

Small RNAs from MITE-derived stem-loop precursors regulate abscisic acid signaling and abiotic stress responses in rice

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SUMMARY

Small silencing RNAs (sRNAs) are non-coding RNA regulators that negatively regulate gene expression by guiding mRNA degradation, translation repression or chromatin modification. Plant sRNAs play crucial roles in various developmental processes, hormone signaling, immune responses and adaptation to a variety of abiotic stresses. miR441 and miR446 were previously annotated as microRNAs (miRNAs) because their precursors can form typical stem-loop structures, but are not considered as real miRNAs because of inconsistency with some ancillary criteria of the recent guidelines for annotation of plant miRNAs. We tentatively rename them small interfering (si)R441 and siR446, respectively, in this study. It has recently been shown that the precursors of siR441 and siR446 might originate from the miniature inverted-repeat transposable element (MITE) *Stowaway1*. In this report, we show that, in contrast with Dicer-like (DCL)3- and RNA-dependent RNA polymerase (RDR)2-dependent MITE-derived ra-siRNAs, siR441 and siR446 are processed by *OsDCL3a* but independent of *OsRDR2*, indicating that siR441 and siR446 are generated from single-stranded stem-loop precursors. We also show that abscisic acid (ABA) and abiotic stresses downregulate the expression of siR441 and siR446 but, surprisingly, increase the accumulation of their precursors in rice plants, implying that processing of siRNA precursors is inhibited. We provide evidence to show that this defective processing is due to increased precursor accumulation *per se*, possibly by intermolecular self-pairing of the processing intermediate sequences, thus hindering their normal processing. Functional examinations indicate that siR441 and siR446 are positive regulators of rice ABA signaling and tolerance to abiotic stress, possibly by regulating *MAIF1* expression.

Keywords: small RNA, miniature inverted-repeat transposable element, stem-loop precursor, abscisic acid and abiotic stresses, rice.

INTRODUCTION

Small silencing RNAs (sRNAs) are fundamental, sequence-specific regulatory elements of eukaryotes that mediate endogenous gene silencing. Based upon their origins and structures, four classes of sRNAs have been discovered in plants: microRNAs (miRNAs) and three types of small interfering RNAs (siRNAs), including *trans*-acting siRNAs (ta-siRNAs), natural *cis*-antisense transcripts-derived siRNAs (nat-siRNAs), and repeat-associated siRNAs (ra-siRNAs). One major difference between miRNAs and siRNAs is

that miRNAs result from the processing of a single-stranded hairpin precursor, while siRNAs are generated from long double-stranded RNAs (dsRNAs). Some siRNAs are derived also from single-stranded hairpin precursors. Plant miRNAs are processed from primary miRNA transcripts through two sequential cleavages by Dicer-like1 (DCL1). The biogenesis of ta-siRNAs is initiated by miRNA-mediated cleavage of non-coding transcripts. The cleaved RNAs are copied into dsRNAs by RNA-dependent RNA polymerase 6 (RDR6), and

are processed by DCL4 into phased siRNAs from the end defined by miRNA-mediated cleavage. The production of ra-siRNAs requires activity of DCL3, RDR2, and polymerase (Pol) IV, a plant-specific DNA-dependent RNA polymerase (Chen, 2009).

Plant sRNAs play crucial roles in various developmental processes. For example, loss-of-function mutants of any components in miRNA or ta-siRNA biogenesis exhibit dramatic and pleiotropic developmental defects in Arabidopsis and rice (Liu *et al.*, 2005; Mallory and Vaucheret, 2006; Chen, 2009; Wu *et al.*, 2009, 2010). A number of validated miRNA or ta-siRNA targets are indeed essential for plant growth and development (Jones-Rhoades *et al.*, 2006; Mallory and Vaucheret, 2006; Chen, 2009). Moreover, sRNAs are known to be key regulatory molecules in plant hormone signaling, immune responses and adaptation to a variety of abiotic stresses (Sunkar *et al.*, 2007; Voinnet, 2008; Liu and Chen, 2009).

Obviously, the important and ubiquitous roles of plant sRNAs entail their spatio-temporal level and activity to be controlled delicately. The expression and activity of plant sRNA can be regulated at several layers along the sRNA pathway. For example, a significant amount of data has accumulated concerning the regulation at the transcriptional level of *MIR* genes (Xie *et al.*, 2005; Megraw *et al.*, 2006; Liu *et al.*, 2009). Besides, regulations at the levels of plant miRNA processing and action have been reported. Arabidopsis DCL1 and AGO1, two key players in global miRNA biogenesis and action respectively, have been shown to be feedback-regulated by their cognate miRNAs (Xie *et al.*, 2003; Vaucheret *et al.*, 2004). Recently, it was found that the RNA transcripts of the non-protein coding gene *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*) could outcompete for and thus dampen the activity of miR399 by mimicking the miR399 target sequence (Franco-Zorrilla *et al.*, 2007). In sharp contrast with the study of miRNA regulations, very little is known about the regulatory mechanisms of controlling siRNA activity.

Two sRNAs (UACCAUCAUAUAAAUGUGGGAAA and CAUCAUAUGAAUAUGGGAAUUGG) were identified by cloning from rice small RNA libraries and annotated as miR441 and miR446, respectively, because their precursors can form typical stem-loop structures (Sunkar *et al.*, 2005). However, miR441 and miR446 are not appreciated as *bona fide* miRNAs according to the recent criteria for annotation of plant miRNAs (Meyers *et al.*, 2008), because other sRNA species are produced from the same precursors (Wu *et al.*, 2009; Li *et al.*, 2010). In this study, we name them siR441 and siR446, respectively. It has recently been shown that the precursors of siR441 and siR446 might originate from the miniature inverted-repeat transposable element (MITE) *Stowaway1* (Piriyapongsa and Jordan, 2008). MITEs are a class of short non-autonomous DNA transposons derived from the full-length DNA transposons (TEs) (Feschotte *et al.*,

2002), and produce 24-nt ra-siRNAs dependent on DCL3 and RDR2 (Kuang *et al.*, 2009). We found that in contrast with MITE-derived ra-siRNAs, siR441 and siR446 are processed by *OsDCL3a* but are independent of *OsRDR2*, indicating that siR441 and siR446 are generated from single-stranded stem-loop precursors. We further demonstrated their expression patterns and functions in abscisic acid (ABA) signaling and response to abiotic stresses in rice.

RESULTS

siR441 and siR446 are processed by *OsDCL3a* independent of *OsRDR2*

Rice has six DCL proteins of which *OsDCL1*, *OsDCL3a* and *OsDCL4* represent close-related orthologs of *AtDCL1*, *AtDCL3* and *AtDCL4*, respectively (Margis *et al.*, 2006). DCL orthologs between Arabidopsis and rice have similar functions in their processing of different types of small RNAs. For example in rice, like in Arabidopsis, *OsDCL1* and *OsDCL3a* are required for the production of canonical miRNAs (cmiRNAs) and 24-nt sRNAs, respectively, whereas *OsDCL4* is involved in the biogenesis of ta-siRNAs (Liu *et al.*, 2005, 2007; Wu *et al.*, 2010). In order to see which *OsDCL*(s) is responsible for the biogenesis of siR441 and siR446, we analyzed their accumulation in the published *OsDCL1*, *OsDCL3a* and *OsDCL4* RNAi-knock-down rice lines. The results, shown in Figure 1, indicate that the expression of siR441 and siR446 was not affected in the *Osdc11* and *Osdc14* plants but was greatly reduced in the *Osdc13a* plant lines (Figure 1a,b), indicating that the processing of siR441 and siR446 is performed mainly by *OsDCL3a* activity.

In order to check if 24-nt siR441 and siR446 are MITE-derived ra-siRNAs, we detected their accumulation in two published *OsRDR2* RNAi-knock-down rice lines (Wu *et al.*, 2010). The results showed that the accumulation of siR441 and siR446, like the cmiRNAs, was affected slightly in the *OsRDR2* RNAi-knock-down rice lines. In contrast, the accumulation of siRNAs from the intergenic region cluster2 was reduced to barely detectable levels (Figure 1a), as previous reported (Wu *et al.*, 2010). Taken together, these results indicate that siR441 and siR446 are a class of DCL3-processed 24-nt siRNAs and are independent of RDR2, suggesting that siR441 and siR446 are derived from single-stranded hairpin precursors.

In order to confirm that siR441 and siR446 are derived from single-stranded precursor sequences, we performed an *in vitro* processing analysis in which protein extracts from 30-day-old rice calli were used as a source of sRNA-processing proteins and incubated with the *in vitro*-transcribed 86-nt siR446 precursor sequence. The results showed that siR446 precursor was correctly processed into 24-nt sRNA (Figure 1c). Taken together, the above findings indicate that siR441 and siR446 are generated from MITE-derived stem-loop precursors by *OsDCL3a*.

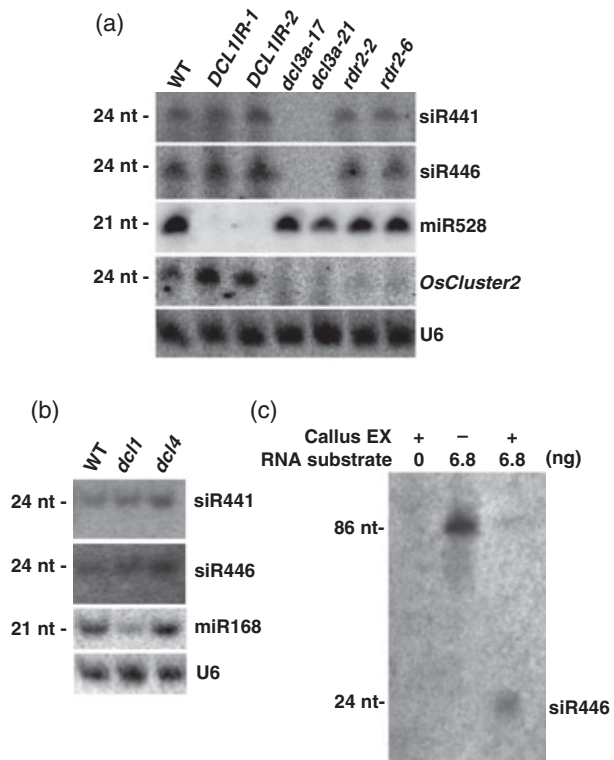


Figure 1. siR441 and siR446 are processed by *OsDCL3a* independent of *OsRDR2*.

(a, b) Accumulation of siR441 and siR446 was analyzed in wild-type rice (WT) and RNAi-knock-down rice plants for *OsDCL1* (*DCL1IR-1*, *DCL1IR-2* and *dcl1*), *OsDCL3a* (*dcl3a-17* and *dcl3a-21*), *OsDCL4* (*dcl4*) and *OsRDR2* (*rdr2-2* and *rdr2-6*) by small RNA gel blot hybridization. miR528, miR168 and *OsCluster2* served as *OsDCL1*-dependent or *OsDCL3a*- and *OsRDR2*-dependent control. The hybridized signals for U6 RNA (U6) are shown as a loading control.

(c) 24-nt siR446 was processed from a single-stranded precursor sequence *in vitro*. The *in vitro*-transcribed 86-nt RNA sequence of the predicted siR446 processing intermediate was incubated with (+) or without (-) a rice callus extract (Callus EX). RNA was extracted from the incubation mixtures and subjected to gel blot hybridization probed for siR446.

Accumulation of siR441 and siR446 is down-regulated by ABA and abiotic stresses

It has been shown that the expression of some rice sRNAs is regulated by abiotic stresses and phytohormones, implying

that they have a role in plant stress responses or hormone signaling (Sunkar *et al.*, 2008; Liu *et al.*, 2009). To gain an insight into regulatory functions of siR441 and siR446 in mediating rice responses to stress conditions, we studied their expression profiles in 10-day-old rice seedlings under cold-, drought-, salt- or ABA-treatment conditions. RNA gel blot analyses showed that the accumulation of siR441 and siR446 decreased with these treatments, especially at 12 h after the treatments (Figure 2a–d). The drought condition seemed to have a more profound effect on reducing the levels of siR441 and siR446 than did other conditions, beginning at 1 h after treatment (Figure 2b).

ABA and abiotic stresses increase accumulation of precursors of siR441 and siR446

We have shown that the accumulation of siR441 and siR446 decreased under ABA and abiotic stress conditions (Figure 2). Interestingly, we found that some larger RNA bands could be detected with prolonged exposure and these were enhanced by drought and salt treatments (Figure S1). These signals might represent the processing intermediates of siR441 and siR446 precursors. To check whether the transcriptional levels of siR441 and siR446 precursors are altered by these stresses, we chose three siR441 loci (siR441a–c) and one siR446 locus (previously named miR441a–c and miR446 by Sunkar *et al.*, 2005) to perform semi-quantitative RT-PCR analysis using oligo(dT) as a universal RT primer and locus-specific PCR primers to amplify individual polyadenylated primary precursor transcripts. The results showed that, although the transcription levels of the three siR441 loci and one siR446 locus were very low in normal conditions, treatment with ABA, cold, drought or salt increased the accumulation of all four precursors to different extents, with the drought treatment showing the most prominent enhancing effects (Figure 3a). We also used the *uidA* (*GUS*) reporter to show induction of activity of these genes' promoters by abiotic stress. As shown in Figure 3(b), in *SIR441a*-, *SIR441b*-, *SIR441c*- and *SIR446*-promoter-*uidA* transgenic rice plants, *uidA* mRNA accumulation was markedly increased by drought and salt treatments.

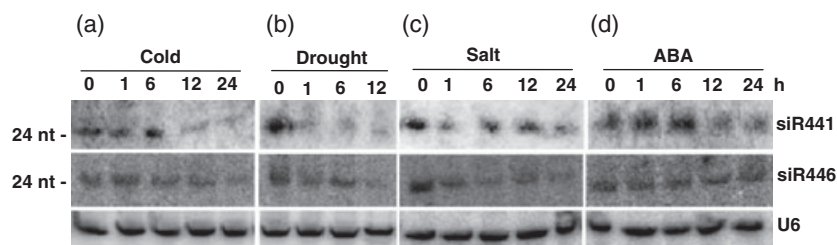


Figure 2. Accumulation of siR441 and siR446 is reduced by ABA and abiotic stresses.

Ten-day-old rice seedlings were treated with cold (4°C) (a); drought (b); salt (200 mM NaCl) (c); or ABA (20 μM) (d). Seedlings were harvested at different time points (0–24 h) for RNA isolation and the RNA samples were subjected to small RNA gel blot hybridization. The hybridized signals for U6 RNA (U6) are shown as a loading control.

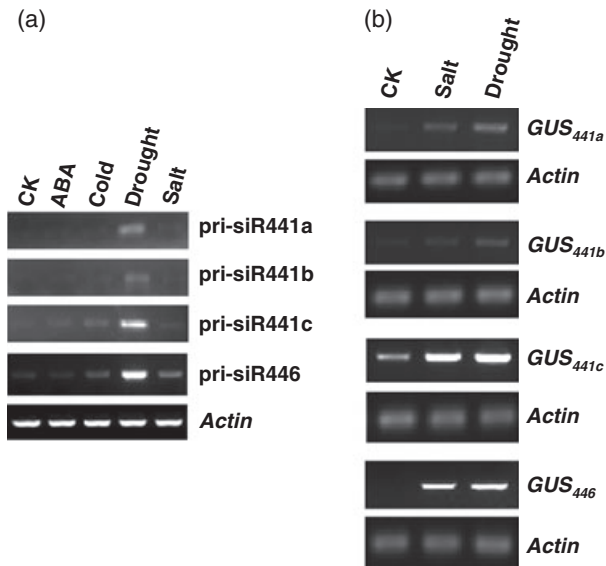


Figure 3. ABA and abiotic stresses upregulate the transcription of *SIR441a–c* and *SIR446*.

(a) RT-PCR analyses of the primary precursors (pri-siR441a–c and pri-siR446). (b) RT-PCR analyses of the *uidA* mRNA accumulation level controlled by a 2-kb promoter fragment of *SIR441a* (*GUS_{441a}*), *SIR441b* (*GUS_{441b}*), *SIR441c* (*GUS_{441c}*) or *SIR446* (*GUS₄₄₆*). For RT-PCR analyses, total RNA was isolated from 10-day-old seedlings of wild-type (a) and transgenic rice plants (b) either untreated (CK) or treated with ABA, cold, salt or drought for 24 h. Oligo(dT) was used as a primer for reverse transcription reactions and a pair of PCR primers specific for each *SIR* gene or the *uidA* mRNA were used for amplification of individual transcripts. The RT-PCR product of the *Actin* RNA served as an internal control.

The above results showed that ABA, cold, drought and salt treatments all up-regulated the transcription of *SIR441a–c* and *SIR446*, hence they increased accumulation of the precursors of siR441 and siR446, but reduced the accumulation of the mature siRNAs, indicating that processing was inhibited by an unknown mechanism(s). It seems unlikely that such a regulation directly involves the key components of the plant sRNA biogenesis machinery, since in Arabidopsis and rice the expression levels of most sRNAs are not notably altered by ABA and abiotic stress (Sunkar and Zhu, 2004; Zhao *et al.*, 2007; Sunkar *et al.*, 2008). In addition, there was experimental evidence indicating that none of the *OsDCL* genes, including *OsDCL3a* which is responsible for biogenesis of siR441 and siR446, showed any alternation in their expression level in response to cold, salt or drought conditions (Kapoor *et al.*, 2008).

Increased expression of precursors reduces siRNAs accumulation

To gain insight into the mechanism controlling the abnormal inverse correlation between the levels of siR441 and siR446 and the levels of their precursors, we took the approach of over-expressing the siRNA precursors in transgenic rice plants. Genomic DNA fragments of approximately 1 kb

harboring the predicted precursor sequences from *SIR441a–c* and *SIR446* were separately cloned downstream of the *Cauliflower mosaic virus* 35S promoter and transformed into rice plants. RT-PCR analyses of the precursor transcripts showed that these *SIR* transgenes were ectopically expressed in transgenic rice plants (Figure 4a–d). Surprisingly, we found that the level of siR441 or siR446 was decreased in *SIR441a–c* and *SIR446*-transgenic plants compared with that in untransformed rice plants (Figure 4a–d), similar to the observation that the expression of siR441 or siR446 was down-regulated in ABA- or abiotic stress-treated wild-type rice plants (Figures 2 and 3). Treatments of *SIR441a*- and *SIR441b*-transgenic plants with ABA, cold, salt or drought stress further reduced the accumulation of siR441 (Figure S2). These results infer that it is the increase in the levels of siRNA precursors, but not other factors, that causes reduced accumulation of siR441 and siR446, suggesting that these siRNA precursors can attenuate the processing of themselves.

siR441 and siR446 positively regulate ABA signaling and abiotic stress responses in rice

ABA is known to regulate a broad range of physiological processes in plant development, e.g. to maintain seed dormancy and to control seedling growth after seed germination. Several positive and negative regulators in ABA signaling in Arabidopsis have been identified through the characterization of ABA-insensitive mutants (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Wasilewska *et al.*, 2008). However, ABA signaling components in rice are largely unknown. We have shown that ABA and abiotic stresses down-regulate accumulation of siR441 and siR446, providing clues for investigation of regulatory roles of these siRNAs in ABA signaling and abiotic stress responses in rice. To address this factor, we used previously described *SIR441a–c* and *SIR446*-transgenic plant lines for the following studies. We checked all the described *SIR441a–c* and *SIR446*-transgenic lines and found that they showed the same phenotype. As representatives, the phenotypes of *SIR441a*- and *SIR446*-transgenic lines are shown here. First, we tested their ABA response during seed germination. In the ABA-free medium, the siR441- and siR446-knockdown plants showed normal seed germination rates, similar to wild-type rice plants. However, in medium containing 3 μ M ABA over 60% of seeds from siR441- and siR446-knockdown plants germinated, while germination of wild-type rice seeds was severely inhibited, i.e. <20% of seeds germinated (Figure 5a), indicating that siR441- and siR446-knockdown plants are less sensitive to ABA than wild-type plants regarding seed germination. Next, we investigated their response to ABA treatment in seedling growth. As shown in Figure 5(b), differences between them and wild type plants were observed mainly in root morpha. In the ABA-free medium, roots of siR441- and siR446-knockdown seedlings

Figure 4. Ectopic expression of *SIR441a*, *SIR441b*, *SIR441c* and *SIR446* in rice. The siRNA primary transcripts and the accumulation levels of siR441 and siR446 in transgenic rice plants were analyzed by RT-PCR and RNA gel blot hybridization respectively. For each *SIR* gene, 5 (a, b) or 3 (c, d) independent transgenic lines were analysed. The total RNA from untransformed rice seedlings (WT) was parallel-analyzed. The hybridized signals for U6 RNA (U6) are shown as an RNA gel loading control.

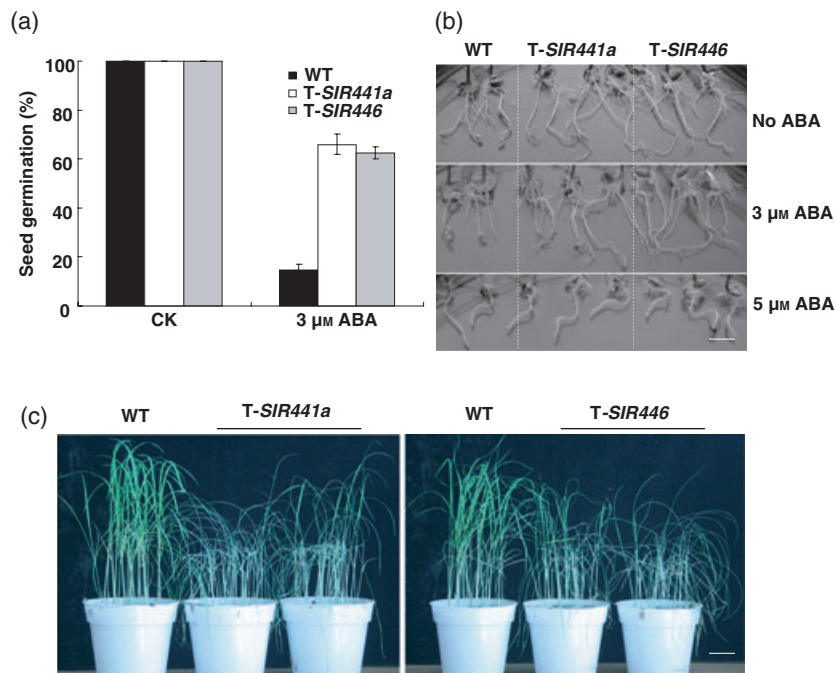
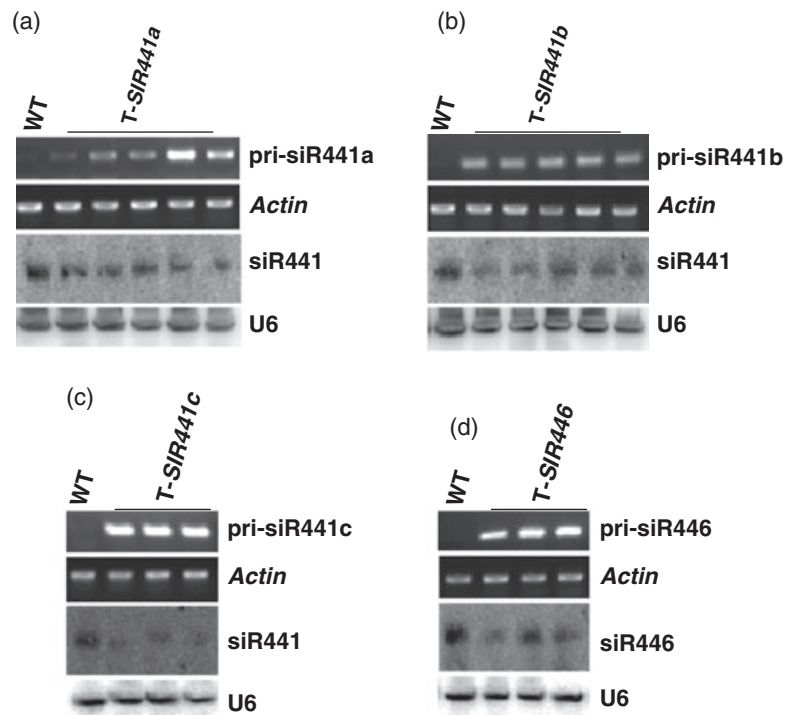


Figure 5. Down-regulation of siR441 or siR446 reduces rice ABA sensitivity and drought tolerance.

(a) siR441- and siR446-knockdown plants are less sensitive to ABA than wild-type plants in seed germination. After 1-day soaking, rice seeds were transferred to ABA-free medium (CK) or 3 μM ABA-containing medium for germination. Germination rate was measured at 3 days after transfer. Error bars represent SE (triplicate measurements; $n = 60$).

(b) Root growth of siR441- and siR446-knockdown seedlings and wild type seedlings in ABA-containing medium at the post-germination stage. Two-day-old seedlings were kept on or transferred from ABA-free medium to the medium with 3 or 5 μM ABA to grow for 5 days. Bar = 2 cm.

(c) Drought response of siR441- and siR446-knockdown seedlings and wild-type seedlings. Seedlings of each genotype were planted in barrels in triplicates with each barrel containing 25 seedlings. Drought stress was initiated at the four-leaf stage and conducted by water-withholding for 12 days followed by 1-day re-watering.

grew slightly faster than those of wild-type plants. In the presence of 3 μM ABA, growth of roots, especially lateral roots, of wild-type plants was severely inhibited, whereas root growth of siR441- and siR446-knockdown plants was hardly affected. In the medium containing 5 μM ABA, growth of adventitious and lateral roots in wild-type plants was totally inhibited but siR441- and siR446-knockdown plants still showed short adventitious and lateral roots. These results indicated that siR441- and siR446-knockdown seedlings are hyposensitive to ABA in root growth comparing with wild-type rice seedlings. Together, the above observations indicate that siR441 and siR446 should act positively in ABA signaling in rice seed germination and seedling growth. We also assessed the siR441- and siR446-knockdown plants in response to abiotic stresses. The results showed that they are more sensitive than wild-type plants to drought stress. Under 12-day water-withholding condition, most of siR441- and siR446-knockdown plants were withered, but wild-type plants grew continuously. After 1-day re-watering, only a small fraction of siR441- and siR446-knockdown plants survived whereas almost all wild-type plants recovered (Figure 5c). Similarly, the siR441- and siR446-knockdown plants exhibited more sensitive phenotypes than wild-type plants in salt or cold treatment (data not shown). These results indicate that siR441 and siR446 play positive roles in rice tolerance to abiotic stresses. The positive roles of siR441 and siR446 in both ABA signaling and abiotic stresses tolerance are consistent with the function of ABA in up-regulating plant responses to various abiotic stresses (Zhu, 2002).

DISCUSSION

In this study, we demonstrate the biogenesis, expression and function of two siRNAs derived from MITE-evolved stem-loop precursors. We showed that siR441 and siR446 are produced by *OsDCL3* and independent of *OsRDR2*. We also indicated that accumulation of siR441 and siR446 is down-regulated by defective processing of their precursors under ABA and abiotic stress conditions, where the precursors are up-regulated. We studied the biological function of siR441 and siR446 by a transgenic approach and found that they are positive regulators of ABA signaling and abiotic stress responses. Our results also showed that siR441 and siR446 (previously annotated miR441 and miR446) have some characteristics similar to that of miRNAs, for example they are generated from single-stranded hairpin precursors like pri-miRNAs and their processing intermediates, like pre-miRNAs, can be detected by RNA gel blot. It is possible that MITE-derived *SIR441* and *SIR446* are on the way becoming *MIRNAs* because MITEs are thought to be a potential source for evolving new *MIR* genes (Piriyapongsa and Jordan, 2008). Notably, the siR441 and siR446 bands detected by RNA blots could contain other homologous sRNAs because they are derived from the MITE *Stowaway1* family, which has many members in rice, or some other *SIR441a-c* and

SIR446-derived siRNAs whose sequences overlap with siR441 and siR446.

siR441 and siR446 may regulate *MAIF1* expression

siR441 and siR446 are the same size (24-nt) with a highly identical sequence of 21 nts when they are aligned staggered by 3 nts, and share five predicted target sequences that are all located in the 3' UTRs of the target genes (Sunkar *et al.*, 2005) including *MAIF1* (Yan *et al.*, 2010). Sequence alignments between *Stowaway1*, the siR441- and siR446-generating loci and the target locus in the *MAIF1* gene revealed a very high homology especially at regions beyond the 5'- and 3'-end of the siRNA precursor sequences (Figure S3), suggesting that *SIR441a-c* and *SIR446* as well as the target sequence are derived from the same MITE. We found that *SIR441a-* and *SIR446*-transgenic rice plants, in which accumulation of siR441 and siR446 decreased, showed the same phenotypes as *MAIF1*-overexpressed rice plants (Yan *et al.*, 2010), suggesting that siR441 and siR446 possibly regulate *MAIF1* expression. It also should be kept in mind that in addition to siR441 and siR446, *SIR441s* and *SIR446* produce other siRNAs (Wu *et al.*, 2009) that might contribute to the regulation of *MAIF1* expression because the homologous regions between *MAIF1* 3' UTR and these *SIR* genes extend beyond the siR441 and siR446 target regions (Figure S3).

To check if mRNA cleavage is a mechanism for siR441 and siR446 to regulate *MAIF1* expression, we detected *MAIF1* expression patterns and mRNA cleavage sites by 5' RACE analysis. We found that down-regulation of siR441 and siR446 levels under abiotic stress conditions was accompanied by an increase in the level of the *MAIF1* mRNA, as assayed by RNA gel blot analysis using a specific region of *MAIF1* 3' UTR as a probe (Figure S4). Our results are in agreement with previous observations by microarray and RT-PCR analysis that drought, salt and cold treatments increased the abundance of the *MAIF1* mRNA (Jain *et al.*, 2007; Yan *et al.*, 2010). Besides the full-length *MAIF1* mRNA, we also detected some shorter RNAs, which may correspond to the 3' cleaved products of the *MAIF1* mRNA (Figure S4). 5' RACE results revealed that the *MAIF1* mRNA was cleaved indeed around the predicted siR441- and siR446-targeted sites, although not at the typical 10th nucleotide of the siRNAs (data not shown). So, we propose that siR441 and siR446 or other *SIR441-* and *SIR446*-derived siRNAs direct cleavage of the *MAIF1* mRNA.

Intermolecular self-pairing of a processing intermediate might be a mechanism for precursor defective processing

Transcription of three paralogous *SIR441* genes as well as the *SIR446* gene is induced under ABA and abiotic stress conditions, especially by drought treatment. Consistent with this induction, ABA- and abiotic stress-responsive *cis*-elements are present in each of the *SIR* gene promoters (data

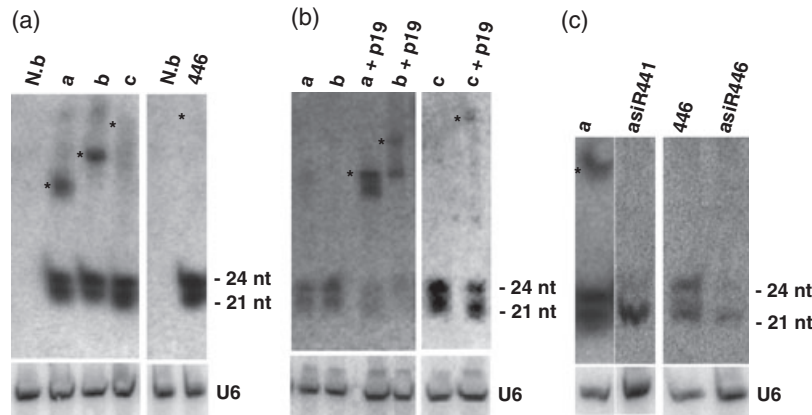


Figure 6. Transient expression of *SIR441a*, *SIR441b*, *SIR441c* and *SIR446* in *N. benthamiana*.

Total RNA samples isolated from *N. benthamiana* leaves agroinfiltrated with the 35S promoter-driven *SIR* gene constructs (a); or co-infiltrated with the 35S-TBSV P19 construct (b); were subjected to RNA gel blot analysis. *a*, *b*, *c*, *446* and *p19* refer to the expression constructs for *SIR441a*, *SIR441b*, *SIR441c*, *SIR446* and P19, respectively. In co-infiltration experiments (b), *Agrobacterium* cells harboring *a*, *b* or *c* were reduced to one-fifth of those used in (a), and mixed with 4-fold of *Agrobacterium* cells harboring *p19*.

(c) 21-nt artificial siR441 (*asiR441*) and siR446 (*asiR446*) were generated from an *Osa-miR528* precursor backbone in agroinfiltrated *N. benthamiana* leaves. The processed products of expression constructs for *SIR441a* (*a*) and *SIR446* (*446*) were included for comparison. For each lane, 15 µg of total leaf RNA was used and the gel blot was probed for siR441: (a) left panel; (b, c) lanes *a* and *asiR441*; or siR446 (a) right panel; (c), lanes *446* and *asiR446*. Total RNA from un-infiltrated leaves (*N. b*) was used as a blank control. Asterisks mark the migration positions of respective processing intermediates. The U6 RNA signals (U6) served as a loading control.

not shown). Interestingly, upregulation of the siRNA precursors by ABA and abiotic stresses accompanies the downregulation of siR441 and siR446. This abnormal inverse correlation between the levels of siRNAs and their precursors implicates an unusual regulation at the siRNA processing steps. We provide evidence to show that increased precursor accumulation causes this defective processing (Figure 4).

The miniature inverted-repeat transposable element (MITE) *Stowaway1* sequence is capable of forming a 77-nt near-perfect inverted repeat (Figure S3). Therefore, the processing intermediates of siR441 and siR446 are near-perfectly self-complementary, as their sequences are highly homologous with that of MITE *Stowaway1* (Figure S3). We suggest that base-pairing between two oppositely orientated molecules of the same processing intermediates of siR441 and siR446 might lead to this precursor-incited defective processing. This suggestion was supported by the results of another set of experiments in which the 35S-*SIR* precursor constructs were transiently expressed in *Nicotiana benthamiana* leaves. We found that all four expression cassettes produced 21-nt and 24-nt sRNAs instead of a single 24-nt sRNA species (Figure 6a). Different degrees of processing were observed among the four precursor molecules: while processing intermediates of siR441a and siR441b were partly processed to 21- and 24-nt sRNAs, processing intermediates of siR441c and siR446 were almost completely converted to the two sRNAs (Figure 6a). Next, we used the tomato bushy stunt virus (TBSV) P19 suppressor as a probe to determine if these 21-nt and 24-nt RNAs are derived from dsRNAs of these processing

intermediates, as TBSV P19 was known to bind and sequester siRNA duplexes and prevent them from incorporating RNA-induced silencing complex (RISC) to direct degradation of target RNAs, which form dsRNAs for producing these siRNAs (Silhavy *et al.*, 2002). Co-expression of TBSV P19 with precursors of siR441a, siR441b or siR441c resulted in a significant reduction in the levels of the 21-nt and 24-nt sRNAs accompanied by a marked increase in accumulation of their processing intermediates (Figure 6b), indicating these two small RNAs are derived from, and *cis*-actingly target, the processing intermediates. This finding in turn suggests that the processing intermediate molecules should form dsRNAs *in vivo* possibly by self-pairing to serve as precursors for siRNAs. Furthermore, we showed that chimeric siR441 and siR446 precursors with a non-self-complementary *Osa-miR528* precursor sequence as a backbone were normally processed to 21-nt artificial siR441 and siR446, respectively, in *Nicotiana benthamiana* (Figure 6c), conversely verifying the self-pairing-based defective processing of the processing intermediates. Based on the above results, we propose that increased accumulation of precursors prevents their regular processing and generation of siR441 and siR446 in response to ABA and abiotic stresses by self-pairing of processing intermediates.

This self-pairing-based defective processing of processing intermediates can explain the regulation of the processing from processing intermediates to siR441 and siR446: under normal conditions, the precursors are at low levels and processed regularly to siRNAs; however, under ABA or abiotic stress conditions, the elevated levels of the precursors cause increasing processing intermediates which

base-pair between themselves, thus preventing them from formation of the hairpin precursor structures, leading to reduced accumulation of siRNAs. It seems that the degree of self-complementarity and the accumulation level of siRNA precursor molecules are two determining factors affecting the defectiveness of processing intermediates. For example, compared with cold, salt or ABA treatments, drought stress had a greater effect on the stimulation of transcription of the *SIR* genes (Figure 3) and the accumulation of processing intermediates (Figure S1), and thus caused a severe reduction in the levels of siR441 and siR446. The same principles could explain the aberrant production of siRNAs when these precursors were over-expressed in Agro-infiltrated *N. benthamiana* leaves. Thus precursors of siR446 and siR441c, which are near-perfectly self-paired, would form stable dsRNAs that in turn were thoroughly processed to siRNAs (Figure 6a). On the other hand, precursors of siR441a and siR441b with imperfect self-complementary sequences would not form perfectly self-paired dsRNAs and thus would be incompletely processed to siRNAs in Agro-infiltrated *N. benthamiana* leaves (Figure 6a). We propose that this self-pairing-based defective processing of precursors might be a mechanism for regulating a subset of plant sRNAs, whose hairpin precursor sequences tend to form dsRNAs by self-pairing before more drift mutations occurred.

EXPERIMENTAL PROCEDURES

Plant materials

Oryza sativa spp. *japonica* cv. Nipponbare was used as the wild-type rice plant and the genetic background for transgenic plants. For analysis of RNA accumulation, 10-day-old rice seedlings were either untreated or treated with ABA or abiotic stresses: for ABA treatment, seedlings were transferred to a medium containing 20 μM ABA; for cold stress, seedlings were transferred to a growth chamber of 4°C; drought treatment was performed by withholding water supply; seedlings were transferred to a medium with 200 mM NaCl for salt stress. Samples were harvested at different time points up to 24 h after stress treatments.

Constructs and generation of transgenic rice plants

To generate siR441- and siR446-ectopic expression vectors, approximately 1 kb fragments of *SIR441a-c* and *SIR446* surrounding the siR441 or siR446 sequence that include the fold-back precursor structures were amplified from the rice genomic DNA. To generate artificial siR441- and siR446-expression vectors, 24-nt siR441 or siR446 sequence was inserted into the OsmiR528 precursor backbone to replace the native miR528 sequence by PCR amplification. These fragments were separately cloned into pCAMBIA1300 downstream of the CaMV 35S promoter. For the *SIR441a-c* and *SIR446* promoter-*uidA* fusion constructs, a approximately 2.0-kb fragment upstream from each of the predicted fold-back precursor sequences was amplified and cloned into pCAMBIA1300 in front of the coding sequence of *uidA*. All the constructs were electroporated into *Agrobacterium tumefaciens* EHA105 to transform rice using the method described by Hiei *et al.* (1994). The primers used for PCR are listed in Table S1.

RNA gel blot analysis

Total RNA was extracted from rice samples with Trizol reagent (Invitrogen, <http://www.invitrogen.com>). For analysis of the *MAIF1* transcripts, total RNA was separated on 1.2% formaldehyde-MOPS agarose gels and blotted onto Hybond-N⁺ membranes (Amersham Biosciences, <http://www.gelifsciences.com>). Ribosomal RNAs, visualized by methylene blue staining, were used to monitor equal RNA loading. Hybridization was performed at 65°C in PerfectHyb Plus buffer (Sigma-Aldrich, <http://www.sigmaaldrich.com>) with a ³²P-labeled probe corresponding to the *MAIF1* 3' UTR sequence downstream of the siR441 and siR446 target sites and non-homologous to the *Stowaway1* sequence. The probe was made by PCR amplification with primers (5'-TTTGCTTGTGTTTCGTGGTATC-3' and 5'-CCCGAGCACTAAGCACATC-3'). For analysis of small RNAs, total RNA or small RNA-enriched samples were separated on a denaturing 17% polyacrylamide gel and transferred electrophoretically to Hybond-N⁺ membranes. Hybridizations for different kinds of small RNAs on the same membrane were performed at 38°C in PerfectHyb Plus buffer successively with 5' end-labeled DNA oligonucleotide probes specific for each kind of small RNA, after stripping off the former probes. Hybridization signals were detected by autoradiography or phosphorimager (GE Healthcare Life Sciences, <http://www.gelifsciences.com>). The sequences of the probes are described in Table S1.

RT-PCR analysis

Total RNA (3 μg) treated with RNase-free DNase I was subjected to reverse transcription using SuperScript[®] III Reverse Transcriptase (Invitrogen) following the supplier's protocol. PCR amplifications were performed for primary transcripts of siR441 and siR446 loci using locus-specific primers flanking the fold-back precursor sequences and for the *uidA* mRNA with *GUS*-specific primers. The amount of input RNA was normalized for each reaction using *actin*-specific primers. The primers used for PCR are listed in Table S1.

In vitro processing of siR446 precursor

In vitro processing of siR446 precursor was performed essentially following the procedures of the *in vitro* rice Dicer activity assay (Liu *et al.*, 2007). Briefly, the rice total protein extract as a source of sRNA-processing proteins was prepared from 30-day-old rice calli and incubated with 86-nt RNA sequence of the predicted siR446 processing intermediate (Figure S3). Synthesis of the siR446 precursor RNA was accomplished by *in vitro* transcription of a T7 promoter-siR446 precursor DNA fusion sequence, which was made by PCR amplification on the rice genomic DNA template using the sense primer (5'-TAATACGACTCACTATAGATTTCCACATTTA-3', the T7 promoter sequence is underlined) and the antisense primer (5'-CCATTTCCATATTCATATTGATGTTAATA-3'). RNA was extracted from the *in vitro* processing reaction mixture and subjected to RNA gel blot analysis as described in Liu *et al.* (2007).

Transient expression in *Nicotiana benthamiana*

For transient expression assay, *Agrobacterium* transformants harboring the designated constructs were grown overnight in the presence of 50 $\mu\text{g ml}^{-1}$ kanamycin, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 20 μM acetosyringone and harvested by centrifugation. Agrobacterial cells were resuspended in 10 mM MgCl₂, 10 mM MES (pH 5.6), and 100 μM acetosyringone to an OD₆₀₀ of 1.0. After incubation at room temperature for 3 h, the agrobacterial cell suspension was pressure-infiltrated into *N. benthamiana* leaves as described in Liu *et al.* (2003). The leaves

were harvested 4 days after infiltration and subjected to total RNA extraction and gel blot analysis as described above.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Accumulation levels of the processing intermediates of siR441 and siR446 precursors are up-regulated by drought and salt treatment.

Figure S2. Accumulation level of siR441 in *SIR441a*- and *SIR441b*-transgenic rice plants under abscisic acid and abiotic stress conditions.

Figure S3. *SIR441* loci, *SIR446* and their target sequence are derived from the MITE *Stowaway1*.

Figure S4. Accumulation of *MAIF1* mRNA is increased under abiotic stress conditions.

Table S1. Primers and probes used in this study.

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