels [20–21]. In contrast to the Type-B ARRs, the Type-A genes *ARR4, 5, 6*, and *7* inhibited the cytokinin-induced changes in gene expression [12].

Few cytokinin signaling components have been identified downstream of the phosphorelay. Monitoring the cytokinin-dependent regulation of gene expression represents an attractive approach to gain access to additional components of cytokinin signaling and effector pathways. Whereas changes in gene expression in response to cytokinin have been observed in various plants, few cytokinin-responsive genes have been reported in *A. thaliana* seedlings [22]. The only large-scale gene expression study focused on root explants that became committed to shoot regeneration on cytokinin-rich induction medium [23]. We therefore aimed at a genome-

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Abbreviations: IPT, isopentenyl transferase; MPSS, massively parallel signature sequencing; DEX, dexamethasone; MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase

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wide observation of changes in gene expression in response to an increase in cytokinin levels in *Arabidopsis* seedlings. We induced the bacterial isopentenyl transferase gene, *IPT*, in *A. thaliana* seedlings. IPT is a key enzyme in the biosynthetic pathway of cytokinins, and it has been shown that its expression in *Arabidopsis* results in an increase of endogenous cytokinin levels and changes in gene expression of two homeobox genes [24–26]. Genome-wide expression profiling using massively parallel signature sequencing (MPSS) allowed the identification of 823 and 917 up- and downregulated genes, respectively, in response to cytokinin.

2. Materials and methods

2.1. Plant material and RNA isolation

Arabidopsis plants (ecotype *Landsberg erecta*) were generated by root transformation with *Agrobacterium tumefaciens* as described [24,26]. Homozygous T3 seedlings of transgenic plants were grown on agar plates containing MS salts, pH 5.7, and 3% sucrose solidified with 0.8% Bactoagar (Gibco BRL, Grand Island, NY, USA) at 22°C under long-day conditions (16 h of light/8 h of dark). After 2 weeks seedlings were transferred to a medium containing 30 μM dexamethasone (DEX, dissolved in DMSO). The induction period was started at 10.00 am. Plants were removed after 6 h as well as 24 h and frozen in liquid nitrogen. Total RNAs for the MPSS experiment were isolated using Trizol reagent (Gibco BRL, Grand Island, USA). Poly(A) RNAs were isolated using a purification kit following the protocols of the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.2. RNA gel blot analysis

Total RNAs for Northern hybridization were isolated using the ATA method and RNA gel blot analysis was performed following standard protocols [27–28]. Each lane contained 10 μg total RNA. Two hundred to two hundred and fifty bp fragments from the 5′ end or 3′ regions of the indicated genes were amplified using rTaq DNA polymerase (Panvera, Madison, WI, USA). The fragments were purified using the Qiaquick Gel extraction protocol (Qiagen, Valencia, CA, USA), verified by sequencing analysis and labeled with [^{32}P]dCTP and [^{32}P]dATP by random priming (Amersham, Arlington Heights, IL, USA).

2.3. Data acquisition and analysis

In vitro cloning of poly(A)-RNA, generation of microbead libraries, sequencing of DNA on microbeads and base calling were performed as described [29–30]. The number of signatures collected (corresponding to the number of mRNA molecules analyzed) was 2 960 880 for the control sample, 2 728 424 for the 6 h sample, and 2 675 509 for the 24 h *IPT*-expressing sample. Four separate sequencing runs were performed for each sample to produce the total number of signatures. Abundance for each distinct signature was counted and normalized in parts per million (PPM) to obtain the estimates of transcript abundance. Differences in expression levels were deemed significant when the ratio of the abundances was 3 and greater or statistically significant at the 0.0001 level as determined on the basis of the total number of signatures collected in each sample using the formula given in [31].

3. Results

3.1. Genome-wide identification of cytokinin-responsive genes

Genes showing cytokinin-responsive expression are good candidates for components of cytokinin signaling pathways. Despite advances in the identification of signal transduction elements downstream of the two-component system, the number of known cytokinin-responsive genes is still rather limited [5,22]. We therefore used MPSS to monitor changes in cytokinin-responsive gene expression on a genome-wide scale [29–30,32]. To increase cytokinin levels in 3–4-week-old *Arabidopsis* seedlings, we induced the expression of a bacterial *IPT* gene in planta, cf. [24,26]. For this purpose, we used transgenic *A. thaliana* lines that carry the *IPT* gene under the transcriptional control of a chemically inducible promoter system [24,26]. After induction of the promoter by DEX, high levels of the *IPT* transcript could be detected (cf. Fig. 2). Total RNAs were isolated from tissue samples after treatment for 24 h. Expression of the *IPT* gene could not be detected in control RNA samples, which consisted of total RNAs derived from plants treated for 24 h with mock and from transgenic vector control plants treated with DEX for 24 h.

Based on a 0.0001% significance level we identified 823 and 917 signature sequences in annotated genes that were up-, and downregulated, respectively, by three-fold or more after 24 h DEX treatment. The normalized number of the frequency of occurrence in PPM which represents a measure of transcript abundance was plotted for the DEX-treated sample versus the one for the control sample to highlight the large number of cytokinin-responsive genes (Fig. 1). Confirmation of gene regulation identified by MPSS has been shown in a similar experiment for highly and lowly expressed genes using different techniques [32]. To verify the results of cytokinin regulation obtained with MPSS, we performed RNA gel blot analysis using as probes 11 cytokinin-dependent genes that may have very diverse functions in the responses of a plant to cytokinin (Fig. 2). These include genes encoding ARR5 (At3g48100), ARR6 (At5g62920), a transporter-like protein (At5g13740), a zinc finger protein (At4g39070), WRKY70 (At3g56400), a protein containing AP2 domain (At1g16060), cystatin B

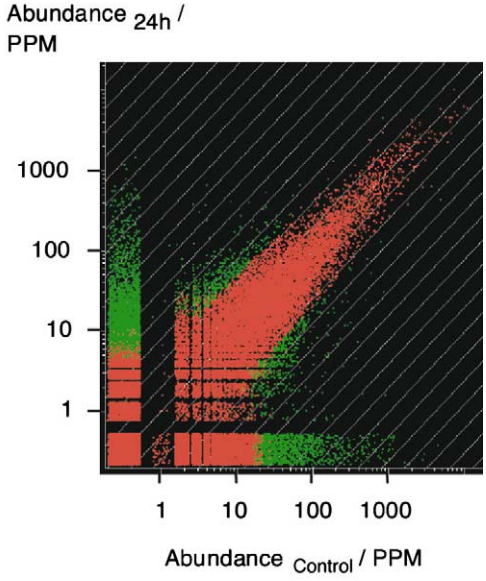

Fig. 1. Changes in gene expression after 24 h of *IPT* induction. The normalized abundance in PPM of each signature derived from the DEX-treated sample (*y*-axis) was plotted on a logarithmic scale versus that derived from the control sample (*x*-axis). The log scaling ends at 1 and signatures with abundance 0 are presented in the lower left corner. For each abundance (PPM) value, points are not placed on a line but allocated to a bar of a certain width that decreases with increasing abundance. Thus, data points are scattered in a bidimensional space to better reflect density. This translates into a wide gap as seen at around 1 PPM because there were few signatures with both 1 or 2 PPM that would normally occupy a certain space either along the *x*- or *y*-axis. Up- or downregulated genes, as defined on the basis of a five-fold ratio and/or a 0.0001% significance level, are shown in green. Genes unaffected by cytokinin are shown in red.

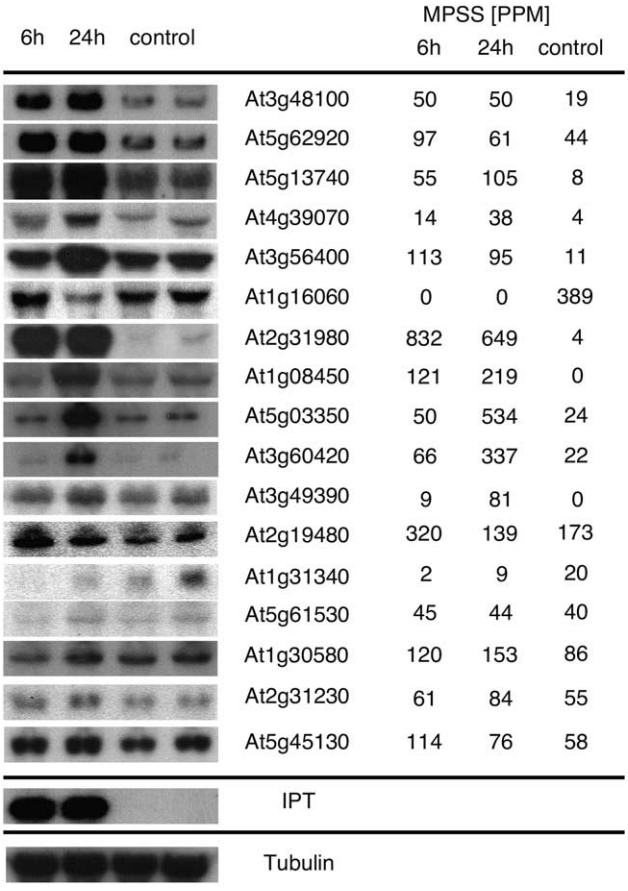

Fig. 2. RNA gel blot analyses of cytokinin-responsive gene expression. Total RNAs from Fig. 1 were probed with DNA fragments of the indicated genes. MPSS data in PPM are given on the right. Expression levels of tubulin were used as loading controls.

(At2g31980), calreticulin (At1g08450), receptor kinase-like protein (At5g03350), a putative protein (A3g60420), a RNA binding-like protein (At3g49390), and a polyubiquitin (At1g31340). The results of RNA gel blot analysis were in good agreement with the MPSS data confirming the observed gene regulations. In addition to cytokinin-regulated genes, we also confirmed the MPSS data of genes that were not affected by *IPT* expression following the 0.0001% significance and at least three-fold change (Fig. 2). These include genes encoding a putative nucleosome assembly protein (At2g19480), a putative GTP-binding protein (At1g30580), a Rho GAP-like protein (At5g61530), an ethylene response factor-like AP2 domain transcription factor (At2g31230), and a ras-related protein (At5g45130).

To investigate the kinetics of cytokinin-responsive gene expression (see below), we also prepared RNA samples from plants after 6 h of DEX induction. This time of induction has been chosen because high levels of cytokinins were observed in a comparable experiment using the same *IPT*-expressing *A. thaliana* transgenic line [24–25]. Expression of the *IPT* gene was monitored to verify the effectiveness of the 6 h DEX treatment (cf. Fig. 2). Based on the same parameters used for the analysis described above, we detected the upregulation and downregulation of 1263 and 911 genes, respectively, after DEX induction for 6 h (Fig. 3). The MPSS data for cytokinin-responsive and cytokinin-insensitive genes were confirmed by RNA gel blot analysis with the exception of At1g16060 and At3g56400 (cf. Fig. 2).

Table 1 summarizes the MPSS data for genes encoding putative elements of the two-component systems (*AHKs* and *AHPs*) as well as Type-A and B response regulators (*ARRs*), and other genes that have been reported to be cytokinin-responsive. We also listed the MPSS data for genes encoding proteins that are involved in cytokinin metabolism, namely IPT and cytokinin oxidases (CKX). Whereas expression of the histidine kinase genes CKI1 and AHK2 was not detected in our experiment, low expression was observed for *AHK3* and *AHK4*. The *AHK4* transcript abundance was slightly increased after 6 h of DEX induction. Previous reports have shown that the application of exogenous cytokinin does not affect the expression level of genes encoding putative AHPs [14–16]. In agreement with these reports, we found that the increase in endogenous cytokinin by inducing the *IPT* gene did not significantly alter the transcript levels of *AHP* genes with the exception of *AHP5*. The MPSS data set was also confirmed by analysis of *ARR* gene regulation. Type-A genes including *ARR4*, *ARR5*, *ARR6*, *ARR7*, and *ARR8* were upregulated in response to cytokinin, whereas Type-B genes including *ARR2*, *ARR10*, and *ARR11* were not affected by the treatment, cf. [17,33–36]. Note that expression of the actin genes *ACT2* and *ACT8* was not affected by *IPT* induction.

3.2. Functional classification of cytokinin-responsive genes

To understand the genome-wide network of cytokinin-dependent regulation of gene expression, we used the classification of the MIPS data to categorize the identified cytokinin-responsive genes (after 24 h of induction) into: cell rescue, defense, ageing (7 genes upregulated/7 genes downregulated); cell growth and division (5/5); cellular communication/signal transduction (22/10); cellular organization (6/8); energy and metabolism (31/60); protein synthesis and destination (11/12); transcription (23/24); transport facilitation (19/12); unclassified (699/779). The classification of up- and downregulated

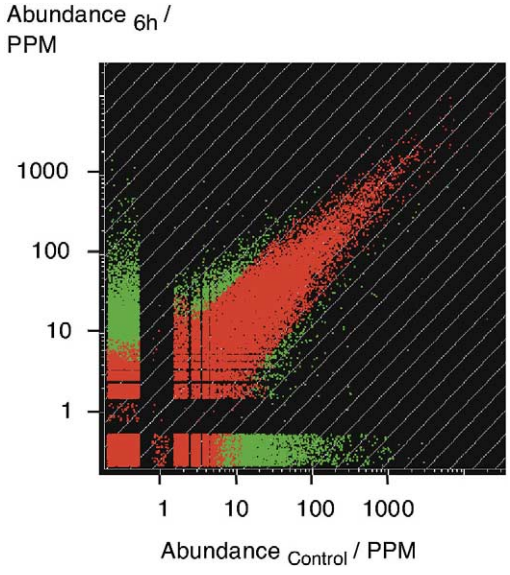

Fig. 3. Changes in gene expression after 6 h of *IPT* induction. Normalized abundances in PPM of each signature derived from the DEX-treated sample (*y*-axis) were plotted on a logarithmic scale versus those derived from the control sample (*x*-axis). Representation is as described in Fig. 1.

Table 1
MPSS data of cytokinin signaling components and cytokinin-responsive genes

Gene/gene ID	Control (PPM)	6 h (PPM)	24 h (PPM)
AHK1, T30B22.27/At2g47430, identical to CKI1	0	0	0
AHK2, MXH1.16/At5g35750	0	0	0
AHK3, F17L21.11/At1g27320	10	9	3
AHK4, T23K3.2/At2g01830 identical to CRE1/WOL	8	37	6
AHP1, MIL23.7/At3g21510	22	30	29
AHP2, MUO10.16/At3g29350	62	61	36
AHP3, K3K3.15/At5g39340	32	32	29
AHP4, T2O4.20/At3g16360	45	84	48
AHP5, F21B7.5/At1g03430	12	44	31
ARR1, 5′ end, MUH15.1/At3g16857	11	6	44
ARR1, 3′ end, K20I9.8/At3g16855	0	0	0
ARR2, FCAALL.287/At4g16110	0	3	0
ARR3, F23H11.25/At1g59940	0	6	1
ARR4, T10O24.8/At1g10470, identical to ATRR1/IBC7	205	405	321
ARR5, T17F15.30/At3g48100, identical to ATRR2/IBC6	19	50	50
ARR6, MQB2.220/At5g62920	44	97	61
ARR7, F14D16.20/At1g19050	13	99	65
ARR8, F13H10.14/At2g41310, identical to ATRR3	31	66	42
ARR9, F24I3.120/At3g57040	42	37	47
ARR10, F10N7.270/At4g31920	16	13	0
ARR11, F12A21.15/At1g67710	2	0	3
ARR12, F13D4.140/At2g25180	3	10	17
ARR13, T20P8.12/At2g27070	0	0	0
ARR14, T8O11.7At2g01760	0	15	15
ARR15, T7D17.15/At2g40670	4	9	7
ARR16, T7D17.15/At2g40670	4	9	7
ARR17, T5P19.30/At3g56380	0	0	0
ARR18, K21I19.60/At5g58080	0	0	0
ARR19, F27J15.4/At1g49190	0	0	0
ARR20, F26K9.100/At3g62670	0	0	0
ARR21, T28J14.150/At5g07210	0	0	0
ARR22, T6K12.10/At3g04280	0	0	0
ARR23, MTG10.140/At5g62120	0	0	0
CAB, F28P10.130/At3g54890	2288	343	1135
PAL1, T1J8.22/At2g37040	236	162	102
CHS, MAC12.28/At5g13930	214	205	315
CHI, T15C9.120/At3g55120	0	0	0
DFR, MJB21.18/At5g42800	0	0	1
CDC2A, T21J18.20/At3g48750	60	71	57
CYCD3, T7H20.160/At5g02110	0	0	0
KNAT1, F9M13.2/At4g08150	7	30	4
STM, F24O1.38/At1g62360	0	0	0
CKX1, T32G6.3/At2g41510	11	0	0
CKX2, F3P11.10/At2g19500	0	0	0
CKX3, MHM17.8/At5g56970	10	16	9
CKX4, T16L4.250/At4g29740	4	6	9
CKX5/6, F1B16.8/At1g75450	3	0	7
CKX7, MAA21.70/At3g63440	0	10	6
IPT1, T26J14.3/At1g68460	0	0	0
IPT2, F15K20.14/At2g27760	0	0	0
IPT3, T20O10.210/At3g63110	0	47	0
IPT4, F22K18.150/At4g24650	0	0	0
IPT5, T16G12.80/At5g19040	0	0	0
IPT6, F2J7.12/At1g25410	0	0	0
IPT7, MDB19.12/At3g23630	7	0	0
IPT8, MVI11.7/At3g19160	0	0	0
IPT9, F28I16.190/At5g20040	37	74	64
FPS2, FCAALL.91/At4g17190	60	16	25
ACT2, MVE11.16/At3g18780	277	370	220
ACT8, F27J15.1/At1g49240	200	262	216

genes is almost identical. The high number of genes in the group 'unclassified' may reflect that only few cytokinin-responsive genes had been identified prior to this study.

3.3. *Identification of gene clusters with similar induction kinetics*

D'Agostino et al. [36] examined the cytokinin-responsive expression of response regulator genes and reported that whereas the transcript levels of some genes were increased and stayed on a plateau for a long period, the induction of others was transient. We therefore followed changes in transcript abundance in response to cytokinin not only after 24 h of *IPT* induction, but also after 6 h. Possible regimes of gene expression are shown schematically in Fig. 4. On the basis of a

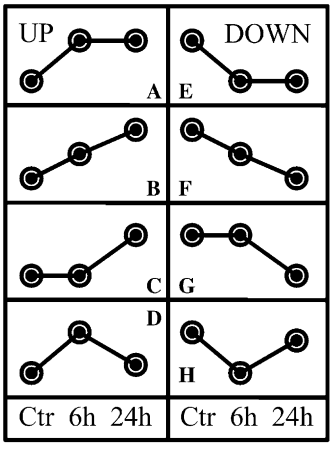

Fig. 4. Schematic view of possible regimes of cytokinin-responsive gene expression after 6 h and 24 h of induction.

significant change after 24 h of *IPT* induction compared to the control condition we grouped genes into six different categories (A–C for upregulation and E–G for downregulation, see also the supporting data). Two-thirds of the regulated genes displayed changes in expression levels only after 24 h but not 6 h of elevated endogenous cytokinin levels (67.7% in C and 66.4% in G). Some genes already reached a plateau of induction or repression after 6 h (24.3% in A and 29.6% in E), whereas others changed in proportion to the induction time (8.0% in B and 4.0% in F). In addition, we grouped genes that were transiently regulated after 6 h, but returned to the base expression level after 24 h of *IPT* induction in two categories (D and H). These genes may be of special interest because their expression appears to be well regulated in a closed interval. Genes in these categories were selected on the basis of two criteria: (i) their expression was not significantly different between the control condition and the 24 h sample and (ii) the transcript level of these genes exhibited at least a three-fold change between the 6 h and the 24 h sample. Strikingly, half of the genes that were upregulated after 6 h of *IPT* induction showed only a transient response to increased endogenous cytokinin levels (49.7% in D). These included genes encoding transcription factors (a homeodomain-like protein (At1g70920), bHLH093 (At5g65640), AP2 domain containing protein RAP2.3 (At3g16770), myb-like proteins (At2g31180, At2g47460, and At3g49850), KNAT4 (At5g11060), Scarecrow 1 (At3g54220) and putative protein kinases (At1g52290, At2g40270, and At4g18950). Fewer genes downregulated by endogenous cytokinin appear to be changed in their expression level only transiently (23.6% in H). These included genes encoding putative transcription factors including a MADS-box-like (At5g23269), a CCAAT-box binding-like (At2g37060), a zinc finger-like (At1g30970), two myb-like (At3g11280 and At3g48920), and a DNA binding-like protein (At1g74500). In addition, we found genes encoding a putative two-component response regulator (At2g01760), a putative chloroplast nucleoid DNA binding protein (At2g03200), a receptor protein kinase-like protein (At4g05200), and a putative protein kinase (At1g25320) in this category. The observed clusters of genes with different modes of regulation suggest that these gene clusters may play distinct roles in cytokinin responses.

Table 2
MPSS data of genes that were induced on cytokinin-rich medium in root explants

Gene/gene ID	Control (PPM)	6 h (PPM)	24 h (PPM)
3 days on cytokinin-rich medium			
At5g15960	52	28	37
At1g54000	0	0	3
At5g15970*	1472	2991	1149
At1g78380	64	69	60
At2g17820*	0	0	19
At3g61850	29	23	14
At5g60890	0	4	2
At1g68640	0	4	6
6 days on cytokinin-rich medium			
At2g40610	234	87	162
At3g54820	2	5	0
At4g24780*	38	32	80
At1g69530*	146	189	319
At3g48100*	19	50	50
At4g34590*	17	40	27
10 days on cytokinin-rich medium			
At4g27730	0	0	0
At4g36430*	26	91	40
At5g02380	0	2	0
At2g18150	119	82	108
At2g19120	0	0	3
At2g32800*	7	48	27
At5g57620	11	2	3
At5g23000	0	7	0
15 days on cytokinin-rich medium			
At5g01530	6290	2185	4486
At1g20620*	6534	9758	4559
At2g05520	70	62	0
At2g41100*	51	105	127
At2g35940	8	6	5
At4g14540	38	13	12

Table 3
MPSS data of genes encoding WRKY transcription factors

Gene ID	Control (PPM)	6 h (PPM)	24 h (PPM)
At2g03340	0	25	20
At4g24240	0	19	12
At2g30250	5	71	27
At4g01250	0	14	0
At1g30650	4	49	22
At4g23810	0	12	53
At4g26640	0	11	0
At3g56400	11	113	95
At1g62300	6	59	54
At1g80840	7	59	27
At5g56270	3	18	61
At5g15130	6	18	1
At5g22570	0	7	48
At5g26170	0	7	26
At1g66550	0	4	25
At2g25000	6	16	35

4. Discussion

Cytokinins are involved in many different processes during the plant life cycle [37]. The diversity of cytokinin-dependent responses suggests that the expression of many genes is regulated by cytokinins. The regulation of some genes by the application of exogenous cytokinin has been reported [22]. Increase in transcript levels of the two homeobox genes *KNAT1* and *STM* was detected in plants that overproduce endogenous cytokinin [25]. To identify global changes in the expression of cytokinin-responsive genes, we increased cytokinin levels in planta by inducing expression of a bacterial *IPT* gene in transgenic *A. thaliana* plants (Fig. 2, cf. [24–25]). For genome-wide expression profiling we used MPSS [29–30], which, compared to other techniques, offers several advantages including high resolution and gene specificity. This method has been successfully applied to monitor changes in ABA-responsive gene expression in *A. thaliana* [32]. In line with the hypothesis that cytokinin action requires extensive changes in gene expression, we identified 823 (1263) up- and 917 (911) downregulated genes at 24 h (6 h) after *IPT* induction, respectively. The cytokinin responses of 17 genes, each in three different conditions, were confirmed by RNA gel blot analysis thus verifying the MPSS data.

Using an oligonucleotide array covering about 8000 genes, Che et al. [23] identified about 250 genes that were upregulated in root explants that became committed to regenerate shoots on cytokinin-rich induction medium. To compare induction of gene expression in root explants and seedlings, we selected 28 genes from our data set that were most prominently induced in root explants on cytokinin-rich medium (table 1 in [23]). About 30% of these genes were also slightly induced by the elevation of endogenous cytokinin in seedlings (marked by asterisks in Table 2). This may indicate that common subsets of genes including *AHK1* and *ARR5* are regulated similarly by cytokinin even at different developmental stages. However, the majority of genes responsive to cytokinin differed between root explants and seedlings. This suggests that plants also exhibit a unique response to increased cytokinin levels depending on the developmental stage.

Members of two-component systems involved in phosphorelay including AHKs, AHPs, and ARRs have been implicated in ethylene and cytokinin signaling in *A. thaliana*. Overexpression of *AHK4* increased cytokinin-responsive gene expression [12]. We showed that *AHK4* is transiently upregulated by endogenous cytokinin within a short interval (Table 1). A higher activity of AHK4 may be achieved by activating transcription of the gene thus enhancing the reception of the cytokinin signal and corresponding downstream changes in gene expression. The cytokinin response of several *ARR* genes has also been studied in detail. Type-A *ARR* genes are induced by *t*-zeatin in leaves of N-starved *Arabidopsis* and by BA in 3-day-old etiolated seedlings [33,36,38]. In our experiment, we increased endogenous cytokinin levels in 2-week-old seedlings and also observed induction of *ARR4*, *ARR5*, *ARR6*, *ARR7*, and *ARR8*; in agreement with D'Agostino et al. [36], *ARR9* was not affected (Table 1). An inhibitory effect of *ARR4*, *ARR5*, *ARR6*, and *ARR7* on cytokinin-dependent induction of *ARR6* has been shown in transient expression assays [12]. It is tempting to speculate that the induction of *ARR4*, *ARR5*, *ARR6*, and/or *ARR7* may play a role in the repression of gene expression by endogenous cytokinin (see list of repressed genes in the supporting data). Differences in cytokinin response were observed for *ARR3* whose transcripts were upregulated in leaves of N-starved *Arabidopsis*, but could not be detected in etiolated seedlings or in 2-week-old seedlings (Table 1, cf. [33,36]). In etiolated seedlings a transient increase in transcript abundance was previously reported for *ARR15* and *ARR16* after 40–60 min of cytokinin treatment. However, under our conditions both genes were cytokinin-insensitive (Table 1 and [36]). According to previous reports the Type-B *ARR* genes *ARR2*, *ARR10*, and *ARR11* did not show a significant change in expression with cytokinin treatment (Table 1 and [17,34]). The chosen examples illustrate that the responses of many genes to cytokinins could be detected irrespective of the tissue examined and the treatment used.

However, different factors may contribute to differences in cytokinin-dependent gene expression: (1) Regulation of genes may vary with the investigated tissue/organ examined. Note that *ARR13* and *ARR17-ARR23* could not be detected in seedlings. These genes may play an important role at a different developmental stage, e.g. during seed development. (2) Cytokinin-sensitivity and the effective cytokinin in the treated plants may depend on the specific cytokinin used. (3) The incubation time may affect gene expression pattern (see *ARR15* and *ARR16*). A detailed kinetics of induction is required to relate changes at the transcriptional level to the plant response.

In addition to the regulation of putative components of the phosphorelay system, cytokinin may control genes involved in its own metabolism on the transcriptional level. Recent work has identified nine *Arabidopsis* genes encoding IPT that are believed to catalyze the key step in cytokinin biosynthesis [39–41]. Our results provide additional data on the responsiveness of *Arabidopsis IPTs* to cytokinin. From the nine genes seven appear not to be expressed at the seedling stage (Table 1) and may therefore function at earlier developmental stages. Both, *IPT3* and *IPT9*, are induced by increases in endogenous cytokinin levels. This upregulation may be a positive feedback mechanism to potentiate cytokinin responses. Interestingly, one of the two *Arabidopsis* genes encoding farnesyl diphosphate synthases, *FPS2*, which use the same substrate as IPTs [42], is downregulated by cytokinin (Table 1). Thus, downregulation of *FPS2* may favor the synthesis of cytokinins.

Six CKX genes whose products catalyze the conversion of active cytokinins into inactive conjugates have also been identified in *A. thaliana* [43]. Our results show that most of the *CKXs* are expressed in seedlings at a very low level. However, they are not regulated by cytokinin at the transcriptional level. The conversion into inactive cytokinin conjugates appears not to be controlled by transcription of *CKXs*.

It is widely accepted that the phosphorelay system underpins cytokinin signaling. It is, however, not conclusively known how this translates into a cellular response. Hwang and Sheen [12] presented a model of a cytokinin signaling circuit mediated by distinct functions of AHP and ARR proteins. In the ethylene signaling pathway, however, mitogen-activated protein kinases (MAPKs) may link the stimulus that is activated by external sensors to cellular functions. The Raf-like MAPK kinase kinase (MAPKKK) CTR1 has been shown to act downstream of the two-component-like receptor ETR1 [44,45]. In our kinetic analysis of cytokinin-responsive gene expression we found that AtMPK5 (At4g11330), AtMPK6 (At2g43790), and AtMKK3 (At5g46440) are transiently upregulated. The expression of these genes appears to be well regulated in a closed interval. Plant-specific transcription factors of the WRKY family have been suggested as possible targets of the MAPK pathway in elicitor-responsive plant defense as transduction of the plant defense signal led to the induction of *WRKY22* and *WRKY29* transcription [46]. Here, we show that cytokinin induces the transcription of 16 members of the WRKY family of transcription factors (Table 3). Note that we did not observe downregulation of any genes encoding WRKY transcription factors. Our data may suggest that the increase in endogenous cytokinin is perceived as a signal by the two-component system, transduced into the nucleus via MAPK signaling, and translated into a response by inducing WRKY trancription factors that activate downstream genes. In line with this hypothesis, most of the WRKY genes were already induced after 6 h of *IPT* induction and may therefore potentiate the signal at an early stage resulting in extended changes in gene expression. Our data provide a genome-wide database of genes that are regulated by this and/or other cytokinin signaling pathways including the two-component circuitry.

5. Supporting data

In the supporting data (doi: 10.1016/S0014-5793(03)01194-3), we provide tables containing 823 (1263) induced and 917 (911) repressed genes 24 h (6 h) after *IPT* induction, respectively. These tables provide the signature sequences and their locations in the genome, the name of the gene, the gene IDs, the putative functional classification, the location of the gene in the genome, the factor of change in gene expression, the normalized abundance in PPM for the control and 6 h and 24 h samples, and the assignment of the gene to a specific cluster on its kinetics of expression. If the abundance in one condition was 0 PPM, it was considered 1 PPM to determine the factor of change.

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