The alteration in the architecture of a T-DNA insertion rice mutant osmtd1 is caused by up-regulation of MicroRNA156f

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Abstract Plant architecture is an important factor for crop production. Some members of microRNA156 (miR156) and their target genes SQUAMOSA Promoter-Binding Protein-Like (SPL) were identified to play essential roles in the establishment of plant architecture. However, the roles and regulation of miR156 is not well understood yet. Here, we identified a T-DNA insertion mutant Osmtd1 (Oryza sativa multi-tillering and dwarf mutant). Osmtd1 produced more tillers and displayed short stature phenotype. We determined that the dramatic morphological changes were caused by a single T-DNA insertion in Osmtd1. Further analysis revealed that the T-DNA insertion was located in the gene Os08g34258 encoding a putative inhibitor I family protein. Os08g34258 was knocked out and OsmiR156f was significantly upregulated in Osmtd1. Overexpression of Os08g34258 in Osmtd1 complemented the defects of the mutant architecture, while overexpression of OsmiR156f in wild-type rice phenocopied plant architecture. Computational and experimental analysis indicated that OsmiR156f controlled plant architecture by mediating plant stature and tiller outgrowth and may be regulated by an unknown protease inhibitor I family protein.

Keywords: Oryza sativa; OsmiR156f; plant architecture; protease inhibitor; T-DNA insertion
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INTRODUCTION

Plant architecture is important for plant morphology and crop production. Branching and plant height are key factors affecting plant architecture. As specialized branches bearing grains, rice tillers are formed on the un-elongated basal internodes and grow independently from the mother culm with its own adventitious roots (Li 1979; Wang and Li 2005). Rice tillering is important for rice ideal plant architecture (IPA) which indicates a rice variety without unproductive tillers, with more grains per panicle, and with thick and sturdy stems. Because IPA plants have more potential for higher yields in rice (Jiao et al. 2009; Miura et al. 2010), it is feasible to increase rice yield by reducing the unproductive tillers in plant breeding.

MicroRNAs (miRNAs) are 21–24 nucleotide RNAs binding to target genes by imperfect base pairing and cause cleavage of target mRNA or repression of translation (Chen 2008). Being regarded as universal regulators of gene expression, many miRNAs have been proved to be essential in regulating plant architecture (Schwab et al. 2005; Xie et al. 2006; Chen 2008; Wang et al. 2011; Fu et al. 2012; Wei et al. 2012). Among them, the microRNA156 (miR156) family is a class of miRNAs playing pivotal roles in plant architecture. The miR156 genes are conserved and highly expressed miRNAs in the plant kingdom. Computational and experimental analysis indicated that miR156s directly regulate the expression of SQUAMOSA-PROMOTER BINDING LIKE (SPL) genes (Rhoades et al. 2002; Xie et al. 2006; Guo et al. 2008). SPL genes encode plant-specific transcription factors containing a DNA-binding motif highly conserved in SQUA promoter-binding protein (SBP) (Shikata et al. 2009). The miR156 and its target SPL genes are involved in various developmental processes. In Arabidopsis, miR156 controls proper pattern formation during early embryogenesis by mediating SPL10 and SPL11 (Nodine and Barte 2010). The
miR156, miR172, and SPL genes (including SPL3/4/5, SPL9, SPL10, SPL15) determine plant development by promoting juvenile/adult phase transition (Chuck et al. 2007; Wu et al. 2009; Jung et al. 2011; Willmann and Poethig 2011; Yang et al. 2011). The miR156-targeted SPL factor SPL9 affects leaf plastochron length and organ size (Wang et al. 2008) and regulates the anthocyanin accumulation during the transition from the leaf to flower formation (Gou et al. 2011). Enhanced miR156b expression leads to branching, altered trichome morphology, and increased seed carotenoid levels through the suppression of SPL15 and other SPL target genes (Wei et al. 2012). In addition, it is also reported that miR156 is a phloem-mobile signal regulating the process of tuberization in potato by targeting StSPL3, StSPL6, StSPL9, and StSPL13 (Bhogale et al. 2014). In switchgrass, overexpression of miR156 genes improves biomass by changing apical dominance and floral transition through suppressing target SPL genes (Chuck et al. 2011; Fu et al. 2012).

The rice genome contains 12 miR156 genes, named OsmiR156a to OsmiR156l, and 19 SPL genes, named OsSPL to OsSPL19, respectively. Except 11 OsSPLs (OsSPL2, OsSPL3, OsSPL4, OsSPL7, OsSPL11, OsSPL12, OsSPL13, OsSPL14, OsSPL16, OsSPL17, and OsSPL18), no other genes contained OsmiR156 mature complementary sequences in rice genome, suggesting that OsmiR156 specially targets OsSPL genes in rice (Xie et al. 2006). Among the 12 miR156 genes in rice, 10 members (OsmiR156a to OsmiR156j) produce the same mature miRNA sequence, implying that a complex regulation network exists between OsSPL and the OsmiR156 family. Indeed, previous studies have suggested that different OsSPL genes have a diverse temporo-spatial expression patterns in rice. OsSPL7, OsSPL12, OsSPL14, OsSPL16, OsSPL17, and OsSPL18 were predominantly expressed in young panicles; OsSPL2, OsSPL4, OsSPL8, OsSPL10, OsSPL11, and OsSPL15 were highly expressed in stem, leaf sheath, and young panicles; while OsSPL1, OsSPL3, OsSPL5, OsSPL6, OsSPL9, and OsSPL13 were expressed in all tissues. Overexpression OsmiR156d or OsmiR156h in rice resulted in increased miRNA levels of three OsSPL genes (OsSPL2, OsSPL12, and OsSPL13) in the flag leaves, two OsSPL genes (OsSPL16 and OsSPL18) in the panicles, and one OsSPL gene (OsSPL14) in both flag leaves and panicles (Xie et al. 2006), suggesting that OsSPL genes may be tempo-spatially regulated by OsmiR156.

Although miR156s have been elucidated to regulate diverse processes in plants, the function and regulation of OsmiR156 family genes in rice remain elusive. In this paper, we identified a rice T-DNA insertion mutant Osmtd1 (Oryza sativa multi-tiller and dwarf mutant). The Osmtd1 produced many more tillers and reduced plant height. We located one T-DNA insertion in Os08g34258, an unknown gene encoding a putative protease inhibitor, in Osmtd1. Overexpression of Os08g34258 complemented the multi-tillering and dwarf phenotypes of Osmtd1. We demonstrated that OsmiR156f was upregulated and OsSPL3, OsSPL12, and OsSPL14 were downregulated in Osmtd1. Our findings provided a new layer of understanding on the function and regulation of OsmiR156f in rice architecture establishment.

RESULTS

The Osmtd1 mutant exhibited dwarf and bushy phenotypes

We identified a mutant from a rice T-DNA insertion mutant collection and named it Osmtd1 because the mutant displayed multi-tillering and dwarfism phenotypes. Although no differences between Osmtd1 and wild-type plants were observed at the seedling stage, Osmtd1 produced plant architecture distinct from wild-type at the tillering stage. The phenotypes of Osmtd1 include decreased plant height and increased tiller number (Figure 1A). To further illustrate Osmtd1 phenotypes, we investigated the plant height and tiller number in a time course during plant development. Osmtd1 was shorter than wild-type rice from 45 to 104 d after sowing (DAS). At 75 DAS, the Osmtd1 height was no longer increased, while the wild-type rice further grew to a higher stature (Figure 1B). The analysis of tiller number indicated that there were no obvious differences between wild-type rice and Osmtd1 found at 45 DAS. However, Osmtd1 produced more tillers than wild-type rice after 62 DAS (Figure 1C). Usually, rice tillers are only formed on the un-elongated basal internodes of rice main culm in wild-type rice. However, we observed the outgrowth of tiller buds at the elongated higher internodes in Osmtd1 (Figure 1D). In addition, the grain number was reduced and the primary panicle branches were shorter in Osmtd1 (Figure 1D).

It has been suggested that dwarfism may be the secondary effect of the formation of more tillers, because removal of tillers rescues plant height in some rice mutants (Zou et al. 2006). To evaluate whether dwarf phenotype is caused by the excessive tillers in Osmtd1, we removed the new axillary tiller buds of wild-type or Osmtd1 every day from tillering stage to reproductive stage, and measured the plant height. The results showed that removal of tillers did not increase the height of Osmtd1, but did increase the height of wild-type plants (Figure 1E), indicating that the dwarf trait of Osmtd1 was independent from multi-tillering and the mutation caused the high tillering and dwarfism simultaneously.

The phenotypes of Osmtd1 were caused by a single T-DNA insertion

As Osmtd1 was obtained from a T-DNA mutant collection, we first test whether the phenotypes of Osmtd1 could be resulted from T-DNA insertion. We crossed Osmtd1 with the wild-type rice cultivar Zhonghua 11 (ZH11) and then analyzed the F1 plants. We found that the heterozygous Osmtd1 displayed moderate phenotypes in terms of the plant height, tiller number, length of main panicle, and the total and filled grain number (Table 1), indicating that Osmtd1 was a semi-dominant mutant. The T-DNA insert harbor a bar resistance gene conferring the transgenic plants resistant to the herbicide phosphinothricin (PPT). We then investigated whether the PPT resistance could be cosegregated with the phenotypes of Osmtd1. We generated an F2 population by crossing F1 plants to ZH11. The progenies in the F2 population were either PPT-resistant or PPT-sensitive. We found that all the PPT-resistant plants displayed moderate dwarf and multi-tillering phenotypes, while all the PPT-sensitive plants had no difference from wild-type ZH11. We further generated the F3 populations. We analyzed the segregation ratio of PPT-resistant plants to PPT-sensitive ones using the F2 populations and found that the ratio is statistically 3:1 (Table 2). Phenotype analysis showed that all the PPT-resistant plants in the F2 populations displayed strong or moderate phenotypes in plant architecture, while the PPT-sensitive plants showed no phenotypes (Table 2), indicating that the phenotypes...
Figure 1. The phenotypes of Osmt1

(A) Osmt1 was significantly shorter and had more tillers than the wild-type rice Zhonghua 11 (ZH11). (B) The plant height (cm) and (C) the tiller number are showed at different days after sowing. (D) The outgrowth of the higher node tiller in Osmt1. (E) The influence of rice plant height after removing axillary tiller buds in Osmt1 and ZH11. Plant height of Osmt1 did not increase after removing the tillers while the wild-type rice ZH11 did. Bar, 10 cm. All data and pictures were examined using the plants grown in the pots.
showed that a clear Os08g34258-GFP band was observed.

Os08g34258 may be cosegregated with PPT-resistance conferred by T-DNA insertion. Furthermore, we observed that the ratio of the plants without phenotypes, the ones with moderate phenotypes and the ones with strong phenotypes, is approximately 1:2:1 (Table 2), suggesting that the phenotypes of Osmtld were possibly caused by a single mutated locus.

The T-DNA insertion was located in an unknown gene
To reveal the molecular base of the phenotypes in Osmtld, we first identified the T-DNA flanking sequence by thermal asymmetric interlaced polymerase chain reaction (TAIL–PCR) (Liu and Chen 2007). A BLAST search on the Rice Genome Annotation Project website showed that the T-DNA insertion was localized in the middle of an unknown gene, Os08g34258. Os08g34258 has no introns and OsmiR156f was located in the 3.3 kb downstream of it (Figures 2A, S1). A database search showed that Lex_Os08g34249 had the same sequence as Os08g34258 in rice genome. Reverse transcription (RT)-PCR analysis revealed that Os08g34258 was possibly knocked out in Osmtld because the T-DNA was inserted in the exon of Os08g34258 and the trace of product may be derived from Os08g34249 (Figure 2B). Os08g34258 encodes a putative protein containing only 67 amino acid residues and a conserved domain shared by the protease inhibitor I family (potato inhibitor type I). This protein family contains a class of small protease inhibitors widely spreading in plants and has been found in potato, tomato, barley, pumpkin, buckwheat, Solanum nigrum, and so on (Melville and Ryan 1972; Svendsen et al. 1980; Plunkett et al. 1982; Krishnamoorthi et al. 1990; Hartl et al. 2010; Wang et al. 2011). No functions of this kind of protease inhibitor have been reported in rice. To first test whether Os08g34258 could encode a protein, we cloned Os08g34258 and generated the construct 35Spro-Os08g34258-GFP in which Os08g34258 was fused with GFP and driven by the CaMV 35 S promoter. We transformed 35Spro-Os08g34258-GFP into tobacco leaves. Western blotting showed that a clear Os08g34258-GFP band was observed in the isolates from leaves transformed with 35Spro-Os08g34258-GFP but not in the control, indicating that Os08g34258 is a real gene (Figure 2C). We then generated the construct 35Spro-Os08g34258 in which Os08g34258 was driven by the CaMV 35 S promoter. We transformed 35Spro-Os08g34258 into Osmtld and obtained 31 transgenic rice plants which recovered to the architecture of wild-type plants (Figure 2D), indicating that disruption of Os08g34258 by T-DNA insertion caused the defects of plant architecture in Osmtld. To further demonstrate the roles of Os08g34258 in rice architecture, we performed RNA interference (RNAi) to knockdown Os08g34258 in wild-type ZH11. In the RNAi plants, we indeed observed the dwarfism and multi-tillering phenotypes similar to those observed in Osmtld (Figure S2), indicating that Os08g34258 may play important roles in plant architecture.

OsmiR156f was upregulated in Osmtld
The OsmiR156 family gene OsmiR156d and OsmiR156h play essential roles in plant architecture (Xie et al. 2006). We found that Osmtld displayed similar phenotypes as those observed of Osmtld may be cosegregated with PPT-resistance conferred by T-DNA insertion. Furthermore, we observed that the ratio of the plants without phenotypes, the ones with moderate phenotypes and the ones with strong phenotypes, is approximately 1:2:1 (Table 2), suggesting that the phenotypes of Osmtld were possibly caused by a single mutated locus.

| Lines | Plant height | Effective tillers | Length of main panicles | Total grain numbers | Filled grain numbers |
|-------|--------------|------------------|------------------------|---------------------|---------------------|
| ZH11  | 104.2 ± 5.2  | 11.2 ± 1.8       | 21.6 ± 1.16            | 165.2 ± 4.21        | 157.4 ± 3.3         |
| Osmtld| 68.6 ± 3.3   | 28.4 ± 6.6       | 13.6 ± 0.42            | 42.2 ± 7.33         | 41.6 ± 6.66         |
| ZH11/Osmtld F₁ | 86.4 ± 4.3 | 17.7 ± 7.7       | 18.4 ± 1.49            | 92.3 ± 18.4         | 88.9 ± 18.2         |
| Osmtld/ZH11 F₁ | 85.5 ± 3.1 | 19.3 ± 6.7       | 17.4 ± 1.49            | 82.5 ± 13.4         | 78.2 ± 15.2         |
| ZH11/Osmtld/ZH11 R | 88.3 ± 2.5 | 15.3 ± 4.4       |                        |                     |                     |
| ZH11/Osmtld/ZH11 S | 103.7 ± 2.2 | 8.7 ± 3.4        |                        |                     |                     |
| Osmtld/ZH11/ZH11 R | 89.3 ± 4.0 | 17.2 ± 6.1       | 17.8 ± 3.03            | 103.4 ± 14.7        | 96.6 ± 11.13        |
| Osmtld/ZH11/ZH11 S | 101.2 ± 1.8 | 6.8 ± 1.1        | 21.4 ± 0.66            | 172.8 ± 8.14        | 162.6 ± 8.65        |

Data were means ± SE. R indicated resistant lines and S indicated sensitive lines to the herbicide phosphinothricin (PPT). ZH11, Zhonghua 11.

### Table 2. The χ² analysis using F₂ population from the cross of Osmtld and ZH11

| Lines          | Resistance | Sensitive | χ²   |
|----------------|------------|-----------|------|
|                | Trial      | Theoretical | Trial | Theoretical |      |
| Osmtld/ZH11 I  | 321        | 245       | 240.75 | 76         | 80.25 | 0.354 | 76 | 80.25 | 163 | 160.5 | 82 | 80.25 | 0.313 |
| Osmtld/ZH11 II | 131        | 97        | 98.25  | 34         | 32.75 | 0.043 | 34 | 32.75 | 63  | 64.5  | 34 | 32.75 | 0.128 |
| Osmtld/ZH11 III| 74         | 58        | 55.5   | 16         | 18.5  | 0.631 | 16 | 18.5  | 40  | 18.5  | 18.5 | 18.5  | 0.63  |
| Osmtld/ZH11 IV | 165        | 121       | 123.75 | 44         | 41.25 | 0.20  | 44 | 41.25 | 86  | 82.5  | 35 | 41.25 | 1.42  |
| Osmtld/ZH11 Total| 691      | 521       | 518.25 | 170        | 172.75| 0.082 | 170| 172.75| 352 | 344.5 | 169 | 172.75| 0.27  |
| ZH11/Osmtld I  | 334        | 245       | 250.5  | 89         | 83.5  | 0.426 | 89 | 83.5  | 157 | 167   | 88 | 83.5  | 1.206 |
| ZH11/Osmtld II | 142        | 102       | 106.5  | 40         | 35.5  | 0.645 | 40 | 35.5  | 69  | 71    | 33 | 35.5  | 0.817 |
| ZH11/Osmtld III| 87         | 68        | 65.25  | 19         | 21.75 | 0.629 | 19 | 21.75 | 51  | 43.5  | 17 | 21.75 | 2.81  |
| ZH11/Osmtld IV | 229        | 166       | 171.75 | 63         | 57.25 | 0.667 | 63 | 57.25 | 115 | 114.5 | 51 | 57.25 | 1.28  |
| ZH11/Osmtld Total| 792      | 581       | 594    | 211        | 198   | 1.053 | 211| 198   | 392 | 396   | 189 | 198   | 0.723 |

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Figure 2. An unknown gene, Os08g34258, was involved in the mutant phenotypes

(A) The T-DNA insertion was located in the middle of a putative gene, Os08g34258, which is approximately 3.3 kb upstream of the OsMIR156f gene on the eighth chromosome in the Osmtdr1 mutant. (B) Os08g34258 was possibly knocked out in Osmtdr1. (C) Transient expression of Os08g34258 in tobacco leaves showed that Os08g34258 encodes a protein by western blotting. The controls were the samples from the non-transformed tobacco leaves. (D) Overexpression of the Os08g34258 gene complemented the phenotypes of Osmtdr1.
in the OsmiR156f or OsmiR156h overexpressing rice plants. Considering that the Os08g34258 gene is located in the 3.3 kb upstream of OsmiR156f (Figure 2A), we hypothesized that OsmiR156f may be regulated in Osmtd1. To test the hypothesis, we analyzed the expression level of OsmiR156f. Interestingly, the transcripts of the mature OsmiR156f were increased significantly in Osmtd1 (Figure 3A). We further determined that the transcripts of the OsmiR156f precursor were increased in Osmtd1 (Figure 3B), suggesting that the upregulation of OsmiR156f may be the molecular base of multi-tillering and dwarfism in Osmtd1.

To further confirm that the excessive production of OsmiR156f accounts for the phenotypes of Osmtd1, we generated the construct UBQpro-OsmiR156f in which the OsmiR156f precursor was driven by the UBIQUITIN promoter from maize (Cornejo et al. 1993). We transformed UBQpro-OsmiR156f into ZH11. Among the 217 UBQpro-OsmiR156f transgenic lines, more than 180 plants displayed dwarfism and multi-tillering similar to those observed in Osmtd1. Some lines showed severe phenotypes with more than 100 tillers (Figure 3C). Similar to Osmtd1, the axillary tiller buds in higher internodes were also observed in UBQpro-OsmiR156f transgenic lines (Figure 3C). We then analyzed the expression level of OsmiR156f in the UBQpro-OsmiR156f transgenic line, Osmtd1, or wild-type ZH11. The results showed that the OsmiR156f expression level was highly increased in the UBQpro-OsmiR156f transgenic line and in Osmtd1, when compared with that in wild-type ZH11. These results demonstrated that OsmiR156f was critical for rice tillering and plant height.

**OsmiR156f regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes at a post-transcriptional level**

It has been suggested that SPL genes are the direct targets of miR156 (Rhoades et al. 2002; Xie et al. 2006; Guo et al. 2008). To determine which OsSPLs were specifically degraded by OsmiR156f, we analyzed the expression level of all the 19 OsSPLs in Osmtd1 and an UBQpro-OsmiR156f transgenic line by quantitative PCR (qRT–PCR) (Figure 4). The results showed that only eight SPL genes (OsSPL1, OsSPL3, OsSPL6, OsSPL9, OsSPL12, OsSPL14, OsSPL15, and OsSPL19) were detected in young culms. Among them, four SPL genes (OsSPL3, OsSPL12, OsSPL14, and OsSPL19) were downregulated significantly in UBQpro-OsmiR156f transgenic plants, two genes (OsSPL14 and OsSPL19) were downregulated obviously in Osmtd1, three genes (OsSPL1, OsSPL6, OsSPL15) had no changes at the expression level in both UBQpro-OsmiR156f and Osmtd1, and one gene OsSPL9 was unexpectedly upregulated in UBQpro-OsmiR156f and Osmtd1. Among the four genes downregulated in UBQpro-OsmiR156f or Osmtd1, OsSPL3, OsSPL12, and OsSPL14 were putative target genes of OsmiR156f (Xie et al. 2006), suggesting that OsmiR156f may specifically regulate OsSPL3, OsSPL12, and OsSPL14 in the young culms.

**DISCUSSION**

Plant architecture is crucial for yield in rice. The development of IPA has been proved to facilitate rice yield increase (Jiao et al. 2010; Miura et al. 2010). Plant height and tiller number are the two most important agronomic traits for IPA in rice. In this study, we identified a new semi-dominant rice mutant, Osmtd1, that displayed dwarfism, multi-tillering, late flowering, and shortened panicle size. Molecular analysis suggested that Os08g34258 encoding a protease inhibitor I family protein and the miRNA gene OsmiR156f were affected in Osmtd1. Overexpression of Os08g34258 complemented the phenotypes of Osmtd1, while over-expression of OsmiR156f in the wild type recapitulated abnormal phenotypes of Osmtd1. Our findings established the important roles of Os08g34258 and OsmiR156f in control of plant architecture in rice.

Rice tillering is affected by various factors including the ambient temperature, the plant density, the axil position, and the plant age. Genetic analysis has identified important genes essential for tiller development. The temporal and spatial accumulation of these regulators determined the proper development of rice tillers. MOC1 initiates axillary buds and promoted tiller outgrowth. MOC1 encodes a putative CRAS family nuclear protein regulated by 26 S proteasome degradation (Li et al. 2003; Lin et al. 2012; Xu et al. 2012). OsPUP7 has a dominant effect on rice tiller number by regulating the transport of cytokinins (Qi and Xiong 2013). Whereas, OsTBRII encoding a TCP transcription factor represses axillary bud activity (Takeda et al. 2003). Several factors involved in strigolactone (SL) signaling and biosynthesis have been found to be important for controlling rice architecture. D3 is a homolog of Arabidopsis MAX2/ORE9 possessing leucine-rich repeat (LRR) and F-box domain and is an important component in SL signaling in control of rice tillering (Ishikawa et al. 2005). D53 functions as a repressor of SL signaling and affects axillary bud outgrowth (Jiang et al. 2013; Zhou et al. 2013). D14 encodes a proposed SL receptor for perception in SL signaling and inhibits rice tillering (Arite et al. 2009; Nakamura et al. 2013). HTD1, D27, and D10 all function in SL biosynthesis and control rice tillering (Zou et al. 2006; Arite et al. 2007; Lin et al. 2009). Some factors including LAZY1, LPA1, and PROG1 regulate the angle of rice tillering (Li et al. 2007; Jin et al. 2008; Tan et al. 2008; Wu et al. 2013). In this paper, we identified a new gene, Os08g34258, that may also participate in the regulation of rice tillering and plant height. Os08g34258 encodes a protease inhibitor I family protein which serves as a defensive compound against pathogens. The protease inhibitor inactivates the hydrolytic cleavage ability of proteases which are important pathogenic substances secreted by insects and pathogenic microorganisms. We first discovered the roles of the protease inhibitor I family protein in the development of plant architecture. This protein is completely different from the above-mentioned known factors which are transcription factors, signaling components, and enzymes in SL biosynthesis, indicating that Os08g34258 may use a new mechanism to control plant architecture.

The miRNA miR156 family contains 12 members in rice and is highly conserved in plants. Some members of this gene family have been identified to play essential roles in plant development. Constitutive expression of miR156 homologous genes in different plants causes similar morphological changes including increased number of axillary buds and dwarfism, late flowering, and low fertility (Schwab et al. 2005; Xie et al. 2006; Chuck et al. 2007, 2014). In this paper, we demonstrated that the mutant Osmtd1 and OsmiR156f overexpression lines showed dramatic phenotypes similar to those...
Figure 3. OsMiR156f was upregulated in Osmtd1

(A) OsMiR156 was upregulated in the leaves or culms of Osmtd1. UBIQUITIN1 was used as the internal control. (B) The expression level of pri-OsMiR156f was increased in the leaves of Osmtd1 or a UBQpro-OsmiR156f transgenic line. (C) A UBQpro-OsmiR156f transgenic line displayed a bushy phenotype (left) and the outgrowth of axillary tiller in the elongated nodes (right). (D) The comparison of phenotypes of a UBQpro-OsmiR156f transgenic line, Osmtd1, and Zhonghua 11 (ZH11). (E) The relative expression level of Os-miR156 was identified by quantitative reverse transcription polymerase chain reaction in a UBQpro-OsmiR156f transgenic line, Osmtd1, and ZH11.
observed in the overexpression lines of OsmiR156d or OsmiR156h (Xie et al. 2006). However, we found that OsSPL3, OsSPL12, and OsSPL14, but no other OsSPLs, were targeted by OsmiR156f, indicating that different OsmiR156 genes may have differentiated functions in the regulation of rice development. In addition, we unexpectedly found that the expression level of OsSPL9 was significantly increased in the OsmiR156f overexpression lines, suggesting that the regulation between OsSPL family genes and OsmiR156 family may be complicated.

In the Osmtd1 mutant, the expression level of Os08g34258 was possibly knocked out, while the expression level of OsmiR156f was increased. Interestingly, when we searched the rice database, we found that Loc_Os08g34249 had the same sequence as Loc_Os08g34258. So, it is possible that Loc_Os08g34249 may also be expressed and may have redundant function with Loc_Os08g34258. However, the expression level of Loc_Os08g34249 is rather low, so it is reasonable that disruption of Loc_Os08g34258 may lead to obvious phenotypes in rice. The generation of double knockout mutants would be very important for ultimately revealing the function of Loc_Os08g34258 and Loc_Os08g34249 in the future. The fact that either knockout of Os08g34258 or overexpression of OsmiR156f led to multi-tillering and short stature in Osmtd1 implied that Os08g34258 may regulate the abundance of OsmiR156f, although we cannot provide enough evidence to connect them at present. Especially, OsmiR156f is only located in approximately 3.3 kb downstream of Os08g34258. It is possible that the T-DNA insertion may cause both knockout of Os08g34258 and overexpression of OsmiR156f. Further experiments will be needed to verify the possible relationship between OsmiR156f and Os08g34258. We hypothesize that two mechanisms may exist if OsmiR156f were regulated by Os08g34258 during rice tillering. First, Os08g34258 may control the OsmiR156f transcriptions by indirectly regulating unknown transcription factors which control the transcripts of OsmiR156f. Second, as Os08g34258 encodes a putative protease inhibitor, it may regulate the OsmiR156f abundance by negatively affecting the process of miRNA biogenesis. In plants, there are at least two important and critical steps involved in cleavage or dicer processing during miRNA biogenesis: the processing of pri-miRNAs to pre-miRNAs, and further processing to miRNA-miRNA* duplex by DCL1 and its interacting partners (Kurihara and Watanabe 2004; Qi et al. 2005; Kurihara et al. 2006; Chen 2008). Based on the fact that both protease and DCL1 perform a similar role in cleavage of their targets, the protease inhibitor encoded by Os08g34258 may affect the activities of DCL1 or other enzymes during miRNA biogenesis. The investigation of the interactions between Os08g34258 and the components in the miRNA pathway including DCL1, HYL1 (Kurihara et al. 2006), SE (Lobbes et al. 2006; Yang et al. 2006), HEN1 (Yu et al. 2005), AGO (Baumberger and Baulcombe 2005; Qi et al. 2005, 2006), and HASTY (Bollman et al. 2003; Park et al. 2005) would provide further evidence to prove the possible implications of Os08g34258 in regulation of miRNA biogenesis in the future.

Figure 4. The regulation of OsSPLs by OsmiR156f
The expression level of OsSPL genes in the young culms from an UBQpro-OsmiR156f transgenic line, Osmtd1, or Zhonghua 11 (ZH11) was revealed by quantitative reverse transcription polymerase chain reaction. The expression levels of the genes in ZH11 were set to 1.0. The error bars represent the standard deviation of three biological replicates.

MATERIALS AND METHODS

Plant materials
A multi-tillering and dwarf rice mutant Osmtd1 was obtained by a T-DNA insertion mutant collection. The plasmid including maize Ds sequence and a bar gene (Wang et al. 2000) was induced in Oryza Sativa spp. japonica cv. ZH11. Agronomic traits were investigated in detail between Osmtd1 and ZH11 at different developmental stages. Genetic analysis was carried out using standard methods.
out using Osmt1 or ZH11 as pollen donors to obtain F1 hybrids of Osmt1 × ZH11 or ZH11 × Osmt1. Reciprocal crossing experiments were carried out between F1, hybrids and Osmt1 or ZH11. The agronomic traits of plant height, tiller number, and panicle length were examined in different hybrid populations.

Constructs and transformation
To generate 35Spro-Oso8g34258, in which the Os08g34258 gene was driven by the CaMV 35S promoter, the coding region of Os08g34258 was amplified from spp. japonica (cv. Nipponbare) genomic DNA using primers 5'-ATG AGC CAG AAG TGG TGG C-3' and 5'-ACA CAT GAA GTA CGG GGC CCC C-3'. The fragment was cloned into the EcoRI V site of pbLueScript SK+ (pBS) (designated pBS-inh) and was sequenced. The overexpression construct of 35Spro-Oso8g34258 was generated by ligation of the Kpn I/Xba I digested fragment from pBS-inh and vector pgGT11 digested by the same restriction enzymes (Tao et al. 2013). 35Spro-Oso8g34258 was then transformed into the mutant Osmt1.

To generate 35Spro-Oso8g34258-GFP, the coding region of Os08g34258 was amplified using the primers 5'-CAC CAT GAG CCA GAA GTC GTC G-3' and 5'-ACC GAT GAC GGG AAT TTT GA-3'. The fragment was cloned into the vector pENTRY-D-TOPO (Invitrogen, San Diego, CA, USA) to generate pENTRY-Os08g34258. The 35Spro-Os08g34258-GFP was generated by LR reaction between pENTRY-Os08g34258 and pK7FWG2. 35Spro-Oso8g34258-GFP was infiltrated into the leaves of tobacco by Agrobacterium tumefaciens Os08g34258. The 35Spro-Os08g34258-GFP was generated by ligation of the Kpn I/Xba I digested fragment from pBS-inh and the Ubiquitin promoter released from pBS-pUBQ with Hind III/BamHI digestion was ligated to obtain UBQpro-OsmiR156f. UBQpro-Oso8g34258-RNAi and UBQpro-OsmiR156f were then transformed into ZH11. The A. tumefaciens-mediated transformation was employed as previously reported (Liu et al. 1998).

Polymerase chain reaction analysis
The T-DNA flanking sequence in Osmt1 was isolated by TAIL-PCR method (Liu and Chen 2007). Three specific primers (5'-CGA TTA CGG TAT TTA TCC GTG TCG C-3', 5'-GGT ACC GGT ATA TCC GTG TTT CG-3', and 5'-GAG GTA TTT TAC CGA CCG TTA CGG-3') complementary to the Os sequence were used as described (Liu et al. 2007). The T-DNA insertion site was obtained by searching the T-DNA flanking sequence in the rice genome using BLAST (Basic Local Alignment Search Tool) software.

To perform RT-PCR, qRT-PCR, and Stem-loop RT-PCR, total RNA was isolated using Trizol reagent (Invitrogen) and removed DNA contamination with RNase-free DNase I (Invitrogen). M-MLV kit (Invitrogen) was used according to the manufacturer's instructions to synthesize first-strand cDNA for PCR.

Reverse transcription PCR was performed using the 2x Taq Master Mix (CWbio), and normalized using ACTIN1 as a standard. Quantitative RT-PCR experiments were performed using Power SYBRGreen Master Mix (QIAGEN, Hilden, Germany) and 7300 qRT-PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocols. UBQUITIN1 expression was used as an internal control. Three biological replications were analyzed as described previously (Xie et al. 2006). Stem-loop RT–PCR was employed to detect Os-miR156 abundance as reported previously (Varkonyi-Gasic et al. 2007). The primers used for testing Oso8g34258 expression were 5'-ATG AGC CAG AAG TGG TGG C-3' starting from the ATG codon and 5'-ACC GAC GGG CCT GTC GTC GA-3' at the position 130 bp from the ATG codon. The primers for testing pri-OsmiR156f were 5'-CTT CCC TTC GAC AGA AGA GC-3' and 5'-GCC GAC ACC TGT ATC ATC A-3'. The primers used for different OsSPLs and internal control genes are as follows: OsSPL1, 5’-ACC GAA AAG AAG CCG AGC CA-3’, 5’-TGG GGT GGC CCC TGC ATG AT-3’, OsSPL3, 5’-CTG CCC GTG TTT ATG GGA GCC-3’; 5’-GCA GAA GTC TGC ATG TGG-3’, OsSPL6, 5’-GCT ACA TGC TGC CCT GCG GAC-3’, 5’-GCC TCT CGC TTT CCT CTG CA-3’, OsSPL9, 5’-TGC GTC TCT GCA CCG TTA GGC-3’; 5’-TCT ACG GCC GAA AAA-3’, OsSPL10, 5’-GCC TCT GGC GGA GCC CCA AA-3’, OsSPL12, 5’-CGG TTC ACC GGA GGA GGC-3’, 5’-AGC GGC AGG TGC TCT CG-3’, OsSPL14, 5’-CCG GTG TCT GGC GCA CCA AA-3’, OsSPL15, 5’-CAT GGC GGA GCC GGC TCC AG-3’, OsSPL19, 5’-CTG TCG GCC TTA GAT CCG CCG TCC-3’, OsSPL19, 5’-TCA GCC ACC TTC CCG GAA CCC-3’; 5’-TGC GGT TGC GGT TGT GGT AA-3’, ACTIN1, 5’-GTC TAT GTA GGT CCG CAT CCA G-3’, 5’-AAT GAG TAA CCA GCC TGC TTC A-3’; and UBQUITIN1, 5’-GTC GCC AGT AAG TCC TCA GGC-3’, 5’-ACA ATG AAA CGG GAC ACG-3’.

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