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ORIGINAL ARTICLE

Microarray analysis of *Arabidopsis* plants in response to allelochemical L-DOPA

Anna Golisz · Mami Sugano · Syuntaro Hiradate · Yoshiharu Fujii

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Abstract Velvetbean (*Mucuna pruriens*) plants impede the growth of neighboring plants. One compound, 3-(3′,4′-dihydroxyphenyl)-L-alanine (L-DOPA), is responsible for the allelopathic capacity of velvetbean. This compound is an active allelochemical that decreases root growth of several plant species. In mammals, L-DOPA is a well-known therapeutic agent for the symptomatic relief of Parkinson's disease. However, its mode of action in plants is still not well understood. To address such issues, gene expression in *Arabidopsis thaliana* plants, which had been exposed to L-DOPA, was analyzed using DNA microarrays. After 6 h of L-DOPA exposure, the expression of 110 genes was significantly upregulated, and the expression of 69 genes was significantly downregulated. These induced genes can be divided into different functional categories, mainly on the basis of subcellular localization, metabolism, and proteins with a binding function or cofactor requirement. Based on these results, we suggest that L-DOPA acts by two mechanisms: it influences amino acid metabolism and deregulates metal homeostasis, especially that of iron, which is required for the fundamental biological processes of all organisms.

Keywords Allelopathy · 3-(3′,4′-Dihydroxyphenyl)-L-alanine · DNA microarray · Gene expression · Mucuna

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Abbreviations

ASP2	Aspartate aminotransferase
bHLH	Basic helix-loop-helix
EMB2454	Embryo defective, zinc ion binding
FER	Ferritin
FRO	Ferric reduction oxidase
L-DOPA	3-(3′,4′-Dihydroxyphenyl)-L-alanine
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NRAMP	Natural resistance-associated macrophage protein
OPT	Oligopeptide transporter
PAL	Phenylalanine ammonia-lyase
POD	Peroxidase
ZIF	Zinc induced facilitator

Introduction

Allelopathy is a phenomenon of chemical interaction among plants. Plants with allelopathic capabilities play important roles in agricultural production. Natural toxins, including allelochemicals that suppress or eliminate competing plant species near the source plant, have been extensively studied because of their agricultural potential as herbicides (Dayan et al. 2000). A component of velvetbean (*Mucuna pruriens* L. DC.), 3-(3′,4′-dihydroxyphenyl)-L-alanine (L-DOPA), is an allelochemical (Fujii et al. 1991). Because velvetbean has special abilities such as weed smothering, tolerance to pests, suppression of the nematode population, and soil improvement in its physical structure, the usage of plants could drastically reduce the application of artificial chemicals to agricultural crops (Fujii 2003). Fresh velvetbean leaves contain from 0.5 to

1.5% L-DOPA (by weight), and the yield of fresh leaves and stems from velvetbean plants ranges from 20 to 30 t ha^{-1} (Fujii et al. 1991, Fujii 1999). Therefore, velvetbean could produce 100–450 kg ha^{-1} of L-DOPA. L-DOPA is quite unstable in soils; especially those with a high pH value have been shown to possess the ability to eliminate L-DOPA through transformation and adsorption reactions, resulting in the detoxification of L-DOPA (Hiradate et al. 2005; Furubayashi et al. 2005, 2007; Dayan and Duke 2009). The phytotoxic activity of L-DOPA in the absence of soil has been observed at concentrations as low as 1×10^{-4}–1×10^{-5} M (Fujii 2003; Nishihara et al. 2004; Furubayashi et al. 2005; Hiradate et al. 2005). Concentrations in this range are high enough to reduce the growth of neighboring plants and to reduce the rate of root growth in several plant species (Fujii et al. 1991; Hachinohe et al. 2004; Nishihara et al. 2004). Velvetbean seeds also contain high concentrations of L-DOPA (6–9%) (Damodaran and Ramaswamy 1937; Rehr et al. 1973), which acts as a chemical barrier against insect attacks (Bell and Janzen 1971; Premchand 1981).

In the mammalian brain, L-DOPA is the precursor of the neurotransmitter dopamine and is the most effective therapeutic agent for the symptomatic relief of Parkinson's disease (Mercuri and Bernardi 2005). In animal skin, hair, feathers, and fur as well as in insect cuticle, L-DOPA is oxidized to dopaquinone and finally converted to melanin (Fujii 2003).

In plants, it is a precursor of melanin and many alkaloids, catecholamines, flavonoids, and phenylpropenoids (Hahlbrock and Scheel 1989; Pattison et al. 2002). According to Hachinohe and Matsumoto (2005, 2007), the phytotoxicity of L-DOPA in barnyard grass and lettuce is attributed to the oxidative damage caused by reactive oxygen species and/or free radical species that are generated from the melanin synthesis pathway. Soares et al. (2007) suggested that L-DOPA-induced inhibition in soybean roots occurs because of a cell wall stiffening process related to cross-linking between cell wall polymers that occurs during lignin production.

Little is known about the function of allelochemicals and the defensive responses of plants to them. *Arabidopsis thaliana* L. has been a good model for studying plant responses to allelochemicals and other environmental toxins (Baerson et al. 2005). Moreover, microarray techniques have become a standard tool for genome-wide monitoring of gene expression. Hence, the present work uses microarrays to analyze the gene expression profiles of *A. thaliana* L. plants exposed to L-DOPA. To the best of our knowledge, this is the first study to evaluate gene responses to this compound using microarrays. This study may lead to a better understanding of the mode of action of L-DOPA in plants.

Materials and methods

Plant material, growth conditions, and experimental treatment

Seeds of *A. thaliana* L. (Col-4, source NASC N933), bought from Lehle Seeds Company (Round Rock, TX, USA) were sterilized for 1 min in 70% ethanol, followed by the application of 1% sodium hypochlorite with one drop of Tween 20 (Sigma–Aldrich Group, St. Louis, MO, USA) for 6 min, after which they were rinsed ten times with distilled water. Sterilized seeds were placed on 1% solidified agar (Nacalai 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, Osaka, Japan) in an Agripot (Kirin, Tokyo, Japan). The seeds in the Agripots were cold treated (4°C) for 3 days in darkness and then transferred to a growth chamber. Plants were maintained in the growth chamber on a schedule of 16 h of light (22°C) and 8 h of dark (20°C) (Boyes et al. 2001; Baerson et al. 2005). After 20 days in the growth chamber, the plants were removed from the agar and transferred to a solution culture in distilled water containing 100 mg kg^{-1} (of water) of L-DOPA (Nacalai Tesque). This concentration reflects specific activities as determined in previous studies (Fujii et al. 1991). The effective concentration of the compound to induce half-maximum inhibition for lettuce in water was 50 mg kg^{-1}. However, we used a higher concentration because we had a shorter time of exposure. As a control, plants were transferred in parallel to distilled water. After 6 h of treatment, plants were collected and immediately frozen in liquid nitrogen, and then stored at −80°C prior to analysis. Each treatment was replicated three times in each of the three independent experiments.

Microarray data analysis

Total RNA from both the treated and the control 20-day-old *Arabidopsis* plants was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of total isolated RNA were assessed by spectrophotometry and gel electrophoresis, respectively. RNA was amplified and labeled as described in the GeneChip Expression Analysis Technical Manual, Rev. 5 (Affymetrix, Santa Clara, CA, USA). Total RNA (1 μg) was converted into double-stranded cDNA using the One-Cycle cDNA Synthesis Kit (Affymetrix). In vitro transcription reactions were performed using a GeneChip IVT labeling kit (Affymetrix), which includes T7 RNA polymerase and biotin-labeled ribonucleotides. The cDNA (15 μg) was hybridized to an Affymetrix GeneChip Arabidopsis ATH1 Genome

Array. The array was incubated for 16 h and then automatically washed and stained using the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The genome array was scanned using a GeneChip Scanner 3000 7G. Expression analysis was performed using GeneSpring Viewer 5.2 software (Agilent Technologies, Santa Clara, CA, USA). We defined genes as responsive when transcripts were detected in at least two of the three independent experiments (biological replicates) and when the signals were significantly different ($P \leq 0.05$) as compared to the control plants.

Quantitative real-time RT-PCR analysis

Total RNA from both the treated and the control 20-day-old *Arabidopsis* plants was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). The synthesis of cDNA was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The kits were used according to the manufacturers' instructions. A primer pair was used to amplify the constitutively expressed control gene encoding elongation factor 1α (EF1α; At5g60390; Becher et al. 2004). The primers for quantitative real-time RT-PCR were designed using Primer Express software (v 2.0, Applied Biosystems, Foster City, CA, USA). The sequences of all primers are presented in Table 1.

Quantitative real-time RT-PCR was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green. cDNA was diluted 1:50 with nuclease-free water. Reactions were performed in 20 µl of liquid, containing 9 µl of qPCR Master Mix (Eurogentec, Liege, Belgium), 0.6 µl of SYBR Green (Eurogentec), and 0.5 µmol of forward and reverse primers (Operon Biotechnologies, Tokyo, Japan). The following standard thermal profile was used: 2 min at 50°C, 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 technical replicates, and the resulting data were analyzed using ABI Prism 7000 SDS Software. For the calculation of the threshold cycle (C_T) values, the auto-C_T function was used. For further calculations, the mean value of each triplicate was used. To normalize the target gene expression, the difference between the C_T of the target gene and the C_T of EF1α (constitutive control) for the respective template was calculated (ΔC_T value). To calculate fold changes in gene expression, the ΔC_T value was calculated as follows: $\Delta C_T = C_T$ (target gene) $- C_T$ (constitutive control gene). Relative transcript levels were calculated as $1{,}000 \times 2^{-\Delta C_T}$.

Results

The results demonstrated that L-DOPA influenced gene expression in *A. thaliana* plants. Microarray analysis showed that the expression of 110 genes was significantly upregulated, and that the expression of 69 genes was significantly downregulated ($P \leq 0.05$). Among the upregulated genes, 32 were induced ≥ threefold as compared with their expression in the control plants (Table 2). The highest levels of expression were for an expressed protein of unknown function (At1g47400) and for a protein bHLH (At5g04150) with fold changes of 27.67- and 16.90-fold changes, respectively. Besides these proteins (transcription factor), L-DOPA affected the expression of another bHLH protein (At3g47640) and the expression of the WRKY

Table 1 Primers used for quantitative real-time RT-PCR assays

Target gene	Primer sequence	Amplicon size (bp)
At5g60390 elongation factor	F 5′-TGAGCACGCTCTTCTTGCTTTCA-3′	76
	R 5′-GGTGGTGGCATCCATCTTGTTACA-3′	
Atlg05250 peroxidase	F 5′-CACACCATTGGAATCTCTAGTTGC-3′	64
	R 5′-CCTTTCCTGTGAAGTTGTAGAGACG-3′	
Atlg47400 expressed protein	F 5′-CAAGAGATTTGACCATGCTTCC-3′	83
	R 5′-CACCACCATTCTCACTATATGCC-3′	
At3g20340 expressed protein	F 5′-ACGTCCATGACTTGACCTTCC-3′	65
	R 5′-GTACCCGCGATTGTTCAGG-3′	
At4g23810 WRKY family transcription factor	F 5′-TTTAGGCGCCAAATTCCC-3′	65
	R 5′-CCCAACAGTTTTGTGTGCTACG-3′	
At5g01600 ferritin FER1	F 5′-AACTCCACCCTATCGTCTCACC-3′	74
	R 5′-CTCCATTGCATATAAAGCATCTCC-3′	
At5g04150	F 5′-TGTACTCTTCACTTCGTGCTCTCT-3′	61
basic helix-loop-helix (bHLH) family protein	R 5′-GGAATGCTCAGCTTCCTCTTTTGA-3′	

Table 2 Genes significantly ($P \leq 0.05$) induced > threefold after a 6-h exposure to L-DOPA in *Arabidopsis thaliana* plants

ID_Affymetrix	Gene Title_Affymetrix	Fold-change	*P* value
Atlg47400	Expressed protein	27.67	0.00
At5g04150	Basic helix-loop-helix family protein (bHLHIOl)	16.90	0.00
At5g53450	Protein kinase (ORG1)	9.08	0.00
At4g23810	WRKY family transcription factor (WRKY53)	7.84	0.01
At4gl6370	Oligopeptide transporter family protein (OPT3)	7.60	0.00
At2g43620	Chitinase, putative	7.11	0.00
Atlg77120	Alcohol dehydrogenase (ADH)	6.67	0.01
At3g47640	Basic helix-loop-helix family protein (bHLH047)	6.06	0.00
At5gl3740	Zinc-induced facilitator (ZIF1)	5.51	0.00
At3g18290	Embryo defective (EMB2454), zinc ion binding	5.19	0.00
At4gl4400	Ankyrin repeat protein	5.07	0.03
At3g27060	Ribonucleoside-diphosphate reductase small chain	4.54	0.00
At2gl8680	Expressed protein	4.32	0.00
Atlg35710	Leucine-rich repeat transmembrane protein kinase	4.31	0.02
At2g30770	Cytochrome P450, CYP71A13	4.20	0.01
At3g02550	LOB domain protein	4.20	0.02
Atlg23020	Ferric-chelate reductase (FR03)	4.06	0.01
Atlg66880	Serine/threonine protein kinase family protein	3.91	0.00
Atlg21120	O-methyltransferase, putative	3.57	0.00
At2gl6060	Non-symbiotic hemoglobin 1	3.51	0.01
At4g23280	Protein kinase, putative	3.39	0.02
At2g46440	Cyclic nucleotide-regulated ion channel	3.38	0.02
Atlg64400	Long-chain-fatty-acid CoA ligase, putative	3.31	0.01
At4g16890	Disease resistance protein	3.29	0.05
At3g56360	Expressed protein	3.27	0.01
Atlg48300	Expressed protein	3.23	0.00
At2gl4610	Pathogenesis-related protein 1	3.22	0.05
At5g67330	NRAMP4 metal ion transporter	3.20	0.00
Atlg72060	Expressed protein	3.13	0.02
Atlg26420	FAD-binding domain-containing protein	3.10	0.01
Atlg64980	Expressed protein	3.08	0.01
At5g02580	Expressed protein	3.04	0.03

family transcription factor (At4g23810) with 6.06- and 7.84-fold changes, respectively (Table 2). Furthermore, more than ten upregulated genes were responsible for metal homeostasis, especially iron homeostasis. Apart from the genes listed above, other notable genes included OPT (At4g16370), FRO (At1g23020), and NRAMP4 metal ion transporter (At5g67330) with 7.60-, 4.06-, and 3.20-fold changes, respectively (Table 2). In addition, two other notable genes were two alcohol dehydrogenase genes (At1g77120, At1g64710) with fold changes of 6.67 and 2.26, respectively. These two genes are involved in the response to osmotic stress (TAIR database http://www.arabidopsis.org/index.jsp). Furthermore, other upregulated genes included those involved in the defensive response to biotic stress such as ankyrin repeat protein (At4g14400), disease resistance protein (At4g16890), and pathogenesis-related protein 1 (At2g14610; Table 2). It is possible that the phytotoxicity of L-DOPA influences the expression of genes involved in responses to both abiotic and biotic stresses.

The upregulated genes were distributed into 17 functional categories (Fig. 1a) based on FunCat assignments available through the MIPS *A. thaliana* database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). The largest categories were "subcellular localization", "protein with binding function or cofactor requirement", and "metabolism", representing 30.8, 25.2, and 25.2% of all functions assigned, respectively. Also noteworthy functional categories were "cellular transport, transport facilities, and transport routes" (17.7%), "cell rescue, defence, and virulence" (14.0%), and "transcription" (3.7%; Fig. 1a).

The 69 downregulated genes were distributed into 16 functional categories based on FunCat database (Fig. 1b).

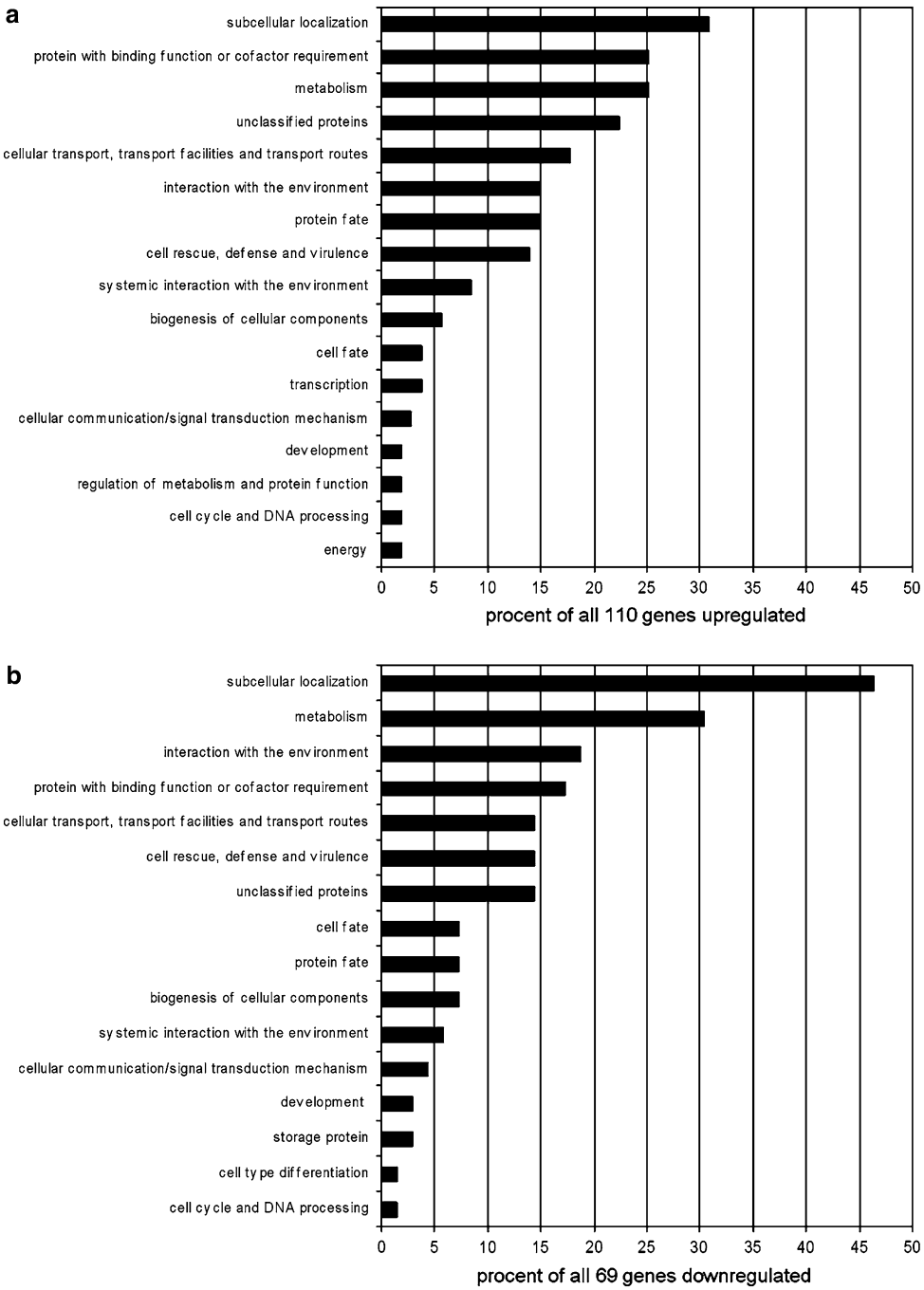

Fig. 1 Genes that were significantly upregulated (**a**) and downregulated (**b**) in *Arabidopsis* plants after 6 h of exposure to 100 mg kg^{-1} of L-DOPA. Genes were assigned to various functional categories based on the FunCat scheme devised by Munich Information Center for Protein Sequences

The main categories were "subcellular localization" and "metabolism", representing 46.3 and 30.4% of all functions assigned, respectively. Among these downregulated genes were those induced by photo-oxidative stress (TAIR database http://www.arabidopsis.org/index.jsp), such as FER1 (At5g01600) and expressed protein (At3g20340), with 0.28- and 0.40-fold changes, respectively (Table 3). Moreover, ferredoxin–nitrate reductase (At2g15620), which is involved in the second step of nitrate assimilation (TAIR), was also downregulated with a 0.47-fold change. The protein product of this gene functions in chloroplasts as an electron carrier in the photosynthetic electron transport chain. Notably in the presence of L-DOPA, more than ten genes that function in the transport of ions such as zinc (At4g36050), copper (At3g43670 and At4g14940), calcium (At1g76650), and ferric iron (At2g40300 and At3g56090; Table 3) were downregulated, suggesting that L-DOPA influences ion transport and homeostasis in plants. Notably six peroxidase genes that are involved in the response to oxidative stress were also downregulated following L-DOPA exposure. These include At1g05250, At4g30170, At4g08390, At4g26010, At5g15180, and At2g37130 with

Table 3 Selected genes that were significantly ($P \leq 0.05$) downregulated in *Arabidopsis thaliana* after a 6 h exposure to L-DOPA

ID_Affymetrix	Gene title_Affymetrix	Fold-change	*P* value
Atlg05250	Peroxidase 1/2	0.18	0.00
At4g30170	Peroxidase 45	0.27	0.01
At5g01600	Ferritin 1 (FER1)	0.28	0.05
At4g08390	L-Ascorbate peroxidase, stromal	0.31	0.03
At2g40300	Ferritin, putative (FER4)	0.34	0.04
At3g56090	Ferritin, putative (FER3)	0.36	0.05
Atlg09240	Nicotianamine synthase, putative	0.37	0.01
At4g26010	Peroxidase 44, putative	0.37	0.03
At3g20340	Expressed protein	0.40	0.00
At4g14940	Copper amine oxidase, putative	0.40	0.04
At5gl5810	Peroxidase 56, putative	0.44	0.00
Atlg76650	Calcium-binding EF hand family protein	0.47	0.01
At2g15620	Ferredoxin-nitrate reductase, putative	0.47	0.05
At5g65870	Phytosulfokmes 5 (PSK5)	0.47	0.00
At5g51010	Rubredoxin family protein	0.49	0.02
At3g43670	Copper amine oxidase, putative	0.50	0.03
At2g46650	Cytochrome b5, putative	0.50	0.04
At4g36050	Endonuclease/exonulease/phosphatase family protein	0.50	0.02
Atlg60960	Metal transporter, putative (IRT3)	0.50	0.04
At2g47550	Pectinesterase family protein	0.50	0.03
At5g47500	Pectinesterase family protein	0.50	0.03
At2g37130	Peroxidase 21	0.50	0.04
At5g67070	Rapid alkalinization factor (RALF) family protein	0.50	0.03

0.18-, 0.27-, 0.31-, 0.37-, 0.44-, and 0.50-fold changes, respectively (Table 3). Moreover, two important factors for plant growth were also downregulated: one was phytosulfokines 5 (At5g65870), which encodes a unique plant peptide growth factor, with a 0.47-fold change and the second was rapid alkalinization factor (At5g67070) with a 0.50-fold change; the protein encoded by this gene has an essential role in the physiology of *Arabidopsis*, where it regulates growth and development (Table 3).

To verify the microarray results, quantitative real-time RT-PCR assays were carried out for three upregulated and three downregulated genes (Fig. 2). The fold changes for a given gene were very similar for both the methods. In the case of the upregulated genes, both microarray and real-time RT-PCR results showed that the relative transcript level of expressed protein (At1g47400) was the highest among other tested genes as compared with the control sample, while the transcript with the greatest reduction in expression was peroxidase (At1g05250) for both methods (Fig. 2).

Discussion

In the past few decades lots of allelochemicals have been identified but the mode of actions of many of them are not yet clear. In the present study, we focused on the analysis of transcriptome response in *Arabidopsis* plants that were exposed to the allelochemical L-DOPA. In this experiment, phytotoxic compounds were applied directly to water to avoid the barrier that produces soil or agar. Thus, plants directly absorbed the allelochemicals through the roots. The plants were exposed to L-DOPA for only a short time (6 h) to study the primary response. Based on previous studies (Nakajima et al. 1999; Hachinohe and Matsumoto 2005, 2007; Soares et al. 2007), we expected that the response of plants will affect genes involved in oxidative stress, the pathway of melanin synthesis, lipid peroxides, phenolic compounds, and lignification, and that they will also influence on amino acid metabolism.

It has been suggested that all catechol-like compounds, including L-DOPA, may have a similar mode of action (Fujii 1999). Microarray analyses revealed that the gene expression profiles of *Arabidopsis* plants that were exposed to the catechol compounds, such as rutin, gallic acid, and fagomine differ from those described in the present report (Golisz et al. 2008).

According to current literature, the mode of L-DOPA action in plants differs depending on its source and the treatment conditions. Dose/response relationships and time of exposure are crucial to allelopathy studies (Belz et al. 2007). The phytotoxic effects of L-DOPA that occur in the

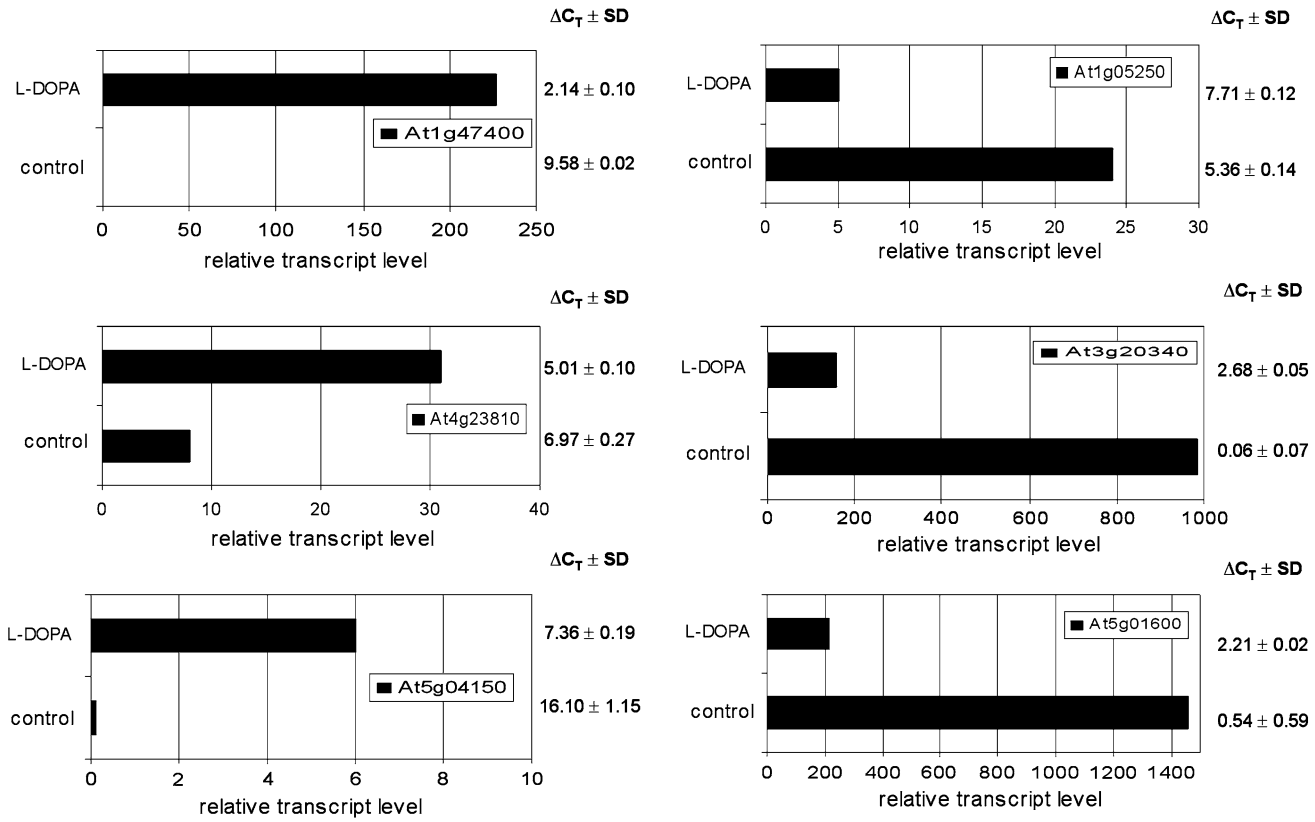

Fig. 2 Quantitative real-time RT-PCR analysis of expression of six selected genes encoding expressed protein (At1g47400), WRKY family transcription factor (At4g23810), bHLH family protein (At5g04150), peroxidase (At1g05250), expressed protein (At3g20340), and FER1 (At5g01600) in *Arabidopsis thaliana*. The transcript levels in plants were assessed by the quantitative real-time RT-PCR following the 6-h exposure of L-DOPA the 20-day-old plants. The ΔC_T values and the relative transcript levels were means of three technical replicates. The ΔC_T values were calculated as follows: ΔC_T (target gene) = C_T (target gene) − C_T (constitutive control gene: EF1α), where C_T is the cycle number at which the PCR product exceeded a set threshold. Relative transcript levels (RTL) were calculated as follows: RTL = 1,000 × $2^{-\Delta C_T}$

roots of lettuce and barnyard grass after 5 days of exposure at a concentration of 10^{-4} M, are due to oxidative damage caused by reactive oxygen species and/or free radical species generated by the melanin synthesis pathway (Hachinohe and Matsumoto 2005, 2007). In the roots of cucumber after 8 days of treatment with 250–500 μM L-DOPA, the amount of tyrosine and phenylalanine increased remarkably (Nakajima et al. 1999). Similar results were found in soybean roots, where PAL (EC 4.3.1.5) and POD (EC 1.11.1.7) activities, phenolic compounds, and lignin content increased, whereas length and fresh and dry weight of the roots decreased after 24 h of treatment with 0.1–1.0 mM L-DOPA (Soares et al. 2007). These results are consistent with our finding that L-DOPA influenced the amino acid metabolism of phenylalanine and tyrosine. Compared with previous studies (Nakajima et al. 1999; Hachinohe and Matsumoto 2007; Soares et al. 2007), our treatment was relatively short (only 6 h); nevertheless, despite this short period of exposure to L-DOPA, an increased expression of genes that play an important role in amino acid metabolism, such as tyrosine aminotransferase (At5g36160 and At5g53970; 1.27- and 1.17-fold change; EC 2.6.1.5) and all five types of aspartate aminotransferase, particularly ASP2 (At5g19550; 1.52-fold change; EC 2.6.1.1) was observed. Moreover, the tryptophan synthase genes At3g54640 and At4g27070 (EC 4.2.1.20) were notably affected with 1.62- and 1.46-fold changes, respectively. However, the expression level of the gene that encodes PAL was almost unchanged as compared with that of control plants (data not shown).

Our results substantiated the findings of previous studies (Nakajima et al. 1999; Hachinohe and Matsumoto 2007; Soares et al. 2007) that L-DOPA affects amino acid metabolism; however, this current research shows that the response of *Arabidopsis* plants to L-DOPA is similar to the response that follows iron deficiency. After L-DOPA treatment, the regulation of metal homeostasis, especially iron homeostasis, was disrupted in *Arabidopsis* plants. Iron is an essential nutrient for all organisms. Ferric reduction oxidase (FRO) is the enzyme responsible for the plasma membrane Fe(III) chelate reductase activity that is induced by iron deficiency in *Arabidopsis* (Thimm et al. 2001;

Grotz and Guerinot 2006). In our results, FRO3 (At1g23020) in treated plants was upregulated 4.06-fold compared with that in control plants (Table 2), which is in accordance with published reports of iron deficiency effects on the expression of this gene (Wintz et al. 2003; Puig et al. 2007; Buckhout et al. 2009). AtFRO3 may possibly function both in iron metabolism in roots and in iron reduction on the root surface (Wu et al. 2005). FRO is able to reduce copper (Mukherjee et al. 2006). Therefore, under conditions of low copper, the expression of FRO3 would be induced, as occurred in our experiment. Iron deficiency also induces rapid changes in zinc homeostasis (Buckhout et al. 2009) including increased ZIF1, which is responsible for transport of Zn ligands into vacuoles. These results suggest that L-DOPA influences not only on iron homeostasis but also copper and zinc homeostasis. The upregulation of FRO3 under L-DOPA stress correlated with many essential genes that are regulated by iron deficiency: transcription factors (At5g04150-bHLH101, At3g47640-bHLH047, At3g18290-EMB2454); transporters (At4g16 370-OPT3, At5g67330-NRAMP4, At5g13740-ZIF1), and expressed protein (At1g47400, At3g56360, At1g48300) (Fig. 3). These results from our study correspond well with a previous analysis by Buckhout et al. (2009) of gene transcripts that were upregulated by early iron deficiency. In our results, the highest upregulated genes were expressed protein (At1g47400) and transcription factor bHLH101 (At5g04150) belonging to a subgroup of bHLH Ib genes (Table 2). Both of them were strongly up regulated by iron deficiency in the roots and leaves of *Arabidopsis* (Wang et al. 2007; Buckhout et al. 2009).

In response to iron deficiencies, plants accumulate metal transporters of the NRAMP family, such as AtNRAMP1, AtNRAMP3, and AtNRAMP4 (Grotz and Guerinot 2006). Metal transporters of the NRAMP family have the ability to transport both iron and manganese (Lanquar et al. 2005, 2010). In our results, AtNRAMP4 (At5g67300) was upregulated 3.20-fold compared with the control plants (Table 2). This gene accumulates in both the roots and shoots of *Arabidopsis* and plays a role in the intracellular transport of iron, specifically in mobilizing iron from the vacuole (Grotz and Guerinot 2006). Furthermore, OPT family members are also regulated by metals, and AtOPT3 is highly induced during iron deficiency (Wintz et al. 2003; Wu et al. 2005; Stacey et al. 2008; Buckhout et al. 2009). In our data, AtOPT3 (At4g16370) was 7.84-fold higher in *Arabidopsis* plants treated with L-DOPA compared with that in the control plants (Table 2).

Another iron-storage protein is ferritin, which is repressed during iron starvation (Puig et al. 2007). The FER repressor is ubiquitinated and degraded after iron treatment in a process in which nitric oxide plays a key role (Puig et al. 2007). Excess iron is trapped with oxygen in the ferritin mineral to minimize radical chemistry and reactive oxygen species. The FER family is central to the natural regulation of iron in cells (Hintze and Theil 2006; Ravet et al. 2009). The absence of ferritin leads to a strong deregulation of the expression of several metal transporter genes in the stem, the over-accumulation of iron in reproductive organs, and a decrease in fertility (Ravet et al. 2009). *Arabidopsis* possesses four ferritin family members that are differentially expressed in response to various

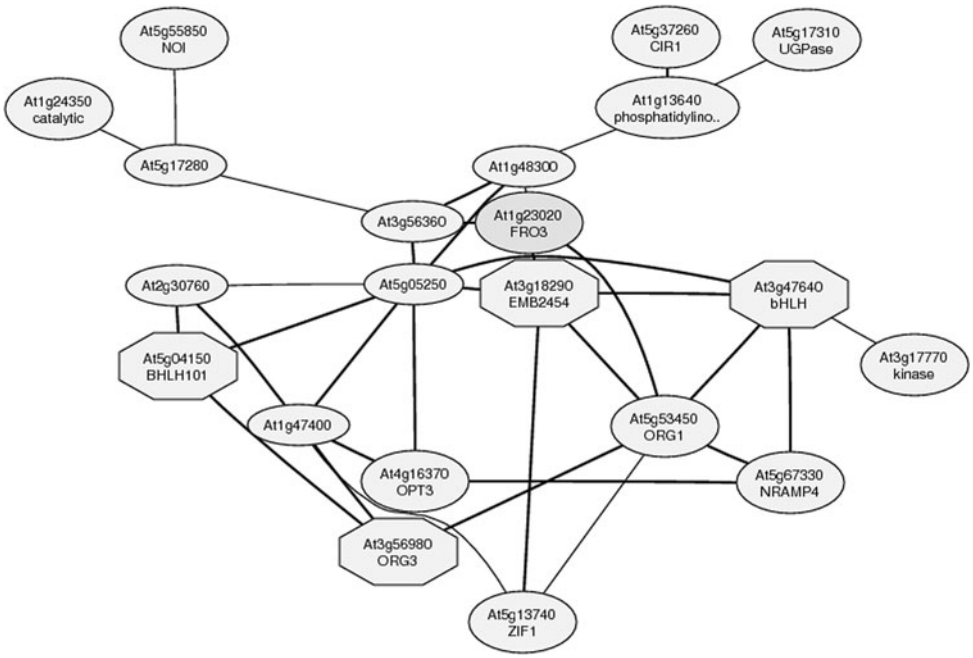

Fig. 3 Co-expression of selected upregulated genes by network of ATTED-II (http://atted.jp/) in response to L-DOPA treatment in *Arabidopsis thaliana* plant

environmental signals and during the course of plant growth and development (Petit et al. 2001a, b). AtFer1 and AtFer3 accumulate in response to high iron and oxidative stress (Petit et al. 2001a; Ravet et al. 2009). In our results, AtFer1 (At5g01600), AtFer3 (At3g56090), and AtFer4 (At2g40300) were downregulated (Table 3), which is in agreement with published reports of iron deficiency (Thimm et al. 2001; Wintz et al. 2003). In the absence of ferritin, plants have higher levels of reactive oxygen species and increased activity of enzymes involved in their detoxification (Ravet et al. 2009).

The results of this study show that the allelopathic effect of L-DOPA may be acting via its effects on amino acid metabolism and/or on the regulation of metal homeostasis, not only that of iron but also that of zinc and copper. L-DOPA seems to be a promising allelochemical for the control of weeds because of its influence on many fundamental biological processes (i.e., photosynthesis) in plants.

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