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)- 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 sRNA activity.

Two sRNAs (UACCAUAUAUAAAUGUGGAAG and CAUCAUAUGAAUUGGGAAUGG) 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 (Piriyaoponsa 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 Osdcl1 and Osdcl4 plants but was greatly reduced in the Osdcl3a 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.
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 transcriptional 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 3b), in *SIR441a*, *SIR441b*, *SIR441c* and *SIR446-promoter-uidA* transgenic rice plants, *uidA* mRNA accumulation was markedly increased by drought and salt treatments.

**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 a role for sRNAs in mediating rice responses to stress conditions. To gain an insight into regulatory functions of siR441 and siR446, 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).

**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 (DCL1R-1, DCL1R-2 and dcl1), OsDCL3a (dcl3a-17 and dcl3a-21), OsDCL4 (dc4) 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.

**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.
and the levels of their precursors, we took the approach of inverse correlation between the levels of siR441 and siR446 to gain insight into the mechanism controlling the abnormal accumulation. Increased expression of precursors reduces siRNAs. To address this factor, we used previously described siRNAs in ABA signaling and abiotic stress responses in rice. 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–c 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 μM 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 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–c 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 μM 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 analyzed. 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 OsDCCL3 and independent of OsDRDR2. 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 Stotaway1 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 Stotaway1, 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...
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 oriented 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 siRNA 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 $S/R$ 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 siRNAs, 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 had a greater effect on the stimulation of transcription of the $S/R$ 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 siRNAs, whose hairpin precursor sequences tend to form dsRNAs by self-pairing before more drift mutations occurred.

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**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 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.gelifesciences.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 Stowaway-1 sequence. The probe was made by PCR amplification with primers (5′-TTTCCCTGGTTTCTGGTATC-3′ and 5′-GCCGAGCACTAACTACATC-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 probe. Hybridization signals were detected by autoradiography or phosphorimager (GE Healthcare Life Sciences, http://www.gelifesciences.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′-TAATCGACTCACTATAGATTTTTTCCCATATT-3′, the T7 promoter sequence is underlined) and the antisense primer (5′-CAATTCGCACTATGGATGATAATA-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 μg ml−1 kanamycin, 10 mm 2-(N-morpholino)ethanesulfonic acid (MES) and 20 μM acetosyringone and harvested by centrifugation. Agrobiological 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 agrobiological 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.

REFERENCES


