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Original Paper

Root-specific induction of early auxin-responsive genes in *Arabidopsis thaliana* by *cis*-cinnamic acid

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Abstract *cis*-Cinnamoyl glucosides are the allelochemicals in Thunberg's meadowsweet (*Spiraea thunbergii*). The essential chemical structure responsible for the bioactivity of *cis*-cinnamoyl glucosides, *cis*-cinnamic acid (*cis*-CA), strongly inhibits the root growth of several plant species; however, its mode of action has not been characterized at the gene expression level. We conducted a time–course microarray analysis of gene expression in Arabidopsis in response to 20 μM *cis*-CA. Comparison of the microarray profiles revealed a 10-fold upregulation of several auxin-responsive *GRETCHEN HAGEN-3* (*GH3*) genes and *LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE* (*LBD*) genes from 2 h to 6 h post-treatment. Two early auxin-responsive gene families, the *Aux/IAA* family (*IAA1*, *IAA5*) and the *GH3* family (*GH3.1*, *GH3.2*, *GH3.3*), and an *LBD* gene (*LBD16*) were markedly upregulated at 2 h after treatment in the roots, but not in the shoots, of Arabidopsis and remained highly expressed for 4 h. The influence of an exogenous application of *cis*-CA on the indole-3-acetic acid pathway strongly suggests that a root-targeted induction of auxin-responsive genes is involved in the *cis*-CA-mediated plant growth inhibition.

Key words: Auxin, *cis*-cinnamic acid, Gene Ontology enrichment analysis, *LBD* gene family, *GH3* gene family, *Aux/IAA* gene family, *Spiraea thunbergii*.

Plants produce diverse secondary metabolites as defense substances against potential enemies such as herbivorous predators or pathogens that are distributed in their natural habitat. Some of these bioactive products, called allelochemicals, which are released into the environment, regulate the growth of neighboring plants (Harborne 1993). The application of allelochemicals for weed control has been pursued because it would reduce the risk of environmental contamination by persistent biodegradable substances (Beaudegnies et al. 2009; Mitchell et al. 2001; Rice 1995). While screening natural chemical alternatives to synthetic herbicides, Morita et al. (2005) found a high inhibitory activity against lettuce in the leaves of Thunberg's meadowsweet (*Spiraea thunbergii*), a popular garden shrub, and in its allied species, *S. cantoniensis* and *S. pruniflora* (Rosaceae). They identified the inhibitory compounds from *S. thunbergii* as *cis*-cinnamoyl glucosides, 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose and 6-*O*-(4′-hydroxy-2′-methylenebutyroyl)-1-*cis*-cinnamoyl-β-D-glucopyranose (Hiradate et al. 2004). They further elucidated the essential chemical structure responsible for the growth inhibition by these compounds as *cis*-cinnamic acid (*cis*-CA). The growth inhibitory activity on lettuce root by *cis*-CA (EC_{50} values: 3×10^{-6} M) was strong, even when compared with abscisic acid (EC_{50} values: 1.5×10^{-5} M) when measured under identical conditions (Hiradate et al. 2005). The presence of natural *cis*-CA in plants has also been reported in *Brassica parachinensis* (Yin et al. 2003). The prominent plant growth inhibitory activity and the natural origin of *cis*-CA imply that it has great potential as an alternative to synthetic herbicides.

Some studies have approached the physiological roles of *cis*-CA from the perspective of plant phenylalanine metabolism. Synthetic *cis*-CA promotes cell elongation and inhibits root growth (Wong et al. 2005; Yang et al. 1999). It has long been thought to act as an auxin-analog because of their similar effects (van Overbeek et al. 1951).

Abbreviations: auxin response factor, ARF; auxin-responsive element, AuxRE; *cis*-cinnamic acid, *cis*-CA; dimethyl sulfoxide, DMSO; half-maximal inhibition effective concentration, EC_{50}; Gene Ontology, GO; indole-3-acetic acid, IAA; Quantitative real-time PCR, qRT-PCR.

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Yet, in a study using auxin-insensitive mutants (*aux1* and *axr2*), Wong et al. (2005) suggested that the mode of action of *cis*-CA was different from that of auxin. Guo et al. (2011) identified two *cis*-CA-upregulated Arabidopsis genes, *MLPL1* (AT2G01520) and *MLPL2* (AT2G01530). DNA microarray analysis could serve as an effective tool to investigate the mode of action of *cis*-CA, considering that it has been used to monitor changes in response to other allelochemical stresses in the transcription state of individual Arabidopsis genes on a whole-genome scale (Baerson et al. 2005; Golisz et al. 2011). In the present study, we reveal for the first time the Arabidopsis genes upregulated by exogenous *cis*-CA, using a DNA microarray and Gene Ontology enrichment analysis. Then, we compare the responses of the auxin-responsive genes to exogenous *cis*-CA with their responses to exogenous indole-3-acetic acid (IAA). Finally, we analyze gene expression in response to exogenous *cis*-CA in the shoots and roots of Arabidopsis to clarify whether *cis*-CA plays an identical role to IAA in plant tissues.

Materials and methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* L. (Col-0, Inplanta Innovations Inc., Yokohama, Japan) were sterilized in 70% ethanol for 1 min, 2% sodium hypochlorite with 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) for 8 min, and were then rinsed 3 times with sterilized distilled water. The sterilized seeds were placed on 0.8% agar (Nakalai Tesque, Inc., Kyoto, Japan) with 0.5×Murashige and Skoog Plant Salt Mixture (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) and 1% sucrose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a sterilized Petri dish (90-mm diameter). The seeds were held at 4°C overnight in darkness and then transferred to a growth chamber. Emerging plants were maintained in the growth chamber under a schedule of 16-h light (22°C) and 8-h dark (20°C). After 14 d in the growth chamber, seedlings were removed from the agar and equilibrated at 22°C in 20 ml distilled water for 1 h. They were then transferred to 20 ml of distilled water containing 20 μM *cis*-CA with 0.0125% dimethyl sulfoxide (DMSO) and incubated under light. *cis*-CA was artificially synthesized according to the method of Abe et al. (2012). Plants were collected at 0 (control), 2, and 6 h and immediately frozen in liquid nitrogen and stored at −80°C before analysis. For comparison, other seedlings were treated with 20 μM IAA, which was supplemented with 0.0125% DMSO, in the above described manner. Each experiment was performed twice.

Root growth inhibition assay

The root growth inhibition assay of *cis*-CA was performed on agar plates (2% agar with 0.5×Murashige and Skoog Plant Salt Mixture containing 1% sucrose) containing various concentrations of *cis*-CA, and the germinated seeds were transferred to vertically oriented agar plates. After 4 d in a growth chamber under a schedule of 16 h light (22°C) and 8 h dark (20°C), root length was measured using a SZH dissecting microscope (Olympus, Tokyo, Japan) and the image measuring software SensivMeasure (Mitani, Fukui, Japan). The required half-maximal inhibition effective concentrations (EC_{50}s) were calculated using the probit method from SPSS for Windows ver. 11.0.1 J statistical software (SPSS Japan Inc., Tokyo, Japan).

Isolation of total RNAs and microarray analysis

Total RNA was extracted from seedlings with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted RNAs were checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The two-color spike mix was added to the total RNA, and the RNA was labeled with a Quick Amp Labeling Kit, two-color (Agilent Technologies), according to the manufacturer's protocol. Fluorescent cRNA was generated from total RNA. Briefly, 500 ng of RNA was reverse-transcribed using MMLV reverse transcriptase and an oligo(dT) primer containing the T7 promoter, and subsequently transcribed in vitro using T7 RNA polymerase, resulting in Cy3-labeled (control) and Cy5-labeled (*cis*-CA-treated) cRNAs. The cRNAs were purified using RNeasy Mini Spin columns (Qiagen) and then quantified with a NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Mixtures of 825 ng of Cy3-labeled and Cy5-labeled cRNAs were co-hybridized at 65°C for 17 h on an Agilent Technologies 4×44K Arabidopsis (v4) 60-mer oligo-microarray. The slides were then washed and the fluorescence intensity detected using an Agilent G2505B Scanner. Two independent biological replicates were assayed.

The fluorescence intensity of individual spots on scanned images was quantified and corrected for background noise using Feature Extraction software (Agilent Technologies). To ensure a high quality of analysis, only features that passed three criteria were analyzed. Features flagged in the Feature Extraction software as non-uniform (IsFeatNonUnifOL and IsFeatPopnOL), saturated (IsSaturated), or low signal (IsWellAboveBG) were omitted, and the remaining features were further filtered by the Feature Significance test at $p<0.01$. Total RNA profiling data were normalized via linear and lowess methods followed by spike-in normalization using a Two-Color RNA Spike-In Kit (Agilent Technologies). The ratio of the intensity in the *cis*-CA-treated sample (Cy5) to that in the control sample (Cy3) was calculated for each gene. We defined a gene as responsive when the ratio of both biological replicates was greater than three to one. Ratios shown are the average of the two independent experiments. In this manuscript, the gene ID number and abbreviation are from The Arabidopsis Information Resource website (TAIR: http://www.arabidopsis.org/). We used the web-based toolkit AgriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) for the Gene Ontology (GO) enrichment analysis (Du et al. 2010). Each expression pattern was analyzed with the Singular Enrichment Analysis program

and tested by a binomial test model with a false discovery rate of <0.01.

Accession numbers for microarray data in National Center for Biotechnology Information (NCBI)

Microarray data are available through Gene Expression Omnibus (GEO) Database in NCBI (http://www.ncbi.nlm.nih.gov/geo/). The accession numbers in the GEO databases are GSE37862 and GSE37899.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) analysis was performed as described (Golisz et al. 2011) to investigate the expression of particular genes in shoots and roots over time, and to validate our microarray data sets. Plants were treated with 20 μM *cis*-CA, 20 μM IAA, or nothing (control). They were sampled at 2 h and 6 h post-treatment, divided into roots and shoots, and immediately frozen in liquid nitrogen. Total RNAs were isolated with an RNeasy Plant Mini Kit. cDNAs were synthesized in a 10 μl reaction volume from 0.2 μg of total RNA in a Primescript Reverse Transcriptase Reagent Kit (Takara Biotech Inc.) following the manufacturer's instructions. PCR used the Power SYBR Green PCR Master Mix (Applied Biosystems). A primer pair was used to amplify the constitutively expressed control gene encoding elongation factor 1α (EF1α; At5g60390; Becher et al. 2004). Primers were designed using a website program (Universal Probe Library for Arabidopsis, https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000).

The following primers were used: EF1αF: 5′-TGAGCACGC TCTTCTTGTTTCA-3′, EF1αR: 5′-GGTGGTGGCATCCAT CTTGTTACA-3′, ydk1F: 5′-GTTTGCTTCCGGTCTCCTC-3′, ydk1R: 5′-CACGAGCAAGTTCCTTCCA-3′, GH3.1F: 5′-AAC TTATGCCGACCATTAAAGAA-3′, GH3.1R: 5′-TCTAGA CCCGGCACATACAA-3′, GH3.3F: 5′-CAGGTGACTGGA TTCTACAA-3′, GH3.3R: 5′-AAGTAAGAGCGATGCGTTCT-3′, LBD16F: 5′-GCTCGTCTTCATGACCCTGT-3′, LBD16R: 5′-GCCTTCATTTGCATGACTTG-3′, PUCHIF: 5′-TCATCA TGAGTTTGGTTCGTG-3′, PUCHIR: 5′-GCAGCAGCACCA TAGTTCG-3′, IAA5F: 5′-TGAAGACAAAGATGGAGATTG G-3′, IAA5R: 5′-TCCAAGGAACATTTCCCAAG-3′, IAA1F: 5′-GCTCCTCCTCCTGCAAAA-3′, IAA1R: 5′-GGAGCTCCG TCCATACTCAC-3′.

qRT-PCR was performed on the ABI StepOnePlus Real Time PCR System (Applied Biosystems) using SYBR Green. cDNA was diluted 1 : 30 with nuclease-free water. Reactions were performed in 20 μl containing 10 μl of qPCR Master Mix (Applied Biosystems), 2 μl of first-strand cDNA, and 0.2 μM of forward and reverse primers (Operon Biotechnologies, Tokyo, Japan). The thermal profile was 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Each sample was analyzed in three biological replicates, and the resulting data were analyzed using ABI StepOne^{TM} software version 2.1. A melting curve analysis was performed for each primer set to verify the presence of a single melting peak after amplification. For the calculation of the threshold cycle (C_T) values, the auto-C_T function was used. To normalize the target gene expression, the difference between the C_T of the target gene and the C_T of the constitutive control, *EF1α*, for the respective template was calculated (ΔC_T value). Relative transcript levels were calculated as $1000\times2^{-\Delta CT}$.

Results and discussion

Arabidopsis growth inhibitory activity of cis-CA

After treating Arabidopsis seedlings with exogenous *cis*-CA, concentration-dependent root growth inhibition was observed (Figure 1A), and adventitious roots and root hairs were induced (Figure 1B). Two individuals out of 18 produced adventitious roots at 4 μM of *cis*-CA, and 4 out of 13 at 8 μM. We estimated the EC_{50} of *cis*-CA as 2.98 μM, which was similar to the value of 1.62 μM from a previous dose–response bioassay using lettuce (Hiradate et al. 2005). Thus, we set the exposure concentration at 20 μM, which we expected to be high enough to promote *cis*-CA-related gene expression in a short time.

DNA microarray and Gene Ontology (GO) analysis

After 2 h of *cis*-CA treatment, 201 genes were upregulated and 36 genes downregulated. Among the

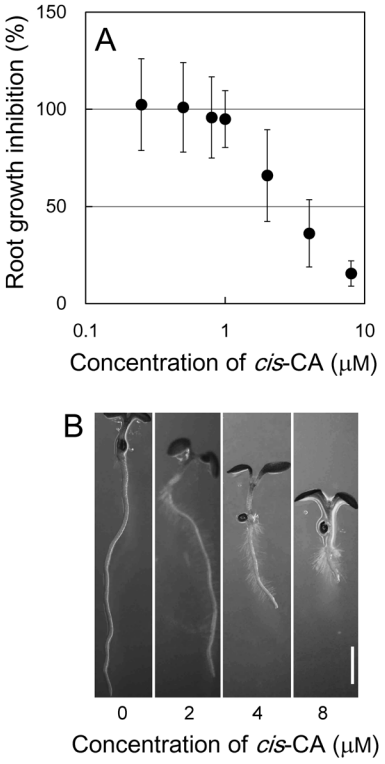

Figure 1. Effect of *cis*-cinnamic acid (*cis*-CA) on the growth of *Arabidopsis thaliana* seedlings. (A) Inhibitory activity of *cis*-CA on the elongation of Arabidopsis roots. Error bars indicate standard deviation of the means ($n>13$). (B) Phenotype of a 4-d-old seedling after exposure to 0, 2, 4 and 8 μM *cis*-CA. Bar=2 mm.

Table 1. Gene Ontology enrichment analysis of *Arabidopsis thaliana* genes upregulated 2 h after treatment with *cis*-cinnamic acid (*cis*-CA).

GO term	Ontology [a]	Description	No. in input list	No. in BG/Ref	*p* value[b]	False discovery rate
GO: 0010200	BP	Response to chitin	9	151	1.5E-07	5.0E-05
GO: 0042221	BP	Response to chemical stimulus	32	2085	3.9E-07	6.5E-05
GO: 0010033	BP	Response to an organic substance	24	1342	6.8E-07	7.6E-05
GO: 0003700	MF	Transcription factor activity	30	2173	6.2E-06	7.4E-04
GO: 0009743	BP	Response to carbohydrate stimulus	9	240	6.9E-06	5.8E-04
GO: 0009733	BP	Response to auxin stimulus	10	360	2.9E-05	0.002
GO: 0030528	MF	Transcription regulator activity	30	2417	4.1E-05	0.0025

[a] BP, biological process; MF, molecular function. [b] $p<1.0E-04$.

Table 2. Top 30 highest upregulated *Arabidopsis thaliana* genes ranked by the change ratio 2 h after treatment with *cis*-cinnamic acid (*cis*-CA).

ID No.	Description	Change ratio
AT4G37390	*YDK1* (*GH3.2*); indole-3-acetic acid amido synthetase	83.24
AT2G23170	*GH3.3*; indole-3-acetic acid amido synthetase	67.77
AT2G14960	*GH3.1*	56.80
AT1G69930	*ATGSTU11* (*A. thaliana* glutathione S-transferase)	55.09
AT1G76640	Calmodulin-related protein, putative	48.07
AT2G26400	*ARD*/*ATARD3*; acireductone dioxygenase	37.52
AT1G14550	Anionic peroxidase, putative	34.72
AT2G45760	*BAP2* (BON association protein 2)	33.06
AT2G23060	GCN5-related N-acetyltransferase (*GNAT*) family protein	26.16
AT1G28480	*GRX480*; thiol-disulfide exchange intermediate	25.23
AT3G42658	Transposable element gene	24.94
AT4G37295	Unknown protein	23.89
AT5G05220	Similar to hypothetical protein [*Vitis vinifera*]	21.71
AT5G52670	Heavy-metal-associated domain-containing protein	21.15
AT3G22910	Calcium-transporting ATPase, plasma membrane-type, putative	20.55
AT5G06080	*LBD33* (LOB domain-containing protein 33)	19.01
AT5G42380	*CML37*/*CML39*; calcium ion binding	18.65
AT2G47520	AP2 domain-containing transcription factor, putative	17.45
AT4G16820	Lipase class 3 family protein	17.05
AT1G56240	*ATPP2-B13* (phloem protein 2-B13); carbohydrate binding	16.72
AT4G37710	VQ motif-containing protein	16.16
AT1G80590	*WRKY66* (WRKY DNA-binding protein 66); transcription factor	15.83
AT5G22570	*WRKY38* (WRKY DNA-binding protein 38); transcription factor	15.80
AT2G42440	LOB domain protein 17 / (*LBD17*)	15.75
AT5G40000	AAA-type ATPase family protein	15.60
AT1G69920	*ATGSTU12*	15.53
AT3G51680	Short-chain dehydrogenase/reductase (*SDR*) family protein	15.30
AT5G43650	basic helix-loop-helix (*bHLH*) family protein	15.26
AT4G36950	MAPKKK21; ATP binding/protein kinase	14.93
AT4G37710	VQ motif-containing protein	14.87

upregulated ones, GO analysis annotated 196 genes. Singular Enrichment Analysis identified 23 GO terms as significant. The genes associated with response to chitin, response to chemical stimulus, response to an organic substance, transcription factors, response to carbohydrate stimulus, response to auxin stimulus, and transcription regulators were over-represented in the 196 genes (Table 1). The only GO category that is directly related to acute toxicity is the response to auxin stimulus.

The three genes that upregulated the most 2 h after the *cis*-CA treatment are in *GRETCHEN HAGEN-3* (*GH3*) family (*GH3.1*, *GH3.2*, and *GH3.3*); these genes encode enzymes that conjugate amino acids to IAA for auxin homeostasis (Table 2) (Staswick et al. 2005). In addition, some *LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE* (*LBD*) genes, which respond to auxin and regulate lateral root formation (Lee et al. 2009a), were upregulated by more than 10-fold. Six hours after treatment with *cis*-CA, the transcripts of members of both auxin-responsive families (*GH3.1*, *GH3.2*, *GH3.3*, *GH3.5*/*WES1*, *DFL1*/*GH3.6*, *LBD16*, *LBD17*, *LBD18*, *LBD29*, and *LBD33*) remained persistently high (Table 3). In contrast, *Aux*/*IAA* genes, which are upregulated early by IAA (Woodward and Bartel 2005), responded only slightly to *cis*-CA. In total, 20 to 30% of the genes which were upregulated by one of the two chemicals, *cis*-CA and IAA, also responded to the other (Figure 2). It should also be noted that four IAA-

Table 3. Change ratios of *Arabidopsis thaliana* auxin-responsive genes upregulated in response to *cis*-cinnamic acid (*cis*-CA) or indole-3-acetic acid (IAA) treatments.

ID No.	Gene name	*cis*-CA		IAA
		2 h	6 h	6 h
AT2G14960	*GH3.1*	56.8	91.7	40.7
AT4G37390	*GH3.2* (*YDK1*)	83.2	122	39.8
AT2G23170	*GH3.3*	67.8	68.7	136
AT4G27260	*GH3.5*/*WES1*	3.61	8.67	9.63
AT5G54510	*DFL1*/*GH3.6*	2.62	6.28	11.2
AT4G14560	*IAA1*	1.89	2.39	35.1
AT1G15580	*IAA5*	3.13	3.85	151
AT3G15540	*IAA19*	3.75	6.75	19.6
AT2G46990	*IAA20*	3.11	4.31	26.5
AT4G32280	*IAA29*	1.86	8.91	60.7
AT3G62100	*IAA30*	4.48	6.22	21.8
AT2G01200	*IAA32*	1.01	6.19	19.0
AT2G42430	*LBD16*	13.3	21.9	14.4
AT2G42440	*LBD17*	15.6	58.75	49.7
AT2G45420	*LBD18*	4.02	11.5	4.89
AT3G58190	*LBD29*	—[a]	164.7	18.67
AT5G06080	*LBD33*	19.01	27.87	82.40
AT5G18560	*PUCHI*	—[a]	29.45	17.82

[a] Only one trial responded to *cis*-CA treatment. *LBD29*: 1.64, 21.5; *PUCHI*: 6.51, 1.75.

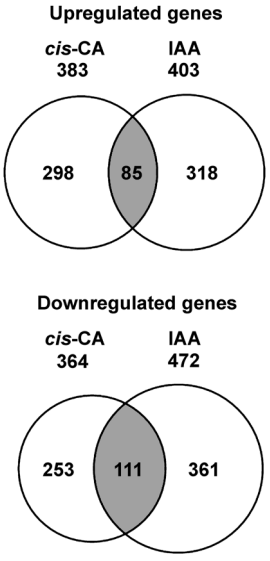

Figure 2. Venn diagrams of the numbers of *Arabidopsis thaliana* genes responding to *cis*-cinnamic acid (*cis*-CA) or indole-3-acetic acid (IAA) 6 h after treatment. The numbers within the overlapping regions of the circles indicate genes that responded to both treatments.

upregulated genes (AT1G01520: *myb* family transcription factor; AT1G69570: Dof-type zinc finger domain-containing protein; AT2G46830: CCA1; and AT3G21150: zinc finger family protein) were downregulated by the *cis*-CA treatment. These observations implied that the physiological function of *cis*-CA is distinguishable from that of auxin.

The *GH3* family is one of the best-characterized auxin-responsive gene families in Arabidopsis. It was first isolated from *Glycine max* as an early auxin-inducible gene (Hagen et al. 1984) and responds rapidly to exogenous applications of auxin. The *GH3* promoter often contains an auxin-responsive element called AuxRE, which binds auxin response factors (ARFs) to regulate auxin-related transcription. AuxREs contain the consensus sequence 5′-TGTCTC-3′ (Guilfoyle and Hagen 2001). A gain-of-function mutation of *GH3.2* selected by activation tagging caused a dwarf phenotype, short hypocotyl and primary roots, and a reduced number of lateral roots (Takase et al. 2004). In addition, the overexpression of *GH3.5* exhibited reduced growth and altered leaf shape (Park et al. 2004), and the overexpression of *DFL1*/*GH3.6* caused a dwarf phenotype (Nakazawa et al. 2001). Because *GH3* genes have important functions in regulating the growth of seedlings, they might be targets of *cis*-CA.

LBD genes encode proteins harboring a plant-specific lateral organ boundary domain; 42 *LBD* genes have been found in Arabidopsis (Iwakawa et al. 2002; Shuai et al. 2002). Their biological roles are not well understood, but some functions have been identified using loss-of-function studies. ARF7 and ARF19, which regulate lateral root formation, activate *LBD16* and *LBD29* directly and *LBD33* secondarily (Okushima et al. 2007). By using a double-knockout mutant, Lee et al. (2009a) showed that *LBD16* and *LBD18* regulate lateral root formation. Interestingly, the target genes of ARF7 and ARF19, *GH3.2* (Takase et al. 2004), *LBD16*, *LBD18*, *LBD29*, and *LBD33* (Lee et al. 2009b; Okushima et al. 2007), tend to be upregulated rapidly after *cis*-CA treatment. At present, it is unclear whether ARF7 and ARF19 are involved in the exogenous *cis*-CA upregulation of the *GH3.2* and *LBD* genes. Loss-of-function studies using single and double knockout mutants of *ARF7* and *ARF19*, now in progress, will answer these questions.

Time-course qRT-PCR analysis

We further focused on the *Aux*/*IAA*, *GH3* family, and *LBD* family genes, based on the results of the DNA microarray analysis. A qRT-PCR analysis over time revealed that 2 h post-exposure to *cis*-CA, the expression of *IAA1*, *IAA5*, and *GH3* genes was upregulated in roots at levels comparable to those treated with IAA, and the levels were maintained for four hours (Figure 3). The shoot expression levels of these genes, 2 h after treatment with *cis*-CA, in contrast, were negligible (Figure 3A). Conversely, there was shoot expression in these genes, except for *GH3.2*, from 2 to 6 h after treatment with IAA (Figure 3B). These results indicate that *cis*-CA, like IAA, induces the expression of *Aux*/*IAA*, *GH3*, *LBD* and *PUCHI* genes; however, unlike IAA, the response is root-specific. Two explanations are possible for the root-specificity of response to *cis*-CA. Either the *cis*-CA signal transduction is limited to the roots, and/or

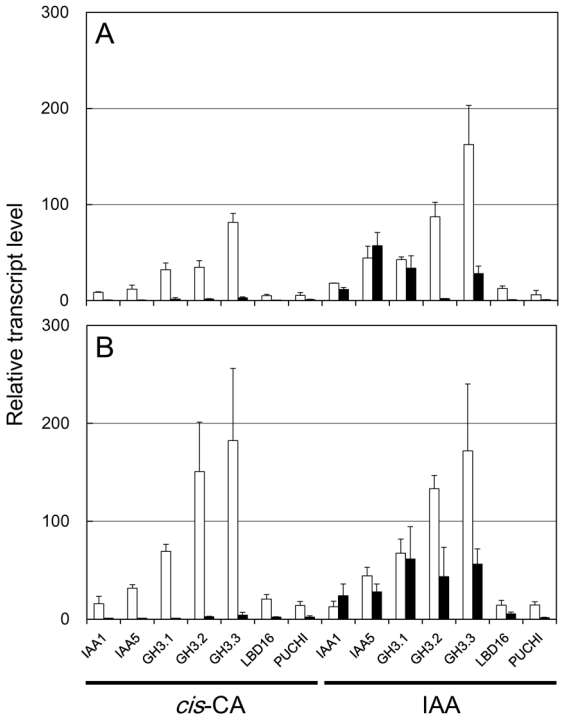

Figure 3. Quantitative real-time PCR analysis of expression of seven auxin-responsive genes encoding IAA1, IAA5, GH3.1, GH3.2, GH3.3, LBD16, and PUCHI in *Arabidopsis thaliana* after treatment with *cis*-cinnamic acid (*cis*-CA) or indole-3-acetic acid (IAA). (A) Relative transcript levels in shoots or roots 2 h after exposure to *cis*-CA or IAA. (B) Relative transcript levels in shoots or roots 6 h after exposure to *cis*-CA or IAA. Black and white bars indicate the transcript levels in shoots and roots, respectively. Each experiment used three biological replicates. Error bars indicate a standard error of the mean (n=3).

the mechanism of *cis*-CA transportation is substantially different from that of IAA. The difference could be supported by a previous study reporting that *AUX1*, which encodes an auxin influx transporter, did not react to *cis*-CA. In addition, an *AUX1* knockout mutant showed an unaltered response to *cis*-CA but decreased sensitivity to IAA (Wong et al. 2005).

The DNA microarray analysis showed that the *Aux*/*IAA* genes responded only slightly to *cis*-CA (Table 3). The *Aux*/*IAA* family genes respond to auxin within 5 to 30 min (Abel and Theologis 1996). Aux/IAA proteins interact with ARFs that bind to AuxREs in the auxin-responsive gene promoters and negatively modulate auxin-regulated gene expression as transcriptional repressors through heterodimerization with ARF transcription activators (Woodward and Bartel 2005). In the qRT-PCR analysis, we focused on *IAA1* and *IAA5*, which are notably upregulated early in response to auxin (Lee et al. 2009b). Their root expression levels in response to *cis*-CA were unexpectedly comparable to their expression levels in response to IAA. In brief, they were upregulated by *cis*-CA in the qRT-PCR analysis (Figure 3), although they were hardly upregulated in the microarray analysis (Table 3). These observations are contradictory to each other, but since total RNAs of whole seedlings were used for the microarray analysis, the detection sensitivity might have been less than in the tissue-specific gene analysis.

Whether *cis*-CA and auxin function in identical manners in plant tissues has been a controversial subject (van Overbeek et al. 1951; Wong et al. 2005). Recent reports favor the idea that the responses are distinguishable (Guo et al. 2011; Wong et al. 2005), and this idea was supported by our data. First, a low percentage of the genes expressed responded to both *cis*-CA and IAA. Second, several IAA-upregulated genes were downregulated by *cis*-CA. Finally, *cis*-CA induced early auxin-responsive genes only in the roots in a tissue-specific gene analysis. The present study suggests the activation of some early auxin-responsive genes by *cis*-CA. This is the first demonstration of gene responses to *cis*-CA, although its mode of action has long been disputed (Guo et al. 2011; van Overbeek et al. 1951; Wong et al. 2005; Yang et al. 1999). It is necessary, however, to conduct further experiments using mutants that are defective in auxin signaling in order to clarify that responses to *cis*-CA in gene expression are actually dependent on auxin signaling pathway. The present study will serve as a base for future studies on the interactions between *cis*-CA and its receptors, and on the translated proteins monitored in this study. Various derivatives of *cis*-CA, which aim at enhancing bioactivity and utility in gene-expression analyses, are under way (Abe et al. 2012).

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