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Combinatorial Microarray Analysis Revealing *Arabidopsis* Genes Implicated in Cytokinin Responses through the His→Asp Phosphorelay Circuitry

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In *Arabidopsis thaliana*, the immediate early response of plants to cytokinin is formulated as the multistep histidine kinase (AHK)→histidine-containing phosphotransmitter (AHP)→response regulator (ARR) phosphorelay signaling circuitry, which is initiated by the cytokinin receptor histidine protein kinases. In the hope of finding components (or genes) that function downstream of the cytokinin-mediated His→Asp phosphorelay signaling circuitry, we carried out genome-wide microarray analyses. To this end, we used a combinatorial microarray strategy by employing not only wild-type plants, but also certain transgenic lines in which the cytokinin-mediated His→Asp phosphorelay signaling circuitry has been genetically manipulated. These transgenic lines employed were ARR21-overexpressing and ARR22-overexpressing plants, each of which exhibits a characteristic phenotype with regard to the cytokinin-mediated His→Asp phosphorelay. The results of extensive microarray analyses with these plants allowed us systematically to identify a certain number of genes that were up-regulated at the level of transcription in response to cytokinin directly or indirectly. Among them, some representatives were examined further in wild-type plants to support the idea that certain genes encoding transcription factors are rapidly and specifically induced at the level of transcription by cytokinin in a manner similar to that of the type-A *ARR* genes, which are the hallmarks of the His→Asp phosphorelay signaling circuitry. Several interesting transcription factors were thus identified as being cytokinin responsive, including those belonging to the AP2/EREBP family, MYB family, GATA family or bHLH family. Including these, the presented list of cytokinin-up-regulated genes (214) will provide us with valuable bases for understanding the His→Asp phosphorelay in *A. thaliana*.

Keywords: *Arabidopsis thaliana* — Cytokinin — Microarray — Phosphorelay — Signal transduction

Abbreviations: AHK, *Arabidopsis* histidine kinase; AHP, *Arabidopsis* histidine-containing phosphotransmitter; ARR, *Arabidopsis* response regulator; bHLH, basic helix–loop–helix; DMSO, dimethylsulfoxide; FC, fold change; GA, gibberellin; MJA, methyl jasmonate; RT–PCR, reverse transcription–polymerase chain reaction.

Introduction

Cytokinins are a class of plant hormones which are implicated in nearly all aspects of plant growth and development, including cell division, shoot initiation and light responses (Mok and Mok 2001). In *Arabidopsis thaliana*, during the last few years, the immediate early response of plants to cytokinin has been formulated as the multistep histidine kinase (AHK)→histidine-containing phosphotransmitter (AHP)→response regulator (ARR) phosphorelay signaling circuitry, which is initiated by the cytokinin receptor histidine protein kinases (AHK2, AHK3 and AKH4/CRE1/WOL) (for recent reviews, see Hutchison and Kieber 2002, Hwang et al. 2002, Schaller et al. 2002, Heyl and Schmulling 2003, Kakimoto 2003). The currently consistent model of this cytokinin-mediated His→Asp phosphorelay signaling mechanism involves four principal steps (for a review, see Sheen 2002): (i) cytokinin receptors (AHKs) sense the signal (Inoue et al. 2001, Suzuki et al. 2001, Ueguchi et al. 2001a, Ueguchi et al. 2001b, Yamada et al. 2001), and phosphorylate phosphotransfer intermediates (AHPs) (Suzuki et al. 1998); (ii) phospho-AHPs move into the nucleus and donate the phosphoryl group to type-B ARRs (Hwang and Sheen 2001, Imamura et al. 2001); (iii) phosphorylated type-B ARRs serve as transcriptional activators, resulting in rapid induction of type-A *ARR* genes (Hwang and Sheen 2001, Sakai et al. 2000, Sakai et al. 2001, Hosoda et al. 2002); and (iv) accumulated type-A ARRs somehow act as repressors that mediate a negative feedback loop in the signaling circuitry (Hwang and Sheen 2001, Kiba et al. 2003, To et al. 2004). To support this scenario, several lines of forward and reverse genetic evidence are further accumulating (Mahonen et al. 2000, Kiba et al. 2002, Osakabe et al. 2002, Suzuki et al. 2002, Imamura et al. 2003, Mason et al. 2004, Tajima et al. 2004). In particular, the mutant plants lacking all three cytokinin receptors were examined recently (Higuchi et al. 2004, Nishimura et al. 2004). Such cytokinin receptor-less and/or cytokinin-insensitive plants are viable, but dwarf and sterile, implying that the AHK-mediated phosphorelay signaling circuitry plays important roles for plant development. Nevertheless, the results also imply that the His→Asp phosphorelay may not be the sole signaling pathway that primarily propagates the cytokinin signal, and there may be alter-

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native His→Asp phosphorelay-independent signaling pathway(s). In any event, this model plant has 10 members of type-A ARRs and 11 members of type-B ARRs (Imamura et al. 1998, Imamura et al. 1999, Mason et al. 2004, Tajima et al. 2004, To et al. 2004). Thus, the proposed scenario, in which anonymous ARR members are implicated, does not necessarily describe the whole picture of the His→Asp phosphorelay (for recent reviews, see Grefen and Harter 2004, Mizuno 2004). In short, clarification of the comprehensive picture of the His→Asp phosphorelay network is still at a very early stage. As an approach to this end, here we carried out genome-wide microarray analyses in the hope of finding components (or genes) that function downstream of the cytokinin-mediated His→Asp phosphorelay signaling circuitry.

It has been postulated that the type-A *ARR* genes are directly activated by the type-B ARR transcription factors through the cytokinin-mediated His→Asp phosphorelay signaling circuitry (Bradstatter and Kieber 1998, Kiba et al. 1999, D'Agostino et al. 2000, Sakai et al. 2001). Are there any other primary targets of the cytokinin-responsive signaling circuitry? An advanced tool that would allow us to identify such cytokinin-regulated genes is DNA microarrays. In fact, several instances of microarray analyses with reference to cytokinin in *A. thaliana* have already been reported (Che et al. 2002, Hoth et al. 2003, Rashotte et al. 2003, Kiba et al. 2004). The results of such microarray analyses revealed a large number of cytokinin-associated genes, which include all those genes that were affected by cytokinin not only primarily, but also secondarily and indirectly. Also, they possibly include cytokinin-regulated genes, whose expression is regulated in a manner independent of the His→Asp phosphorelay. Therefore, the downstream target genes of the cytokinin-mediated His→Asp phosphorelay have not yet been fully specified. Here we used a sophisticated microarray strategy to look for such cytokinin-regulated genes with special reference to the His→Asp phosphorelay. To this end, we carried out a sort of combinatorial microarray analysis, which was conducted by employing not only wild-type plants, but also certain transgenic lines in which the cytokinin-mediated His→Asp phosphorelay signaling circuitry has been genetically manipulated. The employed transgenic lines were ARR21-overexpressing and ARR22-overexpressing lines, both of which have been established and characterized previously (Kiba et al. 2004, Tajima et al. 2004). ARR21 is a representative of the type-B ARR transcription factors, whereas ARR22 is a response regulator that is distinctive from the type-A and type-B ARR family members. Each of these transgenic plants exhibits a characteristic phenotype with regard to cytokinin responses in plants and explants, as reported previously (Kiba et al. 2004, Tajima et al. 2004). Employing these plants with different genetic backgrounds with regard to the His→Asp phosphorelay signaling circuitry, combinatorial microarray analyses were carried out with such plants treated or not with cytokinin. The results allowed us to compile a certain number of genes, the expression of which was assumed to be up-regulated in plants in response to cytokinin in a manner dependent on the His→Asp phosphorelay. To support this view, certain representative genes were examined further in terms of cytokinin responses in wild-type plants and cultured cells.

Results

Logic behind combinatorial microarray analyses

To gain global insight into the cytokinin-mediated signal transduction in *A. thaliana*, we previously adopted the microarray system of Affymetrix GeneChip (Arabidopsis ATH1 Genome Array, Affymetrix, Santa Clara, CA, U.S.A), containing >22,500 probe sets representing approximately 24,000 genes, as described (Kiba et al. 2004). To extend this further, in this study, combinatorial microarray analyses were carried out with three different types of plants, each of which has a characteristic genetic background with respect to the cytokinin-mediated His→Asp phosphorelay signaling circuitry. They were wild-type ecotype Columbia (Col) and two transgenic lines (designated as ARR21-C-ox and ARR22-ox, respectively), as explained below.

ARR21 is a representative of the type-B ARR transcription factors. We previously constructed a transgenic line overexpressing the C-terminal DNA-binding domain of ARR21 (Tajima et al. 2004), showing that this transgenic line (designated as ARR21-C-ox) displayed a characteristic phenotype that was indicative of 'aberrant activation of the cytokinin-mediated His→Asp phosphorelay'. Such an event is evidenced here by the result of the cytokinin-dependent callus formation (greening and/or shooting) assay (Fig. 1A). ARR21-C-ox explants were capable of forming green calli on the medium containing a very low concentration of cytokinin (0.005 μM of *t*-zeatin), under which conditions wild-type explants could not form green calli. On the other hand, ARR22 is another response regulator, which is distinctive from the type-B ARR family members. We previously constructed a transgenic line overexpressing the full length of ARR22 (designated as ARR22-ox) (Kiba et al. 2004), demonstrating that this transgenic line showed a characteristic phenotype that was indicative of 'severe attenuation of the cytokinin-mediated His→Asp phosphorelay'. This event is also evidenced here by the result of the cytokinin-dependent callus formation assay with ARR22-ox explants (Fig. 1A). In sharp contrast to ARR21-ox, ARR22-ox explants could not form green calli even on the medium containing a sufficient amount of cytokinin (5 μM *t*-zeatin).

These transgenic plants, together with the wild-type (Col) plants, were analyzed with the Affymetrix GeneChip, as follows. After these plants (21-day-old young seedlings) were either treated for 3 h with cytokinin or left untreated, RNA samples were prepared, and triplicate microarray data were collected statistically for each. The raw data for wild-type and ARR22-ox plants, and the results of preliminary analyses have already been presented in previously (Kiba et al. 2004). Taking the data for ARR21-ox together, here these vast amounts of

data were analyzed collectively and intensively, based on the following new viewpoints (see Fig. 1A). (i) In wild-type plants treated with cytokinin, certain cytokinin-associated genes were up-regulated. (ii) Certain His→Asp phosphorelay-associated genes were constitutively activated (or up-regulated) in ARR21-C-ox plants even without cytokinin treatment. (iii) Activation of certain His→Asp phosphorelay-associated genes was attenuated in ARR22-ox plants even if they were treated with cytokinin. Taken together, the microarray data were treated with three different procedures (or three different combinations), as schematically shown (Fig. 1B, upper part). First, the microarray data of wild-type plants treated with cytokinin were compared with those of wild-type plants not treat with cytokinin. This would reveal 'cytokinin-up-regulated genes (group-A genes)'. Secondly, the microarray data of ARR21-C-ox plants without cytokinin-treatment were compared with those of wild-type plants without cytokinin-treatment. This would specifically reveal 'His→Asp phosphorelay-associated genes (group-B genes)', because such genes in question have already been activated in ARR21-C-ox plants even without cytokinin treatment. Finally, the microarray data of wild-type plants treated with cytokinin were compared with those of ARR22-ox plants treated with cytokinin. This would also alternatively reveal 'His→Asp phosphorelay-associated genes

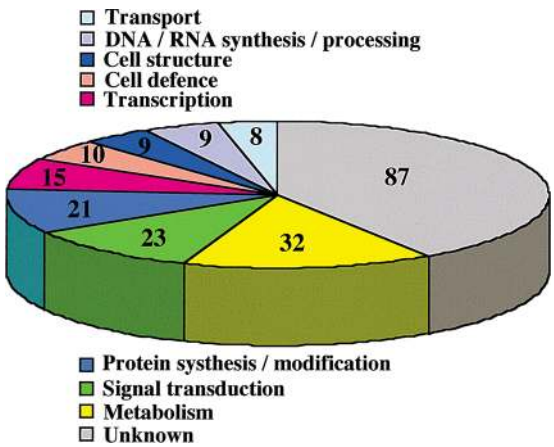

Fig. 2 Classification of 214 cytokinin-up-regulated genes. The identified genes were categorized into certain groups, based on their predicted and/or putative functions, such as 'signal transduction', 'transcriptional regulation' (Tables 1 and 2, see the Functional categories column).

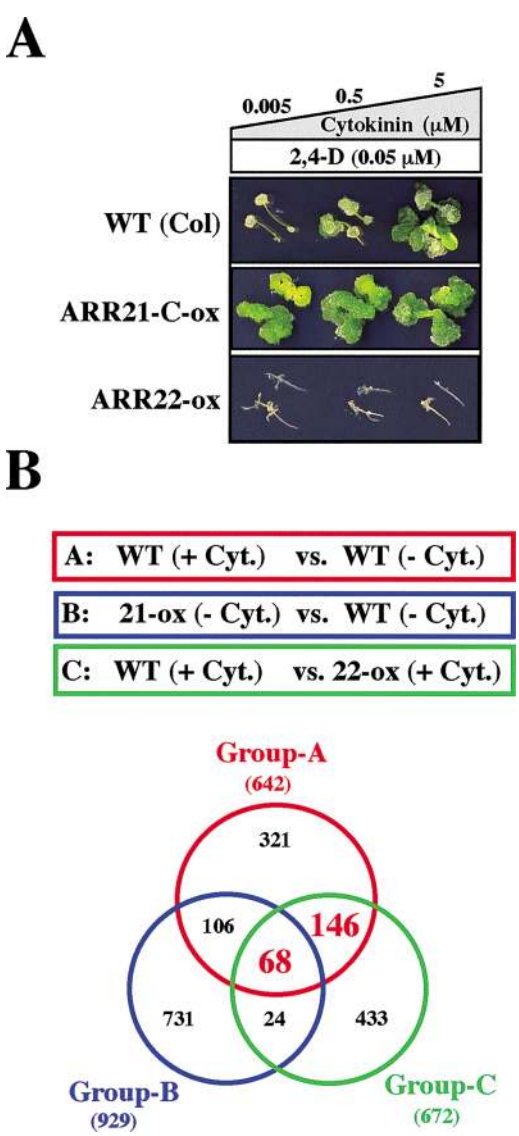

Fig. 1 Combinatorial microarray analyses with ARR21-C-ox and ARR22-ox transgenic plants. (A) Green callus formation assays in response to cytokinin. Wild-type (Col, WT), ARR21-C-ox and ARR22-ox were grown under dim light for 7 days. Then, their hypocotyls were cut and placed on MS-agar plates, containing different concentrations of *t*-zeatin and 2,4-D (2,4-dichlorophenoxyacetic acid), as indicated. After incubating for 30 days, each representative callus was collected and photographed. (B) Employing these plants (abbreviated as WT, 21-ox and 22-ox), 21-day-old seedlings were treated or not with cytokinin (*t*-zeatin) for 3 h. RNA samples were prepared, and then subjected to extensive microarray analyses. They were treated in three different ways, as schematically shown (upper part). First, the microarray data of wild-type young seedlings treated with cytokinin were compared with those of wild-type young seedlings not treated with cytokinin (group-A genes). Secondly, the microarray data of ARR21-C-ox young seedlings without cytokinin treatment were compared with those of wild-type young seedlings without cytokinin-treatment (group-B genes). Finally, the microarray data of wild-type young seedlings treated with cytokinin were compared with those of ARR22-ox young seedlings treated with cytokinin (group-C genes). The statistical outcomes of such combinatorial microarray analyses are shown schematically (lower part). We calculated the mean values of fold change (FC) for each transcript (FC = 2 means a 2-fold change of a given transcript in a given combination). The genes giving FC > 2 were collected into the group-A cytokinin-up-regulated genes (642 genes, red circle-A). Similarly, 929 genes were grouped into group-B (blue circle-B), whereas 672 genes were grouped into group-C (green circle-C). A total of 68 genes were commonly grouped into A∩B∩C. In addition, 106 genes were grouped into A∩B, whereas 146 genes were grouped into A∩C. Other details are given in the text.

(group-C genes)', because such genes were severely attenuated in ARR22-ox even when they were treated with cytokinin. In short, these three combinatorial procedures would allow us to compile the genes that were commonly grouped into 'A∩B∩C ' that would include most probable candidate genes that are regulated by cytokinin in a manner dependent on the His→Asp phosphorelay (Fig. 1B), as will be considered further in the next section.

Data mining

The statistical outcomes from such a combinatorial microarray analysis are shown schematically (Fig. 1B, lower part). We calculated the mean values of 'fold change (FC)' for each transcript (FC = 2 means a 2-fold change of a given transcript in a given combination). In the case of group-A genes, for instance, the signal intensity of a given gene in wild-type plants treated with cytokinin was divided by the signal intensity of the same gene in wild-type plants without cytokinin treatment (triplicate experimental data for each). The genes giving FC > 2 were collected into the group-A cytokinin-up-regulated genes (642 genes, red circle-A in Fig. 1B). Similarly, 929 His→Asp phosphorelay-associated genes were grouped into group-B (blue circle-B in Fig. 1B), whereas 672 His→Asp phosphorelay-associated genes were grouped into group-C (green circle-C in Fig. 1B). These results indicated that a large number of genes were more or less affected at the level of transcription (or mRNA stability) in response to cytokinin in wild-type plants. Also, the expression profiles of transcripts were markedly changed in ARR21-C-ox and ARR22-ox, as compared with those in wild-type plants. We were not surprised by these results because these putative His→Asp phosphorelay-associated genes most probably include all those genes that were affected by cytokinin (or genetic manipulations) primarily, but also secondarily and/or indirectly. Furthermore, it was not easy to inspect and evaluate such a huge number of genes one by one (this is a general problem with regard to microarray analyses). However, the number of genes that were commonly grouped into 'A∩B∩C ' was relatively limited, consisting of 68 genes (Fig. 1B). In addition, 106 genes were grouped into 'A∩B', 146 genes were grouped into 'A∩C', whereas 24 were grouped into 'B∩C' (note that these numbers do not include the genes in A∩B∩C). To obtain more refined data, we must repeat the microarray analyses more intensively. Meanwhile, however, close inspection of these selected genes should provide us with more specific insight into the cytokinin-up-regulated genes with special reference to the His→Asp phosphorelay, as rationalized above.

Critical evaluation of data

Our manipulated data are not necessarily comprehensive and/or they might be highly biased. We thus first needed to evaluate our data by challenging them with a strict criterion. This was done based on the well-documented fact that the type-A ARR family genes consisting of 10 members are the hallmarks of immediate-early cytokinin-responsive genes. If our logic behind the combinatorial microarray analysis was rational, all the type-A family genes must be recovered in A∩B∩C. The fact was that five out of eight type-A-ARR members were recovered in A∩B∩C (*ARR5*, *6*, *9*, *15* and *16*). The other three type-A ARRs were found in A∩C (*ARR4*, *7* and *8*). *ARR3* was also included in A∩B∩C, but the signal intensity was lower than the critical threshold, whereas *ARR17* was not detected on the chip in these experiments. In other words, most of the type-A ARR genes (*ARR3*, *4*, *5*, *6*, *7*, *8*, *9*, *15* and *16*) were up-regulated in wild-type plants treated with cytokinin, but none of them were up-regulated in ARR22-ox plants treated by cytokinin, thereby being grouped into A∩C. The majority of them (except for *ARR4*, *7* and *8*) were up-regulated in ARR21-ox plants without cytokinin treatment, thereby being grouped into A∩B∩C. It was reported previously that the cytokinin receptor (*AHK4/CRE1/WOL*) gene and another histidine kinase *AtHK1* gene are also induced by cytokinin (Kiba et al. 2004). These two genes were also recovered in A∩B∩C. Taken together, we were quite confident that our data mining was meaningful.

We thus suggest that 68 genes in A∩B∩C are primary candidates for the cytokinin-up-regulated genes in question, and that 146 genes in A∩C should also be included in such candidates, as considered above. More specifically, these genes are most probably closely relevant to the cytokinin-mediated His→Asp phosphorelay signal transduction. Other cytokinin-up-regulated genes in group-A (but not in group-C) might be regulated by cytokinin in a manner independent of the His→Asp phosphorelay, or indirectly. It is also possible that only a subset of cytokinin-regulated genes is misexpressed in ARR21-C-ox. Although these remaining 427 genes in group-A are also interesting, we did not inspect these in detail at present in order to make the context (or objective) of this study simple. However, for those who are interested in such whole microarray data, they will be available in the *Arabidopsis* microarray databases of the RIKEN Plant Science Center (Yokohama, Japan).

A summarized view of combinatorial microarray analyses

In summary, the objective of this study is the compilation of the cytokinin-associated genes, which are closely relevant to the His→Asp phosphorelay (see Introduction). Based on the consideration above, the 68 A∩B∩C cytokinin-up-regulated genes are listed in Table 1, and the 146 A∩C genes are also listed in Table 2. In these lists, each gene is indicated by an AGI-Code (http://mips.gsf.de). The 106 A∩B and 24 B∩C genes might also contain some intriguing genes, but they were not inspected further in this study, as considered above. However, these lists of A∩B and B∩C genes will be available at the www site (see http://www.agr.nagoya-u.ac.jp/%7emicrobio/). Also, we could formally compile 'cytokinin-down-regulated genes' in wild-type young seedlings. Nevertheless, it was not easy to analyze these genes logically in close relation to the

Table 1 Microarray analyses of cytokinin-up-regulated genes (A∩B∩C) of wild-type (Col), ARR21-C-ox and ARR22-ox plants

Affymetrix no.	AGI code	Remarks	Functional categories	FC		
				Group-A	Group-B	Group-C
266606_at	At2g46310#	AP2 domain-containing transcription factor	Transcription	10.6	9.8	5.7
262212_at	At1g74890#	**ARR15**	Signal transduction/regulation	9.2	8.6	27.2
256245_at	At3g12580	Heat shock protein 70	Cell defense	7.8	2.0	6.3
266078_at	At2g40670#	**ARR16**	Signal transduction/regulation	7.8	7.5	27.9
259388_at	At1g13420	Sulfotransferase family protein	Unknown	7.3	11.3	26.6
247406_at	At5g62920	ARR6	Signal transduction/regulation	7.3	2.7	150.5
248332_at	At5g52640	Heat shock protein 81–1	Cell defense	7.3	3.1	3.6
248434_at	At5g51440	Mitochondrial small heat shock protein (HSP23.5-M)	Cell defense	6.1	8.3	2.5
266687_at	At2g19670	Protein arginine *N*-methyltransferase	Protein synthesis/modification	5.9	3.0	3.8
252374_at	At3g48100#	**ARR5**	Signal transduction/regulation	5.8	14.4	44.2
254080_at	At4g25630	Fibrillarin 2 (FIB2)	DNA/RNA synthesis/processsing	5.4	2.5	2.5
260331_at	At1g80270	DNA-binding protein	Unknown	5.4	2.1	3.1
261664_s_at	At1g18320	Mitochondrial import inner membrane translocase subunit family protein	Unknown	5.3	3.5	2.5
247575_at	At5g61030	RNA-binding protein	Unknown	4.9	3.6	3.5
258965_at	At3g10530	Transducin family protein/WD-40 repeat family protein	Unknown	4.8	2.6	2.5
258316_at	At3g22660	rRNA processing protein-related	Unknown	4.7	2.6	2.1
250753_at	At5g05860	UDP-glucuronosyl/UDP-glucosyl transferase family protein	Cell structure	4.6	4.3	8.8
257774_at	At3g29250	Short-chain dehydrogenase/reductase (SDR) family protein	Metabolism	4.6	3.5	18.0
251195_at	At3g62930	Glutaredoxin family protein	Cell defense	4.5	2.5	4.7
251538_at	At3g58660	60S ribosomal protein-related	Protein synthesis/modification	4.5	2.1	2.6
252625_at	At3g44750	Histone deacetylase (HD2A)	Protein synthesis/modification	4.3	2.8	2.8
267004_at	At2g34260	Transducin family protein/WD-40 repeat family protein	Unknown	4.3	2.5	2.6
251740_at	At3g56070	Peptidyl-prolyl *cis–trans* isomerase	Protein synthesis/modification	4.2	2.6	2.0
256288_at	At3g12270	Protein arginine *N*-methyltransferase family protein	Protein synthesis/modification	4.0	2.3	2.0
246809_s_at	At5g27140	SAR DNA-binding protein	Transcription	3.9	2.0	3.1
253598_at	At4g30800	40S ribosomal protein S11 (RPS11B)	Protein synthesis/modification	3.9	2.7	2.5
250762_at	At5g05990	Mitochondrial glycoprotein family protein/MAM33 family protein	Unknown	3.8	2.1	2.4
264806_at	At1g08610	Pentatricopeptide (PPR) repeat-containing protein	Unknown	3.8	2.0	2.8
260824_at	At1g06720	Expressed protein	Unknown	3.7	2.1	2.2
254493_at	At4g20020	Expressed protein	Unknown	3.6	2.2	2.7
253696_at	At4g29740#	Cytokinin oxidase family protein	Metabolism	3.6	2.5	7.0
258397_at	At3g15357	Expressed protein	Unknown	3.6	3.5	2.9
250758_at	At5g06000	Eukaryotic translation initiation factor 3G	Protein synthesis/modification	3.5	2.2	2.1
251029_at	At5g02050	Mitochondrial glycoprotein family protein/MAM33 family protein	Unknown	3.5	2.4	2.2
256299_at	At1g69530#	Expansin (EXP1)	Cell structure	3.5	2.7	3.0
250679_at	At5g06550	Transcription factor jumonji (jmjC) domain-containing protein	Signal transduction/regulation	3.4	2.2	2.5
247453_at	At5g62440	Expressed protein	Unknown	3.3	2.0	2.0
251355_at	At3g61100	Expressed protein	Unknowm	3.3	2.0	3.6
260157_at	At1g52930	Brix domain-containing protein	Unknowm	3.3	2.2	2.0
266456_at	At2g22770	Basic helix–loop–helix (bHLH) family protein	Transcription	3.3	3.9	2.6
253777_at	At4g28450	Transducin family protein/WD-40 repeat family protein	Unknown	3.3	2.2	2.0
253949_at	At4g26780	Co-chaperone GrpE family protein	Protein synthesis/modification	3.2	2.5	2.4
267497_at	At2g30540#	Glutaredoxin family protein	Metabolism	3.2	1.9	6.2
248460_at	At5g50915	Basic helix–loop–helix (bHLH) family protein	Transcription	3.1	9.5	8.6
259227_at	At3g07750	3′ Exoribonuclease family domain 1-containing protein	DNA/RNA synthesis/processsing	3.1	2.6	2.1
263599_at	At2g01830#	**AHK4/CRE1/WOL**	Signal transduction/regulation	3.1	2.7	8.6
246395_at	At1g58170	Disease resistance-responsive protein-related	Cell defense	3.0	17.1	4.4
250994_at	At5g02490	Heat shock cognate 70 kDa protein 2 (HSC70–2)	Cell defense	3.0	2.5	3.2
256060_at	At1g07050	CONSTANS-like protein-related	Signal transduction/regulation	3.0	3.0	2.5
245392_at	At4g15680	Glutaredoxin family protein	Metabolism	2.9	3.1	4.4
264470_at	At1g67110#	Cytochrome P450	Metabolism	2.9	2.3	6.6

Table 1 Continued

Affymetrix no.	AGI code	Remarks	Functional categories	FC		
				Group-A	Group-B	Group-C
262594_at	At1g15250	60S ribosomal protein L37 (RPL37A)	Protein synthesis/modification	2.8	2.0	2.2
246825_at	At5g26260	Meprin and TRAF homology domain-containing protein	Unknown	2.8	4.6	3.6
253215_at	At4g34950	Nodulin family protein	Signal transduction/regulation	2.8	2.2	2.5
262704_at	At1g16530#	LOB domain protein 3(LBD3)	Signal transduction/regulation	2.8	4.0	34.3
256890_at	At3g23830	Glycine-rich RNA-binding protein	Unknown	2.6	2.0	2.2
258079_at	At3g25940	Transcription factor S-II (TFIIS) domain-containing protein	Transcription	2.6	2.0	2.2
265442_at	At2g20940	Expressed protein	Unknown	2.6	2.1	2.2
248657_at	At5g48570	Peptidyl-prolyl *cis–trans* isomerase	Protein synthesis/modification	2.4	2.4	3.3
259347_at	At3g03920	Gar1 RNA-binding region family protein	Unknown	2.4	2.0	2.2
251665_at	At3g57040#	**ARR9**	Signal transduction/regulation	2.2	2.1	8.0
257793_at	At3g26960	Expressed protein	Unknown	2.2	5.5	2.9
260294_at	At1g63660	GMP synthase [glutamine-hydrolyzing]	Metabolism	2.2	1.9	2.2
262608_at	At1g14120	2-oxoglutarate-dependent dioxygenase	Unknown	2.2	4.6	2.9
264790_at	At2g17820#	**AtHK1**	Signal transduction/regulation	2.1	3.9	2.6
267012_at	At2g39220#	Patatin family protein	Unknown	2.1	3.0	2.2
249567_at	At5g38020	*S*-adenosyl-L-methionine:carboxyl methyltransferase family protein	Metabolism	2.0	2.5	5.4
261016_at	At1g26560	Glycosyl hydrolase family 1 protein	Metabolism	2.0	2.3	2.3

Detailed procedures of microarray analyses are given in Materials and Methods. Briefly, to identify putative cytokinin-up-regulated genes, the raw data collected for wild-type (Col), ARR21-C-ox and ARR22-ox plants were compared with each other (see Fig. 1B), using a comparative analysis algorithm in MicroArray Suite 5.0 software (Affymetrix). Each gene on the Affymetrix GeneChip is assigned an Affymetrix number, each of which is also designated as the more general AGI code (see http://mips.gsf.de/proj/thal/db/index.html). The fluctuation ratio of the transcript level was calculated for each gene, and they were expressed as fold change (FC). The results are listed at the right hand side of this table. For these identified genes, brief remarks are noted (see http://www.tigr.org/tigr-scripts/ and http://arabidopsis.org/servlets/), and accordingly they are classified into arbitrary functional categories. In the column of remarks, the phosphorelay-associated genes (e.g. *ARRs*) are in bold. Rashotte et al. (2003) have previously reported certain cytokinin-inducible genes, based on their microarray analyses. The cytokinin-up-regulated genes consistently identified by both us and Rashotte et al. are marked by # in the column of the AGI codes.

data from ARR21-ox and ARR22-ox. Therefore, in this study, we intended to focus only on cytokinin-up-regulated genes.

Data inspection of cytokinin-up-regulated genes

As mentioned earlier, a few instances of microarray analyses with reference to cytokinin responses in *A. thaliana* have been reported (Hoth et al. 2003, Rashotte et al. 2003). Taking these previous data into consideration, we first roughly inspected the 214 cytokinin-associated genes in Tables 1 and 2. Of 214 genes, 26 (12%) were reported previously as cytokinin-up-regulated genes (they are denoted by # in Tables 1 and 2, see the AGI code column). They include 10 His→Asp phosphorelay-associated genes (type-A *ARRs*, *AHK4* and *AtHK1*), as mentioned above (see columns highlighted in bold in Tables 1 and 2). Beside these, they include an expansin (At1g69530), a glutaredoxin family protein (At2g30540), a cytochorome P450 (At1g67100), a cytokinin oxidase family protein (At4g29740), an RNA polymerase family protein (At3g57660) and a major intrinsic (MIP) family protein (At4g19030). In addition, the *AMP1* gene (At3g54720) encoding a glutamate carboxypeptidase and the *ACR4* gene (At1g69040) encoding an ACT domain-containing protein were also included, both of which have been characterized previously as cytokinin-responsive genes (Helliwell et al. 2001, Hsieh and Goodman 2002).

To gain further insight into these 214 genes, they were categorized into certain groups, based on their predicted and/or putative functions, such as 'signal transduction', 'transcriptional regulation', 'protein synthesis/modification', 'metabolism', etc. (Tables 1 and 2, see the Functional categories column). Although such a classification is somewhat arbitrary, the results provided us with a rough idea about the putative cytokinin-responsive genes (Fig. 2). Many (87 genes) of them showed no characteristic feature (i.e. unknown), while many others (32 genes) are implicated in metabolism. Interestingly, a certain number of genes (23 genes) appear to be related to signal transduction, whereas some others (15 genes) seem to be implicated in transcriptional regulation.

Examination

The number of candidates (214 genes) was still too large to examine them critically and closely one by one. Also, they possibly contain a certain number of false-positive candidates. To gain an idea with regard to these issues, of these 214 genes, we tentatively focused on the selected set of 28 genes (14 genes from A∩B∩C and 14 genes from A∩C) (Table 3). The criteria for this selection were somewhat arbitrary. (i) The well-known *ARR* and *AHK* genes were excluded. (ii) Genes that appear to be related to signal transduction and transcriptional regulation

Table 2 Microarray analyses of cytokinin-up-regulated genes (A∩C) in wild-type (Col) and ARR22-ox plants

Affymetrix no.	AGI code	Remarks	Functional categories	FC	
				Group-A	Group-C
248725_at	At5g47980	Transferase family protein	Unknown	12.7	27.9
265147_at	At1g51380	Eukaryotic translation initiation factor 4A	Protein synthesis/modification	12.4	3.7
254235_at	At4g23750#	AP2 domain-containing transcription factor	Transcription	9.4	101.6
254907_at	At4g11190#	Disease resistance-responsive family protein/dirigent family protein	Cell defense	8.8	406.4
250504_at	At5g09840	Expressed protein	Unknown	8.4	3.1
251476_at	At3g59670	Expressed protein	Unknown	8.2	3.2
255278_at	At4g04940	Transducin family protein/WD-40 repeat family protein	Unknown	6.3	2.4
257267_at	At3g15030	TCP family transcription factor	Transcription	6.1	5.5
259466_at	At1g19050#	**ARR7**	Signal transduction/regulation	5.9	48.5
264404_at	At2g25160	Cytochrome P450	Metabolism	5.7	29.2
248727_at	At5g47990	Cytochrome P450	Metabolism	5.0	30.6
262516_at	At1g17190	Glutathione *S*-transferase	Cell defense	5.0	3.0
253975_at	At4g26600	Nucleolar protein	Unknown	4.6	2.2
252305_at	At3g49240	Pentatricopeptide (PPR) repeat-containing protein	Unknown	4.5	2.4
258505_at	At3g06530	BAP28-related	Unknown	4.5	2.3
249203_at	At5g42590	Cytochrome P450 71A16, putative (CYP71A16)	Metabolism	4.4	5.7
257516_at	At1g69040	ACT domain-containing protein (ACR4)	Signal transduction/regulation	4.3	4.9
254732_at	At4g13750	Expressed protein	Unknown	4.2	2.5
257652_at	At3g16810	Pumilio/Puf RNA-binding domain-containing protein	Unknown	4.2	2.1
247946_at	At5g57180	Expressed protein	Unknown	4.1	3.4
252273_at	At3g49660	Transducin family protein/WD-40 repeat family protein	Unknown	4.1	2.6
257487_at	At1g71850	Expressed protein	Unknown	4.1	2.6
264118_at	At1g79140	Expressed protein	Unknown	4.1	3.1
247168_at	At5g65860	Ankyrin repeat family protein	Unknown	4.0	2.9
248045_at	At5g56030	Heat shock protein 81–2 (HSP81–2)	Cell defense	4.0	2.1
263236_at	At1g10470#	**ARR4**	Signal transduction/regulation	4.0	61.1
266418_at	At2g38750#	Annexin 4 (ANN4)	Signal transduction/regulation	4.0	4.6
251593_at	At3g57660#	DNA-directed RNA polymerase family protein	DNA/RNA synthesis/processsing	3.9	2.2
265154_at	At1g30960	GTP-binding protein (ERG)	Signal transduction/regulation	3.9	2.3
251800_at	At3g55510	Expressed protein	Unknown	3.8	2.5
258545_at	At3g07050	GTP-binding family protein	Unknown	3.8	2.2
266510_at	At2g47990	Unknown protein	Unknown	3.8	2.4
265326_at	At2g18220	Expressed protein	Unknown	3.7	2.4
250222_at	At5g14050	Transducin family protein/WD-40 repeat family protein	Unknown	3.6	2.9
248729_at	At5g48010	Pentacyclic triterpene synthase	Metabolism	3.6	10.3
249755_at	At5g24580	Copper-binding family protein	Unknown	3.6	7.3
259248_at	At3g07770	Heat shock protein-related	Protein synthesis/modification	3.6	2.6
266419_at	At2g38760	Annexin 3 (ANN3)	Signal transduction/regulation	3.6	4.0
263841_at	At2g36870	Xyloglucan:xyloglucosyl transferase	Metabolism	3.5	5.4
264131_at	At1g79150	Expressed protein	Unknown	3.5	2.2
266801_at	At2g22870	Expressed protein	Signal transduction/regulation	3.5	2.2
249874_at	At5g23070	Thymidine kinase	Metabolism	3.4	2.7
247600_at	At5g60890	Receptor-like protein kinase (ATR1) (MYB34)	Transcription	3.3	2.9
248728_at	At5g48000	Cytochrome P450	Metabolism	3.3	11.8
250304_at	At5g12110	Elongation factor 1B α-subunit 1	Protein synthesis/modification	3.3	24.8
256797_at	At3g18600	DEAD/DEAH box helicase	DNA/RNA synthesis/processsing	3.3	2.3
259759_at	At1g77550	Tubulin–tyrosine ligase family protein	Protein synthesis/modification	3.3	2.4
264357_at	At1g03360	Exonuclease family protein	Unknown	3.3	2.4
264895_at	At1g23100	10 kDa chaperonin	Protein synthesis/modification	3.3	2.7
251668_at	At3g57010	Strictosidine synthase family protein	Metabolism	3.2	84.4
256675_at	At3g52170	Expressed protein	Unknown	3.2	4.2
259736_at	At1g64390	Endo-1,4-β-glucanase	Metabolism	3.2	5.2
261768_at	At1g15550	Gibberellin 3-β-dioxygenase (GA_4)	Metabolism	3.2	3.7
251791_at	At3g55500	Expansin (EXP16)	Cell structure	3.2	3.3
251830_at	At3g55010	Phosphoribosylformylglycinamidine cyclo-ligase (PUR5)	Metabolism	3.2	2.5
252202_at	At3g50300	Transferase family protein	Unknown	3.2	4.6

Table 2 Continued

Affymetrix no.	AGI code	Remarks	Functional categories	FC	
				Group-A	Group-C
255685_s_at	At4g00600#	Tetrahydrofolate dehydrogenase/cyclohydrolase	Metabolism	3.2	2.5
258166_at	At3g21540	Transducin family protein/WD-40 repeat family protein	Unknown	3.2	2.1
260585_at	At2g43650	Sas10/U3 ribonucleoprotein (Utp) family protein	Protein synthesis/modification	3.2	2.7
246765_at	At5g27330	Expressed protein	Unknown	3.1	2.5
254079_at	At4g25730	FtsJ-like methyltransferase family protein	Unknown	3.1	2.0
257702_at	At3g12670	CTP synthase, putative/UTP-ammonia ligase	DNA/RNA synthesis/processsing	3.0	2.1
265429_at	At2g20710	Pentatricopeptide (PPR) repeat-containing protein	Unknown	3.0	2.2
266237_at	At2g29540	RNA polymerase I(A) and III(C) 14 kDa subunit (RPAC14)	DNA/RNA synthesis/processsing	3.0	2.5
266934_at	At2g18900	Transducin family protein/WD-40 repeat family protein	Unknown	3.0	2.4
245770_at	At1g30240	PELP1-related	Unknown	3.0	2.1
246282_at	At4g36580	AAA-type ATPase family protein	Unknown	3.0	4.0
249528_at	At5g38720	Expressed protein	Unknown	3.0	2.1
253880_at	At4g27590	Copper-binding protein-related	Unknown	3.0	4.9
256368_at	At1g66800	Cinnamyl-alcohol dehydrogenase family/CAD family	Metabolism	3.0	5.2
259444_at	At1g02370	Pentatricopeptide (PPR) repeat-containing protein	Unknown	3.0	3.0
263594_at	At2g01880	Purple acid phosphatase (PAP7)	Protein synthesis/modification	3.0	3.5
264974_at	At1g27050	Homeobox-leucine zipper family protein	Transcription	3.0	2.1
246457_at	At5g16750	Transducin family protein/WD-40 repeat family protein	Unknown	2.9	2.0
253073_at	At4g37410	Cytochrome P450	Metabolism	2.9	8.2
263119_at	At1g03110	Transducin family protein / WD-40 repeat family protein	Unknown	2.9	2.5
264731_at	At1g62150	Mitochondrial transcription termination factor-related/mTERF-related	Unknown	2.9	2.2
250538_at	At5g08620	DEAD box RNA helicase (RH25)	DNA/RNA synthesis/processsing	2.8	2.5
256070_at	At1g13730	Nuclear transport factor 2 (NTF2) family protein	Transport	2.8	2.7
257891_at	At3g17170	Ribosomal protein S6 family protein (RFC3)	Protein synthesis/modification	2.8	2.2
251856_at	At3g54720	Glutamate carboxypeptidase (AMP1)	Signal transduction/regulation	2.8	5.2
257131_at	At3g20240	Mitochondrial substrate carrier family protein	Transport	2.8	2.2
263824_at	At2g40360	Transducin family protein/WD-40 repeat family protein	Unknown	2.8	2.0
264529_at	At1g30820	CTP synthase	Metabolism	2.8	3.6
256341_at	At1g72040	Deoxynucleoside kinase family	Metabolism	2.7	2.6
256978_at	At3g21110	SAICAR synthetase (PUR7)	Metabolism	2.7	2.2
249814_at	At5g23840	MD-2-related lipid recognition domain-containing protein	Unknown	2.6	2.4
250201_at	At5g14230	Ankyrin repeat family protein	Unknown	2.6	4.3
257294_at	At3g15570	Phototropic-responsive NPH3 family protein	Signal transduction/regulation	2.6	3.0
258181_at	At3g21670	Nitrate transporter (NTP3)	Transport	2.6	3.2
261226_at	At1g20190	Expansin, putative (EXP11)	cell structure	2.6	2.2
263371_at	At2g20490	Nucleolar RNA-binding Nop10p family protein	Unknown	2.6	2.4
263382_at	At2g40230#	Transferase family protein	Unknown	2.6	11.1
246265_at	At1g31860	Histidine biosynthesis bifunctional protein (HISIE)	Metabolism	2.6	2.2
246461_at	At5g16930	AAA-type ATPase family protein	Unknown	2.6	2.1
249830_at	At5g23300	Dihydroorotate dehydrogenase, mitochondrial/DHOdehase (PYRD)	Metabolism	2.6	2.2
264818_at	At1g03530	Expressed protein	Unknown	2.6	2.5
266372_at	At2g41310#	**ARR8**	Signal transduction/regulation	2.6	5.7
267586_at	At2g41950	Expressed protein	Unknown	2.6	2.4
247246_at	At5g64620#	Invertase/pectin methylesterase inhibitor family protein	Protein synthesis/modification	2.5	6.5
254016_at	At4g26150	Zinc finger (GATA type) family protein	Transcription	2.5	2.4
261439_at	At1g28395	Expressed protein	Unknown	2.5	2.5
261972_at	At1g64600	Expressed protein	Unknown	2.5	2.2
262838_at	At1g14960	Major latex protein-related/MLP-related	Metabolism	2.5	13.0
262943_at	At1g79470	Inosine-5′-monophosphate dehydrogenase	Metabolism	2.5	3.0
253558_at	At4g31120	Skb1 methyltransferase family protein	Unknown	2.5	2.4
258003_at	At3g29030	Expansin (EXP5)	Cell structure	2.5	4.7
247942_at	At5g57120	Expressed protein	Unknown	2.4	2.0
254606_at	At4g19030 #	Major intrinsic (MIP) family protein	Transport	2.4	8.8
256983_at	At3g13470	Chaperonin	Protein synthesis/modification	2.4	2.3
259846_at	At1g72140	Proton-dependent oligopeptide transport (POT) family protein	Transport	2.4	3.2
247980_at	At5g56860	Zinc finger (GATA type) family protein	Transcription	2.4	3.1

Table 2 Continued

Affymetrix no.	AGI code	Remarks	Functional categories	FC	
				Group-A	Group-C
261898_at	At1g80720	Mitochondrial glycoprotein family protein/MAM33 family protein	Unknown	2.4	2.2
262112_at	At1g02870	Expressed protein	Unknown	2.4	2.4
263255_at	At1g10490	Expressed protein	Transport	2.4	2.3
248646_at	At5g49100	Expressed protein	Unknown	2.3	4.7
248753_at	At5g47630	Acyl carrier family protein/ACP family protein	Metabolism	2.3	2.9
260235_at	At1g74560	Nucleosome assembly protein (NAP) family protein	DNA/RNA synthesis/processsing	2.3	2.1
262230_at	At1g68560	α-Xylosidase (XYL1)	Metabolism	2.3	2.6
249354_at	At5g40480	Expressed protein	Unknown	2.2	2.2
251586_at	At3g58070	Zinc finger (C2H2 type) family protein	Transcription	2.2	4.0
254475_at	At4g20440	Small nuclear ribonucleoprotein-associated protein B	DNA/RNA synthesis/processsing	2.2	2.0
254909_at	At4g11210#	Disease resistance-responsive family protein/dirigent family protein	Cell defense	2.2	6.6
263677_at	At1g04520	33 kDa secretory protein-related	Unknown	2.2	2.6
266770_at	At2g03090	Expansin (EXP15)	Cell structure	2.2	5.7
266790_at	At2g28950	Expansin (EXP6)	Cell structure	2.2	7.8
245965_at	At5g19730	Pectinesterase family protein	Cell structure	2.2	2.7
249347_at	At5g40830	Expressed protein	Unknown	2.2	2.2
263549_at	At2g21650#	Myb family transcription factor	Transcription	2.2	45.3
248723_at	At5g47950	Transferase family protein	Unknown	2.1	4.7
248921_at	At5g45950	GDSL-motif lipase/hydrolase family protein	Unknown	2.1	8.0
250192_at	At5g14520	Pescadillo-related	Transcription	2.1	2.0
252272_at	At3g49670	Leucine-rich repeat transmembrane protein kinase	Signal transduction/regulation	2.1	4.6
253090_at	At4g36360	β-Galactosidase	Metabolism	2.1	2.6
253372_at	At4g33220	Pectinesterase family protein	Cell structure	2.1	2.0
256343_at	At1g72090	Radical SAM domain-containing protein	Unknown	2.1	2.2
260676_at	At1g19450	Sugar transporter family protein	Transport	2.1	2.1
249920_at	At5g19260	Expressed protein	Unknown	2.1	2.8
259378_at	At3g16310	Mitotic phosphoprotein N′ end (MPPN) family protein	Unknown	2.1	2.1
248527_at	At5g50740	Copper chaperone (CCH)-related	Unknown	2.0	7.0
250160_at	At5g15210	Zinc finger homeobox family protein/ZF-HD homeobox family protein	Transcription	2.0	2.3
258404_at	At3g17465	Ribosomal protein L3 family protein	Protein synthesis/modification	2.0	2.1
257483_at	At1g49620	Cyclin-dependent kinase inhibitor 7 (ICK7)	Signal transduction/regulation	2.0	3.1
252536_at	At3g45700	Proton-dependent oligopeptide transport (POT) family protein	Transport	2.0	5.8
261055_at	At1g01300	Aspartyl protease family protein	Protein synthesis/modification	2.0	2.8
266264_at	At2g27775	Expressed protein	Unknown	2.0	2.1

Detailed procedures of microarray analyses are given in Materials and Methods. Other details are the same as those given in the footnotes for Table 1.

were preferentially selected. For instance, they are the gene (At2g46310) encoding an AP2 domain transcription factor, the gene (At4g26150) encoding a zinc finger (GATA) family transcription factor, the gene (At5g50915) encoding a basic helix–loop–helix (bHLH) protein and the gene (At3g49670) encoding a transmembrane protein kinase. (iii) In addition, some other apparently interesting genes were also selected. For instance, they include: the gene (At1g16530) encoding a LOB domain protein (LBD3), the gene (At4g29740) encoding a cytokinin oxidase family protein, the gene (At3g44750) encoding a histone deacetylase (HD2A) and the gene (At1g15550) for a gibberellin 3-β-dioxygenase (GA_4).

Each transcript of the selected 28 genes was first examined, as follows. Wild-type plants (21-day-old young seedlings) were treated or not with cytokinin for 3 h. These experimental conditions were exactly the same as those used for the microarray analyses. For each, the corresponding transcripts were specifically detected by reverse transcription–polymerase chain reaction (RT–PCR) amplification, followed by Southern blot hybridization. The following genes were also analyzed as references: the *ARR5* and *ARR15* genes (positive reference), and the *ACT8* gene (internal loading reference). Based on the results, the fluctuation of each transcript in response to cytokinin was calculated (Table 3). Of 28 genes, 21 genes were significantly up-regulated in response to cytokinin (FC > 1.5), as expected. The FC values calculated for the others (five genes) were not so significantly high. For some other genes (At5g06550 and At3g25940), their specific transcripts were not detected under our experimental conditions. Thus, the 21 cytokinin-up-regulated genes were selected further. To see the cytokinin responsiveness of these genes more critically, the time course of induction of these transcripts in response to cytokinin was

Table 3 A set of putative cytokinin-induced genes

Genes (AGI code)	Cytokinin response [a]		Remarks
	Fold	Type	
ACT8	1.1		Actin (control)
ARR5	4.0		Type-A response regulator
ARR15	8.1		Type-A response regulator
At1g13420	5.0	I	Sulfotransferase family protein
At2g46310	4.8	I	AP2 domain-containing transcription factor
At4g26150	3.9	I	Zinc finger (GATA) family transcription factor
At1g16530	3.8	I	LOB domain-containing protein (LBD3/ASL9)
At4g23750	3.7	I	AP2 domain-containing transcription factor
At4g29740	3.1	I	Cytokinin oxidase family protein
At5g50915	3.0	III	bHLH family transcription factor
At2g21650	2.9	I	MYB-domain-containing protein
At5g48570	2.4	II	Pepetidyl-prolyl *cis–trans* isomerase
At2g38750	2.3	I	Annexin (ANN4)
At3g44750	2.2	II	Histone deacetylase (HD2A)
At3g49670	2.1	II	LRR transmembrane protein kinase
At2g38760	2.1	I	Annexin (ANN3)
At1g69040	2.0	I	ACT domain-containing protein (ACR4)
At2g22770	1.8	I	bHLH family transcription factor (AtbHLH20)
At3g58070	1.8	III	Zinc finger (C2H2) family transcription factor
At1g49620	1.6	II	Cyclin-dependent kinase inhibitor (ICK7)
At1g07050	1.6	III	CONSTANS-like protein
At4g20020	1.6	III	DAG-like protein
At1g15550	1.5	II	Gibberellin 3-β-hydroxylase (GA_4)
At2g22870	1.5	II	GTP-binding protein
At1g80270	1.4	–	Putative DNA-binding protein
At3g15030	1.3	–	TCP family transcription factor
At5g60890	1.2	–	Receptor-like protein kinase (ATR1)
At5g26260	1.2	–	Meprin/TRAF-homology protein
At1g74560	1.2	–	Nucleosone assembly protein (NAP)
At5g06550	ND	–	Putative transcription factor
At3g25940	ND	–	Putative transcription factor

[a] Details are described in the text.

examined by preparing RNA samples at short intervals (10–360 min after cytokinin treatment). They were analyzed by RT–PCR-aided Southern blot hybridization with each specific probe (Fig. 3, and see Table 4). The results were also examined quantitatively (Fig. 4, only some representative results are shown).

Of the 21 genes tested, 11 genes were rapidly induced at the level of transcription by cytokinin in a manner similar to the case of the *ARR5* and *ARR15* genes (these genes were classified as type-I in Fig. 3) (see also Fig. 4). Another six genes were also up-regulated by cytokinin to a considerable extent (type-II in Fig. 3), but the responses to cytokinin were rather slow and less evident (see At1g49620 in Fig. 4). The cytokinin responsiveness of the remaining four genes was not clear, because the levels of their transcripts fluctuated even in plants without cytokinin treatment (see At5g50915 in Fig. 4). These results suggested that the solvent (dimethylsulfoxide; DMSO) itself somehow transiently affected the expression of some genes tested (classified as type-III in Fig. 3), which should be excluded from the final lists. Taken together, of 21 genes tested, at least 17 were confirmed to be cytokinin-up-regulated genes, the transcripts of which are accumulated when wild-type plants were treated by cytokinin externally.

Cytokinin responses in T87 cultured cells

As mentioned above, we treated whole plants (young seedlings) with cytokinin (as an external stimulus) to see the responses. In such plants, many direct and indirect events would occur at the level of transcription. Some events would be dependent on the cytokinin-mediated His→Asp phosphorelay signaling circuitry per se, but others might be independent. To gain a more specific insight into this issue, we employed an

Arabidopsis cultured cell line (T87 cells) that previously has been shown to have the ability to respond to cytokinin (Yamada et al. 2004). When we considered the fact that the immediate early induction of the type-A ARR family genes (e.g. *ARR5*) was observed in T87 cells, it was reasonably assumed that the AHK-dependent His→Asp phosphorelay circuitry is indeed propagated in the cultured cells in response to cytokinin. It was thus of interest to examine the expression profiles of the cytokinin-up-regulated genes (e.g. 11 type-I genes in Table 3) in T87 cultured cells. The results of Northern blot hybridization showed that three genes (Ar2g46310, At1g16530 and At2g21650) responded to cytokinin in the cultured cells as rapidly and markedly as in the case of the type-A *ARR5* genes (Fig. 5). Others (At4g23750, At4g 29740, At2g38750 and At2g38760) also responded to cytokinin, but the responsiveness was less striking (data not shown). Transcripts of other genes (e.g. At4g26150) were hardly detected in T87 cells, while others (e.g. At2g22770) were expressed constitutively regardless of cytokinin treatment (Fig. 5). It was thus tempting to speculate that the three genes (Ar2g46310, At1g16530 and At2g21650) are possibly the primary targets of the cytokinin-mediated His→Asp phosphorelay. In any case, this cultured cell system will also provide us with insight into properties of the cytokinin-responsive genes identified in this study.

Specificity of hormone response

Finally, it would be of interest to examine whether the expression of cytokinin-up-regulated genes identified in this study is regulated specifically by cytokinin. Perhaps some of them may also be controlled in response to other hormones. To address this issue preliminarily, some representative genes were examined in this respect (certain type-I genes in Fig. 3). RNA samples were prepared from young seedlings (20 d old), which were treated for 1 and 3 h by each of the following hormones (100 μM each): *t*-Z (*trans*-zeatin), BA (6-benzylaminopurine), 2,4-D (2,4-dichlorophenoxy acetic acid), ACC (1-aminocyclopropane-1-carboxylic acid), GA_3 (gibberellin A3), ABA (abscisic acid), BR (brassinosteroid), MJA (methyl jasmonate) and SA (methyl salicylate). The RNA samples were analyzed by semi-quantitative RT–PCR with appropriate primers (Fig. 6). Transcripts of the genes tested (At1g69040, At2g46310, At4g26150 and At4g23750) were indeed induced specifically by cytokinins (*t*-zeatin and BA), but not by other hormones. Interestingly, transcripts of At2g38750 and At2g38760 were accumulated in response to cytokinins, but also in response to ABA and MJA (even more markedly). It may be noted that these genes, located on the chromosome next to each other, encode homologous members of the annexin family (see Table 3). At4g26150 appears to respond somehow transiently to DMSO (or spraying). In any case, the results of Fig. 6 are consistent with the views deduced from the microarray analyses of this study. However, this line of experimentation must be carried out more extensively for other genes in Tables 1 and 2.

Discussion

During the last few years, the immediate early response to cytokinin in *A. thaliana* was formulated as the multistep AHK→AHP→ARR phosphorelay signaling circuitry. However, clarification of the comprehensive picture of the His→Asp phosphorelay network is still at a very early stage. Assuming that most type-A and type-B ARRs (if not all) are involved in the cytokinin signaling, a number of general questions arise, for instance: (i) during plant development, where, when and how do they play their cytokinin-associated roles; and (ii) in addition to type-A *ARR* genes, are there any other primary targets of type-B ARRs. To address these issues, we must identify the cytokinin-associated signaling components (or genes) that function downstream of the cytokinin-mediated His→Asp phosphorelay signaling circuitry. An advanced tool that would allow us to identify such cytokinin-regulated genes is DNA microarrays. Within the last few years, in general, plant researchers have already begun to explore the impact of microarray analyses on fundamental plant biology, and numerous experimental microarray data are now available not only in the literatures, but also in the *Arabidopsis* public databases (see http://arabidopsis.org/links/microarrays.jsp). However, such microarray data are often very extensive and quite complicated. In other words, proper data mining is not so easy. In fact, several instances of microarray analyses with reference to cytokinin in *A. thaliana* have already been reported (Che et al. 2002, Hoth et al. 2003, Rashotte et al. 2003, Kiba et al. 2004). In our previously experiment (Kiba et al. 2004), wild-type and ARR22-ox plants were treated with cytokinin, and changes in the profiles of transcripts were simply examined to reveal certain genes that were up-regulated and/or down-regulated in response to cytokinin in wild-type plants. The results of such microarray analyses revealed a large number of cytokinin-associated genes, which most probably include all those genes that were affected by cytokinin not only primarily, but also secondarily and indirectly. More critically, they might also include those genes that were affected primarily by cytokinin, but in a manner independent of the His→Asp phosphorelay. Indeed, the downstream target genes of the cytokinin-mediated His→Asp phosphorelay have not yet been fully clarified through these microarray analyses. To gain further insight into such cytokinin-regulated genes, here we took a unique (or sophisticated) approach. The approach is a sort of combinatorial microarray analysis that was conducted by employing not only wild-type plants, but also certain transgenic lines in which the cytokinin-mediated His→Asp phosphorelay has been manipulated appropriately. The results allowed us to compile a certain number of genes (214 genes), the expression of which appears to be regulated through the His→Asp phosphorelay (Tables 1 and 2). To evaluate these data, some representatives of these identified genes were characterized further (Table 3). Of 21 genes characterized, 11 genes (at least) were confirmed to respond rapidly and specifically to cytokinin at the level of transcription in a

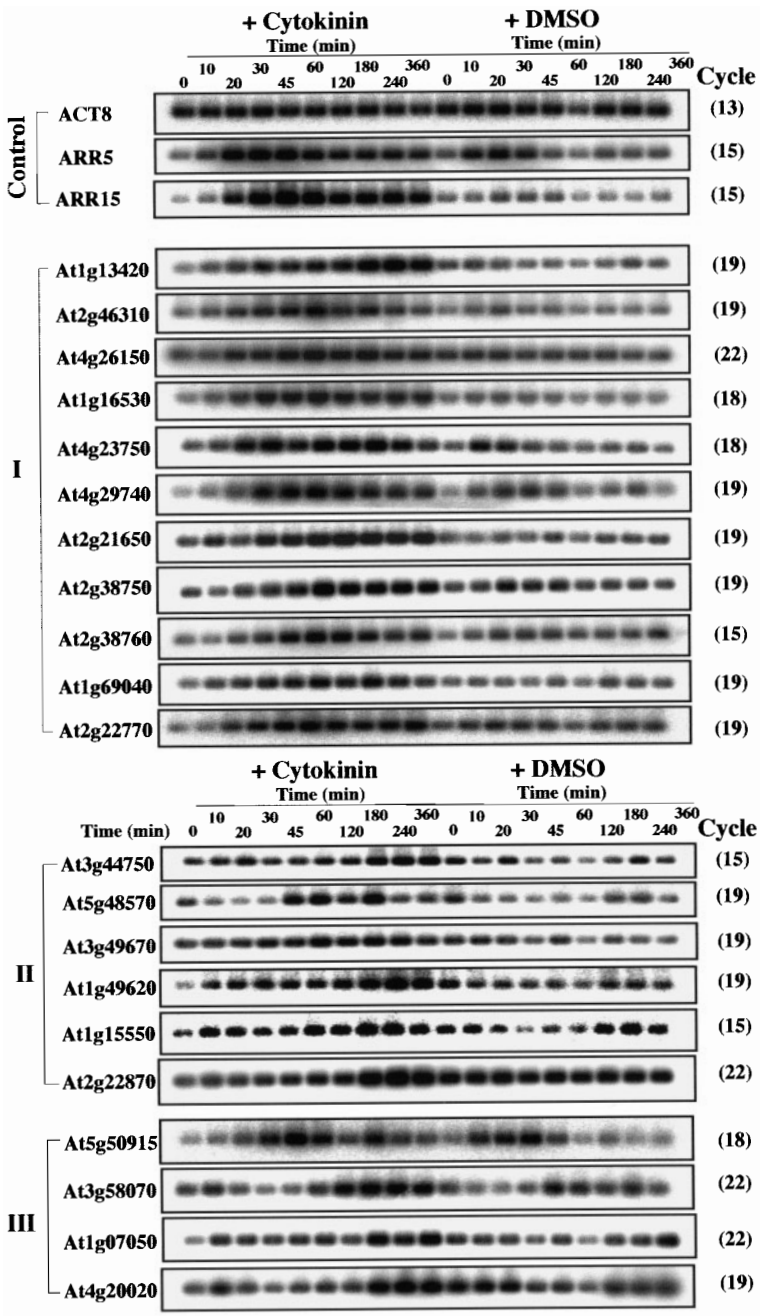

Fig. 3 Expression profiles of cytokinin-up-regulated genes in plants. To see the cytokinin responsiveness of the selected set of genes critically, the time course of induction of their transcripts in response to cytokinin was examined with RNA samples from plants treated or not with cytokinin (*t*-zeatin) for short intervals (10–360 min). They were analyzed by RT–PCR-aided Southern blot hybridization with each specific probe (see Table 4). The results were also summarized in Table 3. The results were also examined quantitatively (see Fig. 4, only some representative results are shown).

manner similar to that of the type-A *ARR* genes (Fig. 3, 4). Furthermore, some of these genes were demonstrated to respond rapidly to cytokinin even in suspension cultured cells (Fig. 5, 6). In these experiments, we intended to focus only on the genes that are relevant to signal transduction and transcriptional regulation, as further discussed below. However, the lists in Tables 1 and 2 will further provide us with valuable information with regard to other cytokinin-responsive genes in *A. thaliana*.

We confirmed that the following 11 genes are up-regulated in response to cytokinin at the level of transcription (or stability of transcripts): At1g13420, At2g46310, At4g26150, At1g16530, At4g23750, At4g29740, At2g21650, At2g38750, At2g38760, At1g69040 and At2g22770. Among these, five genes were inferred to encode DNA-binding transcription factors, as judged by the fact that each protein product contains a characteristic and common DNA-binding domain. At2g46310 encodes an AP2/EREBP (APETALA2/ETHYLENE-RESPONSIVE ELEMENT-BINDING PROTEIN) domain-containing protein, and At4g23750 also encodes another AP2/EREBR family protein (Okamuro et al. 1997, Riechmann and Meyerowitz 1998, Chang and Shockey 1999). At4g26150 encodes a

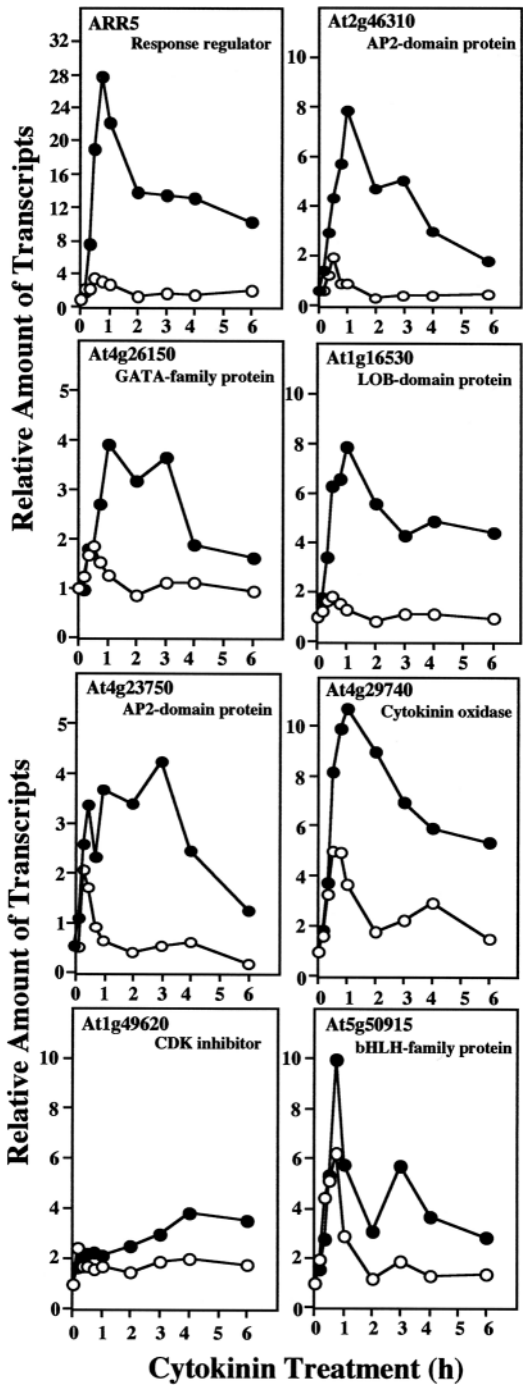

Fig. 4 Expression profiles of cytokinin-up-regulated genes in plants. To see the cytokinin responsiveness of the selected set of genes critically, the time course of induction of their transcripts in response to cytokinin was examined with RNA samples from plants treated or not with cytokinin (*t*-zeatin) for short intervals (10–360 min). They were analyzed by RT–PCR-aided Southern blot hybridization with each specific probe, as shown in Fig. 3. For some representative samples, the results were quantitatively examined, as indicated.

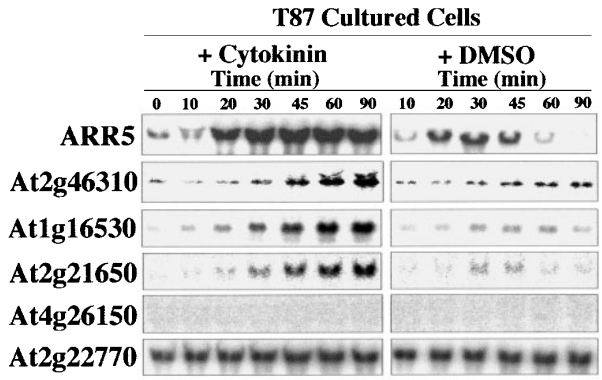

Fig. 5 Expression profiles of cytokinin-up-regulated genes in T87 cultured cells. To see the cytokinin responsiveness of the selected set of genes critically, the time course of induction of their transcripts in response to cytokinin was examined with RNA samples from T87 cultured cell treated or not with cytokinin (*t*-zeatin) for short intervals (10–90 min). They were analyzed by Northern blot hybridization with each specific probe (see Table 4).

zinc finger-containing GATA family protein (Riechmann et al. 2000). At2g21650 encodes a MYB-related family protein (Riechmann et al. 2000, Stracke et al. 2001). At2g22770 encodes a bHLH family protein (Bailey et al. 2003, Toledo-Ortiz et al. 2003). In *A. thaliana*, each of these transcription factor families contains a large number of members that play a variety of roles throughout the plant life cycle (Riechmann et al. 2000). It should be noted that none of these putative cytokinin-associated transcription factors identified in this study has so far been characterized experimentally, except for At2g22770. Recently, it was reported that At2g22770 encoding a bHLH factor (designated NAL1) is implicated in the formation of an ER (endoplasmic reticulum) body (Matsushima et al. 2004). In any case, it is of interest to examine these transcription factors with special reference to cytokinin responses in plants. It is also tempting to speculate that each of these transcription factors might play a role in a cytokinin signaling branch downstream of the His→Asp phosphorelay circuitry.

Besides the putative transcription factors mentioned above, the following genes that were confirmed to be cytokinin responsive are also noteworthy (see Fig. 4, 5). (i) At1g16530 encodes a protein belonging to a large family of plant-specific proteins, namely, LATERAL ORGAN BOUNDARIES (LOB) domain proteins. The LOB family consists of >40 members (also known as the ASYMMETRIC LEAVES2-like protein family) (Iwakawa et al. 2002, Shuai et al. 2002). At1g16530 corresponds to LOB3 (or ASL9) in the compiled list of LOB family proteins. Although no common nature of LOB family proteins is known, the prototype member (LOB, At5g63090) was suggested to play a potential role in lateral organ development in plants. (ii) At4g29740 encodes a member of cytokinin oxidases. The *Arabidopsis* cytokinin oxidase family members that degrade cytokinins have been well characterized (Schu-

Table 4 List of primers used in this study

Genes	Forward primers	Reverse primers
ACT8	5′-GTCGCTGTCGACTACGAGCAAG	5′-CTGTGGACAATGCCTGGACCTGC
ARR5	5′-AGCGGTTACTCAGAGTCTCAT	5′-CTTAAAAGCTCTTTCCTCAGCT
ARR15	5′-CATCTGTTTTGTTGTTTACCTTCCCGAGAG	5′-GGTGAGCATTAGAATCTAGACTTACATAGTTG
At1g13420	5′-AGGAACTTGAAGGAAGAAGAAG	5′-CGTACACGATCTTGCAAGGAG
AT2G46310	5′-CGTTATGTGGATGAGATCAGG	5′-TTTGGTAACAAGGACTGGTGG
At4g26150	5′-ACTCCTCTTTGGAGAAGTGGT	5′-CTAGCTATGAGGGCTTATGGT
At1g16530	5′-CAAAAGGGTCACAGACACGGAA	5′-ATGTCGATGTCACTGTAGAAG
At4g23750	5′-ACGAATTCTCCGGCATTTCTT	5′-AAACTTTCTCCGGTTCCGTTT
At4g29740	5′-TATCTCGGCAGACGGGACTTA	5′-TGGGTGCATGATCCAACGCAA
At5g50915	5′-TAAGTGAGCGGATGAGGACTC	5′-AAGAAACTCACGAAAGCAGGC
At2g21650	5′-AGTTACTAAACAATGGCATCAGG	5′-ACATCTCTCTGAAGTGATTCTCG
At5g48570	5′-CTGTAACCTGAATGATGCAGCT	5′-CTCTGAAACAGTAACACACACC
At2g38750	5′-AAGAAGCCGTGGAGAAGGATGA	5′-CCAAACGGTAGAGAAAACGCAA
At3g44750	5′-CTGTAGCTAAACCAAAGGCTAA	5′-ACTCCAACTGGCTCTCTTTCAT
At3g49670	5′-TCGATTCCAACTTCGAAGCTCA	5′-TAAAAGATCCGGTGGACTTCCT
At2g38760	5′-ATGCTACGTGAAGCCATAGAGA	5′-CAAACTGGAGTTGGAATACAATG
At1g69040	5′-CCATGGAAGTGTTGATACTGAG	5′-GAAACCTCACAGCTAAGTTAAG
At2g22770	5′-GAGTGGTGACCAAGAAAATGG	5′-ACTAAACCGAGTGATCTGGTC
At3g58070	5′-CTTACAGTTACCGTCATTACCC	5′-TCGATGCCGTGAATCTTACACA
At1g49620	5′-TGGCTTACTCGGTTTCAGATTC	5′-ACTTTAGCACTGTCTCTTCTTC
At1g07050	5′-CGAGACCAACACAATCATTCTA	5′-TTTTGCCCCTTTTAAGTCTCTC
At4g20020	5′-TCCAAAGCTTTCCAGAATACTC	5′-TACATCCTTTGCTTAGCCTCTT
At1g15550	5′-CCTCTCAACGATTTCCGTAAAC	5′-CAAACTAACTAGTAGATCACACA
At2g22870	5′-GTTGCTCTTACTTCTAAGAAACC	5′-TGATTCAAAACCGACCTTTTCGA
At1g80270	5′-GGACAGAAGAGCTTCTTCTAGT	5′-ATGAGATACTTCTTGCAAGCAG
At3g15030	5′-CACAACTATCATCATCAGCATC	5′-TTAGTTTCGATTGTCAATGGCG
At5g60890	5′-TTTACGTACTACTTAGGGTATTC	5′-TGCTTCAACCGCTTCTTGAGA
At5g26260	5′-TTCGTTTCTCTACCTTGGAAGAT	5′-ACAAGCATCAACAACGTTGTCG
At1g74560	5′-ATGCCAAAGATGTGAAATCTGG	5′-ATAGAAGCAAGCCAGCAATGTG
At5g06550	5′-GCAGAGAAAGTTCCGGTTTTG	5′-GATATGACAAAGAGCCAGTGG
At3g25940	5′-CAATTGAGACATGTAGAGATGAGT	5′-GGTTCTAATGATGGCACTGAATC

mulling et al. 2003). The enzymes might be implicated in the cytokinin signaling. (iii) At1g69040 was previously reported as a cytokinin-responsive gene, which encodes an ACT domain repeat protein family (designated as ACR4) (Hsieh and Goodman 2002). The function of this protein is not known, although the ACT domain is postulated to serve as an amino-binding site in bacteria (Aravind and Koonin 1999). (iv) It may also be noteworthy that two annexin genes were identified in this study (ANN3, At2g38760; and ANN4, At2g38750). In this study, we suggested that the expression of these homologous genes is controlled not only by cytokinin, but also by ABA and MJA (Fig. 6). In *A. thaliana*, there are seven *ANN* genes, which were hypothesized to be involved in the Golgi-mediated secretion of polysaccharides (Clark et al. 2001). (v) Finally, some other cytokinin-responsive genes (type-II in Table 3) might also be interesting in terms of cytokinin signaling: they include, At3g49670 encoding a leucine-rich repeat protein kinase; At1g49620 encoding a cyclin-dependent protein kinase inhibitor; and At1g15550 encoding GA_4.

In the His→Asp phosphorelay circuitry, type-B ARRs serve as transcriptional activators, resulting in the rapid induction of type-A *ARR* genes (Sakai et al. 2000, Hwang and Sheen 2001, Sakai et al. 2001, Hosoda et al. 2002, Imamura et al. 2003). In this study, we identified several other genes which are induced in response to cytokinin as rapidly as in the case of type-A *ARR* genes (Fig. 4). It was thus of interest to know whether these newly identified genes are also the direct targets of type-B ARR transcription factors. A common sequence-specific DNA-binding domain (GARP motif) is conserved in the C-terminal sequences of type-B ARRs. Both ARR1 and ARR2 bind in vitro to the core sequence of 5′-AGATT-3′ (Sakai et al. 2000, Sakai et al. 2001). The three-dimensional structure of the GARP motif of ARR10 has a Myb-related helix–turn–helix structure, which also recognized in vitro the same (or related) core DNA sequence (Hosoda et al. 2002). Taking this core 5′-

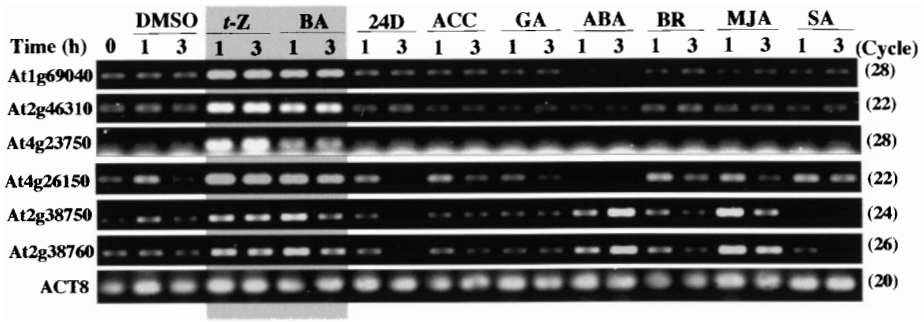

Fig. 6 Specificity of the hormone response. RNA samples were prepared from young seedlings (20-d-old, whole plants), after they were treated for 1 and 3 h by each of the following hormones (100 μM each) in DMSO (control): *t*-Z (*trans*-zeatin), BA (6-benzylaminopurine), 24D (2,4-dichlorophenoxy acetic acid), ACC (1-aminocyclopropane-1-carboxylic acid), GA (gibberellin A_3), ABA (abscisic acid), BR (brassinosteroid), MJA (methyl jasmonate) and SA (methyl salicylate), as indicated. These samples were analyzed by semi-quantitative RT–PCR with appropriate primers (see Table 4). It should be noted that the amplification cycles of the PCR were varied for one RNA sample to another. Furthermore, several (at least four) different cycle conditions were adopted for a given RNA sample (from 15 to 30 cycles). After optimizing the cycles in each case, the quantitative representative is shown for each gene (cycles applied in the PCR are indicated in parentheses).

AGATT-3′ sequence as a reference, we extensively inspected not only the 5′ upstream sequences of the type-A *ARR* coding genes, but also those of the cytokinin-responsive genes identified in this study. Nevertheless, it was difficult to find any statistically meaningful features (or *cis*-elements) common to the promoter sequences of these genes. Thus, this issue remains to be addressed further.

In summary, the results of combinatorial microarray analyses allowed us to identify a certain number (214) of cytokinin-associated genes, in particular, the expression of which was assumed to be up-regulated in a manner dependent on the cytokinin-mediated His→Asp phosphorelay. It is not certain whether these identified genes with divergent functions are indeed directly relevant to cytokinin responses in plants. However, our primary objective of this study was to compile a reliable and limited list of cytokinin-up-regulated genes with special reference to the His→Asp phosphorelay. As has been implied above, characterization of the compiled genes, if not all, will provide us with new bases for a better understanding of the cytokinin signaling network in *A. thaliana*. A set of transcription factors (see Table 3) is particularly of interest for us to examine one by one in terms of cytokinin responses in plants, and these lines of genetic experiments are currently underway in our laboratory.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col) plants were mainly used. In addition, two transgenic lines (named ARR21-C-ox and ARR22-ox) were employed. These are derivatives of Col, and were established and characterized previously (Kiba et al. 2004, Tajima et al. 2004). In ARR21-C-ox transgenic plants, the cDNA encoding the C-terminal DNA-binding domain of *ARR21* was designed so as to be overexpressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. In ARR22-ox transgenic plants, the cDNA encoding the full length of *ARR22* was overexpressed under the control of the CaMV 35S promoter. These plants were grown mainly with 16 h light/8 h dark fluorescent illumination at 22°C on agar plates containing MS salts and 1% sucrose, unless otherwise noted.

Preparation of RNA

Total RNA was isolated by the method described by Carpenter and Simon (1998), with slight modifications, as described previously (Tajima et al. 2004). When RNA samples were used for RT–PCR amplification and microarray, they were treated with DNase I.

DNA microarray analysis

Total RNA samples were prepared from wild-type plants (Col), ARR21-C-ox (T1 young seedlings) and ARR22-ox plants (T1 young seedlings), which were treated or not with cytokinin, as follows. Wild-type plants were grown on MS-agar plates for 3 weeks, and then sprayed with 20 μM *t*-zeatin, and further incubated for 3 h. ARR21-C-ox and ARR22-ox transgenic plants were selected on MS-agar plates containing 50 μg ml^{-1} hygromycin B for 7 days, and then transferred onto MS-agar plates without hygromycin. After incubation for a further 14 days (total 3 weeks), they were treated with cytokinin, as described for wild-type plants. These treated plant samples were divided into three portions, from which RNA samples were prepared separately. These RNA samples were processed as recommended by the Affymetrix instruction (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetrix). Double-stranded cDNA was synthesized from the isolated RNA (30 μg of total RNA for each) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, U.S.A.) and oligo(dT)24 primer primers flanking a sequence recognized by T7 RNA polymerase (Amersham Biosciences). A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction, using the Bio-Array High-Yield RNA Transcript Labeling Kit (Enzo Life Science, Farmingdale, NY, U.S.A). The reaction product was purified with the use of the RNeasy RNA purification kit (Qiagen, Valencia, CA, U.S.A.). The biotin-tagged cRNA was fragmented and hybridized with DNA chips of Affymetrix ATH1 array (Affymetrix), at 45°C for 16 h. The standard post-hybridization wash and double-stain protocols were adopted on an Affymetrix GeneChip Fluidics Station 400 (Affymetrix). Arrays were scanned on an Affymetrix GeneArray Scanner. The results were quantified using MicroAr-

ray Suite 5.0 software (Affymetrix). It should be noted that RNA processing, hybridization and scanning were carried out independently three times for each RNA sample. Finally, such collected raw data of cytokinin-treated samples were compared with those of non-treated samples, in various combinations (see Fig. 1B), using a comparative analysis algorithm in MicroArray Suite 5.0 software (Affymetrix), by which the FC (i.e. fluctuation ratio of transcript level) was calculated. The differences were classified into: (I), increased; (D), decreased; (NC), not changed, (MI), marginal increase; and (MD), marginal decrease. For instance, we selected those genes showing FC > 2 as cytokinin-up-regulated genes. To select more properly, only those genes assigned as 'P' (present) were selected (according to the Affymetrix flag procedure), and only those genes giving raw values >100 in the cytokinin-treated data were selected. More importantly, only those genes were selected which fulfilled the above criteria in three independent hybridization chip assays.

Semi-quantitative RT–PCR-aided Southern blot hybridization

An RT–PCR kit was used according to the instructions (*Bca*BEST RNA PCR Kit, TAKARA, Kyoto, Japan). To perform semi-quantitative RT–PCR, the procedures were slightly modified, in which the conditions used were primarily 94°C for 30 s (denaturation), 56°C for 45 s (annealing) and 72°C for 90 s (elongation). It should also be noted that the cycles of PCR were varied for each RNA sample, and several different cycles were adopted for a given RNA sample (from 15 cycles up to 19 cycles), in order to amplify double-stranded DNA in a semi-quantitative manner. In such semi-quantitative RT–PCR, the primers used were listed in Table 4. For Southern blot hybridization, DNA was separated in agarose gels (1.2%) containing 2.2 M formaldehyde, then transferred to Hybond-N+ nylon membranes. The fixed membranes were hybridized with ^{32}P-labeled DNA fragments in 6× standard saline phosphate and EDTA (1× SSPE = 0.18 M NaCl, 10 mM phosphate, 1 mM EDTA, pH 7.4), 5× Denhardt's solution, and 0.5% SDS buffer containing 10% dextran sulfate and 100 μg/ml salmon sperm DNA, at 65°C for 18 h. A DNA fragment used as a prove specific to a given gene was amplified with a set of primers, listed in Table 4. The membranes were washed twice with 2× SSPE and 0.1% SDS for 15 min at room temperature, twice with 2× SSPE and 0.1% SDS for 30 min at 65°C, and then with 0.2× SSPE and 0.1% SDS for 30 min at 65°C. The washed membranes were exposed and analyzed with BAS-2000II (Fuji Photo Film, Tokyo, Japan).

RNA preparation and Northern blot hybridization for T87 cells

T87 is an *Arabidopsis* suspension cultured cell line. The growth conditions together with other experimental procedures with regard to this versatile cell line were described in detail previously (Yamada et al. 2004). From T87 cells, RNA samples were prepared by essentially the same procedures according to the conventional ATA method for plants, as described previously (Nakamichi et al. 2003, Nakamichi et al. 2004). They were subjected to Northern blot hybridization, as also described previously (Yamada et al. 2004). For Northern blot hybridization, each specific DNA probe was prepared by PCR (see Table 4), and they were radioactively labeled.

Acknowledgments

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