A 5-methylcytosine DNA glycosylase/lyase demethylates the retrotransposon *Tos17* and promotes its transposition in rice

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DNA 5-methylcytosine (5-meC) is an important epigenetic mark for transcriptional gene silencing in many eukaryotes. In *Arabidopsis*, 5-meC DNA glycosylase/lyases actively remove 5-meC to counteract transcriptional gene silencing in a locus-specific manner, and have been suggested to maintain the expression of transposons. However, it is unclear whether plant DNA demethylases can promote the transposition of transposons. Here we report the functional characterization of the DNA glycosylase/lyase DNG701 in rice. *DNG701* encodes a large (1,812 amino acid residues) DNA glycosylase domain protein. Recombinant DNG701 protein showed 5-meC DNA glycosylase and lyase activities in vitro. Knockout or knockdown of *DNG701* in rice plants led to DNA hypermethylation and reduced expression of the retrotransposon *Tos17*. *Tos17* showed less transposition in calli derived from *dng701* knockout mutant seeds compared with that in wild-type calli. Overexpression of *DNG701* in both rice calli and transgenic plants substantially reduced DNA methylation levels of *Tos17* and enhanced its expression. The overexpression also led to more frequent transposition of *Tos17* in calli. Our results demonstrate that rice DNG701 is a 5-meC DNA glycosylase/lyase responsible for the demethylation of *Tos17* and this DNA demethylase plays a critical role in promoting *Tos17* transposition in rice calli.

Oryza sativa | incision activity | *Tos17* copy number

In eukaryotes, DNA cytosine methylation at carbon 5 of the pyrimidine ring [5-methylcytosine (5-meC)] is an important epigenetic mark that contributes to gene silencing and plays critical roles in development and genome defense against viruses, transposons, and transgenes (1–3). Heavy cytosine methylation usually occurs at heterochromatin and at regions rich in transposons and repetitive DNA (2, 4). Cytosine methylation of DNA, however, is reversible through demethylation (5, 6). DNA demethylation can be passive or active, and active DNA demethylation is catalyzed by one or more enzymes to remove methylated cytosines and can occur independently of DNA replication (7, 8). So far, several models have been proposed to explain mechanisms of DNA demethylation in animals (7, 8). One of the models suggests that active DNA demethylation is mediated at least in part by a base excision repair (BER) pathway where the AID/Apobec family of deaminases convert 5-meC to T followed by G/T mismatch repair through the DNA glycosylase MBD4 or TDG with the involvement of Gadd45a (7–9). Recently, a new study suggested that 5-meC hydroxylase TET1 promotes DNA demethylation in mammalian cells through a process that requires the BER pathway (10). Nevertheless, many aspects of these models have not been confirmed, and how DNA demethylation is carried out in animals remains controversial (7, 8).

In contrast to the uncertainty about the mechanism of DNA demethylation in animals, mechanisms of DNA demethylation in plants are much clearer and widely accepted. In the model plant *Arabidopsis*, research showed that the 5-meC DNA glycosylase/lyase-mediated BER pathway plays critical roles in DNA demethylation process (3, 5, 6, 11, 12). There are four 5-meC DNA glycosylase/lyases [i.e., REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE2 (DML2) and DEMETER-LIKE3 (DML3)] found in the *Arabidopsis* genome, and genetic and biochemical analysis revealed that all four proteins function as DNA demethylases, and are involved in genomic DNA demethylation (1, 3, 5, 6, 12–14). ROS1 is required for the prevention of hypermethylation and transcriptional silencing of a repetitive reporter gene (1). DME was reported to be necessary for endosperm gene imprinting and seed viability (3, 13). Both DML2 and DML3 are required for removing 5-meC from improperly methylated DNA in target sites (12). Biochemical evidence demonstrated that the DNA glycosylase activity of these proteins removes 5-meC from DNA backbone and then the lyase activity cleaves the DNA backbone at the abasic site by successive β- and δ-elimination reactions (3, 5, 6, 12, 14). However, compared with these well-documented 5-meC DNA glycosylase/lyases in *Arabidopsis*, no 5-meC DNA glycosylase/lyase has been well characterized in other plant species.

As one of the most important food crops in the world, rice (*Oryza sativa* L.) has become a model monocot species for functional genomics research. Nipponbare, a completely sequenced japonica rice cultivar, is now widely used for functional analysis of genes. *Tos17*, a well-characterized rice retrotransposon, is extensively used to generate *Tos17* insertional mutants in Nipponbare (15, 16). There are two almost identical *Tos17* copies, named $Tos17^{chr7}$ and $Tos17^{chr10}$ (located on chromosomes 7 and 10, respectively) in Nipponbare (17). $Tos17^{chr7}$ transcripts (~4 kb) solely accumulate in calli and $Tos17^{chr10}$ is inserted into a putative ABC-type transporter gene to form a fusion gene that generates a ~7-kb readthrough transcript (17). Both $Tos17^{chr7}$ and $Tos17^{chr10}$ are highly methylated and immobilized under normal plant growth conditions (16, 17). However, in calli the transposition of *Tos17* is activated and the copy number increases gradually during prolonged callus culture (16). Several recent studies showed that the activation of transcription as well as transposition of *Tos17* in calli is associated with DNA hypomethylation (17–19). However, the

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protein that is directly responsible for the active demethylation of *Tos17* in the rice genome is as yet unknown.

In this report, we identified and characterized a rice DNA glycosylase/lyase, DNG701 (for DNA Glycosylase 701) (http://www.chromdb.org/). Enzymatic activity assays demonstrated that DNG701 is an active 5-meC DNA glycosylase/lyase in vitro. Knockout or knockdown and overexpression of *DNG701* in rice calli and *in planta* showed that DNG701 is critical for *Tos17* demethylation, expression, and transposition in rice. Our demonstration of a host plant factor promoting the transposition of *Tos17* suggests that the retrotransposon *Tos17* may have a beneficial function to the rice genome during evolution.

Results

***DNG701* Encodes a Rice 5-meC DNA Glycosylase/Lyase.** Among the five predicted rice DNA glycosylases (http://www.chromdb.org/), DNG701 and its close paralog DNG704 are most closely related to *Arabidopsis* DML2 and ROS1 (Fig. S1*A*). As with the other four rice DNA glycosylases, DNG701 contains a DNA glycosylase domain immediately followed by an EndIII_4Fe-4S domain, and two putative nuclear localization signals are located at the N terminus of the protein (Fig. S1*B*) (20). Similar to the four *Arabidopsis* and other four rice DNA glycosylases, the DNG701 glycosylase domain contains a helix–hairpin–helix motif and a glycine/proline-rich loop with an invariant aspartic acid (GPD) (Fig. S1*C*). An extremely conserved lysine (K) is located inside one of the highly conserved helices (Fig. S1*C*).

A comparison between the cloned *DNG701* cDNA (GenBank accession no. FJ536320) and its genomic DNA sequence revealed that the ORF of *DNG701* consists of 19 exons and encodes a protein of 1,812 amino acids (Figs. S1*B* and S2*A*). RT-PCR analysis indicated that *DNG701* was ubiquitously expressed in the three tissues (leaves, stems, and roots) of 3-wk-old Nipponbare seedlings (Fig. S2*B*, *Upper*) and four tissues (leaves, stems, roots, and panicles) of 3-mo-old flowering Nipponbare plants (Fig. S2*B*, *Lower*). The fused GFP-DNG701 protein was predominantly localized in the nucleus, whereas the GFP control protein was distributed in both the nucleus and cytoplasm of rice cells (Fig. S2*C*).

The purified recombinant maltose-binding protein-DNG701 (MBP-DNG701) fusion protein had nicking activity against ^{m}CG- and ^{m}CCGG-containing pUBIGUS plasmids, which was generated separately by CG methyltransferase (M.SssI) and by MspI methyltransferase, but did not have any nicking activity against unmethylated pUBIGUS (Fig. S3 *A* and *B*, *Upper*). The purified control protein MBP possessed no nicking activity against either methylated or unmethylated pUBIGUS (Fig. S3*B*, *Lower*). Furthermore, we conducted incision assays against 40-bp double-stranded oligonucleotides containing fully methylated and hemimethylated C^{m}CGG or ^{m}CCGG (Fig. S3*C*) (5). The double-stranded oligonucleotides were produced by annealing ^{32}P-labeled single-stranded oligonucleotides to unlabeled complementary strands (Fig. S3*C*). Incubation of MBP-DNG701 with either C^{m}CGG- or ^{m}CCGG-containing oligonucleotides generated one 16-nt and one 29-nt cleavage products corresponding to fully methylated and hemimethylated sites, respectively, suggesting that the protein has active incision activity in vitro against fully methylated and hemimethylated oligonucleotides (Fig. 1 *A* and *B*). In contrast, MBP showed no incision activity on the two types of oligonucleotides (Fig. 1*A*). With an increase in the amount of MBP-DNG701, more 16-nt and 29-nt cleavage products were produced from the methylated oligonucleotides (Fig. 1*B*). Furthermore, four other types of oligonucleotides, containing either fully methylated or hemimethylated C^{m}CGG or ^{m}CCGG, were incubated with MBP-DNG701 (Fig. S3*C*). The results again showed that MBP-DNG701 had incision activity against the fully methylated or hemimethylated C^{m}CGG- or ^{m}CCGG-containing oligonucleotides, but had no incision activity against unmethylated oligonucleotides (Fig. 1*C*). Taken together, these data showed that DNG701 has active DNA glycosylase/lyase activity against 5-meC–containing DNA in vitro.

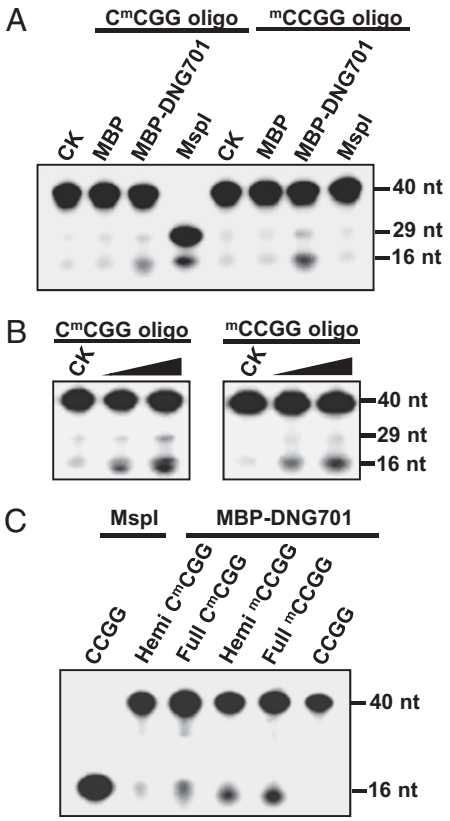

Fig. 1. Enzymatic activity of DNG701 on 5-meC-containing DNA substrates in vitro. (*A*) Incision activity assays of MBP-DNG701 and MBP on C^{m}CGG- and ^{m}CCGG-containing radiolabeled oligonucleotides. One microgram of MBP and MBP-DNG701 proteins and 10 U of MspI were used in this assay. (*B*) Incision activity assays of MBP-DNG701 on C^{m}CGG- and ^{m}CCGG-containing oligonucleotides. In the second and third lanes in each panel, 0.5 and 1 μg of proteins were added, respectively. (*C*) Incision activity assays of MBP-DNG701 on hemimethylated or fully methylated C^{m}CGG- or ^{m}CCGG-containing oligonucleotides. One microgram of MBP-DNG701 protein and 10 U of MspI were used in this assay. CK, no protein added. C^{m}CGG oligo and ^{m}CCGG oligo refer to fully methylated, as well as hemimethylated, oligonucleotides at the internal and external cytosines in CCGG sequence context, respectively. "Hemi" and "Full" refer to hemimethylated and fully methylated oligonucleotides, respectively. CCGG, unmethylated oligonucleotides. The filled triangles above the panels indicate increasing amounts of proteins.

Knockout or Knockdown of *DNG701* Causes DNA Hypermethylation of *Tos17* Retrotranspsons in Rice. To further elucidate the function of DNG701 in rice, we obtained two *Tos17* insertion mutants named *dng701-1* and *dng701-2* (Fig. S2*A*). RT-PCR analysis suggested that *DNG701* is knocked out in both the mutants (Fig. S4, *Left*). Meanwhile, homozygous *DNG701* RNA interference (RNAi) transgenic lines PC5-9 and PC5-10 were selected as well for detailed analysis because the expression of *DNG701* was substantially down-regulated although that of *DNG704* was not affected (Fig. S4, *Right*). DNA gel-blot analysis revealed that there were no apparent DNA methylation changes in the centromeric repeat DNA, 45S rDNA, or Ty1 retrotransposons in both the mutants and RNAi lines, indicating that knockout or knockdown of *DNG701* does not cause global DNA hypermethylation in these regions (Fig. S5*A*). However, substantial DNA hypermethylation was observed in the retrotransposon *Tos17* (Fig. 2*A* and Fig. S6*A*). These results indicated that loss of

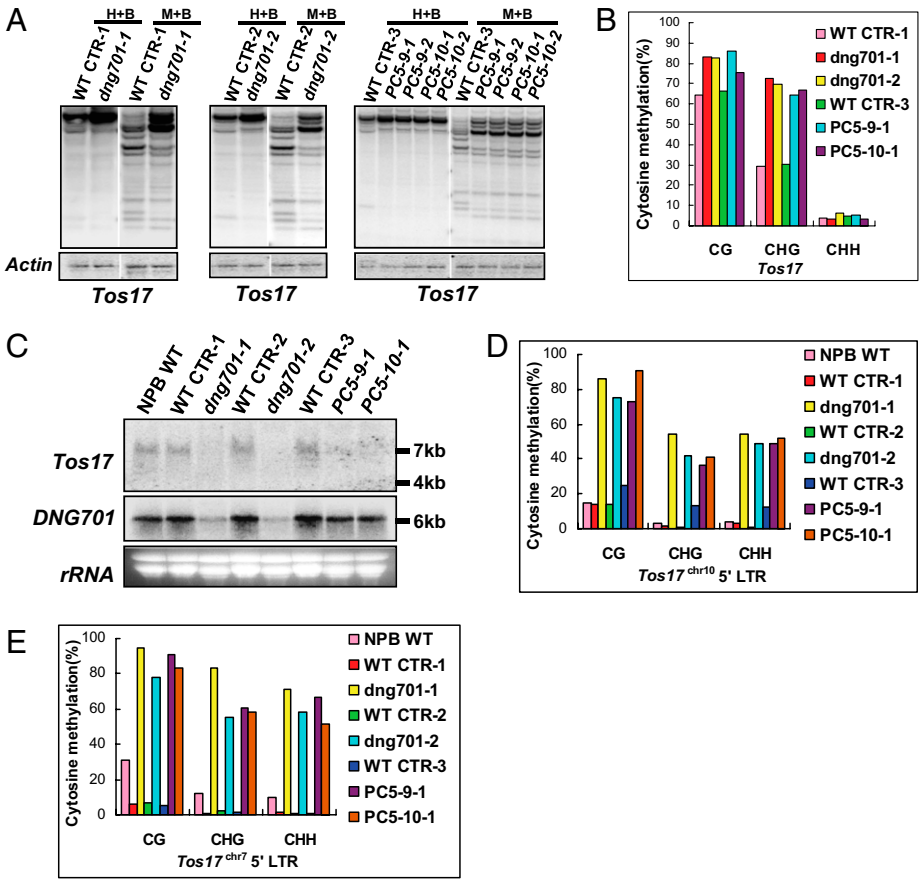

Fig. 2. Impact of knockout or knockdown of *DNG701* on the methylation status of *Tos17*. (*A*) Methylation status of *Tos17* retrotransposons in wild-type controls, mutants, and RNAi lines. A 3.3-kb *Tos17* probe (Probe 1) (Fig. S6*B*) was used for hybridization. The same blots were reprobed with *Actin*. WT CTR-1 and WT CTR-2 are sibling wild-type plants segregated from heterozygous *DNG701/dng701-1* and *DNG701/dng701-2* plants, respectively. WT CTR-3 is a segregated wild-type plant from a hemizygous *DNG701* RNAi transgenic line PC5-9. H+B, HpaII+BstXI; M+B, MspI+BstXI. (*B*) Bisulfite sequencing analysis of methylation status of *Tos17*. *Tos17* region d (Fig. S6*B*) was subjected to bisulfite sequencing. (*C*) Reduced expression of $Tos17^{chr10}$ in the mutants and RNAi lines. A 1.1-kb *Tos17* probe (Probe 2) (Fig. S6*B*) was used for *Tos17* hybridization. NPB WT, Nipponbare wild-type. (*D* and *E*) Bisulfite sequencing analysis of methylation levels of *Tos17* 5′ LTR regions. 5′ LTR regions of $Tos17^{chr10}$ (region b in Fig. S6*A*) (*D*) and $Tos17^{chr7}$ (region c in Fig. S6*A*) (*E*) were assayed.

function of *DNG701* leads to DNA hypermethylation in a locus-specific manner. Bisulfite sequencing results further verified that methylation levels at both CG and CHG (H is A, T, or C) sites of *Tos17* were increased in the mutants and RNAi lines (Fig. 2*B* and Fig. S6*B*).

We then examined expression levels of *Tos17* in the mutants and RNAi lines and found that the expression of $Tos17^{chr10}$ transcripts (~7 kb) was substantially reduced (Fig. 2*C*). The $Tos17^{chr7}$ transcripts (~4 kb) were not detected in either the wild-type Nipponbare or the two mutants and RNAi lines (Fig. 2*C*). To understand if the suppression of $Tos17^{chr10}$ expression is caused by hypermethylation of its promoter, we examined the methylation level of the promoter region of ~1-kb upstream of the translation start site by bisulfite sequencing (Fig. S6*A*). A 202-bp fragment from −445 to −244 was heavily methylated in all of the genotypes and only the CHH methylation levels were slightly increased in the mutants and RNAi lines, suggesting that methylation in this region may not account for the reduction of $Tos17^{chr10}$ expression (Fig. S5*B*). Because the LTR region is known as an enhancer-promoter of LTR retrotransposons and is usually a target of transcriptionally repressive methylation (17, 21), we determined the methylation levels of 5′ LTR regions of $Tos17^{chr10}$ and $Tos17^{chr7}$ and observed much higher DNA methylation levels in the mutants and RNAi lines than in the controls (Fig. 2 *D* and *E*). Taken together, these data indicate that knockout or knockdown of *DNG701* leads to DNA hypermethylation at 5′ LTR as well as coding regions of *Tos17* and transcriptional repression of $Tos17^{chr10}$, suggesting that DNG701 is an important factor for protecting *Tos17* from hypermethylation and silencing. In addition to *Tos17*, we also found that another retrotransposon (LOC_Os07g31690) was hypermethylated in its 3′ terminal region in both the *dng701* mutants, further supporting that DNG701 is an active demethylase/lyase that removes 5-meC at some loci in the rice genome (Fig. S5*C*).

Interestingly, seeds harvested from mature panicles of both mutants fell into two categories: normal seeds and wrinkled seeds (13.66% wrinkled seeds for *dng701-1*, 10.51% for *dng701-2*, and 0.79% for wild-type Nipponbare). The observation suggests that *DNG701* is involved in seed development of rice (Fig. S7).

Overexpression of *DNG701* Leads to DNA Hypomethylation in Rice Calli and Transgenic Plants. To further understand the function of *DNG701* in rice, we overexpressed *DNG701* in Nipponbare under the control of the maize *ubiquitin* promoter. Two independently transformed callus lines, OX-1 and OX-2, were obtained after a 2-mo selection on hygromycin-containing media. Simultaneously, two other independent callus lines, OX-CK-1 and OX-CK-2, which were transformed with the empty vector alone, were obtained and used as controls. Next, each callus line was subdivided into a few smaller callus clones and continuously cultured for another 3 mo. RNA gel-blot analysis demonstrated that the expression levels of $Tos17^{chr10}$ in the OX-1 and OX-2

callus lines were increased when *DNG701* was overexpressed, and low levels of *Tos17*^{chr7} transcripts were detected in all genotypes (Fig. 3*A*). DNA gel-blot analysis revealed that both the OX-1 and OX-2 callus lines showed hypomethylation in *Tos17* relative to the control callus line, which presumably accounts for the enhanced expression of *Tos17*^{chr10} (Fig. 3*B*). This hypomethylation of *Tos17* was further confirmed by bisulfite sequencing analysis (Fig. S8). Interestingly, CG methylation level of the 45S rDNA also declined in the OX-1 and OX-2 callus lines (Fig. 3*C*). Furthermore, methylation levels of the centromeric repeat DNA and Ty1 retrotransposons were also slightly reduced in the overexpression lines (Fig. 3*C*).

DNG701-overexpressing transgenic plants and transgenic controls regenerated from the 2-mo-old transformed calli were used to study the impact of *DNG701* overexpression on *Tos17 in planta*. As shown in Fig. 4*A*, the expression levels of *Tos17*^{chr10} were markedly increased with the overexpression of *DNG701* in the OX-1 and OX-2 transgenic lines. DNA gel-blot analysis and bisulfite sequencing results revealed reduced methylation levels at its promoter region and gene body in the overexpression transgenic plants (Fig. 4 *B–D* and Fig. S6*A*). The transcripts of *Tos17*^{chr7} were not detected in either overexpression line despite the hypomethylation of *Tos17* (Fig. 4*A*). Bisulfite sequencing results of the 5′ LTR regions of both *Tos17*^{chr10} and *Tos17*^{chr7} demonstrated that CG methylation levels in *Tos17*^{chr10} and *Tos17*^{chr7}, and CHG and CHH methylation levels in *Tos17*^{chr7} were reduced in the overexpression plants (Fig. 4 *E* and *F*). Like the overexpression calli, the overexpression plants also showed a remarkably decreased CG methylation in 45S rDNA (Fig. S9). These data suggest that overexpression of *DNG701* has extensive effects on reducing DNA methylation of the rice genome.

DNG701 Is a Critical Factor That Promotes *Tos17* Transposition in Calli. Because *Tos17* is hypomethylated in the OX-1 and OX-2 callus lines, we wondered if transposition of *Tos17* in the two lines was affected. DNA gel-blot analysis was performed to examine *Tos17* copy number in 6-mo-cultured (since callus induction) callus clones derived from the OX-1 and OX-2 callus lines and the control lines. As shown in Fig. 3*D* (*Left*), an apparent increase in the copy number of *Tos17* was observed in the callus clones derived from the OX-1 and OX-2 callus lines in which an average of 6.8 and 6.3 *Tos17* copies were present, respectively. In contrast, there were on average 3, 2, and 3 *Tos17* copies found in the callus clones from Nipponbare, OX-CK-1 and OX-CK-2, respectively (Fig. 3*D*, *Left*).

Furthermore, independent calli from Nipponbare and the *dng701-2* mutant seeds were induced and cultured for 10 mo. Eight independent calli from each material were subjected to DNA gel-blot analysis to investigate the change in *Tos17* copy number. In parallel, leaf samples from both genotypes were used as the controls to assess native copy number of *Tos17*. The results revealed that calli derived from *dng701-2* mutant seeds gained only one additional *Tos17* copy relative to the leaf control (Fig. 3*D*, *Right*). In contrast, calli derived from Nipponbare gained 26 additional *Tos17* copies compared with the leaf control (Fig. 3*D*, *Right*). The increased copy number in these samples was not attributable to incomplete digestion because only a single band was observed when the blot was reprobed with *Tubulin* (Fig. 3*D*, *Right*). Taken together, these results indicate that DNG701 is necessary and also a limiting factor for high transposition activity of *Tos17* in rice calli.

Discussion

Our work demonstrated that DNG701 is an active rice 5-meC DNA glycosylase/lyase, and it is able to process fully methylated

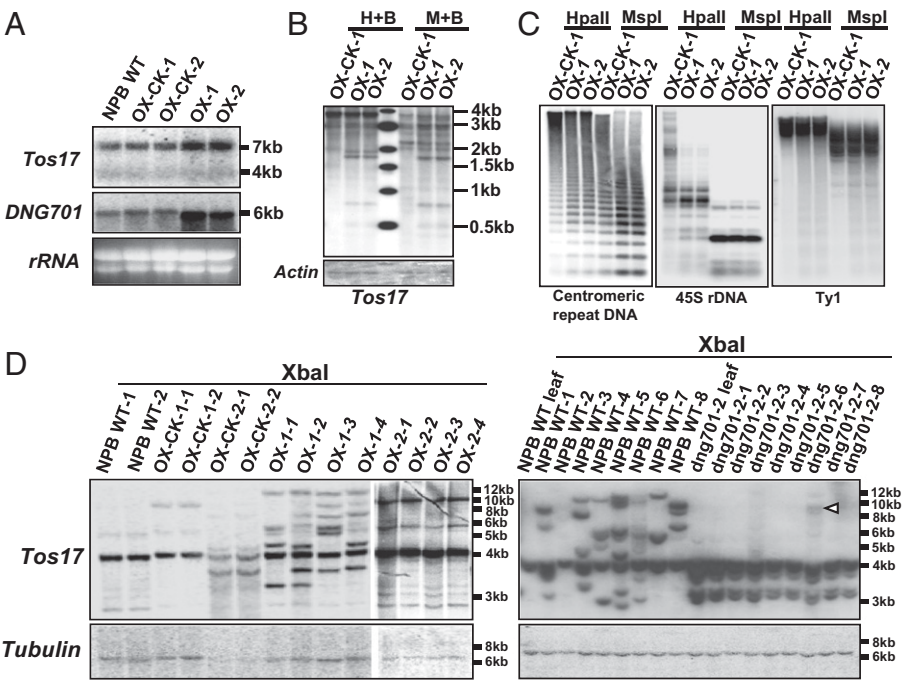

Fig. 3. Effects of *DNG701* overexpression on the DNA methylation and transposition of *Tos17* in rice calli. (*A*) The transcript levels of *Tos17* in *DNG701*-overexpressing calli. Probe 2 was used for *Tos17* hybridization. NPB WT, Nipponbare wild-type calli. OX-CK-1 and OX-CK-2, independent calli transformed with empty vector. OX-1 and OX-2, independent calli transformed with *DNG701*-overexpressing construct. (*B*) Methylation status of *Tos17* in the *DNG701*-overexpressing calli revealed by DNA gel-blot analysis. Probe 1 was used for *Tos17* hybridization. H+B, HpaII+BstXI; M+B, MspI+BstXI. (*C*) DNA gel-blot analysis of methylation status of centromeric repeat DNA, 45S rDNA and Ty1 retrotransposons in the *DNG701*-overexpressing calli. (*D*) Transposition of *Tos17* in the *DNG701*-overexpressing calli (*Left*) and mutant calli (*Right*). The open triangle denotes a newly transposed *Tos17* copy in the *dng701-2* calli. Probe 2 was used for hybridization.

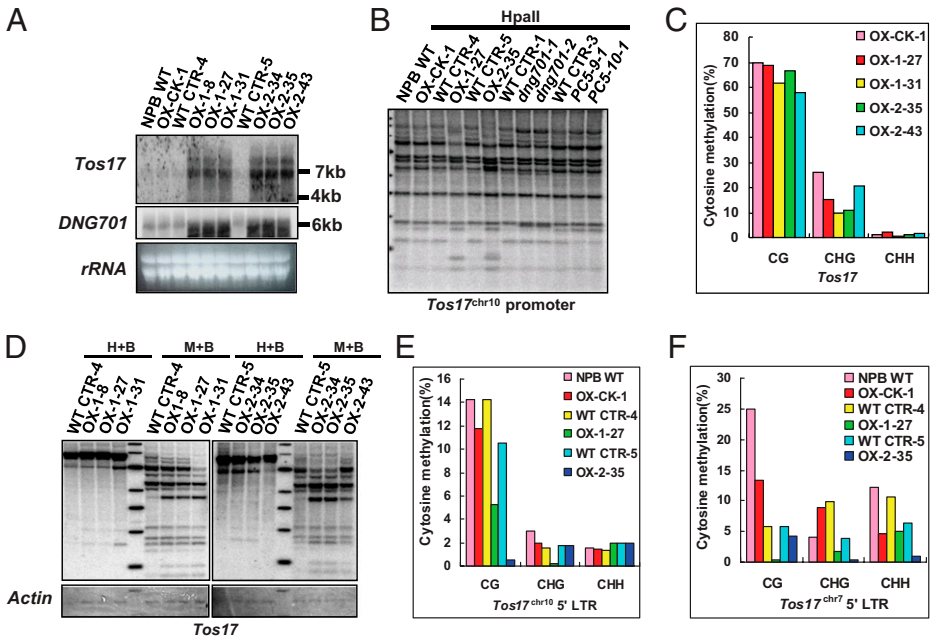

Fig. 4. Effects of *DNG701* overexpression on the DNA methylation of *To17* in transgenic plants. (*A*) The transcript levels of *Tos17* in *DNG701*-overexpressing plants. OX-1–8, OX-1–27, and OX-1–31 are transgenic plants regenerated from 2-mo-cultured OX-1 calli; OX-2–34, OX-2–35, and OX-2–43 are transgenic plants regenerated from 2-mo-cultured OX-2 calli. WT CTR-4 and WT CTR-5, segregated wild-type plants from hemizygous transgenic plants OX-1–27 and OX-2–35, respectively. Probe 2 was used for *Tos17* hybridization. (*B*) Reduced methylation levels of the $Tos17^{chr10}$ promoter in the *DNG701*-overexpressing transgenic plants. A 3.8-kb probe (Probe 3) (Fig. S6*A*) was used for hybridization. (*C* and *D*) Reduction of methylation levels of *Tos17* in the *DNG701*-overexpressing plants as revealed by bisulfite sequencing (region d) (*C*) and DNA gel-blot analysis (*D*). Probe 1 was used for hybridization. H+B, HpaII+BstXI; M+B, MspI+BstXI. (*E* and *F*) DNA methylation levels of $Tos17^{chr10}$ 5′ LTR (region b) (*E*) and $Tos17^{chr7}$ 5′ LTR (region c) (*F*) determined by bisulfite sequencing.

or hemimethylated external or internal 5-meC efficiently (Fig. 1 *A*–*C*). Like the *Arabidopsis ros1* mutant (4), the rice *dng701* knockout mutants show hypermethylation at some transposons, indicating that loss of function of *DNG701* leads to DNA hypermethylation in a locus-specific manner (Fig. 2*A* and Fig. S5*C*). We did not observe apparent methylation changes in the centromeric repeat DNA, 45S rDNA, or Ty1 retrotransposons between *dng701* mutants and wild-type controls (Fig. S5*A*). It is likely that these regions have already been hypermethylated in the wild-type control genomes, and any methylation-enhancing effect of *dng701* on these regions is difficult to detect using methylation-sensitive restriction enzymes. There is no report about the impact of overexpression of *ROS1* or *DML2* on global DNA methylation levels at certain repetitive sequences, although ROS1 was reported to be localized in nucleoplasm and nucleolus that contain many copies of 45S rDNA units (5, 12, 22). Overexpression of *DNG701* in rice calli and plants led to marked hypomethylation of 45S rDNA and slight hypomethylation on centromeric repeat DNA and Ty1 retrotransposons, suggesting that DNG701 is targeted to these regions, and overexpression of *DNG701* presumably has significant dose effect to excessively demethylate them (Fig. 3*C* and Fig. S9). Neither the *Arabidopsis ros1* nor the *dml2* mutant produces wrinkled seeds (1, 12, 14), whereas the rice *dng701* mutants produce a substantial proportion of wrinkled seeds (~10–13% for mutants and ~0.8% for Nipponbare) (Fig. S7). Recently, two studies revealed that both *Arabidopsis* and rice endosperms are hypomethylated in all sequence contexts, and CG methylation levels are partially restored in both gene bodies and repeats in *dme* endosperm, suggesting the participation of DME in endosperm demethylation (23, 24). It is likely that DNG701 is also involved in endosperm demethylation in rice and, unlike its *Arabidopsis* counterparts (DML2 and ROS1), targets developmentally important genes. Compared with *Arabidopsis*, the rice genome contains a substantially higher amount of transposons and other repetitive elements (25, 26). Therefore, it is possible that more genes in rice are subject to regulatory control by repetitive elements, and thus more genes are likely targeted for demethylation by DNG701. It will be of interest to determine the developmentally important target genes of DNG701 in the future.

The relationships among DNA methylation, transcription, and transposition of transposable elements in plant kingdom have been explored extensively (19, 21, 27–29). In *Arabidopsis*, it has been reported that DNA methylation is involved in controlling mobilization of transposons (27–29). In the *ddm1* mutant and the self-pollinated progeny, silent *CACTA* elements and several transposons like *ATGP1* and *ATGP2* are transcriptionally and transpositionally activated because of DNA hypomethylation (28, 29). In the *cmt3 met1* double mutant, transcription and high-frequency transposition of the *CACTA* elements were detected, suggesting that CG and non-CG methylation functions to immobilize transposons (27). Likewise, the reduced DNA methylation levels in rice correlate with activation of transcription and transposition of *Tos17* (17, 18). Other research found that reduced histone H3K9 dimethylation decreases DNA methylation of *Tos17*, leading to its active transcription and transposition in *SDG714* RNAi transformants (19); however, the enzymes directly responsible for demethylation of *Tos17* have remained unclear. Our work suggests that DNG701 is responsible for active DNA demethylation of *Tos17*. In *dng701* mutants, the normally expressed $Tos17^{chr10}$ is silenced, which is accompanied by elevated DNA methylation levels at the 5′ LTR region and gene body (Fig. 2 *A*–*E*). In *dng701* mutant calli, *Tos17* transposition activity is much reduced (Fig. 3*D*, *Right*). In contrast, overexpression of *DNG701* in rice calli and plants not only decreases the DNA methylation levels of *Tos17* and up-regulates $Tos17^{chr10}$ expression, but also promotes transposition of *Tos17* in rice calli (Figs. 3 *A*, *B*, and *D*, *Left*, and 4). Interestingly, the autonomous expression of the $Tos17^{chr7}$ is not affected by its DNA methylation levels, suggesting that DNA hypomethylation

is not a sole determinant for the expression of *Tos17*^{chr7}, and there must be other factors to regulate its expression in rice plants (Fig. 3*A*). We speculate that overexpression of *DNG701* might generate certain immediate triggers to contribute to the transposition of *Tos17*^{chr7}. One possible trigger is the favorable chromatin state/environment. Our results showed that in the overexpression calli the methylation levels of 45S rDNA were obviously decreased, and the methylation levels of centromeric repeats and Ty1 transposons were also slightly reduced, indicating that overexpression of *DNG701* has extensive effects on the methylation levels of callus genome (Fig. 3*C*). Therefore, it is likely that the chromatin state/environment of the overexpression calli might have been altered because of the overexpression of *DNG701*, which may make the chromatin prone to *Tos17* integration. Another possible trigger is posttranscriptional regulation. Overexpression of *DNG701* may enhance translation/reverse transcription of *Tos17* transcripts through an unknown post-transcriptional regulation. Our results suggest that DNG701 is required for *Tos17* demethylation and expression of *Tos17*^{chr10}. Importantly, DNG701 is required for the high transposition activity of *Tos17* in rice calli. Our results also suggest that DNG701 is a limiting factor for *Tos17* transposition. The fact that plants have evolved active DNA demethylases that promote the transposition of transposons implies that the affected transposons also have beneficial effects to the plant species during evolution.

Materials and Methods

Plant Materials. All rice plants used in this study were *Oryza sativa* L. ssp. *japonica* cv. Nipponbare. Details are available in *SI Materials and Methods*.

Cloning of *DNG701* cDNA, Subcellular Localization of DNG701, RNA Gel-Blot and RT-PCR Analysis, and DNA Gel-Blot Analysis. See details in *SI Materials and Methods* and Table S1.

Protein Purification and Enzymatic Activity Assays. The entire *DNG701* cDNA was inserted in-frame into the pMAL-c2× vector (New England Biolabs) to obtain a *malE-DNG701* fusion construct. Expression and purification of the MBP-DNG701 fusion protein and MBP protein were performed as previously described (5). For the nicking activity assays, 200 ng of each of these plasmids was added to nicking reaction buffer (10 mM Tris-HCl, pH7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mg/mL BSA) plus purified MBP-DNG701 or MBP. Reactions were carried out as previously described (5). For incision activity assays, upper strands of the seven oligonucleotides (Fig. S3*C*) were labeled with ^{32}P at the 5′ ends, and then annealed to the corresponding lower strands, as previously described (5). Labeled double-stranded oligonucleotides (1 pmol) were incubated with MBP-DNG701 or MBP or MspI endonuclease in the nicking reaction buffer in a total volume of 10 μL at 37°C for 2 h. The products were separated on 17% denaturing polyacrylamide gels containing 8 M urea, and then the gels were exposed to a PhosphorImager screen. See *SI Materials and Methods* for details.

Genomic Bisulfite Sequencing. Two micrograms of genomic DNA was digested with 20 units of appropriate restriction enzymes (New England Biolabs) for 3 h. Next, the digested DNA was purified by ethanol, treated with a sodium bisulfite solution and desalted, as previously described (19). Subsequently, 4 μL of each recovered DNA sample was subjected to PCR amplification. The PCR products were cloned into the pGEM-T Easy cloning vector (Promega), and 15 to 20 clones were sequenced to calculate the percentage of cytosine methylation in each sample. Details are in *SI Materials and Methods*.

Sequence Alignments and Phylogenetic Analysis. See *SI Materials and Methods* for details.

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