Original Paper

Functional genomics of the Dof transcription factor family genes in suspension-cultured cells of *Arabidopsis thaliana*

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Received October 14, 2008; accepted November 25, 2008 (Edited by Y. Ozeki)

Abstract The Dof (DNA-binding with one finger) genes are members of a family of plant-specific transcription factors that have a highly conserved DNA-binding domain, namely, Dof domain. The Dof domain is a particular class of zinc finger domain that has been demonstrated to bind specifically to DNA sequences with a T/AAAAG core. In the Arabidopsis genomic database, 36 Dof genes have been identified, whereas the functions of most of the members still remain to be studied. Therefore, we attempted to comprehensively and systematically investigate functions of Arabidopsis Dof genes. As the first step, we isolated cDNAs of all the Arabidopsis Dof genes based on the coding sequences identified on the genomic database. Then, we selected genes, which are subjected to further functional analysis, through a phylogenetic analysis, and transformed Arabidopsis cultured cells (line T87) using cDNAs corresponding to the selected genes. After that, we examined transcriptional profiles in the Dof gene-overexpressed calli using an Arabidopsis DNA microarray. From the results, possible involvements of the Dof genes in regulation of metabolic pathways are discussed.

Key words: *Arabidopsis thaliana*, cultured cells, Dof family, metabolic regulation, microarray, transcription factors, transgenic callus.

Harnessing plant metabolites and biomass not only for food, feed, and medicines, but also for feedstock for biofuels and industrial materials, appears promising as a strategy to provide sustainable and renewable resources. Biochemical compositions, yield, and yield stability of these plant materials are subject to transcriptional regulation of a diverse array of genes involved in regulatory networks of many metabolic pathways, growth and differentiation, and response to environmental stimuli. Since transcription factors control gene expression by binding directly or indirectly to the promoters of target genes in a sequence-specific manner to either activate or repress the transcription of downstream target genes, they naturally act as master regulators of many biological processes that are associated with quality and quantity of plant metabolites and biomass. Several pioneering studies demonstrated the potential of transcription factors for the manipulation of complex metabolic pathways in plants (Broun 2004). Therefore, understanding of function of transcription factors is an important step towards to develop the transcription factor-based technology for improvement of value and productivity of the plant materials (Broun 2004; Century et al. 2008; Grotewold 2008).

With the completion of the Arabidopsis sequences, it became possible to identify putative genes for

Abbreviations: CaMV35S, cauliflower mosaic virus 35S; CDS, coding sequence; Dof, DNA-binding with one finger; exGUS, callus ectopically expressing GUS; GUS, β-galacturonidase; LC/PDA/MS, liquid chromatography/photodiode array detection/mass spectrometry; Lhcb, light-harvesting chlorophyll a/b-binding; oxDOF, callus overexpressing Dof; PEPC, phosphoenolpyruvate carboxylase; PCR, polymerase chain reaction; PK, pyruvate kinase; PPDK, orthophosphate dikinase; RT-PCR, transcriptase-mediated polymerase chain reaction.

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This article can be found at http://www.jspcmb.jp/

transcription factors on a genome-wide scale (Riechmann et al. 2000). At that time, it was estimated that only about 7% of the Arabidopsis transcription factors have been genetically and functionally characterized (Riechmann and Ratcliffe 2000). To date, therefore, various reverse genetics tools have been developed to study functions of transcription factor genes, and the functional analysis of transcription factor families in Arabidopsis has been eagerly undertaken (Bi et al. 2005; Okushima et al. 2005; Overvoorde et al. 2005; Weiste et al. 2007; Lee et al. 2008). Most transcription factors can be grouped into gene families based on DNA-binding domains and other conserved features. Members, which are closely related each other in a large family, can be further grouped into subgroups. The members in a subgroup are thought to fulfill similar functions. Therefore, functional analysis of transcription factor genes in a large family is often hampered by the functional redundancy. Knockout and overexpression techniques have been most commonly used. Sometimes, knockout of multiple transcription factor genes has been necessary to exhibit informative phenotypes. In addition, loss-of-function mutation of essential genes by knockout strategy is prone to result in lethal phenotype. Overexpression provides alternative and complementary strategy to knockout analysis. Since overexpression may confer dominant gain-of-function phenotypes, it is less affected by functional redundancy of members of gene families. In addition, essential genes may be more easily studied with such systems because an increase in function may not cause lethality. Thus, the overexpression strategy appears particularly effective based on the unique characteristics and modes of action of transcription factors (Zhang 2003). Furthermore, transcription factor genes that confer phenotypic changes related to beneficial traits by overexpression are easily applied to genetic engineering in economically, agriculturally, and/or ecologically important plant species.

Suspension-cultured cells can be grown as fairly homogeneous populations under strictly controlled culture conditions, such as of temperature, nutrients, pH, growth regulators, light, and abiotic/biotic stress treatments, which are closely related to metabolic regulation in plant cells. Such cells are advantageous for collecting large amount of homogeneous cells and for reducing the complexity of plant tissues consist of various cell types at different stages of growth and differentiation, facilitating obtaining reproducible results. Therefore, the suspension-cultured cells have been widely used for biochemical and molecular biological studies of plant metabolism as simplified experimental systems. Several cell lines of suspension-culture of Arabidopsis, which is a favorable model plant for wide range of functional genomics studies, have been established. Among them, a unique cell line, namely, T87, which has photosynthetic ability under light irradiation (Axelos et al. 1992), have been widely used for biochemical and molecular biological analyses with respect to metabolic regulation, circadian rhythm, and stress response (e.g. Callard et al. 1996; Uno et al. 2000; Takahashi et al. 2001; Nakamichi et al. 2004; Stolc et al. 2005; Nakamura et al. 2007). Thus, this culture system is suitable for functional genomics study of transcription factors in regulation of metabolic pathway.

A technique for transcriptome analysis using microarrays enable comprehensive studies on dynamic cellular processes and their regulation and is an important tool for functional genomics study. The application of the powerful tool has allowed us to follow the changes in global gene expression that define metabolic processes in plants (e.g. Wang et al. 2000; Scheideler et al. 2002; Ko and Han 2004; Lin and Wu 2004; Lloyd and Zakhleniuk 2004; Scheible et al. 2004: Bläsing et al. 2005; Jiao et al. 2005; Tokimatsu et al. 2005). The transcriptome analysis in the transcription factor-overexpressed plants provides useful information to identify genes regulated by the transcription factor and to understand the nature of phenotypes of the transgenic plants (Abe et al. 2003; Vlieghe et al. 2003; Li et al. 2004; Delessert et al. 2005; Lee et al. 2007).

Dof (DNA binding with one finger) transcription factors contain a single C2C2 zinc-finger motif specific to plant as a DNA binding domain (Dof domain) and regulate gene expression through the specific interaction of their DNA binding domain with T/AAAAG sequences in the plant gene promoters (summarized in Yanagisawa 2002, 2004). Dof transcription factors have been shown to be widely distributed in the plant kingdom, including unicellular algae, moss, and vascular plants (Yanagisawa 2002; Lijavetzky et al. 2003; Yanagisawa 2004; Yang et al. 2006; Moreno-Risueno et al. 2007; Shigyo et al. 2007). It was firstly shown that Dof transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize (Yanagisawa 2000). Further studies have suggested that they participate in the regulation of biological processes exclusive to plants such as photosynthetic carbon assimilation, light-regulated gene expression, accumulation of seed storage proteins, germination, dormancy, response to phytohormones, flowering time, guard cell-specific gene expression, and regulation of oil content in seeds (Wang et al. 2007 and references cited therein). Characterization of Dof genes of unknown function by functional genomics in Arabidopsis is the promising approach for uncovering novel functions in regulation of metabolism and production of biomass and plant materials.

In the genome of Arabidopsis, 36 putative Dof genes have been identified (Yanagisawa 2002). Several genes of

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them have been characterized and shown to be involved in the salicylic acid-response, seed germination, phytochrome signaling, flowering, glucosinolate biosynthesis, and phenylpropanoid metabolism (Yanagisawa 2002; Yanagisawa 2004; Imaizumi et al. 2005; Ward et al. 2005; Skirycz et al. 2006), while others remain uncharacterized. Thus, we have performed functional genomics of Arabidopsis Dof genes in order to gain information on their roles in regulation of metabolism and production of biomass and plant materials. As the first step, we isolated cDNAs of all the Arabidopsis Dof genes based on the coding sequences (CDSs) identified on the genomic database. Then, we selected genes, which are subjected to further functional analysis, through a phylogenetic analysis, and transformed Arabidopsis cultured cells (line T87) using cDNAs corresponding to the selected genes. After that, we examined transcriptional profiles in the Dof gene-overexpressed T87 calli using an Arabidopsis DNA microarray. From the results, metabolic pathways controlled by the Dof genes are discussed.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. ecotype Columbia suspension-cultured cells, line T87 (Axelos et al. 1992), was kindly provided by Dr. K Shinozaki (RIKEN, Tsukuba, Japan) and from the Riken BioResource Center (http://www.brc.riken.jp/lab/epd/Eng/). T87 cells were maintained at a 14-day culture interval under continuous illumination (50–100 μmol m^{-2} s^{-1}) at 22°C with shaking at 120 rpm in a JPL medium (pH 5.8; Jouanneau and Péaud-Lenoël 1967; Axelos et al. 1992; Takahashi et al. 2004) containing 1 μM 1-naphthaleneacetic acid. A 2-ml aliquot of cell suspensions, which were filtered through a 1 mm nylon sieve, was transferred into 80 ml of fresh medium in a 300-ml Elrenmeyer flask every 2 weeks.

Cloning of Dof cDNAs into the pENTR entry vector

Total RNA was isolated from one-month-old Arabidopsis plants as described previously (Fukuda et al. 1991) and from 2-weeks-old Arabidopsis plants using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA). Double-strand cDNA was synthesized from 1.0 μg of the total RNA using a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). The polymerase chain reaction (PCR) was performed with KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) or Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). A pair of primers for each Dof mRNA was designed (Table 1) based on the sequence information available from The Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org/). The obtained PCR product was purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), and then cloned into the pENTR entry vector using a pENTR/D-TOPO cloning kit (Invitrogen, Carlsbad, CA, USA). To enable directional cloning, each forward primer contained the sequence 5′-CACC-3′ at the 5′ end, which pairs with the overhang sequence, GTGG, in the pENTR TOPO vector. The coding region of *uidA* gene was amplified by PCR with the attB adaptor using a primer set, 5′-AAAAAGCAGGCTCCATGTTACGTCCTGTAGAAACC-3′, and 5′-AGAAAGCTGGGTTCATTGTTTGCCTCCCTGC-3′, pBI121 as a template, and Platinum *Pfx* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The obtained PCR product was cloned into the pDONR221 entry vector (Invitrogen, Carlsbad, CA, USA). The resultant plasmids were verified by sequencing.

Plasmid construction and Agrobacterium-mediated transformation of T87 cells

The Dof CDS cDNAs and the *uidA* gene, which encodes a β-galacturonidase (GUS), in the entry clones were recombined into the Gateway-based binary vector, pK2GW7 (Karimi et al. 2002), which was provided from Plant Systems Biology (University of Ghent, Ghent, Belgium; http://www.psb.ugent.be/gateway/index.php), using a Gateway LR Clonase Enzyme mix (Invitrogen, Carlsbad, CA, USA). In this vector, Dof or GUS cDNAs are inserted downstream of the cauliflower mosaic virus 35S (CaMV35S) promoter. The reaction was performed at half volume of the reaction solution that recommended in the manufacturer's instruction. Using the resultant plasmids, *Agrobacterium tumefaciens* LBA4404 was transformed according to the method previously described by Cindy and Jeff (1994).

T87 cells were transformed by co-cultivation with *A. tumefaciens* that harbored each plasmid mentioned above according to the previously described methods (Gallego et al. 1999; Ferrando et al. 2001; Forreiter et al. 1997; Nakayama et al. 2000) with several modifications as described bellow. A 4-ml aliquot of 2-week-old T87 cells were filtered through a 1 mm nylon sieve and cultured in 50 ml of JPL medium in a 300-ml Erlenmeyer flask for 4 days. *A. tumefaciens* cells were cultured in 20 ml of YEB medium at 28°C with shaking for a day. The cell suspension (OD600 = 0.6~0.8) was centrifuged at 3,000 rpm for 5 min. The cells were washed with 2 ml of JPL medium and resuspended in 2 ml of JPL medium. A 100 μl of the *A. tumefaciens* cell suspension was added to the precultured T87 cell suspension and co-cultured for 48 h with shaking at 100 rpm. To remove *A. tumefaciens* cells, the T87 cells were washed with JPL medium containing 100 μg ml^{-1} carbenicillin-Na. The T87 cells were precipitated by standing in a 50-ml tube, and the supernatant was removed, and then the cells were resuspended in the 20 ml of JPL medium containing 100 μg ml^{-1} carbenicillin-Na again. This step was repeated more 4 times. The T87 cells were suspended in 50 ml of JPL medium containing 100 μg ml^{-1} carbenicillin-Na and cultured in a 300-ml Erlenmeyer flask for 3 days. Then, the T87 cells were washed by the above-mentioned washing method again. After the cells were suspended in 25 ml of JPL medium containing 100 μg ml^{-1} carbenicillin-Na, a 4-ml aliquot of the cell suspension were spread over the selection medium, JPL medium containing 0.8% agar in addition to 100 μg ml^{-1} carbenicillin-Na and 30 μg ml^{-1} kanamycin, in the rectangle plates and cultured for 3 weeks. Then, cell clusters resistant to kanamycin were picked up and transferred to a fresh JPL

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Table 1. The list of *Arabidopsis* Dof genes whose cDNAs were successfully cloned in this study.

Gene names	Synonims	Loci (AGI codes)	Primers		Cloned CDSs/ORFs
			Forward	Reverse	
AtDof1.1	HPPBF-2b	At1g69570	CACCATGTCTAAATCTAGAGATACGGAGATAAAGTTG	TTATTGTTGCATGCTCTCCCTGAAGTTCA	At1g69570.1
AtDof1.2		At1g21340	CACCATGTTGCCGTACATTGGACACAACAGCTACCAGCAGCA	TCAAATCCCGAACCTGGTAAGATCTGTTG	At1g21340.1
AtDof1.3		At1g26790	CACCATGTCTCAAGTTAGAGATACTCCGG	TTATATGCTCTCTCTGAAGTTCATAGATCTTGCCATGGC	Q9LQX4
AtDof1.4		At1g28310	CACCATGTTTGGCAATTGTGACCAGAACAAGAAGATGCC	TTATATCAAAGAGGTGAGGGAGGATC	30 bp 5′-elongation of At1g28310.2
AtDof1.5	COG1	At1g29160	CACCATGGCGACCCAAGATTCTCAA	TTAACAAGATTGACCATCGGTGTA	At1g29160.1
AtDof1.6		At1g47655[a]	CACCATGCCGTCGGAACCAAACCAAAC	TCACTTAACATCGTTGGTTTGCATTGAGA	At1g47655.1
AtDof1.7	ADOF1	At1g51700	CACCATGCAGGATCTGACGT	TCAATTCTTCTCCATTCTGTTCATAGCT	At1g51700.1
AtDof1.8		At1g64620	CACCATGCCGTCGGAACCAAACCAAAC	TCACTTAACATCGTTGGTTTGCATTGAGA	At1g64620.1
AtDof2.2		At2g28810	CACCATGGTTTTCTCATCCGTCTCAAGCT	TCACATAAGATGCTGGTGATGATGATCG	At2g28810.1
AtDof2.3		At2g34140	CACCATGGCGACTCAAGATTCTCAAGGGA	TCAGCACGATTGACCGTCGGAGTAACACC	At2g34140.1
AtDof2.4		At2g37590	CACCATGGTTTTCTCCTCCATCCAAGCCT	TCAACGAGAAGAGCCGAAAGCAGAAAGAT	At2g37590.1
AtDof2.5	DAG2	At2g46590	CACCATGATGAACGTTAAACCAATGGAGC	TCACCATGAAGATCCTCCTCCTGTAGTAC	At2g46590.1
AtDof3.1	ADOF2	At3g21270	CACCATGCAGGATCCAGCAGCATATTACC	TCAGTTTTTCTCCGCTCTGTTCATAGCCA	At3g21270.1
AtDof3.2		At3g45610	CACCATGGATTATTCTTCGATGCATCAGAATGTG	CTACAATAAAGCACCAGTATTAATGT	At3g45610.1
AtDof3.3	CDF3/HPPBF-2a	At3g47500	CACCATGATGATGGAGACTAGAGATCCAG	CTAAATCTGTTCATGGAAATTGTGTGATC	At3g47500.1
AtDof3.4	OBP1	At3g50410	CACCATGCCGACGTCTGACTCCGGTGAA	TCATTTGAGTGAGTTTCCCGGAGTTGTG	At3g50410.1
AtDof3.5		At3g52440	CACCATGGAGAGAGCAGAGGCCTTGACATCATCGTTTATATGGC	TCATCCATCGGCAGTGAAATCAAAAGGGCTCCAACTTCC	At3g52440.1
AtDof3.6	OBP3	At3g55370	CACCATGGTGGAACGTGCTCGGATCGCAA	TTAGAATGAGAGATGGCCTGTTGAGTTGT	Longet ORF[b] in AF155818
AtDof3.7	DAG1/BBFa	At3g61850	CACCATGGATGCTACGAAGTGGACTCA	TCACCATGAAGATCCTCCTGTATTACTGA	At3g61850.1
AtDof4.1		At4g00940	CACCATGGACCATCATCAGTATCATCATC	CTCCCCTATGCAAGACAAGC	At4g00940.1
AtDof4.2		At4g21030	CACCATGAATAATTTGAATGTTTTTACAA	TTATGATTCATATTCAAATTGCAAC	At4g21030.1
AtDof4.4		At4g21050	CACCATGGATAACTTGAATGTTTTCGCTA	TTATGATTCATGTTCATAGCGTGGTTG	At4g21050.1
AtDof4.5		At4g21080	CACCATGGATAACTTGAATGTTTTCGC	TCAAGGACCATCCTTCTCCAGATGACAAG	At4g21080.1
AtDof4.6		At4g24060	CACCATGGATACGGCTCAGTGGCCACA	TTAGCACCATGATCCTCCACTCAACATCC	At4g24060.1
AtDof4.7		At4g38000	CACCATGACGTCATCCCATCAGAGCAACA	TTAGAGCAGAGCATTATTATTAGCATTAT	At4g38000.1
AtDof5.1		At5g02460	CACCATGGTTTTTTCTTCATTTCCTACTT	GCTTCCACCGCCTATATGATAAGA	At5g02460.1
AtDof5.2	CDF2	At5g39660	CACCATGGCTGATCCGGCGATTAAGCTCT	CTATGAGCTCTCATGGAAGTTTGCTGACC	At5g39660.1
AtDof5.3		At5g60200	CACCATGGATCATTTGTTACAACACCAGGATGT	CTACATTAAAGCACCAGAATT	At5g60200.1
AtDof5.4	OBP4	At5g60850	CACCATGCAAGATATTCATGATTTCTCCA	TCAAGGAAGGTAGAGACCACTCTGATCTT	At5g60850.1
AtDof5.5	CDF1	At5g62430	CACCATGGAGACTAAGTTCTGTTACTACA	TCACATCTGCTCATGGAAATTGATTGAT	AY143830
AtDof5.6		At5g62940	CACCATGGGTCTCACTTCTCTTCAAGTTT	TCAAACCAAGGAGTTTGTTTTAGTGGACG	At5g62940.1
AtDof5.7		At5g65590	CACCATGTCCTCCCATACCAATCTCCC	TCAAGGCAATGCATTATAATGATTAAGATCAGTCC	At5g65590.1
AtDof5.8		At5g66940	CACCATGCCTTCTGAATTCAGTGAATCTC	TCACGCTACGTAGTCTCCAGACACGAA	At5g66940.1

[a] The locus ID has been updated from At1g47650.
[b] ORF, open reading frame.

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medium containing 0.6% agar, 100 μg ml^{-1} carbenicillin-Na and 30 μg ml^{-1} kanamycin in the Petri dish, and cultured for 2 weeks. The individual cluster was assigned to a single transgenic cell line. Three portions of the single cell line were subcultured twice at 2-week intervals. Three clusters of the single cell line was lumped all together and centrifuged with a 50-ml conical tube and a Cellstrainer (Falcon, BD Biosciences, San. Jose, CA, USA) at 3,000 rpm for a second to remove intercellular fluid, and then frozen in liquid nitrogen and stored at −80°C until assayed.

Total RNA was isolated from the cells of every transgenic T87 cell line by the method described previously (Fukuda et al. 1991) or using a Plant RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions, and then treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). To confirm overexpression of Dof or GUS in each transgenic cell line, reverse transcriptase-mediated polymerase chain reaction (RT-PCR) was performed using 1 μg total RNA and SuperScript II RNase H-Reverse Transcriptase (Invitrogen Carlsbad, CA, USA), Phusion (Finnzymes, Espoo, Finland) and the gene-specific primer pairs, which were used for cloning of CDS cDNAs. For each cell line, β-tubulin transcripts were also amplified as a constitutive expression control. Each RT-PCR product was separated by 1.3% (w/v) agarose gel electrophoresis to visualize the amplified DNAs.

Probe preparation and hybridization

Total RNAs, which were isolated from transgenic T87 callus overexpressing Dof (oxDOF) or ectopically expressing GUS (exGUS) using a Plant RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions and treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), were used for microarray analysis. Two micrograms of total RNA was used as the template in each labeling reaction. Fluorescent-labeled targets were prepared using a RNA Transcript SureLABEL Core Kit (Takara Bio Inc., Shiga, Japan) and Cy3-UTP (for oxDOF) or Cy5-UTP (for exGUS) (GE Healthcare UK Ltd., Buckinghamshire, England) according to the manufacturer's instructions. Cy3- and Cy5-labeled antisense RNAs were purified and used for hybridization on a microarray. The microarray analyses were performed with Arabidopsis CHIP ARI3 microarrays with 25,392 300-mer DNA probes (Takara Bio Inc., Shiga, Japan). Prehybridization, hybridization, and washing were performed following the manufacturer's instruction (http://catalog.takara-bio.co.jp/PDFFiles/TX815_j.pdf) with slight modification.

Microarray scanning and data analysis

Fluorescent signals on the microarray were scanned using an Agilent DNA microarray scanner (Agilent Technologies, Santa Clara, CA, USA). The intensity of gene features per array was extracted from scanned microarray images using Feature Extraction 7.5.1 software (Agilent Technologies, Santa Clara, CA, USA), which performs quantification of signal intensities, background subtractions and dye normalization. GeneSpringGX 7.3.1 and 10.0 software (Agilent Technologies, Santa Clara, CA, USA) was used for further expression profiling analysis. Alternatively, fluorescent signals on the microarray were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) and the scanned data were quantified using ArrayVision 8.0 software (Imaging Research, Ontario, Canada). The processed values were referred to as raw data for analysis with GeneSpringGX 7.3.1 software (Agilent Technologies, Santa Clara, CA, USA). Normalization, filtering, detailed expression-profiling analysis of the microarray data were performed with GeneSpringGX 7.3.1 and GeneSpringGX 10.0 (Agilent Technologies, Santa Clara, CA, USA). Ratio data after Lowess normalization were converted to a log base 10 scale and imported into Kappa-View2 and 3 (Tokimatsu et al. 2005; http://kpv.kazusa.or.jp/kappa-view/).

LC/PDA/MS analysis of phenolic compounds

Several clusters of the single cell line of DOF7 callus was lumped all together and centrifuged with a 50-ml conical tube and a Cellstrainer (Falcon, BD Biosciences, San. Jose, CA, USA) at 3,000 rpm for a second to remove intercellular fluid, and then frozen in liquid nitrogen and stored at −80°C until assayed. Extraction and analysis of phenolic compounds were performed according to Tohge et al. (2005). Briefly, the samples were powdered with a mortar and pestle in liquid nitrogen. Phenolic compounds were extracted with 3 ml of extraction solvent (MeOH : HOAc : H_2O, 9 : 1 : 10 v/v) per 1 g fresh weight, and then analyzed using HPLC (Agilent 1100 series; Agilent, Palo Alto, CA, USA). The extract was applied to an ODS column, TSKgel ODS-80TM (4.6×150 mm, 5 μm; TOSOH, Tokyo, Japan) and eluted with a linear gradient of CH_3CN in 0.1% (v/v) TFA/H_2O. The gradient protocol was 0–40.0 min, 10–42%; 40.1–45.0 min, 95%; 45.1–52.0 min, 10% at a flow rate of 0.5 ml min^{-1}. The column temperature was 40°C. To monitor HPLC elution, a photodiode array detector was used in the wavelength range of 200–650 nm. The eluate was introduced on-line to a LCQ Deca XP Plus (Thermo Electron, Waltham, MA, USA), and positive-ion ESI-MS was performed. For ionization, the capillary voltage was set at 35 V, and the capillary temperature at 350°C, the scan range of 200–1500 (*m*/*z*), and tube lens offset 10 V. MS/MS fragmentation was carried out at normalized collision energy 35.0% and isolation width 4.0 (*m*/*z*).

Results and discussion

The functional genomics of Dof family genes in Arabidopsis has been undertaken to gain information of transcription factors that are involved in the regulation of metabolic pathways and biomass production in plant. We searched potential genes of the Dof family on the complete Arabidopsis genome databases, TAIR (http://www.arabidopsis.org/), MIPS *Arabidopsis thaliana* Database (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp), and NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) using BLAST programs (Altchul et al. 1990). We obtained sequence data for 36 Dof genes, which are generally consistent with the pervious reports (Yanagisawa 2002; Lijavetzky et al. 2003), and gene-specific primers for amplification of the predicted CDSs by PCR were designed. The cDNAs were amplified by

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PCR using the gene-specific primers and cloned into the Gateway entry vector. As a result, we obtained the 33 cDNA clones (Table 1). In order to overexpress a Dof protein in Arabidopsis cells, a CDS cDNA in the pENTR entry clone was transferred into the downstream of the CaMV35S promoter of the Gateway-based binary vector, pK2GW7 (Karimi et al. 2002). After Arabidopsis T87 cells were co-cultured with *A. tumefaciens* carrying a CaMV35S : DOF construct, kanamycin-resistant cell clusters were selected and the each cluster was referred to as a transgenic cell line. As a result, the transgenic cell lines for 32 Dof genes were established. As a control, T87 cells were also transformed with a CaMV35S : GUS construct at the same time with the transformation with 35S : DOFs. After overexpression of transgenes in the transgenic cells was confirmed by RT-PCR, three and one representative lines for each transgenic callus of oxDOF and exGUS, respectively, which were concurrently transformed, were selected and subjected to the microarray analysis. Cy3-labeled targets from the every line of the oxDOF callus and Cy5-tragets from the single representative line of the exGUS callus were hybridized on a microarray. The microarray data, which passed the quality check, for 24 Dof gene-overexpressed calli (Table 2) were used for the further analyses. In some cases, only the data for one or two lines of oxDOFs were obtained.

To examine the effect of overexpression of a Dof gene on the metabolic pathway and/or biomass production, we compared the transcriptome profiles in oxDOF callus versus exGUS callus. Figure 1 shows the distribution of the average of log-ratio of expression levels after Lowess normalization for each callus as the box-plots. The box plots are centered at zero and are symmetrically distributed. Therefore, the labeling, hybridization, scanning, and normalization seemed to be properly performed. Figure 2 shows the proportions of up- and down-regulated genes in every oxDOF callus. In many cases, the proportion of down-regulated genes was higher than that of up-regulated. There is no correlation between the degree of fold increase of expression of transgene (Table 2) and the proportions of up- and down-regulated genes in the transgenic calli (Figure 2). The changes in transcriptomic profiles by the overexpression of Dof genes seemed to depend on their individual functional properties, which may result in the changes in the expression of a variety of direct and indirect target genes including transcription factors, rather than the expression levels of them. However, it is unclear whether the changes in transcriptomic profiles were due to transcriptional activation or repression by the overexpressed Dof proteins. The Dof family of Arabidopsis has been grouped into subgroups based on the phylogenetic analysis using the alignment of Dof domain amino acid sequences and conserved motif analysis (Yanagisawa 2002; Lijavetzky et al. 2003). The

Table 2. The list of transgenic calli overexpressiong AtDofs.

Gene names	Callus ID	Groups[a]	No of Lines	oxDOFs (FC)[b]
AtDof1.7	DOF7	A	3	39.46
AtDof5.4	DOF16	A	3	12.04
AtDof2.2	DOF23	B1	2	26.31
AtDof2.4	DOF5	B1	3	n.d.[c]
AtDof3.6	DOF1	B1	3	85.51
AtDof4.7	DOF15	B2	2	39.93
AtDof5.7	DOF26	B2	3	16.3
AtDof1.4	DOF6	B2	3	41.96
AtDof3.2	DOF9	C1	3	n.d.
AtDof5.6	DOF17	C1	3	43.3
AtDof1.8	DOF28	C2.1	3	16.77
AtDof2.5	DOF13	C2.1	2	n.d.
AtDof4.1	DOF3	C2.1	2	n.d.
AtDof4.6	DOF27	C2.1	2	29.27
AtDof1.2	DOF11	C2.2	3	n.d.
AtDof3.5	DOF25	C2.2	3	n.d.
AtDof4.5	DOF4	C3	3	n.d.
AtDof4.2	DOF22	C3	1	n.d.
AtDof1.5	DOF2	D1	3	11.31
AtDof3.3	DOF14	D1	3	5.132
AtDof5.2	DOF21	D1	3	7.502
AtDof1.3	DOF20	D1	3	n.d.
AtDof3.4	DOF10	D2	3	17.66
AtDof1.6	DOF12	D2	3	42.1

[a] Groups classified by Lijavetzky et al. (2003).
[b] Flod change of transgene caliculated from microarray data.
[c] n.d., no data.

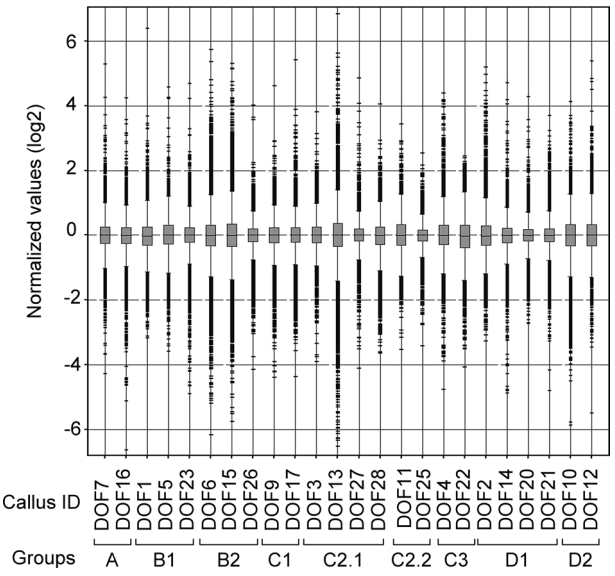

Figure 1 Distribution of fold changes for every gene in the Dof-overexpressed T87 callus. A value of log base 2 of the intensity ratio of signal intensities from an oxDOF callus and its corresponding exGUS callus after Lowess normalization was calculated. Average of the log-ratio value among the lines of every oxDOF callus, except the DOF22, for all genes, which have passed the filtering steps, was plotted as a box-plot. The boxes have lines at the lower quartile, median, and upper quartile values. Whiskers extend to the most extreme value within 1.5 times the interquartile range from the ends of the corresponding box. Each box plot shows the distribution of expression levels from 25th to 75th percentile. The median is shown as a line across the box. Groups according to the classification by Lijavetzky et al. (2003) are indicated.

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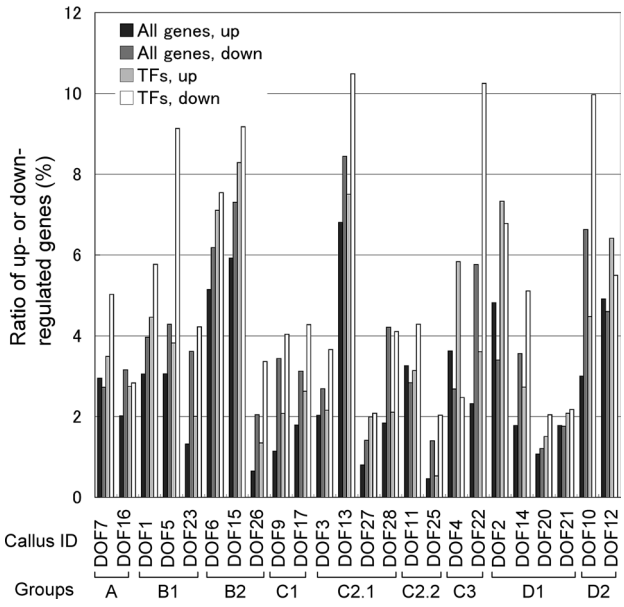

Figure 2 Global changes in gene expression profile in the Dof-overexpressed T87 calli. Percentage of up- (>2 fold) or down-regulated (<0.5 fold) genes in all genes, which have passed the filtering steps, and percentage of up- (>2 fold) or down-regulated (<0.5 fold) transcription factor genes in all transcription factor genes in each oxDOF callus are presented. The values were calculated using average of the fold change among the lines of every oxDOF callus, except the DOF22. Groups according to the classification by Lijavetzky et al. (2003) are indicated.

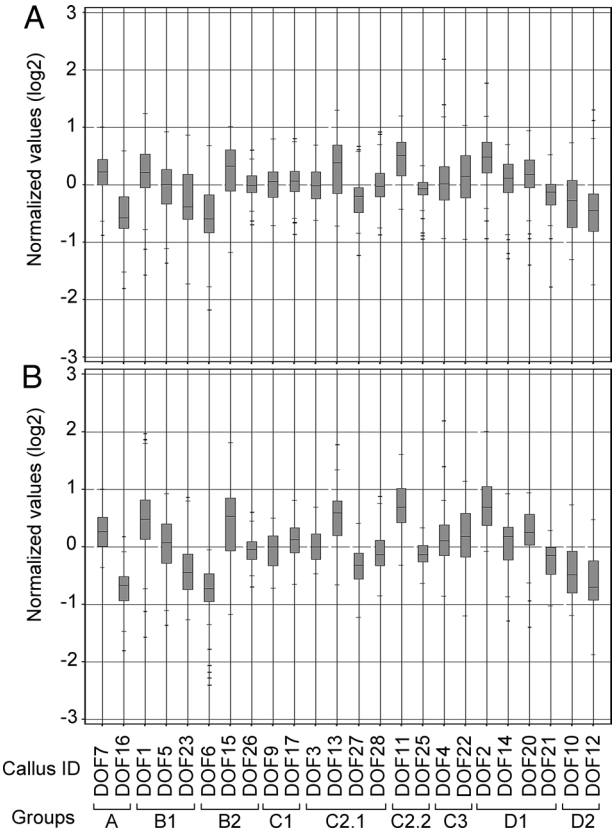

Figure 3. Coordinate up- or down-regulation of photosynthetic genes in the Dof-overexpressed T87 calli. A value of log base 2 of the intensity ratio of signal intensities from an oxDOF callus and its corresponding exGUS callus after Lowess normalization was calculated. Average of the log-ratio value among the lines of every oxDOF callus, except the DOF22, for genes categorized into photosynthesis (A) and photoreaction plus Lhcb proteins (B) was plotted as a box-plot. The boxes have lines at the lower quartile, median, and upper quartile values. Whiskers extend to the most extreme value within 1.5 times the interquartile range from the ends of the corresponding box. Each box plot shows the distribution of expression levels from 25th to 75th percentile. The median is shown as a line across the box. Groups according to the classification by Lijavetzky et al. (2003) are indicated. Gene lists of photosynthesis and photoreaction were adopted from AraCyc (http://www.arabidopsis.org/biocyc/index.jsp).

members in a subgroup of a transcription factor family are generally thought to have similar functions, whereas we did not found such similarity between the expression profiles from the transgenic calli, which are overexpressing Dof genes within a same group.

Since photosynthesis is very important for regulation of production of biomass and plant materials, we inspected changes of expression profiles of genes involved in the pathway in the Dof-expressing calli. As shown in Figure 3A, photosynthetic genes were coordinately up- or down-regulated in the many transgenic calli. Such trend was more obvious in the expression profile of genes involved in light reaction and light-harvesting chlorophyll a/b-binding (Lhcb) proteins (Figure 3B). The light-induced expression of photosynthetic gene is mediated by phytochrome signaling (Kuno and Furuya 2000) and the involvement of Dof genes, such as COG1, OBP3, and DAG1, in regulation of phytochrome signaling has been demonstrated (Papi et al. 2002; Park et al. 2003; Ward et al. 2005). OBP3, which is corresponding to AtDof3.6, was suggested to be a positive regulator of phytochrome signaling (Ward et al. 2005). This is consistent with the present result that photosynthetic genes are up-regulated in the AtDof3.6-overexpressed DOF1 callus. On the other hand, COG1/AtDof1.5-overexpressed Arabidopsis plants revealed attenuation of light-induced morphogenesis and expression of an Lhcb gene, CAB2, which are regulated via phytochrome signaling (Park et al. 2003). However, this is inconsistent with the coordinated up-regulation of Lhcb genes in the AtDof1.5-overexpressed DOF2 callus (Figure 3B). It was demonstrated that the maize Dof1 have positive or negative roles in light-regulated expression of genes involved in the carbon metabolism (Yanagisawa and Sheen 1998). It is plausible that Dof proteins play positive or negative roles selectively in the light-regulated gene expression under different growth and developmental, and environmental stimulus conditions.

Maize Dof genes have been suggested to have roles in expression of genes, such as phosphoenolpyruvate carboxylase (PEPC) and orthophosphate dikinase (PPDK), which are involved in photosynthetic carbon

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Table 3 Expression of nitrate assimilation genes in Dof-overexpressed calli.

Genes[a]	AGI	A[d] D7[b]	B1 D16	B1 D1	B2 D5	B2 D23	B2 D6	C1 D15	C1 D26	C1 D9	C2 D17	C2 D3	C2 D13	C2.1 D27	C2.1 D28	C2.1 D11	C3 D25	C3 D4	D1 D22	D2 D2	D2 D14	D2 D20	D2 D21	D2 D10	D2 D12
Nitrate transport																									
Low-affinity																									
NRT1.1	At1g12110	0.46	−0.51	0.33	−0.66	−1.51	0.72	0.84	−0.48	−0.54	0.52	0.94	0.23	−0.95	−1.05	1.43	−0.59	1.28	n.d.	0.77	−0.06	−0.25	−0.05	−0.01	−0.42
NRT1.2	At1g69850	0.36	−0.07	0.91	0.62	−0.37	0.60	0.72	−0.11	−0.27	0.29	0.80	0.88	0.37	0.21	−0.26	−0.51	0.90	−0.20	0.63	−0.21	0.02	0.32	0.08	0.03
NRT1.3	At3g21670	n.d.[c]	n.d.	−0.55	n.d.	−0.06	−0.50	−0.32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NRT1.4	At2g26690	−2.14	−1.51	−0.63	n.d.	−2.35	−3.44	−3.01	−1.92	−1.99	−2.90	−0.98	−1.68	−1.41	−1.93	−2.23	0.08	−1.63	−0.59	−2.22	−1.20	3.59	1.09	1.59	−1.08
High-affinity																									
NRT2.1	At1g08090	−2.60	−1.63	−0.30	−0.16	−0.26	−3.48	−1.91	0.35	−2.79	−1.87	−0.60	−3.61	−0.16	−2.34	−1.29	1.36	−2.32	n.d.	−1.75	−1.00	0.66	3.26	−2.18	−1.16
NRT2.2	At1g08100	n.d.	n.d.	n.d.	n.d.	n.d.	−1.97	−0.61	n.d.	n.d.	n.d.	n.d.	n.d.	−0.23	n.d.	n.d.	0.59	n.d.	n.d.	n.d.	n.d.	0.37	n.d.	n.d.	n.d.
NRT2.3	At5g60780	n.d.	−1.04	2.76	1.74	−0.33	2.04	5.17	1.11	n.d.	2.09	0.67	0.23	1.72	−0.62	n.d.	1.06	0.81	n.d.	n.d.	−2.14	−1.93	0.00	−2.17	n.d.
NRT2.4	At5g60770	n.d.	n.d.	n.d.	n.d.	n.d.	0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−0.05	n.d.	n.d.
NRT2.5	At1g12940	n.d.	1.43	−0.02	n.d.	1.50	0.65	−0.74	0.36	n.d.	−0.84	n.d.	−1.39	−0.31	−0.29	n.d.	0.04	n.d.	0.02	n.d.	0.66	−0.37	0.33	0.60	n.d.
NRT2.6	At3g45060	1.09	−1.37	0.85	0.54	−0.28	0.13	2.19	0.79	−0.28	−0.35	−0.34	0.21	0.74	0.75	0.50	0.46	−0.45	0.60	0.37	−0.86	−0.72	−0.61	−3.20	−0.92
NRT2.7	At5g14570	0.30	−0.37	1.18	0.52	−0.54	0.81	n.d.	−0.03	n.d.	0.75	0.97	0.05	−0.06	n.d.	0.19	0.08	0.03	n.d.	0.27	n.d.	n.d.	n.d.	0.73	n.d.
Nitrate reduction																									
Nitrate reductase, NR1	At1g77760	n.d.	0.09	n.d.	n.d.	−0.38	1.76	n.d.	0.35	0.57	0.59	n.d.	n.d.	−0.29	−0.15	n.d.	0.50	n.d.	n.d.	n.d.	4.72	−0.49	−1.97	−1.22	n.d.
Nitrate reductase, NR2	At1g37130	0.52	−0.36	−0.44	−0.50	−1.07	1.00	−0.49	−0.24	0.00	0.09	−0.04	−0.86	0.11	−0.16	−0.31	−0.13	0.37	−0.01	−0.04	−0.37	0.19	−0.26	0.84	0.21
Nitrite reductase, NiR	At2g15620	0.21	−0.11	0.66	−1.45	0.10	0.45	−0.72	0.10	−0.18	−0.07	−1.10	−1.40	−0.65	−0.33	0.28	−0.13	n.d.	n.d.	n.d.	n.d.	0.27	0.13	−0.47	n.d.
urophorphyrin III methylase	At5g40850	0.75	0.31	0.08	−0.30	0.18	0.73	−0.17	−0.13	0.45	0.66	−0.15	0.46	0.28	0.15	0.32	−0.22	0.44	0.63	0.28	0.39	0.25	−0.43	−0.05	0.58
Fd-NADP(+) reductase	At5g66810	0.20	0.07	0.12	−0.18	−0.25	−0.44	−0.37	0.05	−0.08	0.16	n.d.	0.27	−0.01	−0.24	−0.38	0.14	n.d.	n.d.	n.d.	0.21	0.07	−0.01	−0.04	n.d.
	At5g66190	0.12	−0.37	0.48	−0.33	−0.26	−0.48	0.37	0.13	−0.08	0.47	0.21	0.66	−0.30	−0.12	0.34	0.10	−0.09	−0.12	0.56	0.41	0.03	−0.20	−0.23	−0.55
	At4g05390	0.23	0.62	−1.04	0.89	0.29	0.58	−0.52	−0.11	0.40	0.26	n.d.	0.20	0.21	0.55	0.51	−0.03	−0.29	0.08	−0.39	−0.23	−0.10	−0.13	0.65	n.d.
	At1g30510	0.05	0.31	0.48	0.29	−0.02	0.66	−0.15	0.08	−0.08	0.14	−0.14	0.58	−0.03	0.10	0.04	0.05	−0.24	0.48	0.08	0.55	0.06	0.18	−0.47	−0.32
	At1g20020	0.42	−0.14	0.72	−0.08	−0.18	−0.66	0.60	0.25	−0.38	n.d.	−0.13	1.09	−0.35	0.27	0.73	n.d.	0.05	−0.06	0.27	n.d.	0.35	0.34	−0.60	−0.36
	At4g32360	−0.11	0.12	−0.52	n.d.	0.06	−0.59	−0.02	0.14	−0.17	−0.07	n.d.	0.09	−0.01	−0.08	n.d.	0.13	n.d.	n.d.	n.d.	−0.13	0.20	0.30	−0.04	n.d.
Ammonium assimilation																									
Glutamate synthase																									
GOGAT1	At5g04140	n.d.	−0.66	−0.20	n.d.	0.44	−0.06	n.d.	0.78	−0.31	−0.07	n.d.	0.40	−0.80	0.55	n.d.	0.33	n.d.	n.d.	n.d.	2.17	0.14	−0.33	−0.21	n.d.
GOGAT2	At2g41220	−0.58	0.10	−0.30	−0.50	0.39	−0.58	−0.49	0.52	−0.37	−0.07	−0.63	−1.18	0.05	−0.25	−0.19	0.38	−1.10	0.29	−1.01	−0.07	0.11	0.27	−0.06	1.92
GOGAT3	At5g53460	−0.24	0.33	0.36	0.04	0.50	0.33	−0.32	0.49	−0.19	0.36	−0.39	−1.50	0.28	−0.34	−0.29	0.60	0.45	n.d.	n.d.	−0.04	−0.23	0.36	−0.46	−0.61
Glutamine synthase																									
GLN1;1	At5g37600	−0.72	−0.46	−0.41	−0.50	−0.89	−1.02	−1.79	−0.17	−0.33	−0.61	−0.48	−1.73	−0.16	−0.41	−0.31	0.03	−0.39	−0.20	−1.04	−0.70	−0.20	0.08	−0.31	−0.93
GLN1;2	At1g66200	−0.59	−0.31	−1.56	−0.74	−0.24	−0.81	−2.30	0.10	−0.13	−0.90	0.06	−1.95	0.20	−0.42	0.28	0.15	−0.05	−0.12	−0.88	−0.83	−0.20	0.07	−0.42	0.37
GLN1;3	At3g17820	0.14	0.39	0.75	0.02	0.50	0.35	−0.02	0.08	−0.17	0.16	0.01	0.61	0.16	0.35	0.08	0.10	0.32	−0.20	0.10	−0.40	−0.78	−0.38	0.18	0.14
GLN1;4	At5g16570	n.d.	1.36	−0.23	n.d.	1.72	1.10	−1.02	0.30	−0.23	−0.23	n.d.	−0.69	0.61	n.d.	n.d.	−0.28	n.d.	−0.55	−0.54	−0.15	−0.55	−0.11	n.d.	n.d.
GLN1;5	At1g48470	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GLN2	At5g35630	−0.30	0.18	0.38	−0.38	0.19	0.41	0.47	0.34	−0.23	0.04	−0.23	0.30	−0.14	−0.11	0.43	0.14	−0.05	0.26	0.05	0.46	0.13	0.25	−0.75	−0.80
ASN1	At3g47340	−1.82	−0.52	−1.00	n.d.	−1.91	−5.18	−4.55	−0.75	−4.21	−2.07	0.13	−6.11	−0.03	−2.92	−1.04	−0.54	n.d.	n.d.	−0.03	−1.15	−0.01	2.33	−1.86	−2.02
ASN2	At5g65010	−0.01	0.36	0.37	0.20	0.13	0.42	0.52	−0.14	−0.20	−0.11	n.d.	−0.22	−0.47	0.14	0.44	−0.20	n.d.	n.d.	0.16	−0.12	−0.21	−0.47	−0.22	n.d.
ANS3	At5g10240	−0.15	0.55	0.73	0.39	0.68	1.72	0.54	0.51	0.44	0.20	n.d.	0.14	−0.23	0.55	n.d.	0.25	n.d.	n.d.	n.d.	0.86	−0.41	−0.18	0.60	n.d.

[a] The gene list is adopted from Bi et al. (2007).
[b] D, DOF.
[c] n.d., no data.
[d] Groups according to the classification by Lijavetzky et al. (2003) are indicated.

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assimilation in maize (Yanagisawa and Sheen 1998; Yanagisawa 2000). In addition, overexpression of maize Dof1 confers enhancement of PEPC and pyruvate kinase (PK) in transgenic Arabidopsis plants (Yanagisawa et al., 2004). In the Dof-overexpressing T87 calli, changes of expression profiles of genes for PEPC, PPDK, and PK were not dramatic. About 2-fold increase in the expression level of AtPEPC1 and AtPEPC3 was observed only in the DOF6 callus (data not shown). In the maize Dof1-overexpressed Arabidopsis plants, increase of glutamine, glutamate, and 2-oxoglutalate were demonstrated (Yanagisawa et al. 2004). By contrast, our preliminary metabolite analysis in the DOF6 callus showed that glutamine and glutamate were unchanged, but 2-oxoglutarte was increased (unpublished result).

It was speculated that Dof transcription factor could play an important role in nitrogen regulation, since Dof-binding motifs, including AAAG core sequence, were found by the computational analysis of putative common nitrogen regulatory *cis*-elements in the promoter regions of co-expressed genes, which were transiently up-regulated after transfer of plants grown under the severe nitrogen-limited condition to the nitrogen-sufficient condition, selected by clustering using transcriptome data under nitrogen stress conditions (Bi et al. 2007). We inspected the changes in expression profiles of genes in the clusters identified by Bi et al. (2007), and coordinate change was not obvious in the expression profiles from the Dof-expressed T87 calli (data not shown). Although it is unclear to date whether Dof genes are directly involved in nitrogen regulation in Arabidopsis, our results show that several genes involved in ammonium assimilation, nitrate reduction, or transport of nitrate were up-regulated more than 2-fold or down-regulated less than 0.5 fold in some Dof-expressed T87 calli (Table 3). A recent report demonstrated that a Dof transcription factor, PpDof5, differentially (positively or negatively) regulates the expression of two genes encoding cytosolic glutamine synthases (Rueda-López et al. 2008). Interestingly, a gene encoding cytosolic glutamine synthase was up-regulated in the DOF16 callus overexpressing AtDof5.4, which is a close relative to PpDof5. Yanagisawa et al. (2004) reported that improved nitrogen assimilation and growth under low-nitrogen conditions could be achieved in maize Dof1-overexpressed Arabidopsis plants via up-regulation of genes involved in carbon metabolism. Thus, some Dof genes may play important roles directly or indirectly in nitrogen metabolism.

The phenylpropanoid pathway, which is a well-studied

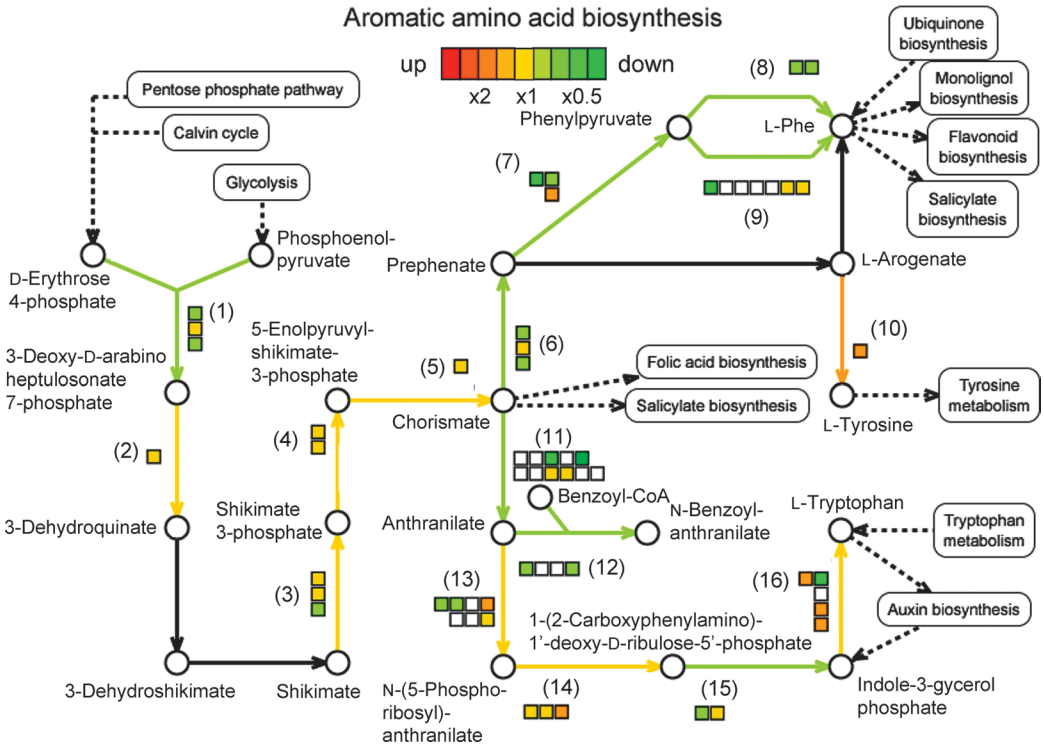

Figure 4. Coordinate down-regulation of shikimate pathway genes in the DOF10 callus. The microarray data for the DOF10 callus were analyzed using the KaPPa-View system (Tokimatsu et al. 2005). For the analysis, the average values of the fold change among the three lines of the DOF10 callus were used. The expression profile of shikimate pathway genes is presented. The white boxes indicate no data. L-Phe, L-phenylalanine. (1) 2-dehydro-3-deoxyphosphoheptonate aldolase, (2) 3-dehydroquinate synthase, (3) shikimate kinase, (4) 5-enolpyruvyl-shikimate 3-phosphate synthase, (5) chorismate synthase, (6) chorismate mutase, (7) prephanate dehydrogenase, (8) histidinol-phosphate aminotransferase, (9) tyrosine transaminase, (10) arogenate dehydrogenase, (11) anthranilate synthase, (12) anthranilate N-benzoyltransferase, (13) anthranilate phosphoribosyltransferase, (14) phosphoribosylanthranilate isomerase, (15) indole-3-glycerol-phosphate synthase, (16) tryptophan synthase.

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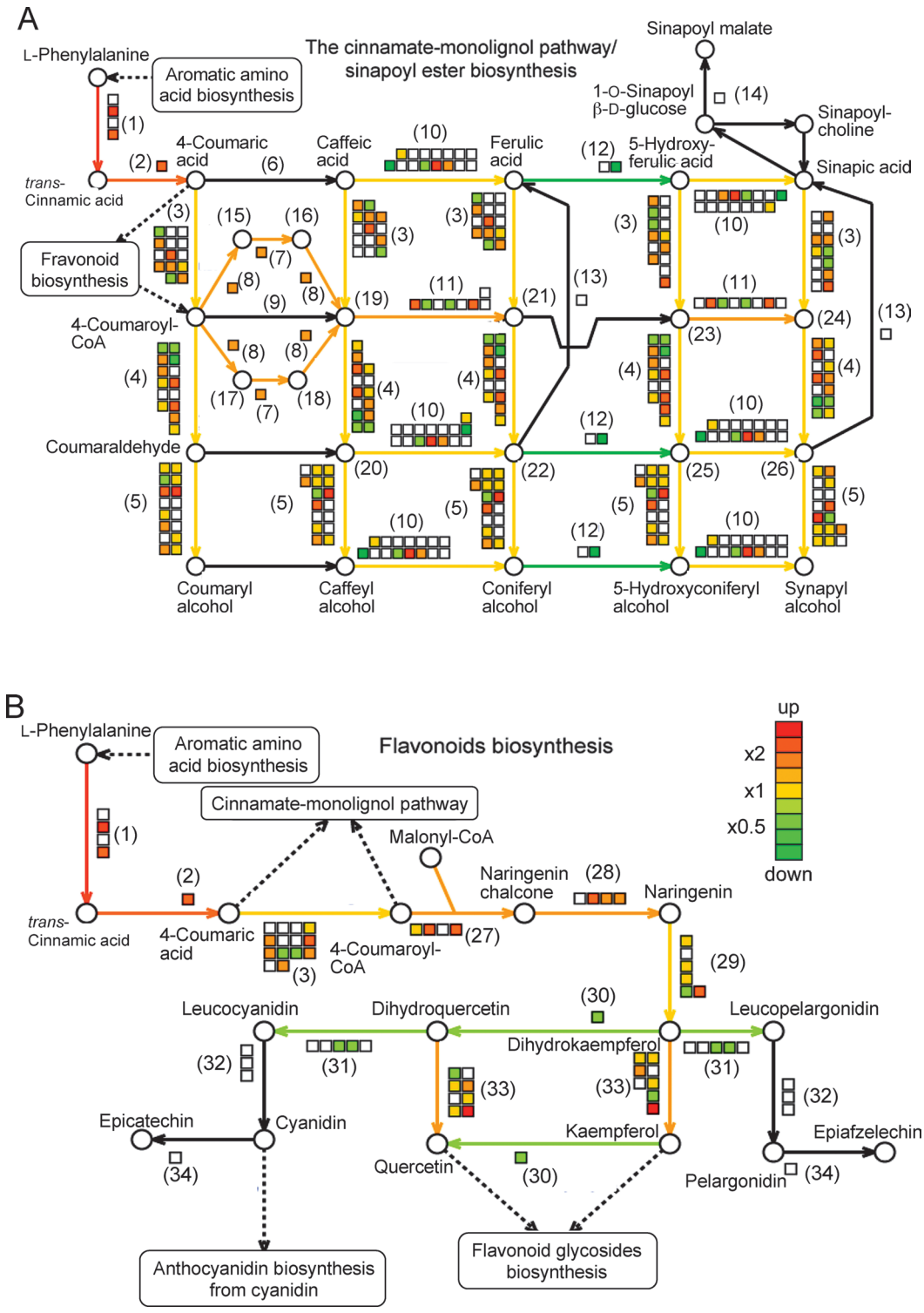

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secondary metabolic pathway in plant, is responsible for the biosynthesis of a wide variety of secondary metabolites, including various phenolic compounds, such as lignin, flavonoids, anthocyanins, and phytoalexins and these metabolites are involved in numerous biological processes and agronomic traits, such as growth and differentiation, and signaling and protection to biotic and abiotic stimuli (Dixon and Paiva 1995; Weisshaar and Jenkins 1998; Schijlen et al. 2004). The precursors of phenylpropanoid pathway, i.e., aromatic amino acids, are supplied from the shikimate pathway, and the supply from this pathway is important for subsequent flux through the phenylpropanoid pathway. So we assessed the effects of overexpression of Dof genes on these pathways using a web-based metabolic pathway analysis tool, KaPPa-View (Tokimatsu et al. 2005), and observed changes in gene expression profiles in several oxDOF calli. For example, multiple genes encoding enzymes for different steps in the shikimate pathway were coordinately down-regulated in the DOF10 callus overexpressing AtDof3.4 (Figure 4). Consistently, our preliminary metabolite analysis showed that phenylalanine, tyrosine, and tryptophan were decreased in this callus (unpublished result). By contrast, genes encoding enzymes for different steps in the flavonoid biosynthesis and the monolignol biosynthesis were differentially regulated in the DOF7 callus, which is overexpressing AtDof1.7 (Figure 5A, B). Such changes in expression profiles suggest that the overexpression of AtDof1.7 gene may affect composition of phenolic compounds. We compared phenolic compound profiles between the DOF7 callus and an exGUS. As shown in Figure 5C, additional peaks, such as peak a, b, c, e, f, and g, were observed in three independent cell lines of the DOF7 callus in comparison with the control callus in contrast to commonly observed peaks, such as peak d. Although these compounds have not been fully identified, the analyzed data suggest that the peak a and g may be a derivative of coniferyl alcohol and a kind of flavonoid, respectively (data not shown).

As described above, the results suggested that Dof proteins share roles in regulation of central metabolism and/or secondary metabolism, i.e. Dof proteins share roles in regulation of flow of carbon metabolism, and

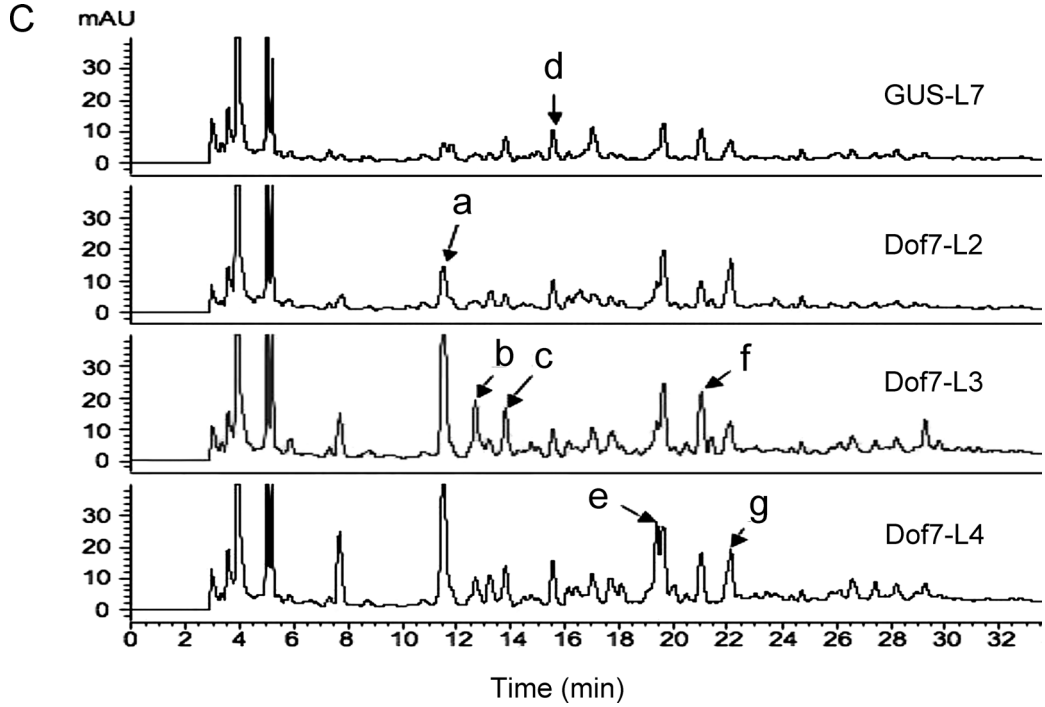

Figure 5 Differential up- and down-regulation of genes of biosynthetic pathway of phenylpropanoids and flavonoids and changes in a phenolic compound profile in the DOF7 callus. A and B, the expression profile of biosynthetic pathway genes of phenylpropanoids (A) and flavonoids (B). The microarray data for the DOF7 callus were analyzed using the KaPPa-View system (Tokimatsu et al. 2005). For the analysis, the average values of the fold change among the three lines of the DOF7 callus were used. The white boxes indicate no data. (1) phenylalanine ammonia-lyase, (2) cinnamate 4-hydroxylase, (3) 4-coumarate-CoA ligase, (4) cinnamoyl-CoA reductase, (5) cinnamyl alcohol dehydrogenase, (6) p-coumarate 3-hydroxylase, (7) p-coumaroyl shikimate/quinate 3′-hydroxylase, (8) hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase, (9) caffeoyl CoA 3-hydroxylase, (10) caffeic acid O-methyltransferase, (11) caffeoyl-CoA O-methyltransferase, (12) ferulate 5-hydroxylase, (13) coniferyl aldehyde dehydrogenase/sinapaldehyde dehydrogenase, (14) sinapoyl glucose:malate sinapoyl transferase, (15) 4-coumaroylquinic acid, (16) caffeoylquinic acid, (17) 4-coumaroylshikimic acid, (18) caffeoylshikimic acid, (19) caffeoyl-CoA, (20) caffealdehyde, (21) feruloyl-CoA, (22) coniferyl aldehyde, (23) 5-hydroxyferuloyl-CoA, (24) sinapoyl-CoA, (25) 5-hydroxyconiferyl aldehyde, (26) sinapaldehyde, (27) chalcone synthase, (28) chalcone isomerase, (29) flavanone 3-hydroxylase, (30) flavonoid 3′-hydroxylase, (31) dihydroflavonol 4-reductase, (32) anthocyanidin synthase, (33) flavonol synthase, (34) anthocyanidin reductase. C, HPLC elution profiles of phenolic compounds from 3 lines of the DOF7 callus and a line of GUS-expressed callus as a control at 280 nm. The peaks (a–g) are described in the text.

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that Dof proteins are also involved directly or indirectly in nitrate metabolism, which is closely linked to carbon metabolism. Therefore, the further functional analyses of Dof genes will provide us useful genes to modulate agronomic traits including quality, yield, and stress tolerance in plants, as it has been shown that the maize Dof1 improve nitrogen assimilation and growth under limited nitrogen condition in transgenic Arabidopsis (Yanagisawa et al. 2004). Recently, it was demonstrated that the soybean Dof genes, GmDof4 and GmDof11, enhance lipid content in the seeds of transgenic Arabidopsis (Wang et al. 2007). Thus, the transcriptome data in the transgenic Arabidopsis calli will provide valuable information for further functional analyses of Dof genes. The microarray data in this study will be available for analysis with KaPPa-View on the website (http://kpv.kazusa.or.jp/kpv3/guestIndex.jsp).

Acknowledgements

The authors thank Dr. Shinozaki (PSC, RIKEN) and RIKEN BioResource Center (Tsukuba, Japan) for providing Arabidopsis T87 cells, and Ms. S. Ito for her technical assistance. This study was supported by New Energy and Industrial Technology Development Organization (NEDO) as part of a project called 'Development of Fundamental Technologies for Controlling the Material Production Process of Plants'.

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