Title

Dicer-like 3 produces transposable element-associated 24-nt siRNAs that control agricultural traits in rice.

Journal

Proceedings of the National Academy of Sciences of the United States of America, 111(10)

Authors

Wei, Liya

Gu, Lianfeng

Song, Xianwei

et al.

Publication Date

2014-03-11

DOI

10.1073/pnas.1318131111

Peer reviewed

Dicer-like 3 produces transposable element-associated 24-nt siRNAs that control agricultural traits in rice

Liya Wei[a,b,1], Lianfeng Gu[a,1], Xianwei Song[a,1], Xiekui Cui[a,b], Zhike Lu[a], Ming Zhou[a], Lulu Wang[a], Fengyi Hu[c], Jixian Zhai[d,e], Blake C. Meyers[d,e], and Xiaofeng Cao[a,2]

[a]State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; [b]College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China; [c]Food Crops Research Institute, Yunnan Academy of Agricultural Sciences, Kunming 650205, China; [d]Department of Plant and Soil Sciences, University of Delaware, Newark, DE 19711; and [e]Delaware Biotechnology Institute, Newark, DE 19711

Edited by James A. Birchler, University of Missouri, Columbia, MO, and approved January 21, 2014 (received for review September 28, 2013)

Transposable elements (TEs) and repetitive sequences make up over 35% of the rice (*Oryza sativa*) genome. The host regulates the activity of different TEs by different epigenetic mechanisms, including DNA methylation, histone H3K9 methylation, and histone H3K4 demethylation. TEs can also affect the expression of host genes. For example, miniature inverted repeat TEs (MITEs), dispersed high copy-number DNA TEs, can influence the expression of nearby genes. In plants, 24-nt small interfering RNAs (siRNAs) are mainly derived from repeats and TEs. However, the extent to which TEs, particularly MITEs associated with 24-nt siRNAs, affect gene expression remains elusive. Here, we show that the rice Dicer-like 3 homolog OsDCL3a is primarily responsible for 24-nt siRNA processing. Impairing *OsDCL3a* expression by RNA interference caused phenotypes affecting important agricultural traits; these phenotypes include dwarfism, larger flag leaf angle, and fewer secondary branches. We used small RNA deep sequencing to identify 535,054 24-nt siRNA clusters. Of these clusters, ∼82% were OsDCL3a-dependent and showed significant enrichment of MITEs. Reduction of *OsDCL3a* function reduced the 24-nt siRNAs predominantly from MITEs and elevated expression of nearby genes. OsDCL3a directly targets genes involved in gibberellin and brassinosteroid homeostasis; *OsDCL3a* deficiency may affect these genes, thus causing the phenotypes of dwarfism and enlarged flag leaf angle. Our work identifies OsDCL3a-dependent 24-nt siRNAs derived from MITEs as broadly functioning regulators for fine-tuning gene expression, which may reflect a conserved epigenetic mechanism in higher plants with genomes rich in dispersed repeats or TEs.

transposon | plant architecture | plant hormone

Transposable elements (TEs) and repetitive sequences make up more than 35% of the rice genome (1). TEs include DNA transposons, which mobilize by a "cut-and-paste" mechanism, and retrotransposons, which mobilize by a "copy-and-paste" mechanism (2, 3). Miniature inverted repeat TEs (MITEs), widespread, short (less than 600 bp), nonautonomous DNA transposons, occur in many plant and animal genomes, including *Arabidopsis*, rice, sorghum, maize, *Caenorhabditis elegans*, and humans (2, 4). These elements contain short terminal inverted repeats and target-site preference (TA or TAA) for target site duplication (2). MITEs are the highest copy-number TEs in rice and are mainly dispersed in the chromosomal arms (i.e., in gene-rich euchromatic regions), especially in the vicinity of genes (5). *Miniature Ping* (*mPing*) was the first discovered active MITE from any organism and the first active DNA transposons from rice (6–8). The copy number of *mPing* is highly polymorphic among *japonica* and *indica* (the two rice subspecies), temperate and tropical *japonica* (two subgroups of *japonica*), as well as domesticated rice (*Oryza sativa*) and ancestral species (*Oryza rufipogon*) (6, 7). In some rice lines, *mPing* elements underwent a massive amplification, with ∼40 new insertions per generation (9). These new *mPing* elements preferentially inserted into the 5′ flanking region of genes and avoided exons (10). These *mPing* elements can either regulate or have no detectable impact on the expression of nearby genes (10). It is proposed that the rapid and recent amplification of a subset of *mPing* confers nearby gene induction upon different stress conditions, implying that new stress-inducible alleles may generate in cultivated populations (10). Rice TEs also include about 14% long terminal repeat (LTR) retrotransposons and 1% non-LTR retrotransposons, which can be further classified as long interspersed elements (LINEs) and short interspersed elements (SINEs) (1). Several LTR retrotransposons, including *Tos10*, *Tos17*, and *Tos19* (11–13), and a non-LTR retrotransposon LINE *Karma*, have been shown to be autonomous and active in rice (14, 15).

The mobilization of TEs provides a major driving force for gene and genome evolution (16). However, TE mobilization can also disrupt genome stability and most organisms have evolved diverse epigenetic mechanisms to repress TE activity. For example, epigenetic mechanisms, including DNA methylation and dynamic histone methylation, control transposon silencing (17, 18). In addition, distinct epigenetic mechanisms regulate different TEs (19).

Significance

The functional relationship of transposons and small RNAs remains an important question in the study of gene expression and its effect on agronomic traits. Here, we use deep sequencing of small RNAs to provide the first evidence that the rice Dicer-like 3 homolog OsDCL3a produces 24-nt small interfering RNAs (siRNAs) predominantly associated with miniature inverted repeat transposable elements (MITEs). These 24-nt siRNAs target genes adjacent to MITEs and act as broadly functioning regulators of gene expression. In particular, OsDCL3a directly targets genes involved in homeostasis of the plant hormones gibberellin and brassinosteroid, thus controlling important agricultural traits. This mechanism of fine-tuning gene expression mediated by MITEs may be conserved in organisms with genomes rich in dispersed repeats or transposable elements.

Author contributions: L. Wei, L.G., and X. Cao designed research; L. Wei, X.S., X. Cui, M.Z., and L. Wang performed research; F.H. and B.C.M. contributed new reagents/analytic tools; L. Wei, L.G., X.S., Z.L., J.Z., B.C.M., and X. Cao analyzed data; and L. Wei, L.G., X.S., and X. Cao wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE50778).

[1]L. Wei, L.G., and X.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: xfcao@genetics.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318131111/-/DCSupplemental.

PLANT BIOLOGY

Plants produce 24-nt small interfering RNAs (siRNAs) from repeats and TEs. These 24-nt siRNAs trigger DNA methylation at all CG, CHG, and CHH (where H = A, T, or C) sites, resulting in H3K9me2 modification. This modification reinforces transcriptional silencing of TEs and genes that harbor or are adjacent to repeats or TEs in *Arabidopsis* (20–25). In *Arabidopsis*, the plant-specific RNA polymerase IV (Pol IV) transcribes heterochromatic regions. RNA-dependent RNA polymerase 2 (RDR2) then synthesizes double-stranded RNA intermediates as precursors for RNase III-class Dicer-like 3 (DCL3) to process into 24-nt siRNAs (26). The siRNAs load into ARGONAUTE 4 (AGO4) and promote heterochromatin formation by DNA and histone methylation at the source loci (26). In *Arabidopsis*, repeats and TEs primarily occur around the centromeres or knob regions; in contrast, the rice genome consists of over 40% heterochromatin, which occurs in discontinuous and less distinct patterns (27, 28). Therefore, in rice more protein coding genes are exposed to repetitive sequences than in *Arabidopsis* (29). Rice contains two homologs of *Arabidopsis* DCL3, OsDCL3a, and OsDCL3b. OsDCL3b produces stamen-specific 24-nt phased small RNAs (30) and OsDCL3a produces 24-nt centromere-associated OsCentO siRNAs, MITE-derived siRNAs for abiotic stress responses, and noncanonical long miRNAs (lmiRNAs) (30–32). These lmiRNAs load into rice AGO4 homologs, and can direct DNA methylation at their target genes for transcriptional gene silencing (31). OsDCL3a activity is required for the biogenesis of miR820, which locates within CACTA DNA transposon and targets *OsDRM2*, the de novo DNA methyltransferase homolog in rice (23, 31, 33, 34). Therefore, OsDCL3a-dependent miR820 produced from a transposon suppresses the host's silencing machinery (33).

High copy-number MITEs are dispersed throughout the rice genome. How the mechanisms regulating MITEs, such as 24-nt siRNAs associated with MITEs, affect gene expression, and thereby contribute to agricultural or developmental traits, remains unclear. Here, we show that *OsDCL3a* is primarily responsible for 24-nt siRNA processing in rice. Reduction of *OsDCL3a* expression levels by RNAi causes a genome-wide reduction of siRNAs and derepresses the heterochromatin status of MITEs, resulting in increased expression of nearby genes. OsDCL3a-dependent 24-nt siRNAs directly target gibberellin (GA) and brassinosteroid (BR) homeostasis-related genes. Our findings thus reveal important roles for OsDCL3a in 24-nt siRNA biogenesis and maintenance of heterochromatin status of siRNA-associated MITEs; suppression of these MITEs influences expression of nearby genes and affects important agricultural traits in rice.

Results

Knockdown of *OsDCL3a* Affects Important Agricultural Traits in Rice. We previously used RNAi to knock down *OsDCL3a* expression, and generated two independent *OsDCL3a* RNAi lines, *3a-3* and *3a-1* (30). These two lines, *3a-3* and *3a-1*, affect phenotypes with a severity correlated with the knockdown level of *OsDCL3a*. Compared with WT (*Nipponbare*), *3a-3* and *3a-1* plants showed significantly reduced plant height at heading stage (Fig. 1 *A* and *B*) and increased bending angle of the lamina joint (Fig. 1*C*). The angle of the flag leaf increased four- to sixfold in *3a-3* and *3a-1* compared with WT (Fig. 1*D*). Furthermore, *3a-3* and *3a-1* plants had smaller panicles than WT, which manifested as reduced primary and secondary panicle branches (Fig. 1 *E–G*). These phenotypes of *3a-3* and *3a-1* plants resemble the reported phenotypes of *ago4ab-1* and *rdr2-2* RNAi lines (Fig. S1) (31).

OsDCL3a-Dependent siRNAs Are Enriched in MITEs. OsDCL3a produces 24-nt unphased, centromere-associated, MITE-derived siRNAs and lmiRNAs (30–32). To provide a genome-wide view of OsDCL3a-dependent 24-nt small RNAs (sRNAs), we

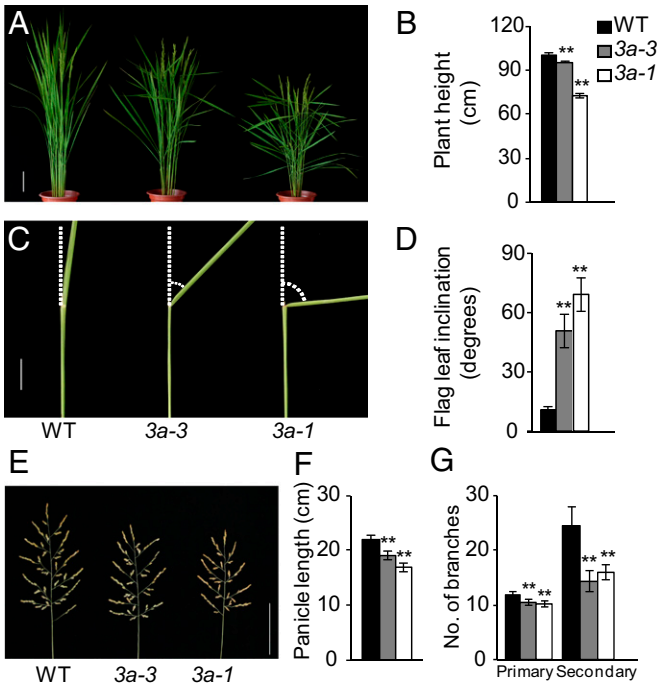

Fig. 1. *OsDCL3a* knockdown plants display pleiotropic phenotypes affecting important agricultural traits. (*A* and *B*) *OsDCL3a* RNAi lines (*3a-3* and *3a-1*) show dwarf phenotypes (*A*) and statistical analysis of plant height (n = 30) (*B*). (*C* and *D*) Flag leaf inclination of WT, *3a-3*, and *3a-1* (*C*) and statistical analysis of leaf angles (n = 30) (*D*). (*E–G*) Panicle morphology of WT, *3a-3*, and *3a-1* (*E*) and statistical analysis of length (*F*) and branches of main panicle (*G*) (n = 30). $^{**}P < 0.01$ with t test. Error bars correspond to the SD of biological repeats. (Scale bars: 10 cm in *A* and *E*; 2 cm in *C*.)

compared small RNA accumulation in WT, *3a-3*, and *3a-1* from the lamina joints of the flag leaf (Fig. S2*A*). We found that in the *3a-3* and *3a-1* lines, ~82% (438,627 of 535,054) of total 24-nt sRNA clusters were reduced by more than threefold, compared with WT (Fig. S2). Further analysis revealed that 76% (333,692 of 438,627) of the OsDCL3a-dependent 24-nt sRNA clusters derive from repeats (Fig. 2*A*). In comparison with the annotation from plant repeat databases (http://plantrepeats.plantbiology.msu.edu/about.html), we identified 304,353 repeat- based OsDCL3a-dependent 24-nt sRNA clusters. These clusters include: MITE DNA transposons (33%, 101,053), other DNA TEs (20%, 59,996), retrotransposons (40%, 122,746, including 18% LTR, 1% non-LTR, and 21% unclassified retrotransposons), and other repeats (7%, 20,558) (Fig. S3*A*). Thus, the OsDCL3a-dependent siRNAs are significantly enriched in MITEs compared with retrotransposons (P = 0.006964, Fisher exact test), and other DNA TEs (P = 0.04709, Fisher exact test).

Effects of OsDCL3a-Dependent siRNAs on Gene Expression. To test the effects of OsDCL3a-dependent siRNAs on the expression of their target genes and TEs, we performed RNA-seq on WT, *3a-3*, and *3a-1*. The correlation of RNA-seq data from *3a-3* and *3a-1* was ~0.95, indicating the reproducibility of two independent samples of RNA libraries (Fig. S3*B*). Among the 859 differentially expressed genes altered by 1.5-fold or more, 679 were up-regulated and 180 were down-regulated in both *3a-3* and *3a-1* plants (Fig. 2*B*). Furthermore, we mapped 24-nt siRNAs and repetitive sequences to gene bodies and found that 24-nt siRNAs are enriched in 2-kb regions upstream of transcriptional start sites (TSS) and downstream of transcriptional terminal sites (TTS), regardless of gene-expression levels (Fig. 2 *C* and *D* and Fig. S3 *C–F*). The abundances of 24-nt siRNAs in the 5′

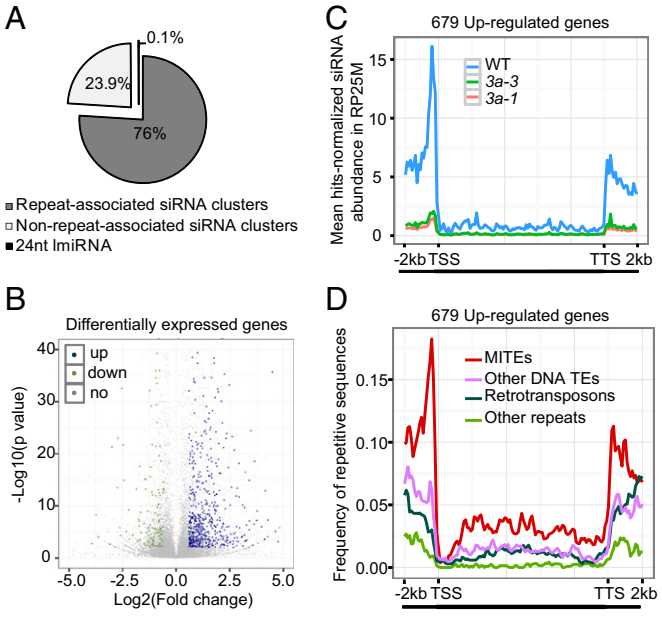

Fig. 2. Distribution of OsDCL3a-dependent 24-nt siRNAs and different classes of repetitive sequences relative to up-regulated genes. (*A*) Pie chart showing the distribution of OsDCL3a-dependent 24-nt sRNAs loci based on Rice Genome Annotation Project Release 7 and miRBase release 20 annotations. (*B*) Differentially expressed genes in *3a-3* and *3a-1*. Blue dots indicate up-regulated genes and green dots indicate down-regulated genes. (*C*) Abundance of 24-nt sRNAs in 2-kb regions upstream and downstream of up-regulated genes, with each gene annotated from the TSS to the TTS in WT (blue), *3a-3* (green), and *3a-1* (red). (*D*) The frequencies of four classes of repetitive sequences, MITEs (dark red), other DNA TEs (pink), retrotransposons (dark green), and other repeats (light green), were plotted in ±2 kb and gene body from TSS to TTS.

and 3′ regions of all genes were significantly lower in *3a-3* and *3a-1* (Fig. 2*C* and Fig. S3 *C* and *E*). The distribution of MITEs, but not other DNA TEs, retrotransposons, or repeats, was highly correlated with OsDCL3a-dependent 24-nt siRNAs (Fig. 2 *C* and *D* and Fig. S3 *C*–*F*). We also noticed that the up-regulated genes are nearly four times more numerous than the down-regulated genes. These data suggest that impairing *OsDCL3a* causes widespread reduction of 24-nt siRNAs from MITEs and other TEs, which mainly result in the up-regulation of nearby gene expression.

OsDCL3a Targets Genome-Wide Gene Expression. Because 24-nt siRNAs usually cause gene silencing, we further examined the functions of these putative OsDCL3a targets by Gene Ontology analysis of the genes up-regulated in the *3a-3* and *3a-1* lines. These genes were enriched in Gene Ontology groups for metabolic processes, including cell wall organization, cell wall macromolecule catabolic process, defense response, GA catabolic process, and BR homeostasis, among others (Fig. S4). Intriguingly, genes for GA catabolic process and BR homeostasis may be associated with the phenotypes of the *OsDCL3a* RNAi lines, such as dwarfism and enlarged leaf angle, which resemble the phenotypes of rice plants with reduced GA and excess BR (35–38).

GA Homeostasis-Related Genes as Direct Targets of 24-nt siRNAs. GAs, diterpenoid plant hormones, control diverse plant developmental processes, including stem elongation, leaf expansion, seed germination, and flowering (39). In rice, two gene clusters encode the enzymes at the branch points between biosynthesis of GA and other labdane-related diterpenoids (Fig. S5); these gene clusters include *CYP76M7* (*cytochrome P450 76 monooxygenase 7*) and *OsKSL7* (*kaurene synthase-like 7*) in one cluster, and *OsCPS4* (*syn-CPP synthase 4*) and *CYP99A3* (*cytochrome P450 A3*) in the other cluster (40). In *3a-3* and *3a-1* lines, the transcript levels of *CYP76M7*, *OsKSL7*, and *CYP99A3* increased with decreasing accumulation of 24-nt siRNAs from MITEs in the 5′ or intron regions of the corresponding genes (Fig. S6). Moreover, *3a-3* and *3a-1* plants produced En/Spm-like TE-derived 24-nt siRNAs in the 5′ region of *OsCPS4*; also, *OsCPS4* mRNA levels increased threefold (Fig. S6*B*). Using bisulfite sequencing and ChIP followed by quantitative PCR (qPCR) assays, we found that DNA methylation of TE regions was not significantly changed, whereas H3K9me2 levels largely decreased (Fig. S6). These results indicate that an *OsDCL3a* deficiency and loss of 24-nt siRNAs causes the up-regulation of genes critical for diterpenoid biosynthesis, which may influence GA biogenesis and therefore reduce plant height.

In addition, *Elongated Uppermost Internode* (*EUI*), which encodes a GA deactivating enzyme, also harbors *OsDCL3a*-dependent 24-nt siRNAs from three MITEs in its introns (Fig. 3 *A*–*C*). *EUI* was up-regulated in *3a-3* and *3a-1* lines along with a decrease in 24-nt siRNAs (Fig. 3 *B* and *D*). Furthermore, we also found that DNA methylation was not significantly altered, whereas H3K9me2 levels decreased in *3a-3* and *3a-1* compared with WT (Fig. 3 *E* and *F*). Overexpression of *EUI* causes severe dwarfism in rice (35, 41) (Fig. S5); thus, the reduction of 24-nt siRNAs from MITEs may elevate *EUI* expression and contribute to the reduced height of *OsDCL3a*-deficient plants. Therefore, MITE-associated 24-nt siRNAs epigenetically regulate GA anabolism and catabolism-related genes, which may affect rice plant height. To prove this hypothesis, we applied exogenous GA_3 and found that the dwarf phenotype of knockdown *OsDCL3a* plants can be rescued, further confirm that OsDCL3a-dependent 24-nt siRNAs regulate GA homeostasis-related gene expression and plant height (Fig. S7 *A* and *B*).

BR Biogenesis-Related Genes as Direct Targets of 24-nt siRNAs. We also found several BR biogenesis pathway genes, including *OsGSR1* (*GAST family gene in rice 1*) and *OsBR6ox* (a rice BR-6-oxidase gene), to be likely direct targets of OsDCL3a. BR and GA pathways function together to regulate many biological processes (39, 42). For example, *OsGSR1* promotes BR synthesis and represses GA20-ox-2, a major enzyme for GA biogenesis in rice (37) (Fig. S5). OsBR6ox catalyzes the C-6 oxidation step in BR biosynthesis (43, 44) (Fig. S5). BR is positively correlated with leaf angle in rice (38, 45). For example, *OsGSR1* RNAi plants show an erect-leaf phenotype similar to plants deficient in BR (37). In *3a-3* and *3a-1* lines, we found fewer 24-nt siRNAs from MITEs, unchanged DNA methylation and reduced H3K9me2 levels in the 5′ region of *OsGSR1* and *OsBR6ox*, and elevated mRNA levels (Fig. S8). Consistent with the enlarged leaf angle, we propose that BR biogenesis-related genes are activated in *3a-3* and *3a-1* lines.

In addition, we also found that two major components of the RNA-directed DNA methylation (RdDM) pathway, namely AGO4a and AGO4b (AGO4ab) and RDR2, also affected GA and BR homeostasis-related genes. In the *ago4ab-1* and *rdr2-2* RNAi background (Fig. 3*D* and Figs. S6 and S8), all GA and BR homeostasis-related genes are up-regulated. This result could be caused by effects on 24-nt siRNA associated MITEs or other TEs, and affect plant height and leaf angle, two important agricultural traits in rice. OsDRM2 is a major component in RdDM and suppressed by OsDCL3a-dependent miR820 at transcriptional and posttranscriptional level (31, 33). We found that, consistent with previous reports that OsDCL3a processes miR820 biogenesis (Fig. S9*A*), *OsDRM2* accumulated approximately twofold higher levels in *3a-3* and *3a-1* lines (Fig. S9*B*), which may increase de novo DNA methylation and counteract the effect of loss of OsDCL3a activity.

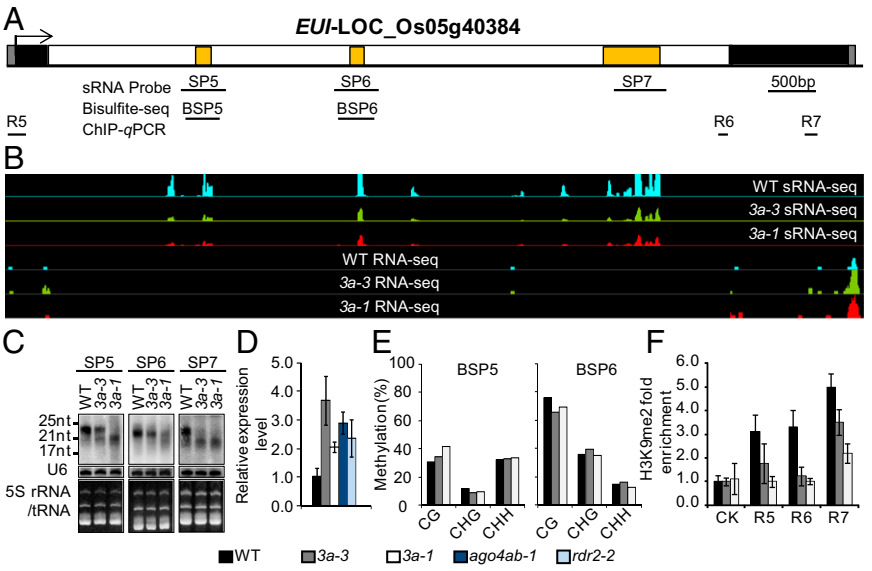

Fig. 3. *EUI* encodes a GA deactivating enzyme and is activated in *OsDCL3a* RNAi lines. (*A*) Schematic representation of *EUI*. The arrowhead indicates the transcription start site. Boxes indicate exons (black), introns (white), UTR regions (gray), and MITEs (yellow). sRNA probes (SP5–SP7), bisulfite sequencing regions (BSP5, BSP6), and the regions used for ChIP-qPCR (R5–R7) are indicated by black lines. (*B*) sRNA-seq and RNA-seq data for *EUI* are shown in WT, *3a-3*, and *3a-1*. (*C* and *D*) Small RNA blot and qPCR validate the sRNA-seq and RNA-seq data, respectively. U6 probe and 5S rRNA/tRNA stained with ethidium bromide were used as small RNA blot loading controls. *eEF1α* was used as an internal reference for qPCR. The level of *EUI* transcription also was detected in *ago4ab-1* and *rdr2-2* lines. Error bars correspond to the SD. (*E*) Bisulfite sequencing analysis of the DNA methylation level of two MITE regions. (*F*) ChIP-qPCR assay detects the chromatin states using anti-H3K9me2. Anti-H3 was used as an internal reference for ChIP-qPCR. Error bars correspond to the SD.

The 24-nt siRNAs Epigenetically Regulate *Os08g19420* and Alter Leaf Inclination. Next, we tested whether 24-nt siRNAs from repeats elevated the expression of nearby genes and affect rice development. We examined *Os08g19420*, whose promoter region contains a Ditto-like MITE and seven tandem repeats, as well as a 3′ end including LTR, En/Spm-like TEs, and a SINE element (Fig. 4*A*). In *3a-3* and *3a-1* lines, the 24-nt siRNAs at above TEs and repeats were strongly reduced and the expression level of *Os08g19420* was higher than WT (Fig. 4 *B–D*). Similar results were also obtained in *ago4ab-1* and *rdr2-2*, indicating that the RdDM pathway is important for protein coding gene expression in rice (Fig. 4*D*). To evaluate whether these 24-nt siRNAs could mediate DNA methylation and H3K9me2 to affect expression of *Os08g19420*, we performed bisulfite sequencing and found a reduction of CHH methylation within the tandem repeats in *3a-3* and *3a-1* lines (Fig. 4*E*). Moreover, we found that the H3K9me2 levels of seven regions of the promoter, gene body, and 3′ end decreased in *3a-3* and *3a-1* (Fig. 4*F*). The downstream gene *Os08g19440* has no transcriptional signals from RNA-seq data (Fig. 4*B*). Thus, a decrease in 24-nt siRNAs originating from 5′ and 3′ ends of *Os08g19420* occurs with a decrease in repressive chromatin markers and an increase in expression of nearby genes.

Additionally, to test whether overexpression of *Os08g19420* could account for the developmental phenotypes in *3a-3* and *3a-1* lines, we transformed the intact *Os08g19420* ORF driven by the *OsActint1* promoter into WT (*Nipponbare*) plants. Four independent transgenic lines with significantly increased *Os08g19420* expression levels showed exaggerated flag leaf angle at heading stage, similar to *OsDCL3a* RNAi lines (Fig. S10 *A–C*). These results demonstrate that 24-nt siRNAs repress *Os08g19420* to control flag leaf inclination in rice.

To dissect the interplay of 24-nt siRNA-mediated regulation and MITE evolution, and to examine how MITE variants differentially affect nearby gene expression, we investigated a MITE in the *Os08g19420* promoter region, examining its effect on gene expression among four rice *japonica* accessions. We noticed that *japonica* accessions from two subgroups, including two *temperate japonica* (TEJ), and two *tropical japonica* (TRJ) accessions, had a MITE-associated polymorphism in the *Os08g19420* promoter regions (Fig. S10*D*). Further analysis showed that MITE partial deletion might cause *Os08g19420* up-regulation in TRJ, but not in TEJ accessions, although we cannot rule out the possibility that the up-regulation of *Os08g19420* was due to different genetic backgrounds (Fig. S10*E*).

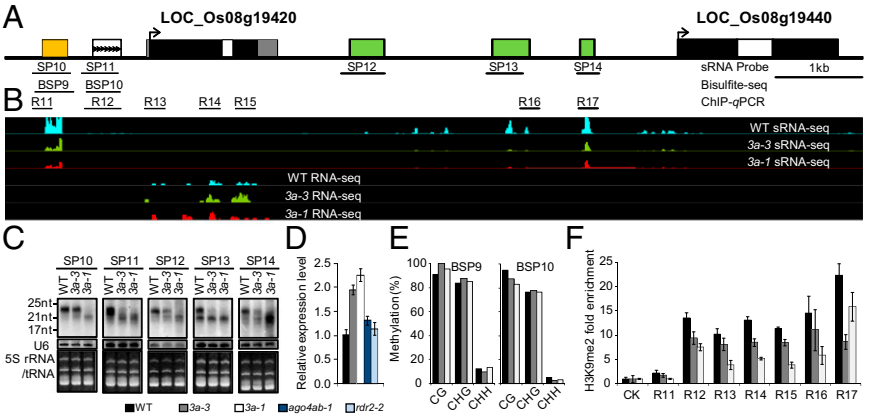

Fig. 4. 24-nt siRNAs from MITEs and tandem repeats regulate expression of a nearby gene, *Os08g19420*. (*A*) Schematic of *Os08g19420* with putative MITE Ditto-like and tandem repeats indicated by yellow boxes and arrows within the white box, respectively. Green boxes indicate LTR, En/Spm-like TEs, and a SINE element from left to right. Boxes indicate exons (black), introns (white), and UTR regions (gray). Black lines show the positions of siRNA probes (SP10–SP14), bisulfite sequencing regions (BSP9, BSP10), and five ChIP-qPCR analysis regions (R11–R17). (*B*) Small RNA-seq and RNA-seq data are shown in the region of *Os08g19420* from WT (blue), *3a-3* (green), and *3a-1* (red). (*C*) Detection of 24-nt siRNAs by small RNA blot in WT, *3a-3*, and *3a-1*. (*D*) qPCR validation of *Os08g19420* expression in WT, *3a-3*, *3a-1*, *ago4ab-1*, and *rdr2-2* plants were normalized using the signal from *eEF1α* gene. The average ± SD values from three biological repeats are shown. (*E*) Bisulfite sequencing analysis of the DNA methylation level of MITE and tandem repeats. (*F*) In WT, *3a-3*, and *3a-1*, the chromatin states are detected by anti-H3K9me2 ChIP-qPCR assays at seven different regions. Anti-H3 was used as internal reference. Error bars correspond to the SD.

Discussion

Our data collectively illustrate the widespread effect of OsDCL3a-dependent 24-nt siRNAs predominantly associated with MITEs and other TEs on the expression of nearby genes. This effect also controls important agriculture traits in rice. Compared with WT, knockdown *OsDCL3a* plants demonstrate a reduction in 24-nt siRNAs from TEs (5′ end, intron, and 3′ end), to a level that triggers genome-wide transcriptional up-regulation. This process results in derepression of GA and BR homeostasis-related genes, potentially accounting for the alterations of important agricultural traits in the RNAi lines (Fig. 5).

In this work, we found that knockdown *OsDCL3a* caused a significant reduction in 24-nt siRNAs and the levels of repressive chromatin marks, such as histone H3K9me2, leading to the activation of expression of nearby genes (Figs. 3 and 4 and Figs. S6 and S8). In addition, knockdown of *AGO4ab* and *RDR2*, two major components of the RdDM pathway in rice, produced a similar phenotype to the *OsDCL3a* knockdown, including derepression of 24-nt siRNA targets (Figs. 3*D* and 4*D* and Figs. S1, S6, and S8). In *Arabidopsis*, 24-nt siRNAs can direct DNA methylation and lead to histone H3K9 dimethylation to maintain TE silencing (46). Therefore, epigenetic regulation mediated by the small RNA pathway is conserved between rice and *Arabidopsis*. In addition, 24-nt siRNAs directly target many genes, including genes involved in plant hormone homeostasis, indicating that this regulatory mechanism plays broad roles in rice development.

We also noticed that the reduction of CHH methylation was only observed in the tandem repeats of the *Os08g19420* promoter in *3a-3* and *3a-1* (Fig. 4*E*). In other MITEs, impaired production of 24-nt siRNAs did not reduce DNA methylation at CG, CHG, and CHH sites (Figs. 3*E* and 4*E* and Figs. S6 and S8). This result could be because of the reduced level of OsDCL3a-dependent miR820, which targets the major de novo methyltransferase, OsDRM2 (31, 34). Indeed, we observed a reduction of miR820 levels and an increase of *OsDRM2* levels in *3a-3* and *3a-1* lines, which may promote de novo DNA methylation and counteract the effect of impaired OsDCL3a activity (Fig. S9). If increased, OsDRM2 activities override the effect of OsDCL3a, which will lead to decreased or unchanged nearby gene expression (Fig. S3 *C–F*). Alternatively, in addition to the RdDM pathway, CHH methylation at long TEs can be mediated in parallel by the nucleosome remodeler DDM1 (Decrease in DNA Methylation1) and another DNA methyltransferase, chromomethylases 2 (CMT2) in *Arabidopsis* (19). The rice CMT2 homolog (*Os05g13780*) also exists (19), which may be responsible for MITE-associated CHH methylation in *OsDCL3a* RNAi lines.

In contrast to rice, in which 24-nt siRNAs affect genome-wide gene expression, in *Arabidopsis* only a few genes (e.g., *FWA*, *SDC*, *FLC*, and *RPP7*) have been shown to be regulated by nearby TEs or repeats (20–22, 25, 47). In addition, unlike knockdowns of *OsDCL3a*, *AGO4a4b*, *RDR2*, and *DRM2* in rice, which display pleiotropic developmental defects, *Arabidopsis* mutants in genes in the RdDM pathway, such as *PolIV*, *RDR2*, and *DCL3*, do not display obvious developmental phenotypes (23, 48, 49). The different effects on development in the two species may relate to the presence of more TEs (like MITEs) or repeats near genes in a complex genome, such as rice. Indeed, in crops such as maize, with complex TE- and repeat-rich genomes, RdDM pathway mutants also show pleiotropic developmental defects (50, 51).

MITEs, the most abundant DNA transposons, are the ultimate genomic parasites and occur very close to genes in rice (5, 52). This raises the question of how MITE insertions affect the behavior of neighboring genes. In this report, we experimentally demonstrated that in rice, OsDCL3a-dependent 24-nt siRNAs are substantially associated with MITEs or other TEs, and to some extent negatively regulate the expression of nearby genes (Figs. 2–4 and Figs. S6 and S8), which is consistent with a recent bioinformatic analysis (53). In addition, MITEs harbor regulatory sequences that may also act as enhancers to up-regulate expression of nearby genes (10, 54). Hence, MITEs might play a dual role in regulation of nearby genes, epigenetically repressing and genetically enhancing gene expression.

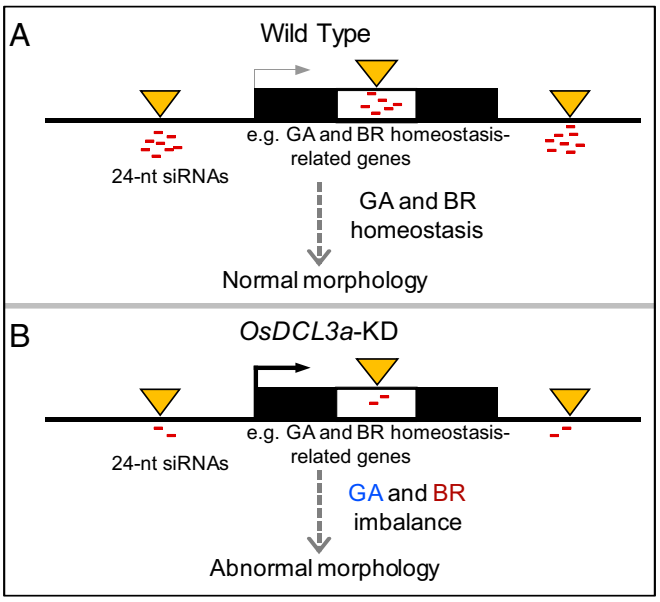

Fig. 5. OsDCL3a-dependent 24-nt siRNAs regulate nearby gene expression and control rice development. In WT plants (*A*), 24-nt siRNAs (red lines) produced from TEs and repetitive sequences (yellow triangles) target nearby genes, including genes involved in GA and BR homeostasis. Normal regulation of GA and BR produces normal morphology. *OsDCL3a* RNAi knockdown (KD) plants (*B*) produce fewer 24-nt siRNAs (red lines), causing increased expression of genes involved in GA and BR homeostasis. Perturbed regulation of GA and BR biosynthetic genes results in an imbalance of GA and BR and causes abnormal morphology.

The polymorphisms in MITE sequences positively correlate with the variation in gene expression (Fig. S10 *D* and *E*). In the buttercup family (*Ranunculaceae*), an MITE inserted into an intron of the petal identity gene *APETALA3-3* (*AP3-3*) results in gene silencing and petals transformed into sepals in apetalous *Nigella* (55). Thus, a burst of MITE activity, as has been observed in some rice strains (10), could generate epialleles for important agricultural traits. These epialleles could be selected and potentially adopted during domestication (56). In rice, epigenetic silencing of the *DWARF1* (*Epi-d1*) cause a metastable dwarf phenotype, whereas *Epi-df* is a gain-of-function of *FERTILIZATION-INDEPENDENT ENDOSPERM 1*(*FIE 1*) epiallele, which shows pleiotropic defects (57, 58). We hypothesize that epialleles might be widespread and important for agricultural traits in rice.

Thus, our results provide evidence showing that 24-nt siRNA-associated MITEs and other TEs globally affect expression of nearby genes and control agricultural traits in rice. With advances in epigenomics and phenomics, it possible to enhance our ability to determine how often epigenetic state changes caused by TEs have been selected for agricultural traits during plant evolution, particularly in crops.

Materials and Methods

The *OsDCL3a* (*3a-1*, *3a-3*), *AGO4a4b* and *RDR2* RNAi lines, as well as TEJ1 (*Nipponbare*), TEJ2 (IRGC 418), TRJ1 (IRGC 17757), and TRJ2 (IRGC 328), were obtained from previous studies (30, 31, 59). Small RNA-seq and RNA-seq were analyzed and validated in WT, *3a-3*, and *3a-1*. Bisulfite sequencing and ChIP-qPCR assay was used to detect the levels of DNA and histone methylation. Details of experimental procedures are described in *SI Materials and Methods*. See Dataset S1 for the primers used in this study.

PLANT BIOLOGY

ACKNOWLEDGMENTS. We thank Dr. Yijun Qi (Tsinghua University) for providing *ago4ab-1* and *rdr2-2* RNAi lines. This work was supported by National Basic Research Program of China Grant 2013CB835200 (to X.S.), Ministry of Agriculture of the People's Republic of China Grant 2013ZX08009-001 (to X.S.), and National Natural Science Foundation of China Grant 31210103901 (to X. Cao). Rice small RNA work in the B.C.M. laboratory is supported by the National Institute of Food and Agriculture, US Department of Agriculture, under Agreement 2012-67013-19396.

1. International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. *Nature* 436(7052):793–800.
2. Feschotte C, Jiang N, Wessler SR (2002) Plant transposable elements: Where genetics meets genomics. *Nat Rev Genet* 3(5):329–341.
3. Lisch D (2013) How important are transposons for plant evolution? *Nat Rev Genet* 14(1):49–61.
4. Han Y, Qin S, Wessler SR (2013) Comparison of class 2 transposable elements at superfamily resolution reveals conserved and distinct features in cereal grass genomes. *BMC Genomics* 14(1):71.
5. Jiang N, Feschotte C, Zhang X, Wessler SR (2004) Using rice to understand the origin and amplification of miniature inverted repeat transposable elements (MITEs). *Curr Opin Plant Biol* 7(2):115–119.
6. Jiang N, et al. (2003) An active DNA transposon family in rice. *Nature* 421(6919): 163–167.
7. Kikuchi K, Terauchi K, Wada M, Hirano HY (2003) The plant MITE *mPing* is mobilized in anther culture. *Nature* 421(6919):167–170.
8. Nakazaki T, et al. (2003) Mobilization of a transposon in the rice genome. *Nature* 421(6919):170–172.
9. Naito K, et al. (2006) Dramatic amplification of a rice transposable element during recent domestication. *Proc Natl Acad Sci USA* 103(47):17620–17625.
10. Naito K, et al. (2009) Unexpected consequences of a sudden and massive transposon amplification on rice gene expression. *Nature* 461(7267):1130–1134.
11. Hirochika H, Sugimoto K, Otsuki Y, Tsugawa H, Kanda M (1996) Retrotransposons of rice involved in mutations induced by tissue culture. *Proc Natl Acad Sci USA* 93(15): 7783–7788.
12. Ding Y, et al. (2007) SDG714, a histone H3K9 methyltransferase, is involved in Tos17 DNA methylation and transposition in rice. *Plant Cell* 19(1):9–22.
13. La H, et al. (2011) A 5-methylcytosine DNA glycosylase/lyase demethylates the retrotransposon Tos17 and promotes its transposition in rice. *Proc Natl Acad Sci USA* 108(37):15498–15503.
14. Komatsu M, Shimamoto K, Kyozuka J (2003) Two-step regulation and continuous retrotransposition of the rice LINE-type retrotransposon Karma. *Plant Cell* 15(8): 1934–1944.
15. Cui X, et al. (2013) Control of transposon activity by a histone H3K4 demethylase in rice. *Proc Natl Acad Sci USA* 110(5):1953–1958.
16. Slotkin RK, Nuthikattu S, Jiang N (2012) The impact of transposable elements on gene and genome evolution. *Plant Genome Diversity,* (Springer, Vienna), Vol 1, pp 35–58.
17. Slotkin RK, Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* 8(4):272–285.
18. Lisch D, Bennetzen JL (2011) Transposable element origins of epigenetic gene regulation. *Curr Opin Plant Biol* 14(2):156–161.
19. Zemach A, et al. (2013) The *Arabidopsis* nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. *Cell* 153(1):193–205.
20. Liu J, He Y, Amasino R, Chen X (2004) siRNAs targeting an intronic transposon in the regulation of natural flowering behavior in *Arabidopsis*. *Genes Dev* 18(23): 2873–2878.
21. Lippman Z, et al. (2004) Role of transposable elements in heterochromatin and epigenetic control. *Nature* 430(6998):471–476.
22. Henderson IR, Jacobsen SE (2008) Tandem repeats upstream of the *Arabidopsis* endogene SDC recruit non-CG DNA methylation and initiate siRNA spreading. *Genes Dev* 22(12):1597–1606.
23. Cao X, Jacobsen SE (2002) Role of the *Arabidopsis* DRM methyltransferases in de novo DNA methylation and gene silencing. *Curr Biol* 12(13):1138–1144.
24. Xu C, Tian J, Mo B (2013) siRNA-mediated DNA methylation and H3K9 dimethylation in plants. *Protein Cell* 4(9):656–663.
25. Zhai J, et al. (2008) Small RNA-directed epigenetic natural variation in *Arabidopsis thaliana*. *PLoS Genet* 4(4):e1000056.
26. Ghildiyal M, Zamore PD (2009) Small silencing RNAs: An expanding universe. *Nat Rev Genet* 10(2):94–108.
27. Cheng Z, Buell CR, Wing RA, Gu M, Jiang J (2001) Toward a cytological characterization of the rice genome. *Genome Res* 11(12):2133–2141.
28. Yan H, Jiang J (2007) Rice as a model for centromere and heterochromatin research. *Chromosome Res* 15(1):77–84.
29. Zhang X (2008) The epigenetic landscape of plants. *Science* 320(5875):489–492.
30. Song X, et al. (2012) Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. *Plant J* 69(3):462–474.
31. Wu L, et al. (2010) DNA methylation mediated by a microRNA pathway. *Mol Cell* 38(3):465–475.
32. Yan Y, et al. (2011) Small RNAs from MITE-derived stem-loop precursors regulate abscisic acid signaling and abiotic stress responses in rice. *Plant J* 65(5):820–828.
33. Nosaka M, et al. (2012) Role of transposon-derived small RNAs in the interplay between genomes and parasitic DNA in rice. *PLoS Genet* 8(9):e1002953.
34. Moritoh S, et al. (2012) Targeted disruption of an orthologue of DOMAINS REARRANGED METHYLASE 2, OsDRM2, impairs the growth of rice plants by abnormal DNA methylation. *Plant J* 71(1):85–98.
35. Zhu Y, et al. (2006) ELONGATED UPPERMOST INTERNODE encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. *Plant Cell* 18(2):442–456.
36. Peng J, et al. (1999) 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* 400(6741):256–261.
37. Wang L, et al. (2009) OsGSR1 is involved in crosstalk between gibberellins and brassinosteroids in rice. *Plant J* 57(3):498–510.
38. Wada K, Marumo S, Ikekawa N, Morisaki M, Mori K (1981) Brassinolide and homobrassinolide promotion of lamina inclination of rice seedlings. *Plant Cell Physiol* 22(2): 323–325.
39. Yamaguchi S (2008) Gibberellin metabolism and its regulation. *Annu Rev Plant Biol* 59:225–251.
40. Swaminathan S, Morrone D, Wang Q, Fulton DB, Peters RJ (2009) CYP76M7 is an ent-cassadiene C11alpha-hydroxylase defining a second multifunctional diterpenoid biosynthetic gene cluster in rice. *Plant Cell* 21(10):3315–3325.
41. Luo A, et al. (2006) EUI1, encoding a putative cytochrome P450 monooxygenase, regulates internode elongation by modulating gibberellin responses in rice. *Plant Cell Physiol* 47(2):181–191.
42. Wang ZY, Bai MY, Oh E, Zhu JY (2012) Brassinosteroid signaling network and regulation of photomorphogenesis. *Annu Rev Genet* 46:701–724.
43. Hong Z, et al. (2002) Loss-of-function of a rice brassinosteroid biosynthetic enzyme, C-6 oxidase, prevents the organized arrangement and polar elongation of cells in the leaves and stem. *Plant J* 32(4):495–508.
44. Mori M, et al. (2002) Isolation and characterization of a rice dwarf mutant with a defect in brassinosteroid biosynthesis. *Plant Physiol* 130(3):1152–1161.
45. Bai MY, et al. (2007) Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. *Proc Natl Acad Sci USA* 104(34):13839–13844.
46. Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11(3):204–220.
47. Tsuchiya T, Eulgem T (2013) An alternative polyadenylation mechanism coopted to the *Arabidopsis RPP7* gene through intronic retrotransposon domestication. *Proc Natl Acad Sci USA* 110(37):E3535–E3543.
48. Chen XM (2009) Small RNAs and their roles in plant development. *Annu Rev Cell Dev Biol* 25:21–44.
49. Henderson IR, et al. (2006) Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning. *Nat Genet* 38(6): 721–725.
50. Erhard KF, Jr., et al. (2009) RNA polymerase IV functions in paramutation in *Zea mays*. *Science* 323(5918):1201–1205.
51. Garcia-Aguilar M, Michaud C, Leblanc O, Grimanelli D (2010) Inactivation of a DNA methylation pathway in maize reproductive organs results in apomixis-like phenotypes. *Plant Cell* 22(10):3249–3267.
52. González J, Petrov D (2009) Genetics. MITEs—The ultimate parasites. *Science* 325(5946):1352–1353.
53. Lu C, et al. (2012) Miniature inverted-repeat transposable elements (MITEs) have been accumulated through amplification bursts and play important roles in gene expression and species diversity in *Oryza sativa*. *Mol Biol Evol* 29(3):1005–1017.
54. Yang G, et al. (2005) A two-edged role for the transposable element Kiddo in the rice ubiquitin2 promoter. *Plant Cell* 17(5):1559–1568.
55. Zhang R, et al. (2013) Disruption of the petal identity gene *APETALA3-3* is highly correlated with loss of petals within the buttercup family (*Ranunculaceae*). *Proc Natl Acad Sci USA* 110(13):5074–5079.
56. Marí-Ordóñez A, et al. (2013) Reconstructing de novo silencing of an active plant retrotransposon. *Nat Genet* 45(9):1029–1039.
57. Zhang L, et al. (2012) Identification and characterization of an epi-allele of FIE1 reveals a regulatory linkage between two epigenetic marks in rice. *Plant Cell* 24(11): 4407–4421.
58. Miura K, et al. (2009) A metastable DWARF1 epigenetic mutant affecting plant stature in rice. *Proc Natl Acad Sci USA* 106(27):11218–11223.
59. Xu X, et al. (2012) Resequencing 50 accessions of cultivated and wild rice yields markers for identifying agronomically important genes. *Nat Biotechnol* 30(1): 105–111.