Gradual Increase of miR156 Regulates Temporal Expression Changes of Numerous Genes during Leaf Development in Rice

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The highly conserved plant microRNA, miR156, is an essential regulator for plant development. In Arabidopsis (Arabidopsis thaliana), miR156 modulates phase changing through its temporal expression in the shoot. In contrast to the gradual decrease in time in the shoot (or whole plant), we found that the miR156 level in rice (Oryza sativa) gradually increased from young leaf to old leaf after the juvenile stage. However, the miR156-targeted rice SQUAMOSA-promoter binding-like (SPL) transcription factors were either dominantly expressed in young leaves or not changed over the time of leaf growth. A comparison of the transcriptomes of early-emerged old leaves and later-emerged young leaves from wild-type and miR156 overexpression (miR156-OE) rice lines found that expression levels of 3,008 genes were affected in miR156-OE leaves. Analysis of temporal expression changes of these genes suggested that miR156 regulates gene expression in a leaf age-dependent manner, and miR156-OE attenuated the temporal changes of 2,660 genes. Interestingly, seven conserved plant microRNAs also showed temporal changes from young to old leaves, and miR156-OE also attenuated the temporal changes of six microRNAs. Consistent with global gene expression changes, miR156-OE plants resulted in dramatic changes including precocious leaf maturation and rapid leaf/tiller initiation. Our results indicate that another gradient of miR156 is present over time, a gradual manner, and miR156-OE attenuated the temporal changes of 2,660 genes. Interestingly, seven conserved plant microRNAs also showed temporal changes from young to old leaves, and miR156-OE also attenuated the temporal changes of six microRNAs. Consistent with global gene expression changes, miR156-OE plants resulted in dramatic changes including precocious leaf maturation and rapid leaf/tiller initiation. Our results indicate that another gradient of miR156 is present over time, a gradual increase during leaf growth, in addition to the gradual decrease during shoot growth. Gradually increased miR156 expression in the leaf might be essential for regulating the temporal expression of genes involved in leaf development.

The microRNAs, a class of 20- to 24-nucleotide small RNAs, play essential roles in developmental regulation in plants and animals. These microRNAs are perfectly or imperfectly complementary to their targeted mRNAs and repress the target genes by mRNA cleavage or translation suppression. A few micro-RNAs and their targeted genes are conserved among plant species and likely regulate basic processes for plants (Axtell and Bartel, 2005; Axtell et al., 2007; Voinnet, 2009). Temporal or spatial accumulation of these conserved microRNAs is essential for proper development in plants. In Arabidopsis (Arabidopsis thaliana), for instance, the adaxial-abaxial patterning of leaves and the radial patterning of stems are regulated by miR165/miR166 (Emery et al., 2003; Juarez et al., 2004). Organ boundaries are regulated by miR164 (Mallory et al., 2004; Baker et al., 2005; Nikovics et al., 2006). The regulation of TEOSINTE BRANCHED/CYCLOIDEA/PCF genes by miR319 and miR159 define the leaf margin and size (Palatnik et al., 2003; Ori et al., 2007). The temporal expression of miR156 and miR172 regulates the vegetative-reproductive phase transitions (Aukerman and Sakai, 2003; Lauter et al., 2005; Schwab et al., 2005; Xie et al., 2006; Chuck et al., 2007; Gandykota et al., 2007; Wang et al., 2008, 2009, 2011).

miR156 is one of the most conserved and highly expressed microRNAs in plants. It has been found in moss, monocotyledons, and dicotyledons. The miR156 targets SQUAMOSA-promoter binding-like (SPL) transcription factor genes in plants. In Arabidopsis, maize (Zea mays), and rice (Oryza sativa), overexpressing miR156 resulted in dramatic morphologic changes, suggesting that miR156 has global regulatory function in plant development (Schwab et al., 2005; Xie et al., 2006; Chuck et al., 2007). In Arabidopsis, miR156 and its target SPL genes define an essential regulatory module in phase change (Wang et al., 2009; Wu et al., 2009), leaf trichome development (Yu et al., 2010), male fertilization (Xing et al., 2010), embryonic patterning (Nodine and Bartel, 2010), and anthocyanin biosynthesis (Gou et al., 2011). The miR156-targeted SPLs regulate the expression of several essential regulators, including...
RESULTS
Gradual Increase of miR156 Expression in Developing Leaves of Rice

Our previous data indicated that miR156 is accumulated at different levels at different stages during the development of the rice panicle (Xie et al., 2006). miR156 showed a much lower expression level in 3-week-old seedlings than in 3-d-old shoots (data not shown), which is similar to observations in Arabidopsis. We also noticed that the miR156 level showed dramatic differences between old and young leaves, which prompted us to investigate the variation of miR156 expression level in the developing or growing leaves from a single plant. In this study, leaves on each tiller were numbered according to their order of emergence, with L1 indicating the first developed leaf (Fig. 1). By checking the level of miR156 in three sequentially developed leaves (L3–L5) from the main tiller of 25-d-after-germination (DAG) rice plants, the miR156 level is approximately 2 times higher in old leaves (L3) than in young leaves (L5; Fig. 1A). The miR156 level increased gradually from L5 to L3. At the tillering stage, when rice boost to branching (40 DAG), miR156 also had a similar expression pattern in the leaves (increased gradually from young to old leaf) both in primary tiller and secondary tiller (L5–L7 in primary tiller and L1–L3 in secondary tiller; Fig. 1B). It is interesting that the difference of relative expression level of miR156 between sequential leaves is always between 20% and 30% (Fig. 2, A and B). At the grain-filling stage at which rice leaves stop growing (100 DAG), miR156 showed a similar expression level in all the green leaves (L1–L15; Fig. 1C). The expression level of miR156 showed a similar dynamic change also in the leaf sheath (Fig. 1, A–C), although the expression level in the sheath was lower than that in the corresponding leaf blade. We further checked the oldest leaves collected at different shoot developmental times, and miR156 had comparable levels in these leaves (Fig. 1D). According to the gradually decreased expression pattern of miR156 in shoot, early emerged or juvenile leaves should have higher levels of miR156 than later-emerged adult leaves have. This model cannot explain the comparable high levels of miR156 in adult leaves that emerged at different shoot stages (Fig. 1, C and D), suggesting that miR156 might have a different temporal expression pattern in developing adult leaves.

To further identify the relationship between miR156 level and leaf developmental time, L4, L6, L8, L9, and flag leaf (L16), with growing times of 3 to 40 d after emergence, were used to check the miR156 level. These leaves were initiated at a comparative adult vegetative stage, except that L4 was from an intermediate juvenile/adult stage. The result clearly showed that miR156 is increased gradually during leaf development (Fig. 2A). In the flag leaf, expression of miR156 was highly correlated ($r^2 = 0.990$) with the time of leaf growth (Fig. 2B). Taking the maximal miR156 level as
100%, miR156 was increased about 10% every 5 d in flag leaf. These data indicated that the miR156 level may positively reflect the developmental time or age of leaves. The difference of miR156 level in different leaves at the same shoot age (Fig. 1) is probably due to leaf age rather than shoot age. However, the miR156 level was lower in leaves than in 3-d-old shoots, which consist of juvenile leaves (Fig. 2C), suggesting that opposite temporal expression patterns of miR156 in developing leaves and shoots (or whole plants) coexist in the rice plant.

Recent studies suggested that some mature small RNAs have different expression features than their primary transcripts (Chitwood et al., 2009). Therefore, we investigated the expression of the precursors of miR156 (pri-miR156) during leaf development by reverse transcription-quantitative (RT-q)PCR. The results indicated that at least pri-miR156d and pri-miR156h have a similar expression pattern to that of miR156 (Supplemental Fig. S1), suggesting that the temporal expression of miR156 in leaves was probably regulated by transcriptional control. We also checked the spatial expression of miR156 by in situ RNA hybridization using a locked nucleic acid (LNA) probe. miR156 was expressed in leaf primordium (P1–P3) and leaf sheath but was not detected in the shoot apical meristem (SAM; Supplemental Fig. S2). In leaf elongating leaves (L3 in 15-DAG seedlings), miR156 was detected in almost all cells (Supplemental Fig. S2). miR156 has no obvious specificity of distribution in leaves.

**Overexpression of miR156 Attenuates the Temporal Expression of miR156-Targeted OsSPL Genes**

In a previous report, we generated miR156-OE transgenic rice lines in which pri-miR156d and pri-miR156h were driven by a maize ubiquitin promoter (Xie et al., 2006). In these studies, homozygous pri-miR156d and pri-miR156h overexpression plants (abbreviated as Md/Mh hereafter) at the T3 or T4 generation were used. By checking age-different leaves from three lines (Md15, Md43, and Mh9), miR156 levels in all the leaves of Md/Mh were higher than levels in the wild type (Fig. 3). In L3 (45 DAG), the miR156 level was 5 times higher in Md/Mh than that in the wild type. In L6 or L7 (45 DAG), miR156 was increased 8-fold in Md/Mh compared with the wild type. Despite high expression, temporal changes of miR156 were less obvious in Md/Mh than in the wild type. In Md/Mh, miR156 was increased only about 30% in the newly emerged leaf compared with early-emerged old leaf. These results suggested that miR156 was overexpressed and the pattern of gradual increase in the wild type was disrupted in Md/Mh.

Figure 1. Expression of miR156 is gradually increased during leaf development. A to C, Expression levels of miR156 in sequentially developed leaves at three different stages: seedling (25 DAG), tillering (40 DAG), and graining (100 DAG). As shown in the photographs of rice plants on the right, the leaf samples were numbered according to the order of emergence from the tiller. The relative expression levels are indicated at the bottom of the blots except for weak bands. The labeled leaves were initiated at the adult vegetative phase, except that L3/L4 were intermediate juvenile/adult leaves of the main tiller. D, Comparison of the maximal miR156 level in leaves at different shoot stages. [See online article for color version of this figure.]
We continued to check whether the temporal expression of miR156 affects its target gene expression. In this assay, sequential leaves from plants at 45 DAG were used. In contrast to the miR156 expression pattern, real-time RT-qPCR indicated that five of the miR156-targeted OsSPL genes (OsSPL3, OsSPL12, OsSPL13, OsSPL14, and OsSPL17) showed obviously higher expressed levels in young leaves than in old ones (Fig. 4). Compared with the wild type, the five target genes showed reduced levels in young leaves of Mh43 (an Md/Mh plant), and the temporal expression patterns of the genes disappeared. The expression of other miR156-targeted OsSPL genes showed no obvious temporal changes both in the wild type and in Mh43 plants. Because miR156 can function by inhibiting target genes at both the transcript cleavage and translational levels in Arabidopsis (Gandikota et al., 2007), it is possible that these OsSPLs may be regulated by miR156 mainly at the translation level. The expression levels of these OsSPL genes were also checked in other Md/Mh lines, and similar results were obtained. These results indicate that the temporal expression patterns of the miR156-targeted OsSPLs were disrupted by miR156-OE.

The Temporal Expression of Numerous Nontarget Genes Was Also Attenuated by miR156-OE

To identify genes whose expression is affected or indirectly regulated by miR156, Affymetrix 57K DNA chips were used to check the transcriptomes of old (L3) and young (L6) leaves (as designated in Fig. 3) of Md/Mh and the wild type at the tillering stage. Compared with the wild type, 3,008 genes were significantly changed in the Md plant (Fig. 5A; Supplemental Tables S1 and S2). Among these genes, 2,233 and 775 genes were up- and down-regulated by miR156-OE, respectively; 2,694 and 407 genes were affected by miR156-OE in the early emerged (L3) and later emerged (L6) leaves, respectively (Fig. 5A). Only 93 genes were affected by miR156-OE in both the L6 and L3 leaves (Fig. 5A). These results suggest that miR156-OE can cause predominant up-regulation of genes mainly in the old leaf.

We further checked the temporal expression of the genes affected by miR156-OE in the wild-type plants. Of the genes up-regulated by miR156-OE, 90% showed increased expression levels in the old leaf compared with levels in the young leaf (such change is called time positive) and 4% of them showed decreased expression (called time negative) in the old leaf (Fig. 5B). Of the genes down-regulated by miR156-OE, 67% and 18% showed time-positive and time-negative expression changes, respectively (Fig. 5B). To indicate more explicitly the temporal changes of the miR156-regulated genes during leaf development, the expression levels of these genes in old leaves were normalized to young leaves of the same plant and log transformed (log-transformed fold change [logFC]). Interestingly, logFC of these genes ranged from $2^6$ to $2^{2}$ in wild type plants but from $-2$ to $-1$ in Md plants (Fig. 5C). Genes with logFC of less than $-5$ were not found in Md, whereas about 300 genes were found with logFC of less than $-5$ in the wild type. In Figure 5D, a logFC-based heat map of these genes clearly shows that miR156-regulated genes have fewer temporal changes in Md than in the wild type. The average logFC values of miR156-regulated genes are $-2.3$ and $-0.8$ in the wild type and Md, respectively. According to gene expression patterns, these genes could be grouped into three clusters (Fig. 5E). In cluster I, 2,660 genes were significantly affected by miR156-OE in old or young leaves, and the logFC values of miR156-OE plants (Md) were lower than those of wild-type plants, suggesting that the
Temporal changes of cluster I genes were significantly attenuated (Fig. 5E). Cluster II included 247 genes without temporal expression changes in wild-type leaves, but these genes showed increased/decreased expression changes in the Md leaf. Cluster III contained 101 genes showing highly dynamic changes in wild-type or Md leaves, but these are distinct from those in cluster I or II. Together, these results indicate that miR156-OE can attenuate the temporal expression changes of numerous genes between old and young leaves.

Ten genes, including six genes showing up-regulation in Md and four genes with distinct temporal expression patterns in the wild type/Md, were selected to confirm their expression changes by RT-qPCR. The results generally agreed with the microarray data (Fig. 6). Some of the arbitrary relative expression values showed little difference between microarray data and RT-qPCR results because of the different data normalization methods used.

**Genes Affected by miR156-OE Are Involved in Diverse Cellular Processes**

Gene Ontology enrichment analysis was performed for all genes regulated by miR156-OE. The results showed that genes related to the cell wall, development, and reproduction were overrepresented (Supplemental Table S3). In addition, genes related to the carbohydrate metabolism process, motor activity, hydrolase activity, transferase activity, catalytic activity, and lipid binding were also overrepresented. Most of the genes up-regulated by miR156-OE were predicted with functions related to metabolism, such as hydrolase activity, transferase activity, and catalytic activity. Genes annotated as transcription regulators, receptors, and kinases were overrepresented in the genes down-regulated by miR156-OE. Among the genes up-regulated by miR156-OE, four genes (LOC_Os01g07630, LOC_Os03g16010, LOC_Os02g09359, and LOC_Os08g17410) are homologous to BAK1 (for BR1-associate protein kinase), which mediates brassinosteroid signaling in Arabidopsis; 13 genes are related to auxin response or signaling, including five OsIAA genes (OsIAA14, OsIAA21, OsIAA27, OsIAA30, and OsIAA31) and five OsSAUR genes (LOC_Os02g24700, LOC_Os04g56680, LOC_Os09g37500, LOC_Os12g41600, and LOC_Os09g37480). Gel blotting of small RNA suggested that miR156 expression was decreased slightly by brassinosteroid treatment but not by other phytohormones (Supplemental Fig. S3). This result implies that miR156 may be involved in the regulation of auxin and brassinosteroid signaling pathways associated with leaf development. Interestingly, stress-responsive genes were overrepresented in the miR156 down-regulated genes (Supplemental Table S3). These data suggested that miR156-OE affected the expression of genes involved in diverse cellular processes related to metabolism, development, and stress responses.

Among the genes up-regulated by miR156-OE, 31 genes were specifically expressed in Md leaves (Supplemental Table S4). Five of them were confirmed by RT-qPCR (Fig. 6). Among these miR156-specific genes, 18 are annotated as unknown function genes. These unknown genes were predicted to encode small polypeptides (less than 200 amino acids) or no-coding RNAs. By searching a small RNA database, three transcripts (AK064111, AK109584, and AK111071) were found with matches of 23- to 24-nucleotide-long small RNAs, which most likely were small interfering RNAs. In addition to these unknown genes, other genes activated by miR156-OE are predicted to function diversely, including stress-related proteins, F-box proteins, zinc finger proteins, and cytochrome P450.

**miR156-OE Affects the Temporal Expression of Some Plant Conserved MicroRNAs**

To determine whether miR156-OE affects other microRNAs, 15 plant conserved microRNAs were investigated for their expression levels in leaves of wild-type and Md plants by using stem-loop RT-qPCR (Shen et al., 2010). Of these microRNAs, eight (miR156, miR159, miR160, miR164, miR169, miR172, miR319, and miR399) showed greater than 3-fold change between young and old leaves (Fig. 7A). These microRNAs can be categorized into three classes according
to their expression patterns in different leaves of the wild type (Fig. 7B). The microRNAs in class I (miR162, miR166, miR167, miR168, miR171, miR393, and miR399) showed strong expression in young leaves with changes of less than 3-fold between young (L6) and old (L3) leaves. miR168 has the most stable expression compared with the others. The microRNAs in both classes II and III showed greater than 5-fold changes between young and old leaves; the microRNAs in class II (miR156, miR159, miR169, and miR319) were strongly expressed in older leaves, whereas those in class III (miR160, miR164, miR172, and miR390) were strongly expressed in younger leaves. The expression patterns of these microRNA clearly show that most microRNAs are expressed temporally during leaf development.

Compared with the wild type, the expression of six microRNAs (miR159, miR164, miR169, miR172, miR319, and miR393) was significantly suppressed (more than 2-fold at least in one leaf; Fig. 7A) in Mh. Interestingly, some of these microRNAs affected by miR156-OE were found only at specific leaf developmental stages. For example, miR164 was dramatically suppressed in young leaves (L5; expanding leaf), miR319 was suppressed specifically in mature leaves, and miR393 was suppressed most obviously in young leaves (Fig. 7A). miR172 was suppressed by miR156 in all leaves except the oldest one, in which miR172 was barely detected. The result of stem-loop RT-qPCR, with miR164 as an example, was confirmed by small gel blotting (Fig. 7C). These results suggest that miR156-OE can also affect the temporal expression of other microRNAs.

miR156-OE Promotes Leaf Initiation and Precocious Maturation

Among the genes affected by miR156-OE, 91% showed expression changes between young and old leaves, and many of them were related to development based on their annotation as indicated above. A previous study showed that miR156-OE plants (Md/Mh) had significantly more leaves and tillers but with a smaller size than wild-type or negative control (NC; no overexpression lines segregated from Md/Mh) plants (Xie et al., 2006). Therefore, we checked the morphologic changes in the miR156-OE plants (Md/Mh) in more detail to determine the role of miR156 in development. In the early vegetative phase (before 35 DAG), no difference was apparent between the Md/Mh plants and wild-type/NC plants. After 35 DAG, the Md/Mh plants were distinct from the wild-type/NC plants in plant height (Fig. 8A), leaf number (Fig. 8B), and tiller number (Fig. 8C). From 35 to 120 DAG, Md/Mh plants showed a 1.5-fold higher rate of leaf initiation than wild-type/NC plants. The maximum leaf initiation rate of Md/Mh was 14 to 17 leaves per day, with 0.3 leaves emerging for a tiller each day on average, which is about 1.5-fold the wild-type value (0.18 leaves per day from one tiller; Fig. 8D). Both Md/Mh and wild-type plants burst in leaf/tiller initiation between 40 and 60 d (Fig. 8D). At the grain-filling stage (120 DAG), the leaf number and tiller number in Md/Mh plants were 250 to 500 and 50 to 100 times, respectively, those in wild-type/NC plants (Fig. 8, B and C). In contrast to the high
speed of leaf initiation, the size of Md/Mh SAM was smaller than that of wild-type/NC plants after the four-leaf stage ($P < 0.05$; Supplemental Table S5). These results imply that overexpression of the miR156 level can accelerate the initiation rates of leaf and tiller.

Because the temporal expression changes of miR156-OE-affected genes were observed mainly in old leaves, we checked the maturation status of these leaves. Except for the early vegetative stage, the width and length of fully expanded Md/Mh leaves at the other stages decreased significantly ($P < 0.01$) compared with wild-type/NC leaves (Fig. 8, E–J). We further checked the maturation status of the Md/Mh leaves using toluidine blue O staining, which has been used to distinguish the cell walls of juvenile and adult leaves in maize (Poethig, 2003). Except for the leaves from the

Figure 5. Summary of miR156-regulated genes in different leaves. A, Venn diagram representation of miR156 up- and down-regulated genes in L6 (later-emerged young leaf) to L3 (early-emerged old leaf). B, Most miR156-regulated genes have temporal expression in the wild type (WT). Time positive indicates that gene expression is increased from L6 to L3 in the wild type; time negative indicates that gene expression is decreased from L6 to L3 in the wild type. C, Distribution of logFC of miR156-regulated genes in the wild type and Md. LogFC indicates the fold change of each gene from young to old leaf in these plants. D, Heat map shows logFC of different plants. Color indicates the value of logFC as shown on the bar at bottom. E, Clusters of miR156-regulated gene expression patterns.
very early vegetative stages, all the other leaves showed no difference in staining (Supplemental Fig. S4, A and B), although the cell size was decreased slightly in Md/Mh mature leaves (Supplemental Fig. S4, C and D). The numbers of trichomes, papilla, and stomata were also obviously decreased in Md/Mh leaves (Supplemental Fig. S5). These results suggest that miR156-OE can result in precocious leaf maturation.

In addition to increased leaf numbers with smaller size, the Md/Mh plants generated more roots with smaller size than did wild-type plants (Supplemental Fig. S6). This result implies that miR156 may also regulate root development, probably by the same mechanism as present in shoots.

**DISCUSSION**

In this study, we found that miR156 increased gradually from young leaves to old leaves in rice after the juvenile stage, and disruption of the miR156 gradient by overexpression of two miR156 precursors attenuated the temporal expression of thousands of genes.
miR156-OE has global impacts on leaf development, including increased initiation rates of tillers and leaves and precocious maturation. Our results suggest that the temporal expression of miR156 has an essential role in regulating leaf and tiller development.

**Two Opposite Gradient Expressions of miR156**

The gradual decrease of miR156 in shoots over plant-growing time regulates phase changing in Arabidopsis (Chuck et al., 2007; Wu et al., 2009). Such a decreased expression of miR156 also exists in rice (Xie et al., 2006; Fig. 2C). We found an opposite gradient of miR156 over time, that is, a gradual increase over the time of leaf growth and maturation in rice (Figs. 1 and 2). At a specific time point, older (or early-emerged) leaves have higher levels of miR156 than younger (or later-emerged) leaves (Fig. 1, A–C), and in a specific leaf generated after the juvenile phase, miR156 is increased gradually over its growing time until it reaches a maximal expression level (Fig. 2, A and B). The maximal levels of miR156 in different adult leaves are comparable (Fig. 1, C and D), which also suggests that miR156 expression in leaves is independent of shoot or plant age after the juvenile stage. It will be interesting to reveal how miR156 regulates plant growth and development through two opposite temporal expression changes over time: decreasing in shoots (or at the whole plant level) and increasing in leaves. Such opposite temporal expression patterns in

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**Figure 8.** miR156-OE changed leaf development. A to D, Dynamic changes of plant height, leaf number, tiller number, and leaf initiation rate of wild-type (WT)/NC and Md/Mh plants (n = 12). Error bars indicate SE. E to J, Comparison of Md/Mh and wild-type/NC plants in leaf shape (length and width) at three representative stages of rice (n = 6). Error bars indicate SD. K, Md/Mh and wild-type/NC plants at the flowering stage (90 DAG). [See online article for color version of this figure.]
shoots and leaves also exist for miR172, which is gradually decreased in leaves from young to old (Fig. 7) and increased gradually in shoots (Zhu et al., 2009). Because unexpanded or young leaves are the major part of young shoots, the gradual reduction of miR156 in shoots is likely due to the decreased average level of miR156 for all the unexpanded or young leaves. The younger leaves with lower miR156 levels may compromise the overall miR156 level in the whole plant. This is indeed the case when we compare the last three leaves from the primary tiller and the secondary tiller (Fig. 1B). Because a growing shoot or plant normally gains more young leaves and miR156 has low levels in young leaves, it is not strange that miR156 has a gradually decreased pattern if it is quantified in shoots or whole plants. Therefore, we propose that the temporal expression pattern in a shoot or whole plant is mainly determined by the temporal expression of miR156 in leaves.

In rice, miR529 shared 70% sequence identity with miR156 (Jeong et al., 2011). However, we did not detect any miR529 in adult rice leaves by small RNA gel blotting, and no cross-hybridization between miR156 probe and miR529 probe was detected either (Supplemental Fig. S7).

miR156-OE Attenuates Temporal Changes of Gene Expression

In the microarray analysis, old and young leaves at the same shoot stage were collected to study the changes of gene expression. Variations between leaf position and emerging time may also affect differential gene expression. Here, we used only two representative leaves to measure the temporal gene expression changes, so the number of genes affected by miR156 during leaf development might be underestimated.

Leaf development is coordinated by a number of temporal/spatial expressions of genes. In rice, approximately half of the expressed genes show temporal changes between old and young leaves, and 30% of their expression is affected by miR156-OE. Besides miR156, some other plant-conserved microRNAs also show temporal expression changes in growing leaves. Regardless of the expression patterns in the wild type, the magnitudes of the temporal changes of these genes have been attenuated in the miR156-OE plants (Figs. 5–7). Therefore, the gradual increase of miR156 during leaf growth and maturation may be essential for maintaining the temporal changes of numerous genes. Nevertheless, high levels of miR156 in Md/Mh leaves, in which the miR156 levels exceeded the maximal levels of miR156 in the old leaves of the wild type, did not completely abolish the temporal changes of most miR156-regulated genes, suggesting that other factors should be involved in maintaining the temporal changes of gene expression.

Because miR156 is increased gradually from young to old leaves, miR156-OE was supposed to suppress gene expression predominantly in young leaves. Unexpectedly, 1,958 genes that have higher expression levels in the young leaves of wild-type plants and were expected to be down-regulated by miR156-OE were actually up-regulated specifically in the old leaves of miR156-OE plants. Related to such unexpected gene expression changes, Md/Mh leaves showed precocious maturation. The unexpected gene expression changes by miR156-OE might result from non-cell-autonomous compensation between leaf maturation and leaf initiation. Non-cell-autonomous compensation has been reported as an important mechanism for leaf development (Kawade et al., 2010). Misexpression of miR156 in different domains of the shoot apex also suggested that miR156 might affect leaf development through a non-cell-autonomous effect (Wang et al., 2008). Because miR156 is increased gradually, it is unclear at which leaf stage miR156 triggers the non-cell-autonomous signal and what the threshold is for the miR156 level to trigger non-cell-autonomous signals.

Temporal Expression of miR156 Affects Genes Involved in Leaf Development

The overall morphology of Md/Mh was different from that of other plastochrone mutants of rice, such as pla1, pla2, pla3, and mori1 (Asai et al., 2002; Miyoshi et al., 2004; Kawakatsu et al., 2006, 2009). Except for smaller leaf size, the number of leaves per tiller (three to four leaves per tiller) in Md/Mh is not changed compared with wild-type/NC plants, but pla1, pla2, pla3, and mori1 continuously generate leaves from each tiller. PLA1 and PLA2 are dominantly expressed in young leaf primordial, whereas PLA3 has higher expression in old leaves than in young leaves according to our microarray data. No difference in expression level was observed for PLA1, PLA2, and PLA3 between miR156-OE and the wild type in leaves of different ages, suggesting that miR156 might act in concert with PLA genes to regulate leaf development.

The expression of miR156 was suppressed by brassinosteroid treatment but not by other phytohormone treatments (Supplemental Fig. S3). Several genes that might be involved in brassinosteroid biosynthesis (e.g. CYP724 and CYP90; Tanabe et al., 2005) or signaling (BAK1 homologous) were affected by miR156-OE (Supplemental Tables S1 and S2). In rice, brassinosteroid deficiency resulted in dwarf stature, erect leaves, and reduced panicle size (Hong et al., 2003; Sakamoto et al., 2006), and Md/Mh plants showed partial similarity to these phenotypes, suggesting that the miR156-initiated regulation networks may have some overlaps with the brassinosteroid signaling-initiated regulation networks. Although auxin treatment did not result in obvious changes of miR156 expression, some auxin-responsive genes, such as OsIAAs and OsSAPs, showed significant changes in expression in miR156-OE leaves (Supplemental Table S1). In addition, miR156-OE also affected the expression of miR164 and miR393 (Fig. 7), whose targets are thought to be involved in auxin signaling (Guo et al., 2005; Navarro et al., 2006). In
addition, the expression of several target genes of miR164 and miR393 in rice showed time-dependent fluctuation in miR156-OE leaves (Supplemental Fig. S8). These data imply that miR156-initiated regulation networks might have some overlaps with auxin signaling-initiated networks. Because auxin is essential for leaf initiation and development, and because auxin levels are also temporally changed in different leaves (Terry et al., 1986; Avsian-Kretchmer et al., 2002), it will be intriguing to check the temporal and spatial interactions between miR156 and auxin signaling, if they exist.

miR156 also temporally regulated the expression of six plant-conserved microRNAs during leaf development. Temporal regulation of miR172 by miR156 was reported in Arabidopsis and maize. miR172 is decreased gradually during leaf development (Fig. 7), whereas a previous study suggested that miR172 is increased gradually in rice shoots during plant maturation (Zhu et al., 2009). Nevertheless, miR156 always has opposite expression patterns compared with miR172. The microRNAs regulated by miR156 might modulate leaf development according to studies in Arabidopsis. For example, both miR164 and miR319 have been suggested to be involved in the control of two distinct leaf developmental events, leaf margin formation and leaf senescence, in Arabidopsis (Palatnik et al., 2003; Mallory et al., 2004; Schommer et al., 2008; Kim et al., 2009). miR156-OE also activated the expression of three small RNA precursors. The function of these no-coding RNAs in rice is still unclear.

Temporal Expression of miR156 Affects Genes Involved in Stress Responses

Leaf age affects susceptibility to abiotic and biotic stresses, and the environmental stresses can also affect leaf growth and initiation. According to our unpublished data and other public microarray data, a number of stress-responsive genes have different expression levels between old and young leaves. As an essential regulator of leaf development, miR156 is also up-regulated by drought, salt, and cold (Shen et al., 2010). The expression levels of many stress-related genes were affected by miR156-OE. For example, drought- or disease-responsive genes, such as dehydration-responsive element-binding proteins (LOC_Os02g45450, LOC_Os06g03670, LOC_Os03g09170, and LOC_Os09g20350), NAC transcription factors (ONAC055 [LOC_Os03g1870] and ONAC122 [LOC_Os11g03300]), and Pathogenesis-related protein1 (LOC_Os07g05370), were down-regulated in old leaves by miR156 (Supplemental Tables S1 and S2). Thus, environmental stresses might modulate plant growth, at least in part, by the miR156-initiated regulation networks.

Taken together, we report a gradually increased gradient of miR156 in the developing leaf, which is conserved in plants and may have an important role in leaf development. Although it is unclear how this gradient is controlled, our data offer novel insights into the function of miR156 and other microRNAs with temporal expression patterns in plant growth and development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of rice (Oryza sativa) transgenic plants were surface sterilized and germinated in Murashige and Skoog medium containing 50 mg L⁻¹ hygromycin. The seedlings were transferred to a greenhouse and grown in polyvinyl chloride tubes. The leaf number, height, and tiller number were measured every 7 d (n = 12). Rice leaf samples used in this study were collected at approximately 2 months. To collect leaves at different developmental stages, plants at the same developmental stage (based on the emergence of the same order leaf on the same day) were selected and the orders of leaves were labeled. Then, the same order leaves were sampled at a specific time point (every 5 d). Each plant was sampled only once. The developmental process and stage system of rice leaf were as described (Bob et al., 2005). For rice cv Zhonghua11 (subspecies japonica), which was used in this study, L1 and L2 are juvenile leaves, L3 and L4 are intermediate juvenile/adult leaves, and later leaves are adult leaves. RNA samples were prepared using TRIzol reagent (Invitrogen) following the manufacturer’s instructions.

Histologic Analysis

The tissues were fixed in formaldehyde-acetic acid immediately after being sliced from plants. Samples were then dehydrated in an ethanol series, embedded in paraffin, and sectioned. The internode samples were treated with 0.1% hydrofluoric acid for 3 d before dehydration. Epidermal peels of rice leaves were prepared using the maize protocol (Chuck et al., 2007). The 1-cm-long segments were collected from the middle of the leaf. Mesophyll was released by digestion with 0.1% pectinase and 0.5% cellulose, stained in toluidine blue O, and photographed with a light microscope. Entire shoot apices were prepared as described (Kawakatsu et al., 2006), and the size of the SAM was measured using ImageJ (version 1.4; http://rsweb.nih.gov/ij/).

For scanning electronic microscopy, dehydrated samples were soaked, infiltrated with isopentyl acetate, dried to the critical point, sputter coated with platinum, and then observed with a scanning electronic microscope (Hitachi S4500).

Real-Time RT-qPCR

A one-tube stem-loop RT-qPCR was applied for the quantification of mature microRNAs. SYBR was adopted to monitor the amplification (Chen et al., 2005; Varkonyi-Gasic et al., 2007; Shen et al., 2010). Briefly, cDNA was synthesized in a 5-μL reaction containing 20 ng of total RNA, 1 × first-strand buffer, 10 μM dithiothreitol, 1 μM appropriate stem-loop RT primer (Supplemental Table S5A; except for U6 RNA, for which a specific primer was used), 1 unit of RNase inhibitor (taqara), and 20 units of SuperScript III (Invitrogen). A pulsed RT reaction (40 cycles of 16°C for 2 min, 42°C for 1 min, and 50°C for 1 s, followed by final reverse transcriptase inactivation at 85°C for 5 min) was performed with an ABI7900 Thermal Cycler (Applied Biosystems). Real-time qPCR was performed with a Prism7500 system using the SYBR Ex Taq kit (Takara). In brief, 12.5 μL of SYBR mix (Takara), 1 μM universal reverse primer (5′-CCAGTCGAGGCTCGAGGTT-3′; except for U6 RNA, for which a specific primer [Supplemental Table S6] was used), and 1 μM appropriate forward primers specific to target microRNA or U6 RNA (Supplemental Table SSA) were added to 96-well plates, and PCR continued in an ABI Prism 7500 (Applied Biosystems). All reactions were performed with three replications. U6 RNA was used as a reference for data normalization. The stem-loop primers were designed as described previously (Chen et al., 2005). The expression levels of miR156 were normalized to U6 RNA and calculated as described previously (Shen et al., 2010).

Real-time RT-qPCR for mRNA detection was performed as described (Xie et al., 2006). The expression levels of the genes of interest were normalized to the reference gene (AK071592; 6-phosphogluconate dehydrogenase), which has stable expression in different leaves, and the relative expression levels were calculated with the 2⁻ΔΔCt method (Livak and Schmittgen, 2001; Chen et al., 2005). Primers for RT-qPCR are listed in Supplemental Table S6.

Microarray Hybridization and Data Analysis

Individual leaves of primary tiller were collected from Md/Mh and wild-type plants. RNA samples were subjected to microarray hybridization after the miR156 level was checked by small RNA gel blot. The Affymetrix 57K rice DNA chip, which contains 57,386 probe sets, was used to measure gene expression in different leaves. Microarray hybridization was performed by
The following DNA oligonucleotides were used: miR156-probe, relative expression level was normalized to U6 or rRNA signals using ImageJ. Stripped blots with no detectable signals were used to detect other probes. The containing 5 nucleotides with membrane. The probes were prepared by end-labeled synthetic DNA oligo-blotted to ensure that the high-molecular-weight RNAs transferred to the above. Transverse and vertical sections (10 μm) were subjected to overnight hybridization at 50°C in hybridization buffer containing 5× SSC, 20 mM NaHPO4 [pH 7.2], 7% SDS, 2× Denhardt’s solution, and 50 μg mL−1 sheared salmon sperm DNA. The blots were washed three times with low-stringency washing solution (3× SSC, 25 mM NaHPO4 [pH 7.5], and 1% SDS) and one time with high-stringency washing solution (1× SSC and 1% SDS). Radiolabeled signals were detected and quantified using the FujiFilm phosphorimager system (FJL-5100; Fuji). The probes were stripped by washing in 1% SDS at 80°C for 30 min and certified by phosphorimagcer. Stripped blots with no detectable signals were used to detect other probes. The relative expression level was normalized to U6 or rRNA signals using ImageJ (version 1.4). The following DNA oligonucleotides were used: miR156-probe, 5′-GTGCTCAGCTCTTCTGTCA-3′; miR164-probe, 5′-GCCAGTGCGCTCTGCTCTCCA-3′; miR529b-probe, 5′-AGCTGTACTCTTCTCTTCTTCT-3′; and U6-probe, 5′-TATCCGCTCAATTATTTCCCGATGT-3′.

In Situ MicroRNA Detection

miRCURY digoxigenin-labeled LNA probes (Exiqon) were used for in situ detection of miR156. Genes specific expressed in miR156-OE leaves. miR156-regulated genes in L6 (young leaf). miR156-regulated genes in L3 (old leaf). Primers used for real-time PCR. Pearson coefficient of microarray data by pairwise comparison.

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