

Identification of drought induced differentially expressed genes in barley leaves using the annealing control-primer-based GeneFishing technique

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Abstract

In the present study, we have used an annealing-control-primer (ACP)-based differential display reverse transcription-polymerase chain reaction (RT-PCR) method to identify drought-stress-induced differentially expressed genes (DEGs) in barley leaves. Using 120 ACPs, a total of 12 up-regulated and 3 down-regulated genes were identified and sequenced. Temporal expression patterns of the 15 up- or down-regulated drought-responsive DEGs were further analyzed by Northern blot analysis. Temporal expression analysis of several candidate genes revealed that within 6 h the mRNA expression was increased and maintained at a high level up to 48h after drought stress. Gradual increases of the mRNA expression of genes encoding dehydrins, receptor kinase and jasmonic acid induced protein suggests that multiple adaptive-mechanisms are involved in plants to cope in drought stress. The possible roles of the identified DEGs are discussed in the context of their putative role during drought stress.

Key words: Barley, GeneFishing technique, Drought stress.

Abbreviations: ACP, annealing-control-primer; DEG, differentially expressed genes; GP, GeneFishing primer.

Introduction

Drought is one of the most serious worldwide problems for agriculture, limiting crop growth and productivity (Passioura, 1996). Four-tenths of the world's agricultural land lies in arid or semi-arid regions. Increasing evidence suggests that drought induces oxidative stress through the production of active oxygen species during stress (Smirnov, 1993). Active oxygen species including superoxide (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl free radical (OH⁻), and singlet oxygen (¹O₂) form in the electron transport systems in chloroplasts and mitochondria. They are highly toxic and can damage many important cellular components, such as lipids, protein, DNA, and RNA (Smirnov, 1993). Osmotic imbalance and membrane damage stability are among the few characteristics that are more frequently recognized during dehydration stress. Analysis of global protein expression revealed that proteins associated with osmotic adjustment, defense signaling and programmed cell death play important roles for drought adaptation of crop plants (Alam et al., 2010a). Accumulation of proline, sucrose and quaternary ammonium derivatives serve as major osmoprotectant molecules (Ibraheem et al., 2011), while heat shock proteins protect cellular proteins from denaturation. The protection of crops against drought-induced damage is a global challenge. Plants can employ numerous physiological and biochemical strategies to cope with adverse conditions by altering the functioning of a number of genes. Consequently, efforts are directed towards a better understanding of the genetic basis of adaptive response of plants to drought and their utilization for breeding purposes (Alam et al., 2010b). More recently, following the increased availability of genome sequencing data, expression profiling has been used to identify genes involved in the adaptive responses to drought and other abiotic stresses (Ozturk et al., 2002; Rabbani et al., 2003; Alam et al., 2010a). An important application of these studies relates to the identification of drought-inducible genes, which

may possess essential or important functions with an effect on tolerance or defense reactions against water loss. Therefore, the identification of genes involved in biotic or abiotic stress responses is a fundamental step in understanding the molecular mechanisms of stress responses and developing transgenic plants with enhanced tolerance to stress. The experimental procedures that have been applied in transcript-profiling studies that mimic drought conditions differ greatly in terms of the dynamics and/or intensity of the water-stress treatments applied. Therefore, it is important to verify the correspondence of changes in expression profiles occurring under different experimental conditions mimicking drought conditions in the field particularly at very initial stage of drought stress. Although several reports have focused on transcriptomic responses of plants exposed to drought or drought like conditions (Ozturk et al., 2002; Rabbani et al., 2003), the present study was designed to identify the very early drought-responsive genes of barley. Barley (*Hordeum vulgare* L.) was chosen for monitoring the changes induced by drought stress in transcript profiles. Barley is moderately tolerant to drought with great economic importance in the world. Recently, an improved, new technique for identifying differentially expressed genes (DEGs) was developed based on annealing control primers (ACPs) (Hwang et al., 2003; Kim et al., 2004). The ACP-based technique focuses on an oligonucleotide primer that anneals with exquisite specificity to the intended template, therefore allowing only the target products to be amplified. GeneFishingTM DEG technique allows for the detection of differentially expressed genes in two or more samples by the random PCR method using Seegene's ACPTM (Annealing Control Primer) technology. This method has been frequently used to identify DEGs in mammalian systems (Kim et al., 2004; Kim et al., 2008). The main goal of this study was to identify novel genes that are differentially regulated upon

exposure to drought stress and thus to provide new insight for the development of plants with enhanced tolerance to drought conditions.

Results and discussion

In order to identify drought-responsive, differentially expressed genes, ACP-based GeneFishing PCR technology was used with a combination of 120 arbitrary primers. Among the 120 GeneFishing primers (GPs), a total of 15 GPs putatively showed differentially expressed DNA bands, with 12 bands increased and 3 bands decreased in intensity in the treated sample compared to the control. The sizes of the bands varied from 225 to 826 bp (Table 1). Among the DEGs, GPs 5, 6, 10, 11, 13, 16, 17, 27, 28, 33, 34 and 36 showed increased or newly detected bands in the treated sample (Fig. 1A). On the other hand, GPs 4, 7 and 49 showed decreased expression (Fig. 1B). The DNA bands were purified from the agarose gels and cloned into TOPO TA cloning vectors for sequencing. The clones were sequenced as described in the Materials and Methods. Sequencing data were confirmed with the barley germplasm database. The sequence similarities of these DEGs are summarized in Table 1. Along with the previously reported drought-responsive genes and/or proteins, such as dehydrins, jasmonate-induced protein, and lipoxygenase 2, some of the novel genes such as PSII 10 kD protein, Glyceraldehyde-3-phosphate dehydrogenase and cold-regulated protein, clone *cortma-ap3* were also identified. According to their putative physiological functions, these identified genes are involved in several processes and/or pathways including stress and detoxification, protein biosynthesis, signaling, and regulatory networks. All the identified DEGs genes were selected to confirm the efficacy of the ACP system, as well as to investigate the temporal mRNA expression patterns of these genes in response to drought stress (Fig. 2). Results of a northern blot analysis were consistent with the ACP-based RT-PCR results for all of the genes at the 12 h to 24 h time point. However, some of the genes, such as those for the PSII 10 kD protein (DEG-6), lipoxygenase 2 (*lox2:Hv:3* gene) (DEG 13), cold-regulated protein (DEG-33) and RTNLB20 (RTNLB20) (DEG 36), showed declining expression until 48 h after treatment (Fig. 2). It is noteworthy that there were slight difference in expression pattern between ACP-based amplification technique and RNA gel blot analysis. This discrepancy may either due to changes in the intensity of individual bands in the gels or to gene family complexity. Nevertheless, the ACP technique allowed the isolation of differentially expressed genes under the conditions tested. Similar phenomenon was also observed in earlier reports (Kim et al., 2004; Manickavelu et al., 2009). Our results clearly indicate that the mRNA levels of drought-induced, differentially expressed genes vary in response to the length of drought treatment. Drought exposure generates oxidative stress in plants. Several non-specific stress responses are also observed that are common to other stresses such as salinity, cold and especially frost. This is due to the fact that the impacts of the stressors trigger similar strains and downstream signal transduction chains. Therefore, in addition to antioxidant enzymes, plants have evolved complex mechanisms in protecting cells against drought condition. Gene upregulation under stress condition indicates their strong needs in cells in order to protect or adapt that adverse condition. In the present study we therefore will put emphasis our interest to the total of 12 up-regulated genes that were identified successfully in barley leaves in response to drought

stress. According to their putative role, these genes are mainly involved in stress and detoxification pathways, and regulatory networks. A discussion of the possible role of these genes in the adaptation of plants to salt-stress conditions follows.

Genes up-regulated under drought stress

DEG-11 and DEG-27 was identified as best linearly upregulated (at 6 h to 48 h time point) transcripts for the biosynthesis of jasmonate and DEG-13 as transcripts for lipoxygenases. Induction of transcripts for the biosynthesis of jasmonate, is known to act as a signal in pathogen defense and under drought conditions (Ozturk et al., 2002; Wierstra and Kloppstech, 2000). Abiotic and biotic signaling pathways often share multiple nodes and their outputs may have significant functional overlap (Alam et al., 2011). In the present study, *LOX2* was up-regulated in response to drought stress in barley leaves (Fig. 1A). It has been reported that *LOX2:Hv:1* is chloroplast-localized, and it might be involved in jasmonate biosynthesis or in the degradation of chloroplastic membrane constituents during a senescence process. Thus, up-regulation of *LOX2* in response to drought stress indicates that *LOX* action leads to membrane degradation and related cell death in drought-induced oxidative conditions as a result of the synthesis of jasmonic acid, which is known to be involved in senescence, or other signaling pathways (Creelman and Mullet, 1997). DEG-17 and DEG-28 and DEG-34 were identified as encoding for *Dhn9* and *Dhn4* genes. Dehydrins (late embryogenesis abundant Group II; D11 family) are an immunologically distinct group of plant proteins which typically accumulate during the late stages of embryogenesis or in response to ABA application, low temperature, or any environmentally imposed dehydrative force such as drought, extracellular freezing, or salinity (Close, 1996). Dehydrin proteins represent one of the potential markers for drought stress (Alam et al., 2010a). *Dhn9*, appears to be orthologous to a *Triticum durum* *Dhn* gene. *Dhn* is believed to be involved in maintaining the integrity of cell structure (Yamaguchi-Shinozaki and Shinozaki, 1994). A comparison of drought resistance of various chickpea species showed enormous differences and from the most resistant *Cicer pinnatifidum*, one dehydrin gene, *Dhn1*, was isolated. Expression of *Dhn1* in *Cicer* is induced by drought and salt stress. Over-expression of *Cicer pinnatifidum* dehydrin *Dhn1* gene in tobacco plants positively affected growth of the seedlings under artificial water stress (Beck et al., 2004). Zhu et al. (2000) reported increased expression of *Dhn* genes during the development of freezing tolerance in a more tolerant barley (*Hordeum vulgare* L.) cv Dicktoo relative to that which occurred in 'Morex', a less tolerant variety. Cellier et al., (1998), using two sunflower (*Helianthus annuus* L.) inbred lines, one tolerant and one susceptible to drought stress, showed a higher accumulation of mRNA transcripts corresponding to *HaDhn1* and *HaDhn2* genes in the tolerant line that was associated with cellular turgor maintenance under drought stress. DEG-10 was identified as encoding gene for receptor kinase. Receptor protein kinases (RPKs) are a diverse group of proteins that span the plasma membrane and allow cells to recognize and respond to their extracellular environment (Van der Geer et al., 1994). They have an extracellular receptor domain that binds signal molecules. Plant receptor kinases play roles in a variety of biological processes including growth, development, hormone

Table 1. Drought-stress induced differentially expressed genes (DEGs) in barley leaves identified by an ACP-based differential display RT-PCR method, Sequencing data were confirmed with the barley germplasm database (<http://shigen.lab.nig.ac.jp/barley/>)

No	GP	DEG No.	Accession No.	Identity	Score	e-value
D-1	4	DEG 3	X12733	Alcohol dehydrogenase gene Adh2	36	2.8E-2
D-2	7	DEG 4	AJ234407	Partial mRNA; clone cMWG0655.	281	4.0E-76
U-3	10	DEG 5	AJ507091	Partial mRNA for NBS-LRR disease resistance	28	2.5
U-4	18	DEG 6	X97771	mRNA for PSII 10kD protein	204	7.0E-53
U-5	31	DEG 10	AY268139	Receptor kinase	30	1.3E-0
U-6	38	DEG 11	X66376	Jasmonate-induced protein	182	1.0E-46
U-7	39	DEG 13	AJ507213	Lipoxygenase 2 (lox2:Hv:3 gene).	34	1.0E-1
D-8	49	DEG 15	M36650	Glyceraldehyde-3-phosphate dehydrogenase	44	2.0E-04
U-9	53	DEG 16	AY268139	BAC 184G9, complete sequece	30	8.1E-1
U-10	54	DEG 17	AF043094	Dehydrin 9 (dhn9) gene, complete cds	500	1.0E-142
U-11	79	DEG 27	X66376	Jasmonate-induced protein	230	5.0E-61
U-12	83	DEG 28	X15287	Dehydrin (<i>Dhn4</i>)	1402	0
U-13	92	DEG 33	AJ291295	Cold-regulated protein, clone cortma-ap3.	353	4.0E-98
U-14	95	DEG 34	AF043094	Dehydrin 9 (dhn9) gene, complete cds	716	0
U-15	100	DEG 36	AY164911	Reticulon	821	0

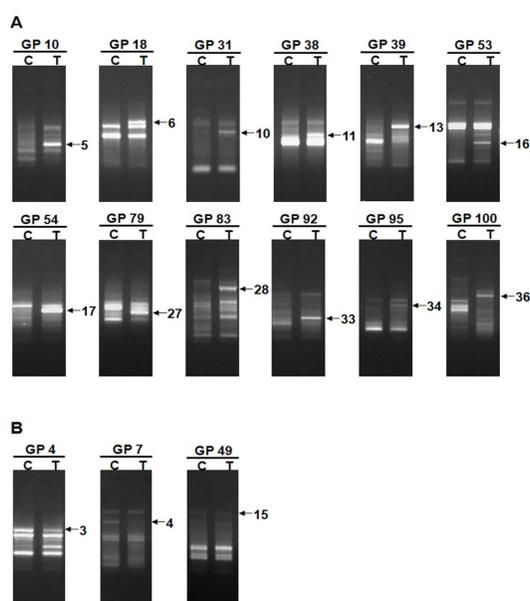


Fig 1. Agarose gel electrophoresis shows results of annealing control primer system for the identification of differentially expressed genes (DEGs) in response to drought stress. The expression of genes were increased (A) or decreased (B) due to treatment (T) compared to control (C). Arrows are indicating the differences (up-regulation or down regulation compared to the control) of the mRNA of the specific gene.

perception, plant-microbe interactions and abiotic stresses such as abscisic acid, waterlogging, drought, and salt stress (Stockinger et al., 1997; Haffani et al., 2004). DEG-33 was identified as encoding gene for cold-regulated protein, clone cortma-ap3. Drought induces some non-specific stress response that are similar to other abiotic stresses such as salinity, cold and osmotic stress. The strategy recites on that the impacts of the stressors trigger similar strains and downstream signal transduction chains. The low-temperature transcriptional activator *CBFI* (C-repeat binding factor) binds to the C-repeat/dehydration-responsive element (DRE with the core sequence CCGAC) of the promoter of cold- and drought-regulated genes (Kasuga et al., 1999; Stockinge et al., 1997). However, the upregulation of cold-regulated protein in response to drought stress is supportive of the above hypothesis in unknown at present. Photosynthesis is one of the most important plant process highly affected by drought stress. DEG-6 was identified as a gene encoding PSII 10 kD protein. In

higher plants, photosystem II is formed by a large number of different polypeptides the majority of which can be assigned to three functional domains, the light-harvesting system, the photochemical reaction centre and the water-splitting activity. In the water-splitting complex, four polypeptides of 33, 23, 16 and 10 kd located in the thylakoid lumen are closely associated (Jansson et al., 1979; Miyao et al., 1989). Ljungberg and coworkers (Ljungberg et al., 1986) have obtained some evidence indicating that the 10 kD protein serves a structural role in the water-splitting complex by providing binding sites for the 23 kd polypeptides. However, limitation of the 10 kD protein on the one hand retards reoxidation of QA- and on the other hand introduces a general disorder into the PSII complex. Up-regulation of PS II 10 protein in response to drought stress suggests that this protein may protect the photosystem machinery against drought-induced ROS. DEG-36 was identified as transcripts for reticulon. Reticulons (RTNs in vertebrates and reticulon-like proteins or RNTIs in other eukaryotes) are a group of

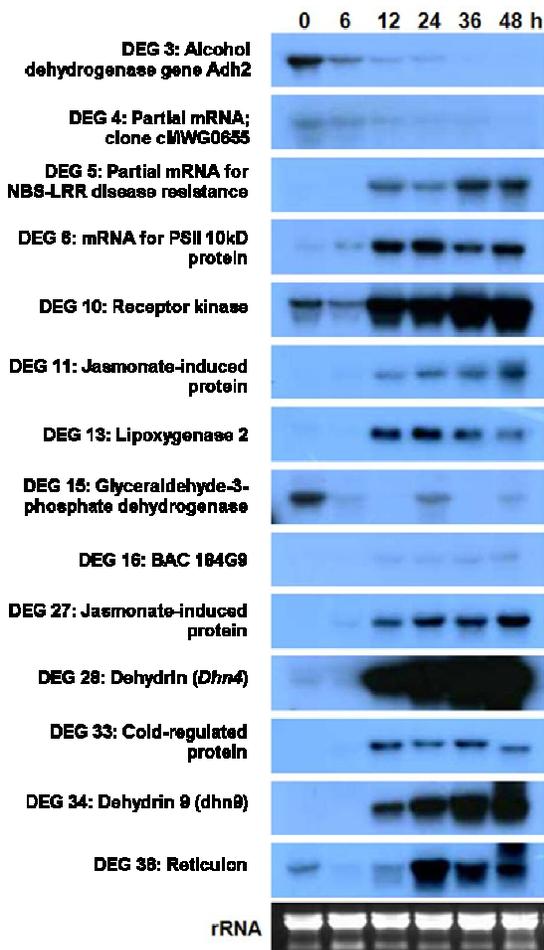


Fig 2. Northern blot analysis of drought-induced differentially expressed genes at different time point (0-48h) obtained by annealing control primer system. Total RNA (10 μ g in each lane) from leaves was separated by 1.2% agarose gel electrophoresis and transferred onto a nylon membrane. Membranes were probed with [32 P] labeled gene-specific probes. Quantity of total RNA loaded in each lane is shown in the bottom panel as a control.

evolutionary conserved ubiquitous proteins found predominantly associated with the endoplasmic reticulum in all eukaryotic organisms examined so far ranging for plants to humans, including yeast and mammalian cells (Nziengui and Schoefs, 2009). Reticulon homologs from nonchordate taxa have been classified into six reticulon-like protein subfamilies (RTNL), including the plant subfamily of RTNLs named RTNLB. The genomes of *Arabidopsis thaliana* and *Oryza sativa* contain 21 and 17 genes, respectively identified 21 sequences encoding RHD-containing proteins from the *A. thaliana* genome in silico (Nziengui and Schoefs, 2009). The overall organization of most AtRTNLBs is reminiscent of those of mammalian RTNs, leading us to suggest that they might share similar functions. While upregulation response of reticulon in drought stress is processes yet to be defined, the similar involvement of reticulon with *Dhn* in membrane stabilization and cell death implies its possible relation in drought response.

Genes down-regulated under drought stress

DEG-15 was identified as transcripts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and DEG-3 for alcohol dehydrogenase 2 (*Adh2*), reflecting the alterations of the glycolytic pathway. GAPDH is an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. The anaerobic induction of maize GAPDH was reported in roots and shoots of tobacco, potato and *Arabidopsis* (Hänsch et al., 2003). Alcohol dehydrogenase (*Adh*) genes are among the best characterized loci in higher plants, both genetically and at the molecular level; the best known of these genes responds to hypoxic and/or anoxic conditions. DEG-3 was identified as *Adh2* (Table 1). Transcriptional activation of the *Adh* gene has also been noticed in response to several environmental stresses including saline conditions (Matton et al., 1990; Baisakh et al., 2008). Consistent with the drought-stress response at the mRNA level, it has also been demonstrated that *Arabidopsis* mutants with defective *Adh* expression showed defective responses to cold and osmotic stresses (Conley et al., 1999). The down-regulation role of *GAPDH* and *Adh2* in response to drought stress is not clearly understood. Indeed, the crosstalk between the fermentative catabolism pathway and drought-tolerance mechanisms remains to be investigated. Although there have been a few transcriptomic analyses carried out to investigate the drought-stress response in barley, in the present study, we have used a new ACP-based, RT-PCR method to identify drought-stress-induced differentially expressed genes in barley leaves. Together with previously identified drought-responsive genes, we identified some candidate genes that regulated at the early drought signaling stage. Further functional studies using forward and reverse genetics will be explored precise role of these genes in response to drought stress. Moreover, these genes could be used as a marker to develop drought resistance/tolerance for crop improvement.

Materials and methods

Plant material and drought treatment

Barley (*Hordeum vulgare* L. cv. Yeongyang Bori) seeds were surface-sterilized and germinated on wet filter paper in a plastic tray for 7 d in a control growth chamber at 24 \pm 1 $^{\circ}$ C (day/night) with a 12 h photoperiod under an irradiance of 350 μ mol m $^{-2}$ s $^{-1}$ and a relative humidity of 60-70%. To initiate drought treatment, the filter papers, on which the seedlings were germinated, were removed and kept on the growth chamber for 24 h. After stress treatment leaves were excised separately and without any treatment were used as controls. Leaf samples were collected then kept at -80 $^{\circ}$ C until RNA extraction.

RNA isolation and synthesis of first-strand cDNAs

Total RNA was isolated from the leaf tissues of treated and control plants using the Plant RNeasy mini kit (Qiagen, CA, USA) and used for reverse-transcriptase-catalyzed first-strand cDNA synthesis. The reverse transcription reaction was performed for 1.5 h at 42 $^{\circ}$ C in a final reaction volume of 20 μ l containing 3 μ g (7.5 μ l) of the purified total RNA; 4 μ l of 5 \times reaction buffer (Promega, Madison, WI, USA); 5 μ l of dNTPs (each 2 mM); 2 μ l of 10 μ M dT-ACPI (5'-CTGTGAATGCTGCGACTACGATIIIIT(18)-3'); 0.5 μ l of RNasin $^{\circ}$ RNase Inhibitor (40 U/ μ l; Promega); and 1 μ l of Moloney murine leukemia virus reverse transcriptase (200

U/μl; Promega). After synthesis, first-strand cDNAs were diluted by the addition of 80 μl of ultra-purified water for the GeneFishing™ PCR.

ACP-based GeneFishing™ reverse transcription chain reaction

Using the GeneFishing™ DEG kit (Seegene, Seoul, South Korea), DEGs were identified by an ACP-based PCR method as described by Lee et al (2009). Second-strand cDNA synthesis was conducted at 50°C (low stringency) during one cycle of first-stage PCR in a final reaction volume of 49.5 μL containing 5 μL (about 50 ng) of the diluted first-strand cDNA, 5 μL of 10× PCR buffer plus Mg (Roche Applied Science, Mannheim, Germany), 5 μL of dNTP (each 2 mM), 1 μL of oligo(dT)₁₅, oligo(dT)₁₅ tail, or oligo(dT)₁₅ ACP (10 μM), 1 μL of 10 μM arbitrary primer (Table 1, 10-mer, tail, or 10-mer ACP) preheated to 94°C and 32 μL of autoclaved Milli-Q. The tube containing the reaction mixture was held at 94°C while 0.5 μL of *Taq* DNA Polymerase (5 U/μL; Roche Applied Science) was added to the reaction mixture. The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5-min final extension at 72°C. The amplified PCR products were separated on 2% agarose gels stained with ethidium bromide, and DEGs were selected visually.

Gene cloning and sequencing

Selected DEGs were extracted from the gel by using the GENCLEAN® II Kit (Q-BIO gene, Carlsbad, CA, USA) and directly cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cloned plasmids were sequenced with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the M13 forward primer (5'-CGCCAGGGTTTTCCAGTCACGA-3') or M13 reverse primer (5'-AGCGGATAACAATTTACACAGGA-3') containing a total of 23 and 24 oligonucleotides, respectively. Sequencing data were confirmed with the GenBank database through the BlastX program of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) and/or the barley germplasm database (<http://shigen.lab.nig.ac.jp/barley/>).

RNA extraction and Northern blot analysis

Northern blot analysis was carried out to investigate the temporal expression of some selected genes in response to drought stress. Leaf samples were harvested 6, 12, 24, 36 and 48 h after treatment. Total RNAs were isolated from the leaf tissues of treated and control plants using a Plant RNeasy mini kit (Qiagen, CA, USA). Ten micrograms of total RNA samples were separated on a 1.2% agarose gel containing formaldehyde. Gene-specific PCR products (DEGs 3,4,5,6,10,11,13,15,16,17,27,28,33,34,36, and 39) were labeled with [α -³²P] dCTP using a random primer labeling kit (GE Healthcare). Northern blot analysis was performed as described previously (Lee et al., 2009).

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