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Article

A long noncoding RNA involved in rice reproductive development by negatively regulating osa-miR160

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ABSTRACT

Long noncoding RNAs (lncRNAs) participate in the regulation of multiple biological processes via diverse manners, one of which is functioning as endogenous target mimics (eTMs) to modulate microRNAs (miRNAs) by competing for their targets. Previously, we have predicted one lncRNA (osa-eTM160) as an endogenous repressor of osa-miR160 and validated the target mimicry ability of osa-eTM160 for ath-miR160 in *Arabidopsis thaliana*, yet the functions of osa-eTM160 in rice remain obscure. Here, we demonstrated that osa-eTM160 attenuated the repression of osa-miR160 on osa-ARF18 mRNAs during early anther developmental stages through the target mimicry manner, therefore to regulate rice seed setting and seed size. These findings revealed the roles of osa-eTM160 in rice, and indicated that lncRNAs with eTM functions may serve as temporal regulators to modulate the effects of miRNAs at specific developmental stages.

1. Introduction

Long noncoding RNAs (lncRNAs) are a class of recently identified regulatory non-coding RNAs usually of more than 200 nucleotides in length. LncRNAs can function at transcriptional level, post transcriptional level or epigenetic level, either by sequence pairing or serving as scaffolds for proteins, therefore to regulate a broad range of biological processes in plants [1–6], especially in response to stresses and reproductive development [2,7–10]. Some lncRNAs possess microRNA (miRNA) binding sites, therefore they can serve as endogenous target mimics (eTMs) to bind with miRNAs and reduce the repression of miRNAs on their targets [11–14].

As one of the first identified plant miRNAs, miR160 targets several auxin response factors (ARFs) to regulate plant growth, root cap formation, seed germination and fertilization [15–17]. In *Arabidopsis thaliana*, the confirmed targets of ath-miR160 include *ath-ARF10*, *ath-ARF16* and *ath-ARF17* [17]. Osa-miR160 also targets ARF family genes, including *osa-ARF18* and *osa-ARF22* [18,19]. A recent study showed that the *osa-ARF18* is essential for the proper growth and organ development of rice [20], yet whether the osa-miR160 and *osa-ARF18* target relationship is also regulated by other components remains unclear.

Previously, we have predicted one lncRNA (osa-eTM160) as an eTM of osa-miR160 and demonstrated the repressor effects of osa-eTM160 on ath-miR160 in *Arabidopsis thaliana* [12]. In this work, we illustrated the biological functions of osa-eTM160 in rice, and demonstrated that osa-eTM160 involved in rice reproductive development by negatively regulating osa-miR160 to enhance *osa-ARF18* expression.

2. Materials and methods

2.1. Plant Materials

The wide-type (WT) *Oryza sativa* L. *ssp. japonica* (Hejiang 19) was planted in the experimental fields in Beijing. The stages of inflorescence were categorized according to panicle length (P1, 0–3 cm; P2-3, 3–10 cm; P4, 10–15 cm; P5, 15–22 cm; P6, 22–30 cm). Mature seed-derived rice callus (Hejiang 19) was used for *Agrobacterium*-mediated gene-transfer as described by Hiei et al. [21]. For each transformation, more than 20 independent transgenic lines were obtained.

2.2. Vector construction

The *osa-eTM160* and *osa-ARF18* full-length sequences were amplified by PCR from cDNA using primers shown in Table S1.

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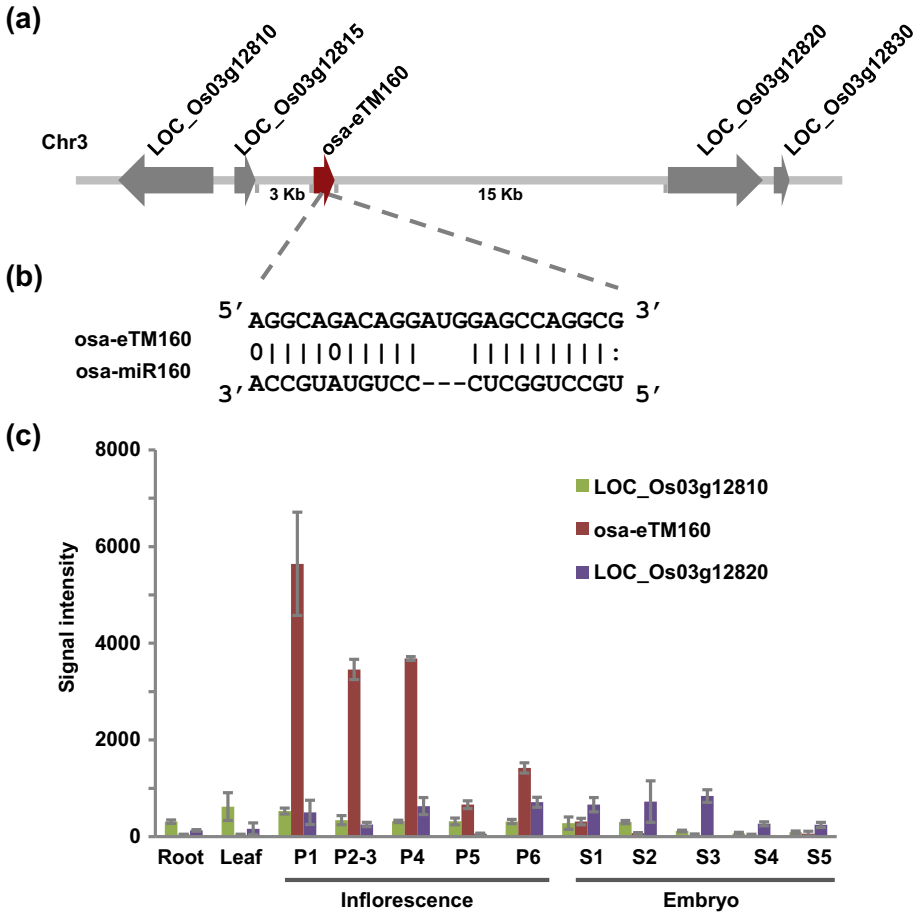

Fig. 1. Genomic location and expression patterns of osa-eTM160. (a) Genomic locus of *osa-eTM160*. (b) Sequence pairing between osa-eTM160 and osa-miR160. Unpaired nucleotides are marked by '0', G-U pairs are marked by ':'. (c) Expression profiles of osa-eTM160 as well as the upstream and downstream genes of *osa-eTM160*, in rice root, leaf, inflorescence stages and embryonic stages. P1, P2-3, P4, P5 and P6 stand for floral transition stage, floral organ development stage, meiotic stage, young microspore stage, vacuolated pollen stage and mature pollen stage, respectively. S1, S2, S3, S4 and S5 stand for early globular stage, middle and late globular stages, morphogenesis stage embryos, maturation stage embryos and dormant embryos, respectively.

The PCR product of *osa-eTM160* or *osa-ARF18* was inserted after the CaMV 35S promoter in the pCAMBIA1300 vector, respectively.

2.3. RT-PCR analysis of gene expression

The total RNA was isolated using Trizol (Invitrogen) from each sample. In the quantitative RT-PCR (qRT-PCR) experiments, 1 μg of total RNAs treated with DNase I (New England Biolabs) was reverse transcribed into cDNA by Moloney Murine Leukemia Virus (New England Biolabs) using poly (dT) oligonucleotides with *osa-NAB* (LOC_Os06g11170) as the internal control [22]. Two-week seedlings were used for qRT-PCR analysis in transgenic plants. SYBR Green PCR Master Mix (Applied Biosystems) was used in all qRT-PCR experiments with primers listed in Table S2. The relative fold expression changes of genes were calculated using the $2^{-\delta\text{-}\delta Ct}$ method [23].

2.4. RT-PCR analysis of miRNA expression

Expression levels of miRNAs were analyzed using All-in-One™ miRNA qRT-PCR Reagent Kits (GeneCopoeia) with primers listed in Table S2. In the qRT-PCR experiments of miRNAs, 1 μg of total RNAs of 2-week seedlings was used in each reaction with U6 snRNA as the internal control. The relative fold expression changes of miRNAs were calculated using the $2^{-\delta\text{-}\delta Ct}$ method [23].

2.5. In silico gene expression analysis

Gene expression data of rice reproductive developmental stages were downloaded from the Gene Expression Omnibus (GEO) Database (GSE6893) and processed using GEO2R. The published rice gene expression data during anther development (GSE13988) was extracted from the Rice Oligonucleotide Array Database (RiceArray).

3. Results

3.1. Osa-eTM160 had high and almost restricted expression in the early reproductive development stage

Osa-eTM160 is a 688 bp long lncRNA transcribed between *LOC_Os03g12815* and *LOC_Os03g12820* of rice chromosome 3 (Fig. 1a). Osa-eTM160 could form near perfect sequence pair with osa-miR160 except for a 3-nt bulge between the 10th and 11th positions of osa-miR160 and two mismatches (Fig. 1b). A gene expression microarray data of rice different developmental stages from the public database showed that the expression of osa-eTM160 was highest at the inflorescence stage P1, then gradually decreased along the inflorescence and embryonic development, became almost undetectable since embryonic stage S2, and was also barely detected in leaves and roots (Fig. 1c). The expression profile of osa-eTM160 was distinct from those of the neighboring

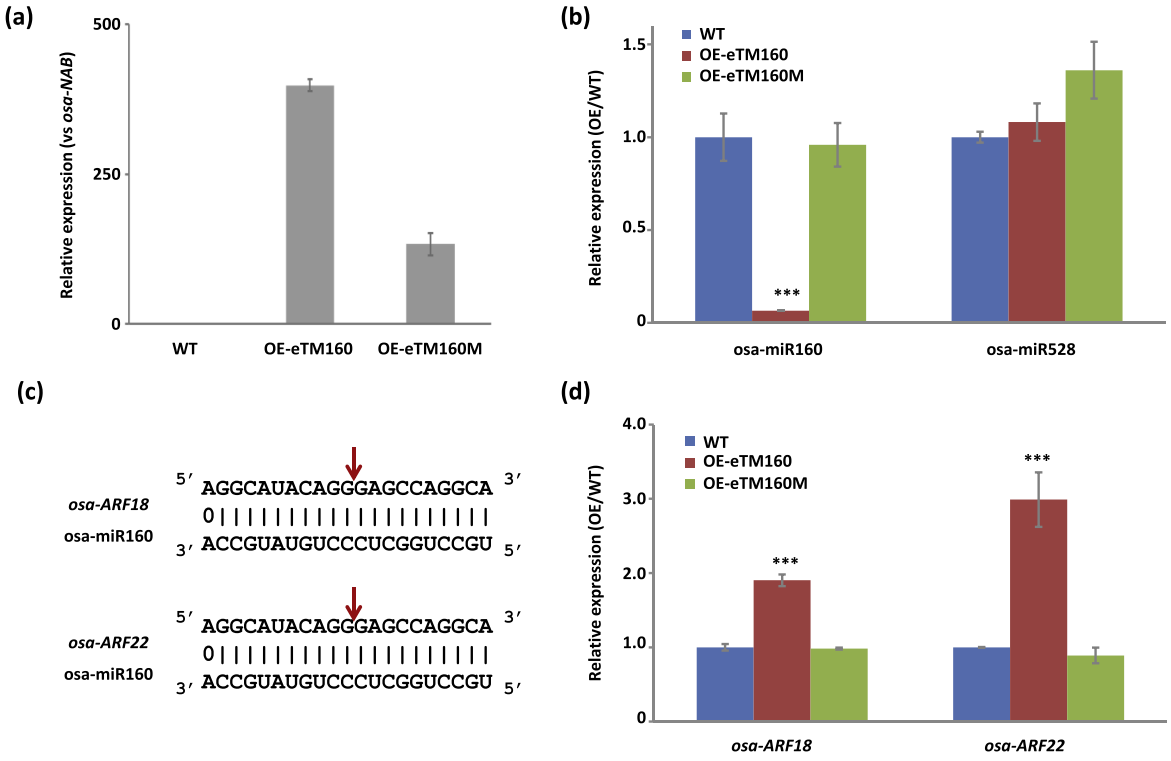

Fig. 2. Osa-eTM160 altered the expression of osa-miR160 and osa-miR160 target genes. (a) Quantitative RT-PCR analysis of osa-eTM160 and osa-eTM160M with designed mutations in wild-type rice (WT), rice overexpressing osa-eTM160 (OE-eTM160), and rice overexpressing osa-eTM160M (OE-eTM160M). (b) Quantitative RT-PCR analysis of osa-miR160 and osa-miR528 in WT, OE-eTM160 and OE-eTM160M rice. (c) Sequence pairing between osa-miR160 and its targets *osa-ARF18* and *osa-ARF22*. The predicted cleavage sites on targets are marked by red arrows. (d) Quantitative RT-PCR analysis of *osa-ARF18* and *osa-ARF22* expression in WT, OE-eTM160 and OE-eTM160M rice. Error bars in all panels represent the standard deviation (SD) of three replicates. $^{***}P < 0.001$ compared with WT using Student's *t*-test.

genes (*LOC_Os03g12810* and *LOC_Os03g12820*) (Fig. 1c), indicating the specific function of osa-eTM160 during the early inflorescence stage. Using qRT-PCR, we also confirmed that osa-eTM160 was mainly expressed in rice inflorescence but not flag leaves (Fig. S1a online), and was highly expressed at the early inflorescence developmental stages (Fig. S1b online).

3.2. Osa-eTM160 functions as endogenous target mimics to negatively regulate osa-miR160

Previously, we reported that overexpression of osa-eTM160 in *Arabidopsis thaliana* could impede the functions of ath-miR160 through the target mimicry manner [12]. To investigate whether osa-eTM160 functions as endogenous target mimics for osa-miR160 in rice, we generated transgenic rice plants overexpressing osa-eTM160 (OE-eTM160) or osa-eTM160 mutant (OE-eTM160M) which was insufficient to pair with osa-miR160 (Fig. 2a, Fig. S2 online). As expected, the expression of osa-miR160 was reduced in OE-eTM160 plants but not in OE-eTM160M plants (Fig. 2b). As the negative control, osa-miR528, which was not predicted to pair with osa-eTM160, showed no significant expression changes either in OE-eTM160 or OE-eTM160M plants (Fig. 2b). These results demonstrated that osa-eTM160 could negatively regulate cellular osa-miR160 abundance via a sequence dependent manner.

Previous studies have identified *osa-ARF18* and *osa-ARF22* as the target genes of osa-miR160 [18,19]. Both *osa-ARF18* and *osa-ARF22* could form perfect complementary sequence pairs with osa-miR160 except for the 3′ end 1 nt mismatch of osa-miR160 (Fig. 2c). Consistent with the expected effects of eTMs, the repression of *osa-ARF18* and *osa-ARF22* was partially released in OE-eTM160 rice, but remained almost unchanged in OE-eTM160M plants (Fig. 2d).

3.3. Osa-eTM160 regulates rice reproductive development

The OE-eTM160 transgenic rice did not show obvious phenotypes in the vegetative developmental stage (Fig. 3a), but failed to open lemma and palea which led to enclosed anthers during reproductive development (Fig. 3b). The OE-eTM160 plants had significantly lower seed setting rate as compared to the WT control (Fig. 3c, d and Table 1). In addition, the OE-eTM160 seeds were smaller than those of WT control in terms of both seed length and seed width (Fig. 3e, f). To the contrary, plants overexpressing osa-eTM160M were normal without any of these defects (Fig. 3a–f), demonstrating the importance of osa-eTM160 sequence in executing its functions.

3.4. Functional and expression consistency between osa-ARF18 and osa-eTM160

As overexpression of osa-eTM160 could enhance the transcript abundance of *osa-ARF18* (Fig. 2d), we speculated that *osa-ARF18* and osa-eTM160 may control the same phenotypes. To verify this hypothesis, we generated several rice lines overexpressing *osa-ARF18* (OE-ARF18). Due to the silencing effects of OE-ARF18 by endogenous osa-miR160, only 20% of OE-ARF18 plants had elevated *osa-ARF18* expression, and all these *osa-ARF18* highly expressed plants showed reduced seed setting rate and smaller seed size (Figs. 4a, b and S4 online), similar to the phenotypes of OE-eTM160. To investigate whether such regulatory relationship

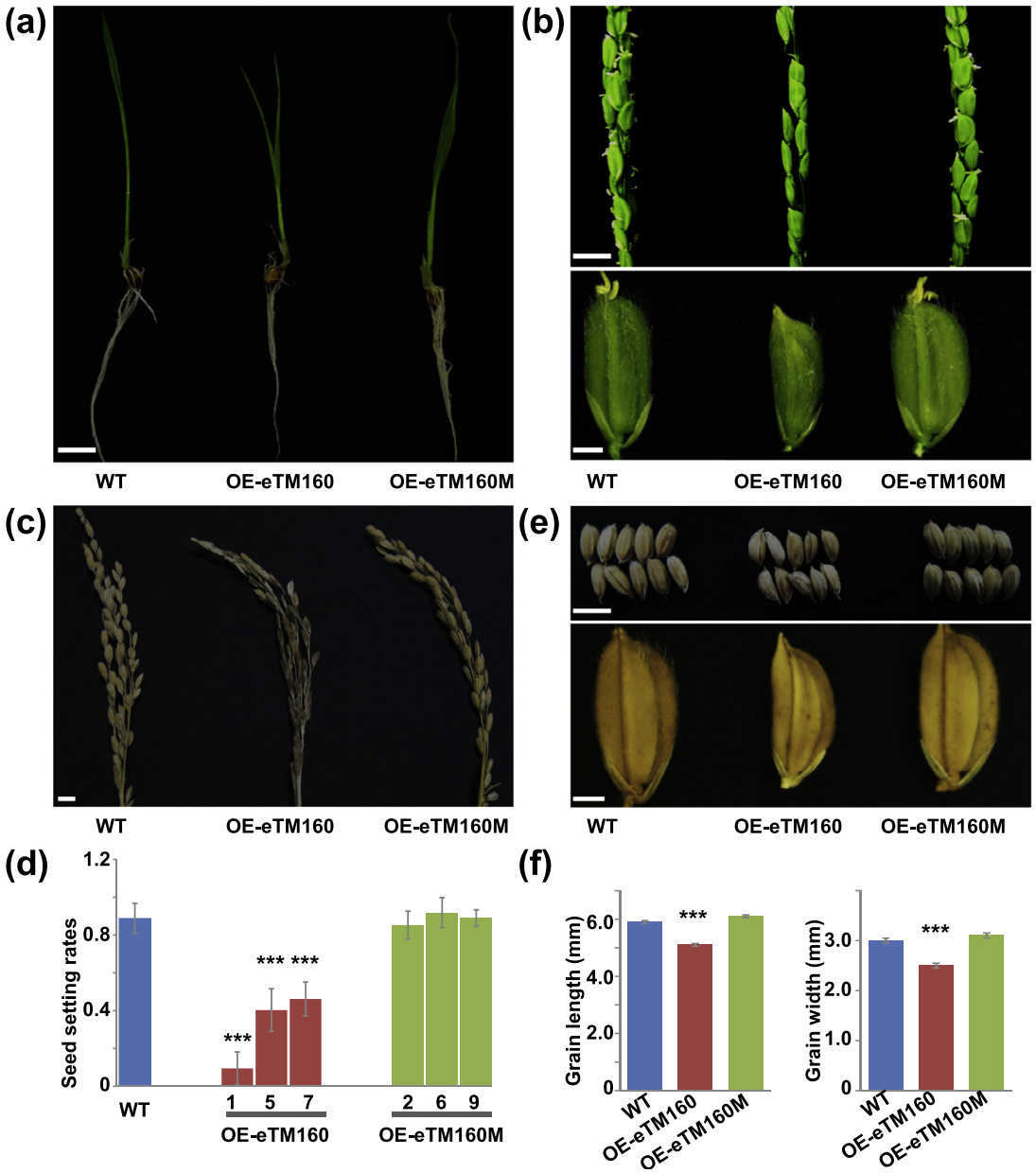

Fig. 3. (Color online) Phenotype analysis of rice overexpressing osa-eTM160 and osa-eTM160M. (a) Seedlings of WT, OE-eTM160, and OE-eTM160M rice plants. Scale bar, 1 cm. (b) Phenotypes of inflorescence (upper panel, scale bar = 1 cm) and spikelet (bottom panel, scale bar = 2 mm). (c) Phenotypes in seed setting rates. Scale bar, 1 cm. (d) Statistical analysis of seed setting rates. (e) Phenotypes in seed size. Scale bar in upper panel, 1 cm. Scale bar in bottom panel, 2 mm. (f) Statistical analysis of seed length and width. Error bars in all panels represent the standard deviation (SD) of three replicates. $^{***}P < 0.001$ compared with WT using Student's *t*-test.

Table 1
The seed setting rates of OE-eTM160 and OE-eTM160M rice.

Lines		Seed setting rates
WT	–	0.8876 ± 0.0795
OE-eTM60	1	0.0946 ± 0.0872
	5	0.4031 ± 0.1133
	7	0.4620 ± 0.0897
	17	0.4265 ± 0.1456
OE-eTM60M	2	0.9178 ± 0.0794
	6	0.8525 ± 0.0739
	7	0.9145 ± 0.0524
	9	0.8893 ± 0.0441

is authentic *in vivo*, we resorted to a public microarray data of rice anther development [GSE13988, 24]. The results showed that *osa-ARF18* and osa-eTM160 had very similar expression profiles, both of which were induced at the early anther developmental stages and gradually reduced along the late anther developmental stages (Fig. 4c), indicating the capability of osa-eTM160 to regulate *osa-ARF18* during anther development.

4. Discussions

As a class of newly identified non-coding RNAs, the functions of majority of lncRNAs are still obscure. It has been shown that some lncRNAs possessing miRNA binding sites, therefore could pair with miRNAs to release the repression of miRNAs on their target mRNAs. Such lncRNAs, which are now called competing endogenous RNAs (ceRNAs) in animals [25,26], were first identified in plants and coined endogenous target mimics (eTMs) [14]. Basing on our prediction, miR160 has eTMs in both *Arabidopsis thaliana*

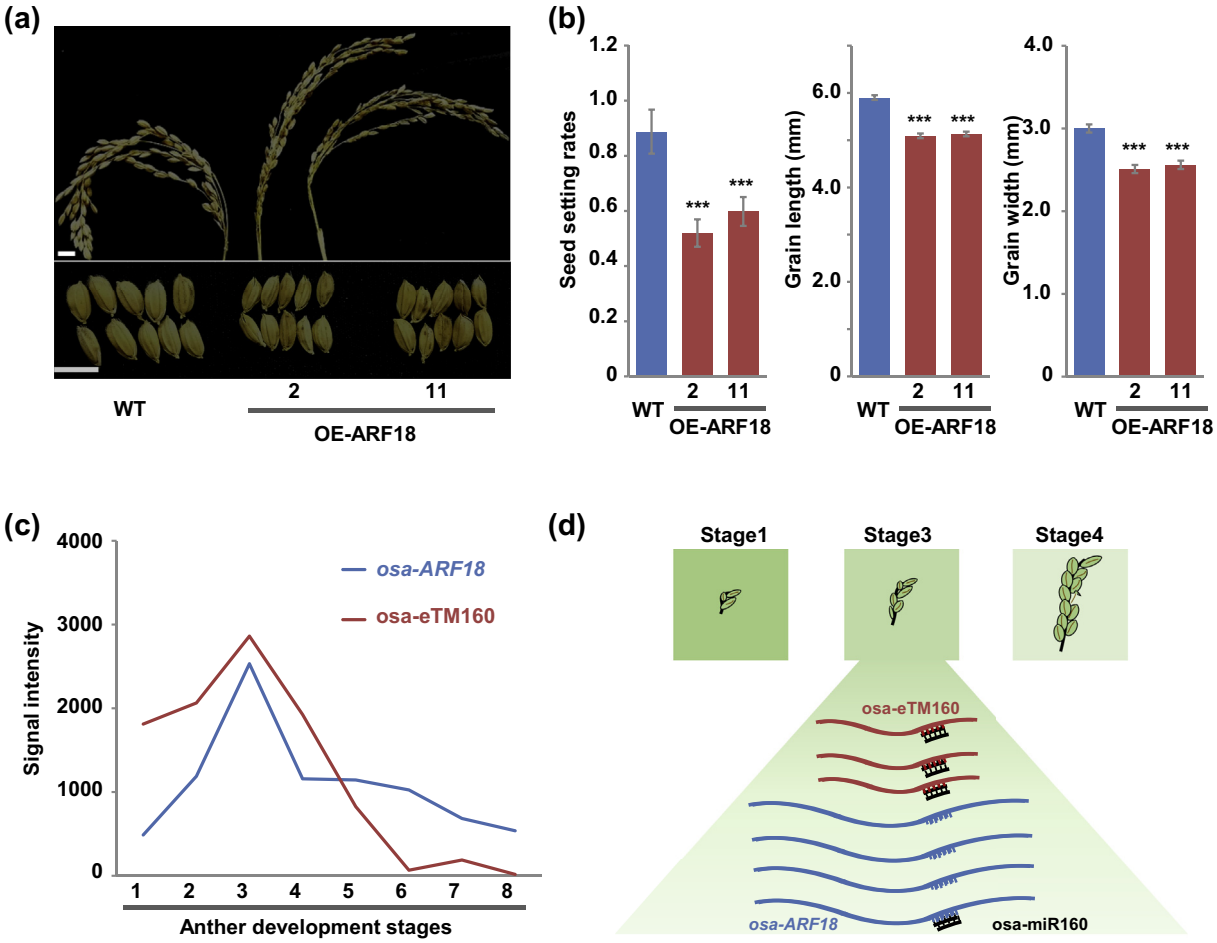

Fig. 4. (Color online) Osa-ARF18 regulates reproductive development. (a) Phenotypes of rice plants overexpressing *osa-ARF18* (OE-ARF18). Two and eleven represent the name of two independent transgenic lines. Scale bar in upper panel, 1 cm. Scale bar in bottom panel, 2 mm. (b) Statistical analysis of seed setting rates (left panel), seed length (middle panel) and seed width (right panel) in WT and OE-ARF18 plants. Error bars in all panels represent the standard deviation (SD) of three replicates. $^{***}P < 0.001$ compared with WT using Student's *t*-test. (c) Expression profiles of osa-eTM160 and *osa-ARF18* during anther development. Numbers along the x-axis stand for hypodermal archesporial cells forming stage, pollen mother cells at pre-meiotic S/G2 stage, pollen mother cells at meiotic leptotene stage, pollen mother cells at meiotic zygotene-pachytene stage, pollen mother cells at meiotic diplotene-tetrad stage, uni-nucleated gametopyte stage, bi-cellular gametopyte stage and tri-cellular mature pollen stage, respectively. (d) A hypothetical working model of osa-eTM160 involved in rice reproductive development. Reproductive stages are defined as described by Fujita et al. [24].

and rice [12]. Previously we have shown that osa-eTM160 could regulate the function of ath-miR160 in Arabidopsis. In this work, we further demonstrated that osa-eTM160 could serve as authentic eTM for osa-miR160 in rice and investigated the functions of osa-eTM160.

MiR160 is a highly expressed and sequence conserved miRNA in plants. Previous studies have shown that repression of ath-miR160 affected both the vegetative growth and reproductive development of Arabidopsis [15–17,27]. However, in our study, when the expression of osa-miR160 was repressed by OE-eTM160, only the reproductive development of rice was effected (Figs. 3, S3 online). These results demonstrated that although with identical sequences, the functions of ath-miR160 and osa-miR160 have diverged in Arabidopsis and rice. Similarly, overexpressing both ath-eTM160 and osa-eTM160 resulted in smaller and serrated leaves in Arabidopsis, whereas in rice, the major defects of OE-eTM160 are reduced seed setting rate and seed size (Fig. 3).

Here we have proven that osa-eTM160 could release the repression of osa-miR160 on its target *osa-ATF18* (Fig. 2).Our results demonstrated that osa-eTM160 negatively regulates osa-miR160 via the target mimicry manner to repress *osa-ARF18*, therefore play roles in rice reproductive development (Fig. 4d). It is intriguing to see that osa-eTM160 and *osa-ARF18* had consistent expression profiles, both were highly expressed during early anther development stages. The restricted expression of osa-eTM160 indicated that eTMs may serve as a temporal regulator for miRNAs, thus could fine-tune the functions of miRNAs according to different needs.

Taken together, our work demonstrated the target mimicry relationship between osa-eTM160 and osa-miR160, and identified the functions of osa-eTM160 in regulating rice reproductive development through altering the expression of *osa-ARF18* via the target mimicry manner. These results added a new layer to the regulatory network of *osa-ARF18*, and could also shed light on the functional studies of other eTMs, especially on their potentials as temporal regulators of miRNAs.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scib.2017.03.013.

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