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Antisense Phenotypes Reveal a Functional Expression of *OsARF1*, an Auxin Response Factor, in Transgenic Rice

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Abstract

OsARF1 is the first full-length member of auxin response factor (*ARF*) gene family to be cloned from monocot plant. Using quantitative RT-PCR this study found that, the transcript abundance of *OsARF1* was significantly higher in embryonic tissues than in vegetative tissues. To investigate the effect of *OsARF1* on the phenotype of rice, a cDNA fragment of *OsARF1* was inserted in inverse orientation to the 35S promoter in vector pBin438 to produce an antisense (AS) construction. The *AS-OsARF1* construct was transferred into rice (*Oryza sativa* L. *japonica*) calli via *Agrobacterium tumefaciens*-mediated transformation. Molecular analysis of transgenic plants showed that the functional expression of *OsARF1* was inhibited at mRNA level efficiently. The *AS-OsARF1* plants showed extremely low growth, poor vigor, short curled leaves and tillered but were sterile. Therefore, the *OsARF1* was shown to be essential for growth in vegetative organs and seed development.

Keywords: auxin response factor 1(ARF1), antisense technology, gene transformation, functional genomics, *Oryza sativa*

Abbreviations: ARF, auxin response factor; RT-PCR, reverse transcriptional polymerase chain reaction; AS-DNA/RNA, antisense DNA/RNA; RACE, rapid amplification of cDNA end.

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Introduction

Auxin is one of the two most important plant hormones, and regulates various growth and developmental processes by controlling the expression of auxin-response genes (Ulmasov *et al*., 1995). Auxin responsiveness is conferred to several genes by conserved promoter elements, termed 'auxin-responsive elements' (AuxRE) AuxRE promoter elements are bound by a new class of plant-specific transcription factors, named auxin response factors (ARFs) (Ulmasov *et al*., 1997a). Because of the extremely low expression of genes encoding ARFs, none were isolated until 1997. The first auxin response factor (*ARF1*) was isolated from *Arabidopsis* (Ulmasov, *et al*., 1997a). It was found that all members of the *ARF* gene family have an amino-terminal DNA-binding domain and most contain a C-terminal region with two conserved domains, which are involved in homo-and hetero-dimerization (Ulmasov *et al*., 1999). In *Arabidopsis*, it has been reported that the ARF proteins are encoded by a gene family with 23 members and some of them have been shown to repress or to activate expression of reporter genes with an AuxRE promoter element (Ulmasov *et al*., 1999a; Remington *et al*., 2004; Okushima *et al*., 2005). Recently, the first full-length *ARF* gene of a monocot plant was cloned from rice (Frank *et al*., 2002). Several rice ARF family transcriptional regulators were also identified homologous to *Arabidopsis ARF1* (Waller *et al*., 2002). From genome sequences many *ARF* genes in *Arabidopsis* and in rice have been isolated (Akila *et al*., 2004; Wang *et al*., 2007). Many of the *ARF* genes mediate up-regulation by auxin but some cause down-regulation by auxin (Ulmasov *et al*., 1999). Different amino acid biases in mediate regions of *ARF* genes family were shown to underlie the ability to cause up or down regulation to downstream genes, by fusion protein experiments (Ozga *et al*., 2002). The protein product of a rice auxin response factor *OsARF1* was shown to be localized in nucleus with the OsARF1: GFP fusion protein (Waller *et al*., 2002).

The data on *ARF* genes family was accumulating rapidly in last few years, but there are still few reports on their effects on phenotype. Most (13/18) *ARF* genes when knocked out in *A. thaliana* plants showed no obvious phenotypic change except the previously identified *arf2/hss*, *arf3/ett*, *arf5/mp*, and *arf7/nph4* mutants (Okushima *et al*., 2005). *arf* mutations can affect gynoecium patterning (ARF3); impaired hypocotyl response to blue light, growth and auxin sensitivity (ARF7); the formation of vascular strands and embryo axis formation (ARF5); suppression of the hookless phenotype and hypocotyl bending (ARF2); hypocotyl

elongation, and auxin homeostasis (ARF8). Some double mutants with a phenotypic single gene knock out family members may show new phenotypes. For example, the *arf7 arf19* double mutant was severely impaired in lateral root formation and showed abnormal gravitropism in both hypocotyls and roots.

To test the functional analysis of a previously cloned gene *OsARF1* we generated transgenic plants expressing an antisense (AS) copy of *OsARF1* cDNA in rice. Using a combination of morphological and molecular analysis, we show that AS-copy of *OsARF1* plants exhibits low growth and sterility.

Materials and methods

Germination assays and tissue culture

Rice callus was induced from the seeds of Zhonghua11 (*Oryza sativa L. japonica*) on MS medium that contained MS salts (Sigma, St Louis, MO, USA), 2 mg/l 2,4-D, B5 vitamins and 3% (w/v) sucrose. The primary callus was dissected from the seeds and placed on the same MS medium for 2 weeks. The subcultured callus was separated from the primary callus. After two weeks, one part of subcultured callus was placed on the same MS medium to regenerate the tertiary callus (designated as embryogenic callus) and another part was placed on the MSD medium (containing MS salt, 2 mg/l 6-BA, 0.5 mg/l kinetin, B5 vitamins and 3% sucrose), on which the callus was cultivated until a large number of green dots were growing (designated as differentiating callus). The embryogenic callus and the differentiating callus were collected at the same time for RNA extraction. The sterilized rice seeds germinated and grew to the seedling stage at 25ºC under a 16-h light/8-h dark photoperiod. After the 2-leaf stage, the seedlings were transplanted into the field. The leaves and roots at the 4-leaf stage were collected. Additionally, at the reproductive stage, the young panicles that were shorter than 5 mm in length were collected and immediately frozen in liquid nitrogen and stored at −80ºC.

RNA extraction and reverse transcription

Total RNA was extracted from different rice tissues by using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Sigma) at 37ºC for 1 h. Total RNA amount was quantified by UV spectrophotometry at 260 nm. All the RNA samples were adjusted to the same concentration for further use. The cDNA strands were generated by reverse transcribing 5 µg of total RNA (50 µl reaction volume) using AMV reverse transcriptase (Takara Biotechnology, Shiga, Japan) at 42ºC for 1 h.

Semi-quantitative RT-PCR

The cDNA samples were diluted to 1/5, 1/20 and 1/100. 1 µl of each dilution and the undiluted sample was used as template for PCR quantification. The actin specific forward primer: 5′-CCATTGGTGCTGAGCGTT-3′ and reverse primer: 5′-TAGGAATGGAAGCTGCGGG-3′ were used to quantify the amount of amplico effective cDNA template. PCR was carried out as follows; 95ºC for 3 min; followed by 30 cycles of 94ºC for 30 sec; 56ºC for 30 sec and 72ºC for 30 sec; finally a extension of 10 min. Precisely 5 µl of each amplification was loaded on 1.5% (w/v) agarose gel and the gel was stained by a 10 µg/ml ethidium bromide solution to visualize the bands. The gel was photographed by UV analysis system FR200A (Mikuni, Tokyo, Japan). The relative density of each band was measured by software Smart view™. The cDNA concentrations were normalized based on the concentration of actin. A pair of primers P-5: 5′-CTGCGTGTTGGAGTCAGGCG and P-2: 5′-CATTTGGTTCAGACTCGACCACAAC that were specific to the DNA binding domain of *OsARF1* was applied for semi-quantitative RT-PCR. Each PCR reaction was performed in a 25 µl aliquot as follows; 95ºC for 3 min; followed by 30 cycles at 94ºC for 30 sec; 60ºC for 30 sec and 72ºC for 30 sec; finally with an extension of 10 min. The following steps were the same as for actin quantification.

Construction of antisense OsARF1 transgenic rice

The original partial cDNA, corresponding to the full length coding sequence from 514 bp to 1190 bp, was inversely inserted between *Bam*HI and *Sal*I site of the binary vector pBin438. Expression was driven by Cauliflower mosaic virus (CaMV) 35S promoter and terminated by the *nos* terminator (Fig. 1A). The recombinant plasmid was transformed into rice embryogenic calli through *Agrobacterium*-mediated transformation as previously described (Sunilkumar *et al*., 1999). Transformed calli were inculcated into selection medium supplemented by kanamycin (100 mg/l) and incubated in the dark at 28 ºC for 2 weeks. Resistant-calli were sub-cultured in differentiation medium to regenerate plantlets. Primary transformants were grown in the greenhouse at 30±2 ºC during the day (14 h light) and 22±2ºC during the night (10 h dark). When the 4th leaf grew, the transformed plantlets were transplanted into the field for phenotypic analysis. The controls were treated under the same conditions.

Northern hybridization analysis

The whole plants at later vegetative stage were collected for RNA extractions. Total RNA (30 µg) of each sample of the transgenic plants and controls were separated by electrophoresis in agarose-formaldehyde gels and transferred to positively charged nylon membranes (Boehringer, Mannheim, Germany). Hybridization was performed with Rapidhyb™ hybridization buffer (Amersham Piscataway, NJ, USA) at 65 °C with a γ – [^{32}P]-dCTP random-labeled probe corresponding to *OsARF1* cDNA. After washing with 2× SSC, 1× SSC and 0.5× SSC washing solutions (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 0.2% (w/v) SDS at 63 °C, membranes were autoradiographed for 16–96 h using an intensifying screen (Biomax, Kodak,Rochester, NY).

Microarray analysis

Microarray analysis was carried out by Capital Biochip Corporation with hybridization tuned for gene expression variation (Lan *et al*., 2004). The RNA samples from 11 independent transgenic plants were quantified and evenly contributed to the RNA population for microarray

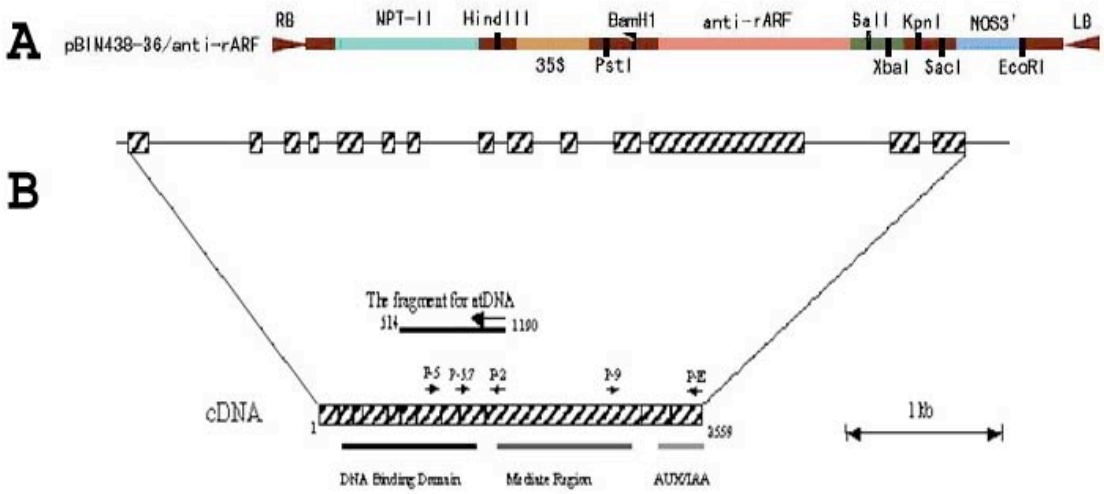

Fig. 1**. A** Construction of binary vector pBin438 harboring the AS-cDNA of *OsARF1* in the antisense orientation driven by *35S* promoter and *Nos* terminator. *Kpn*1 was within the selectable marker gene. **B** Structural features of *OsARF1* gene. The exons and introns are represented by boxes and lines, respectively.

analysis. The RNA of untransformed plants was used as the control. Probe hybridization and scanning of the hybridized microarray slide were done according to the method of Lan, (2004). Hybridization was carried out twice and data were analyzed with ArrayGuage™ version 1.21 (Fujifilm, Tokyo, Japan).

Results and discussion

Structural features of OsARF1 *gene*

A cDNA of a rice *ARF* orthologue was isolated by subtractive suppressed hybridization (SSH), using a cDNA library of young panicles as tester and that of meritstems as driver (data not shown). The 5′-end and 3′- ends were cloned using RACE method (Subudhi and Huang, 1999). By screening a bacterial artificial chromosome (BAC) library of 'IR64 *Indica'* rice variety, a BAC clone containing the genomic DNA region corresponding to the cDNA was identified. The region of approximately 8kb that encompassed the coding region was sequenced by primer walk sequencing. A rice marker RZ797, which mapped on chromosome 11, was within this region locating *OsARF1*. The full-length cDNA of the rice *ARF1* gene was 2,559 bp; consisted of 14 exons; and encoded 852 amino acid polypeptides (Fig. 1B). Similarity searches indicated that the *OsAFR1* gene was most closely orthologous to *Arabidopsis ARF1.* Therefore, '*Oryza sativa* auxin response factor 1' (*OsARF1*; GenBank accession number AJ306306) was named according to the standard nomenclature. The deduced amino acid sequence of *OsARF1* had the typical features of transcriptional factor, with a DNA binding domain (AuxRe) close to its N- terminal, and a protein interaction domain *(*Aux/IAA*)* at its C- terminal. However, the first 50 amino acid at N-terminus and the mediate region between AuxRe and Aux/IAA showed low identities to any known ARF family gene (Fig. 1B).

Expression pattern of OsARF1 gene in specific rice tissues

The expression level of *OsARF1* was extremely low so that it was not detected by Northern hybridization; even in young panicle where the gene was originally isolated. Consequently the *OsARF1* transcript abundance patterns were analyzed using semi-quantitative RT-PCR. The 5′ primer was designed to a region of *OsARF1* sequence that was not conserved in other ARF or *Aux/IAA* genes. *OsARF1* was transcript accumulated in callus and young panicle at much higher amount than in leaf and root. Compared to that in non-differentiated callus, the transcript abundance in differentiating callus dropped significantly (Fig. 2). The results suggested that *OsARF1* could be associated with embryogenesis, because of the higher transcript abundance in young panicles and calli, which both are embryonic tissues.

AS-OsARF1 transgenic rice showed inhibited growth

To investigate the effects of the loss of function of the *OsARF1* gene on rice phenotypic characters, the *AS-OsARF1* vector was constructed and transferred to the rice cells using *Agrobacterium*-mediated transformation technique. The transgeneic plants were detected by PCR using non-intron sequence specific forward primer P-5.7: 5′-AAATGGCGCTCCCTTAAGGTGAG and the reverse primer P-2: 5′-CATTTGGTTC GACTCGACCACAAC. Eleven of 16 regenerated rice plants showed the

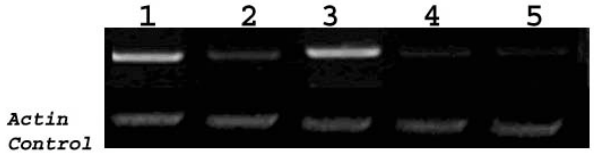

Fig. 2**.** Transcript abundance of *OsARF1* in wild type rice. Lanes were; 1. embryogenic callus; 2. differentiating callus; 3. young panicles; 4. leaves; 5. roots. Lower lanes: *Actin* mRNAas a control.

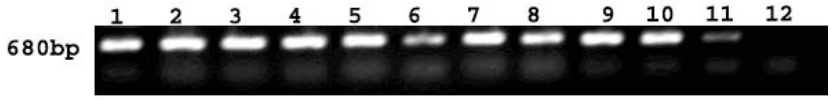

Fig. 3. Detection of *AS-OsARF1* integration by non-intron sequence specific primers. Lane 1–11: *AS-OsARF1* transformed regenerated plants; Lane 12: control plant.

expected amplicons (680 bp) while there was no signal in the control, indicating that the *AS-OsARF1* cDNA was successfully integrated into the genome of transgenic plants (Fig. 3). The *AS-OsARF1* transformed plants showed significantly lower growth and vigor that included smaller leaves and shorter heights, compared to non-transformed plants. Interestingly, the length of the tillering node didn't change significantly in transgenic plants (Fig. 4). Also, the leaves curled across the width. Eight of 11 transgenic plants failed to head and the remaining three were sterile, although they were able to develop to the heading stage 13–15 days later than the non-transformed plants. Hence, no F_2 progeny was obtained. In order to investigate the transcript abundance of *AS-OsARF1*, total RNA was isolated from transgenic and control plants and subjected to RNA gel blot analysis. The result showed that there was extremely low level of mRNA in control and transgenic plants (Fig. 5).

Microarray analysis of regulated genes expression in transgenic plants

To test the changes in gene expression caused by *OsARF1* in transgenic plants, microarray analysis was conducted. Genomic transcript abundances pattern in the transgenic plants was checked with a cDNA microarray containing 14,688 genes arrayed in duplicates that were randomly selected from rice ESTs library (http://microarray.rice.dna.affrc.go.jp). Microarray analysis of the transgenic plants in later vegetative stages showed that 10,325 genes of the total of 14,688 genes were expressed at this stage in transgenic plants (Fig. 6A). The genes whose florescent ratio was < 0.5 or > 2.0 (transgenic/control) were defined as differently expressed genes. By this criterion, 435 genes were differently expressed, 255 of them were down regulated (Fig. 6B) and 180 were up regulated (Fig. 6C). The annotated genes were located in 9 sub-categories of molecular functions in gene ontology (Table 1). Most of the differently expressed genes were categorized among those encoding proteins with catalytic activity. However, the inference of gene function based on homology and annotation may not reflect the real function, thus the classification of these genes was only a reference.

In this study, "knock down" phenotypic analysis was used. Phenotypes were clear and consistent but unlike those seen from ortholgues in *A. thaliana*. Semi-quantitative RT-PCR showed that no transcript of the original *OsARF1* was detected in *AS-OsARF1* transgenic plants, suggesting that the AS-RNA not only inhibited the translation of its target mRNA, but also caused degradation of target mRNA. So, AS-RNA might work in a similar way as RNAi (RNA interference). Recently, it was reported that the expression of *OsARF1* was positively regulated by auxin concentration (Waller *et al*., 2002). However, the expression level of *ARF1* in *Arabidopsis* was relatively consistent regardless the concentration of auxin (Ulmasov *et al*., 1997a). That suggested that the *OsARF1* might not be the exact ortholog of *AtARF1* even though these two genes shared the highest degree of similarity between rice and *Arabidopsis* (Frank *et al*., 2002). Here, *OsARF1* was expressed significantly higher in embryonic tissues (callus and young panicle) than that in non-embryonic tissues (root, leaf and differentiating callus). Therefore the endogenous auxin to cytokinin ratio might be higher in embryonic tissues. In addition, the increasing of the expression of *OsARF1* could be essential to embryogenesis since all the *AS-OsARF1*transgenic plants were sterile. *OsARF1*, as an early response gene in auxin signaling (Waller *et al*., 2002) might play a key role in fertility and regulate quite a few downstream genes associated with growth and reproduction.

The cDNA microarray provided an efficient high throughput approach to investigate the function of a large numbers of genes and their expression in response to environmental effects (Kawasaki *et al*., 2001). The results

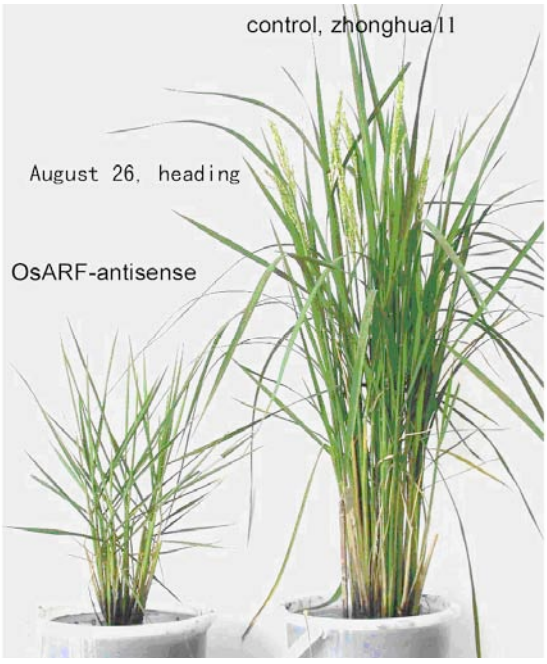

Fig. 4. Comparison of *AS-OsARF1* transgenic rice and a non transgeneic control. Left; *AS-OsARF1* transgenic rice. Right; untransformed control.

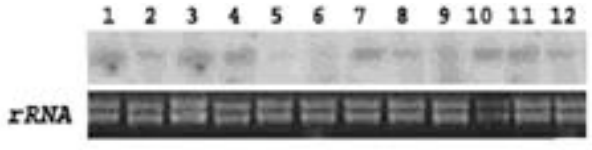

Fig. 5. Abundances of the *AS-OsARF1* mRNA in transgenic rice plants. Upper panel: Lane 1: control; lanes 2–12: 11 transgenic plants. Lower panel: rRNA control.

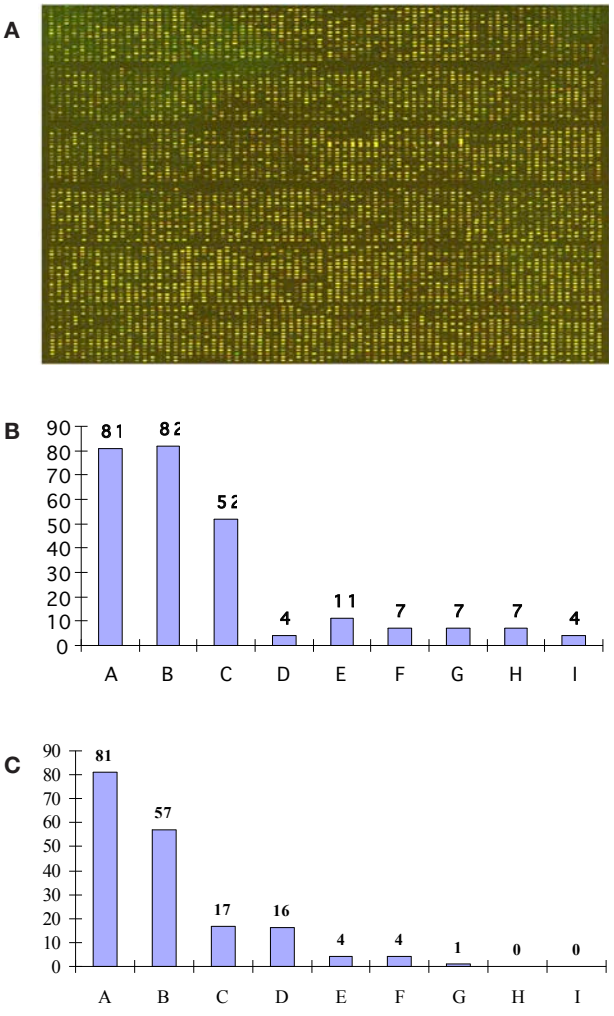

Fig. 6**A.** Microarray analysis of regulated transcript abundance among transgenic plants. Three-color image of a transgenic plant cDNA array. After scanning for each fluorescent dye separately false colour images were superimposed. Red dots correspond to genes higher expressed down-regulation, green dots to genes that are higher expressed up-regulation, and yellow dots represent genes that show no differential expression. **B** Genes decreased in TA in transgenic rice. Alphabetic letters represent indicators in Table 1. **C** Genes increased in TA in transgenic rice. Alphabetic letters represent indicators in Table 1.

here indicated that approximately 3% of the total set of predicted genes and approximately 4% of known to be expressed genes showed significant differences among their transcriptional abundance between the transgenic plants and the controls. The ratio of differently expressed genes was similar among different tissues. Reproductive stage analyses were not possible because all AS plants failed to reach to productive growth stage. It seems likely the knock down of *OsARF1* turned off some key switches for transformation from vegetative stage to the reproductive stage. Perhaps *OsARF1* itself was the switch. Suppression of *OsARF1* expression also caused dramatically growth at vegetative stages, even though its expression was extremely low at this stage in wild type rice. This demonstrates that, perhaps *OsARF1* is crucial to all growth. In comparison the phenotypic effect of *ARF1* in *Arabidopsis* is unknown (Wang *et al*., 2007). In contrast, *OsARF1* was shown to be essential for the developmental growth stages of the life cycle of the rice plant. The pathways and functional mechanisms will be studied further.

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Table 1. Genes with different transcript abundances detected by cDNA microarray.

	Differently expressed genes			
No.	Categorization of expressed genes	Down-regulated genes	Up-regulated genes	Total numbers of expressed genes
A	No significant homology in GenBank	81	81	162
B	Homology to function unknown genes	82	57	139
C	Catalytic activity	52	17	69
D	Structural molecule activity	4	16	20
E	Transporter activity	11	4	15
F	Binding	7	4	11
G	Antioxidant activity	7	1	8
H	Enzyme regulator activity	7	0	7
I	Signal transducer activity	4	0	4
Total		**255**	**180**	**435**

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