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Activation of a rice endogenous retrotransposon *Tos17* in tissue culture is accompanied by cytosine demethylation and causes heritable alteration in methylation pattern of flanking genomic regions

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Abstract *Tos17* is a *copia*-like, cryptic retrotransposon of rice, but can be activated by tissue culture. To study possible epigenetic mechanism controlling activity of *Tos17*, we subjected three rice lines (the parental line cv. Matsumae and two introgression lines, RZ2 and RZ35) that harbor different copies of the element to tissue culture. For each line, we investigated transcription and transposition of *Tos17* in seed plants, calli and regenerated plants, cytosine-methylation status at CG and CNG positions within *Tos17*, effect of 5-azacytidine on methylation status and activity of *Tos17*, and cytosine-methylation states in genomic regions flanking original and some newly transposed copies of *Tos17* in calli and regenerated plants. We found that only in introgression line RZ35 was*Tos17* transcriptionally activated and temporarily mobilized by tissue culture, which was followed by repression before or upon plant regeneration. The activity and inactivity of *Tos17* in calli and regenerated plants of RZ35 are accompanied by hypo- and hyper-CG methylation and hemi- and full CNG methylation, respectively, within the element, whereas immobilization of the element in the other two lines is concomitant with near-constant, full hypermethylation. Treatment with 5-azacytidine induced both CG and CNG partial hypomethylation of *Tos17* in two lines (Matsumae and RZ35), which, however, was not accompanied by activation of *Tos17* in any line. Heritable alteration in cytosine-methylation patterns occurred in three of seven genomic regions flanking *Tos17* in calli and regenerated plants of RZ35, but in none of the five regions flanking dormant *Tos17* in the other two lines.

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Introduction

Retrotransposons, also called class I mobile elements, transpose via reverse transcription of RNA intermediates and are prominent genomic components in many eukaryotes. The most prominent type of retrotransposons in plants is the long terminal repeat (LTR) retroelement that can be further divided into *copia*- and *gypsy*-like classes (Kumar and Bennetzen 1999; Bennetzen 2000). Although LTR retrotransposons usually contain sequences encoding all proteins required for their retrotransposition, the majority of them are largely quiescent during normal growth and development (Bennetzen 1996). Nevertheless, some high-copy retrotransposons in the grass family show constant expression in somatic tissues (Vicient et al. 2001). On the other hand, accumulating evidence indicates that some of the retrotransposons are responsive to, and often transcriptionally and even transpositionally activated by, various biotic and abiotic stresses such as wounding, pathogen attack, cell culture, environmental extremes, and interspecies hybridization (Lucas et al. 1995; Wessler 1996; Hirochika 1997; Grandibastien 1998; O'Neill et al. 1998; Liu and Wendel 2000; Kashkush et al. 2003). Because most of the retrotranspositions are conceivably deleterious to the host's genome, it is not surprising that activity of retrotransposons is often tightly controlled by host factors. Given the often-reversible nature of elements' activity, it is likely that epigenetic mechanisms such as DNA methylation and/or chromatin remodeling might play a decisive role in the host-controlling machinery. DNA methylation has been implicated to have multiple cellular functions in eukaryotes with this epigenetic code,

but the primary role of DNA methylation is likely to serve as a genome defense system, such as taming mobile elements (Yoder 1997; Martienssen and Colot 2001). The finding that transposons and retrotransposons in plants and animals possess a higher methylation level than coding cellular genes (Finnegan et al.1998; Colot and Rossignol 1999) is consistent with this view. Moreover, classical work in maize showed that there exists a close correlation between transposon activity and its methylation states (Chandler and Walbot 1986; Schwartz and Dennis 1986; Banks et al. 1988; Cui and Fedoroff 2002). Recent studies in both plants and animals have further strengthened this correlation, or even provided a causal link between mobile element activity and its DNA-methylation states. For example, it was demonstrated that in maize the methylation states (holo-, hemi-, or unmethylated) of the *Ac/Ds* elements largely determine their transposition competence (Wang et al. 1998; Ros and Kunze 2001). Studies in a wallaby hybrid showed that demethylation is associated with rampant amplification of a retroelement (O'Neil et al. 1998). In *Arabidopsis*, silencing of an introduced retrotransposon (*Tto1*) is correlated with hypermethylation of the element, and genome-wide demethylation (in the *ddm1* background) results in its reactivation and retrotransposition (Hirochika et al. 2000). Another study in *Arabidopsis* showed that the *ddm1* mutation has directly caused transcriptional activation of some retrotransposons and derepression of an endogenous transposon of the *CACTA* family, which is mobilized and leading to a spectrum of new insertions (Miura et al. 2001). Nevertheless, because the *DDM1* gene encodes a putative *SWI2/SNF2*-like chromatin-remodeling factor (Vongs et al. 1993), it is likely that the *ddm1* mutation will primarily affect chromatin conformation, with genome-wide hypomethylation being a secondary effect (Jeddeloh et al. 1999; Singer et al. 2001). Thus, more compelling evidence in *Arabidopsis* that establishes a causal connection between cytosine methylation and transposon silencing comes from studies on single or double mutants of the DNA-methyltransferase genes; it was found that loss-of-function mutation of the genes leads to hypomethylation of transposons and their transcriptional and transpositional activation (Kato et al. 2003).

Tos17 is one of the few characterized, endogenous *copia*-like retrotransposons that are potentially active in plants (Hirochika et al. 1996). Nevertheless, the copy number of *Tos17* is extremely low in all rice cultivars assayed, ranging from one to four (Hirochika 1997). Copy number of the element can be significantly elevated by tissue culture, albeit all copies become inactive again upon plant regeneration (Hirochika et al. 1996). Apparently, activity of *Tos17* is under tight host control, but nature of the controlling mechanism is not clear. In addition, given that changes in activity of a transposon might often affect activity of its nearby host genes (Martienssen 1996; Kashkush et al. 2003), it is also interesting to know what genetic and epigenetic consequences that activated *Tos17* might impose on flanking genomic regions.

The present study was aimed to investigate possible roles of cytosine methylation on *Tos17*'s activity and the potential effects of the element's transcriptional activation and insertion on structure and epigenetic state of the flanking genomic regions. We report that *Tos17* was activated by tissue culture in only one of the three lines, and that under tissue-culture conditions, transcriptional activation and silencing of *Tos17* is accompanied by hypo- and hypermethylation at the inner cytosines and hemi- and full methylation at the outer cytosines of CCGG sites within the element, but 5-azacytidine-induced partial hypomethylation is not accompanied by element activation. We also report that activation of *Tos17* might cause heritable alterations in cytosine-methylation patterns of the flanking genomic regions.

Materials and methods

Plant materials

The three rice lines used include a pure line *japonica* cultivar (Matsumae) and its two derived, genetically homogeneous introgression lines (RZ2 and RZ35) that contain minute amounts of introgressed genomic DNA from wild rice, *Zizania latifolia* (Liu et al. 1999). Based on genome-wide fingerprinting by RAPD, ISSR, and AFLP assays at ~2,000 loci, it was found that both RZ2 and RZ35 contain $\leq$0.01% of *Zizania* genomic DNA (unpublished data). Thus, the three lines are nearly isogenic, being different only by possessing various trace amount of *Zizania* DNA. Because the rice endogenous retrotransposon *Tos17* could be mobilized by *Zizania*-DNA introgression (Liu and Wendel 2000), the two lines used in this study were assumed also to possess increased copy numbers of the element, as was indeed confirmed (see Results).

Calli were induced from germinating seeds of the three lines on Murashige-Skoog solid medium containing 2 mg/l 2,4-D. Embryogenic calli were selected and subcultured on the same medium at 3-week intervals. Plants were regenerated from 6- and 12–14-month-old embryogenic calli on the same medium without 2,4-D. Plants were grown to maturity and selfed to produce progenies.

For 5-azacytidine (Sigma, St. Louis, Mo.) treatments, seeds of the three lines were dehusked and germinated in the absence or presence of the drug at concentrations of 50 μg/ml and 100 μg/ml for 7 days. Genomic DNA and total RNA were isolated from leaves of the drug-treated and control plants.

Preparation of probes for *Tos17* and flanking genomic regions

The probe for *Tos17* is a 666-bp internal fragment of the reverse transcript (RT)/RNaseH region, which was isolated by PCR with specific primers RTP1 (5′-GCTACCCGTTCTTGGACTAT-3′, nucleotide positions 2817–2837) and RTP2 (5′-CTGAAATCGGAGCACTGACA-3′, nucleotide positions 3483–3463) (Fig. 1). Thermal asymmetric interlaced (TAIL)-PCR (Liu et al. 1995) was used to isolate *Tos17*-flanking genomic regions. Original insertion sites of *Tos17* were isolated from uncultured plants of the three lines, while new insertion sites were isolated from a 12-month-old callus-regenerated plant of line RZ35. Three nested *Tos17* 3′-LTR-specific primers, T17TAIL2, T17TAIL3, and T17TAIL4, described by Yamazaki et al. (2001) were used, together with each of the three arbitrary primers, AD1, AD2, and AD3, reported by Liu et al. (1995). PCR products from the third-round amplification were purified and either directly sequenced or ligated to the pTGM-T vector (Promega) and sequenced with vector primers. Similarity searches of the isolated sequences were conducted with BlastX at the NCBI Web site.

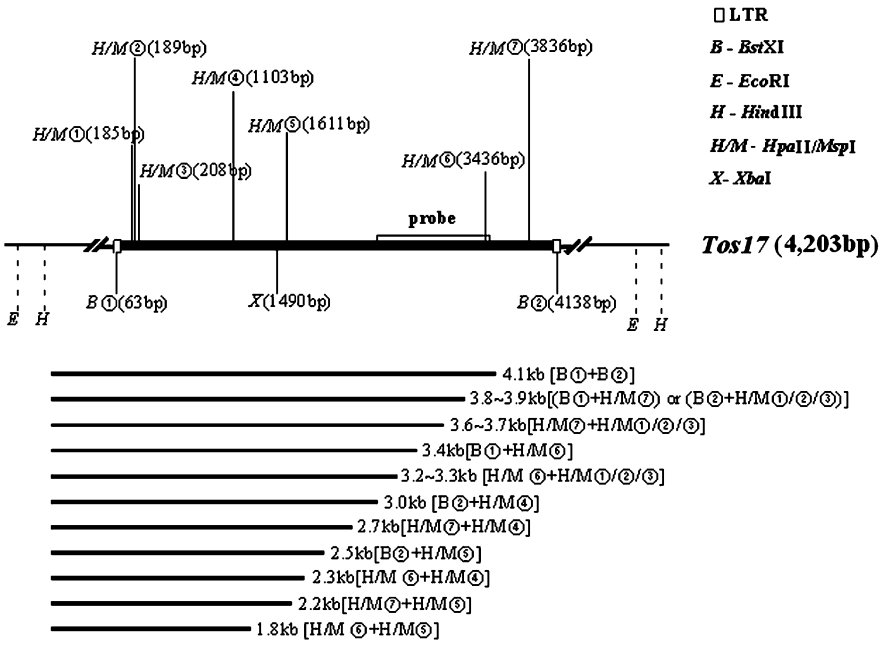

Fig. 1 Restriction map of retrotransposon *Tos17*, based on Hirochika et al. (1996) and on a BAC clone of rice chromosome 10 (nbxb0019M20, accession no. AC087545). Positions of the seven *Hpa*II/*Msp*I restriction sites within *Tos17* are indicated. Positions for *Xba*I that has a single restriction site and *Bst*XI that has one restriction site within each of the long terminal repeats (LTRs) are given. The element does not have restriction sites for *Eco*RI and *Hin*dIII; thus, their restriction sites in the flanking regions are marked by *dashed lines*. The *empty rectangle* denotes the reverse transcriptase (RT) region probe. Depending on methylation status (i.e., digestibility) of the seven CCGG sites, a maximum of 12 hybridizing bands could potentially be generated with *Hpa*II/*Msp*I digestions in the *Bst*XI-delineated fragment, as depicted in the *lower panel* of the figure

Probes for each of the flanking sequences of the *Tos17* 3′ LTR were prepared by digesting the PCR products with proper enzymes selected based on the sequence information. All enzymes chosen were adjacent to, but downstream of, the 3′ LTR of *Tos17* and had unique restriction sites within the sequenced regions. In this way, potential confounding effects of *Tos17* are completely eliminated. Fragments of the expected sizes were gel purified and labeled with α-^{32}P-dCTP by the random priming method.

Southern blot hybridizations

Genomic DNA was isolated from expanded leaves of individual plants (seed plants and callus regenerated) and from calli derived from individual seeds by the CTAB method. Precaution was taken to use leaves at the same developmental stage and calli at the same length of subculture. For *Tos17* copy-number estimation, genomic DNA was digested by each of the three enzymes *Xba*I, *Eco*RI, and *Hin*dIII, which have either a unique restriction site or no site within *Tos17*, and hybridized with the internal fragment of *Tos17* as a probe. For assaying cytosine-methylation status at the CCGG sites within *Tos17*, genomic DNA was cut with *Bst*XI (to delineate *Tos17*) plus either *Hpa*II or *Msp*I (Fig. 1a) and hybridized with the same probe as used for copy-number estimation. For assaying possible structural and methylation changes in *Tos17*-flanking genomic regions, genomic DNA was digested with a methylation-insensitive enzyme (usually the same as the ones used for probe preparation to exclude any confounding effects of *Tos17*, thus ensuring the analyzed regions are confined to flanking regions) alone and the enzyme plus *Hpa*II or *Msp*I and hybridized with each of the flanking sequences as probes.

Digested DNA was run through 1% agarose gel and transferred onto Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech) by the alkaline transfer. Hybridization and washing stringencies were as described (Liu and Wendel 2000); autoradiography was done with the PhosphorImager (Molecular Dynamics, USA).

Northern-blot hybridization and RT-PCR analysis

Total RNA was isolated from leaves of seed-derived or regenerated plants and from 12-month-old calli of the three lines by the Trizol Reagent (Invitrogen) according to manufacturer's protocol. For northern blotting, about 20 μg total RNA for each sample was run through 1% agarose-formaldehyde gels, transferred to a Hybond-N$^+$ nylon membrane, and hybridized against the RT fragment of *Tos17* as a probe. For RT-PCR, the RNA was further treated with DNaseI (Invitrogen), reverse transcribed by the SuperScript RNase H$^-$ Reverse Transcriptase (Invitrogen), and subjected to RT-PCR analysis using the RT region-specific primers that amplify a 666-bp fragment, as described above. A pair of primers (actP1: 5′-CG-TCTGCGATAATGGAACTG and actP2: 5′-TCTGGGTCATCT-TCTCACGA) designed to specifically amplify a 341-bp fragment from cDNA or a 424-bp fragment from genomic DNA (encompassing a 83-bp intron) of the rice actin gene was included in each amplification (multiplexing) as an internal control and as a check for trace DNA contamination. Twenty cycles were used to ensure the amplification was within the linear range. The amplicons were transferred to a Hybond-N$^+$ membrane, and signals were detected by Southern blot hybridization using the RT probe. For comparison of transcript level, element signal was normalized to that of the internal actin by the ImageQuant software.

Results

Tos17 is a *copia*-like retrotransposon that was isolated by Hirochika and colleagues from cDNA of rice calli (Hi-

rochika et al. 1996). Using a cDNA fragment of the RT region of *Tos17* (accession no. D85876) as a query, we searched the GenBank database by BlastN and found a hit of a 139-kb rice BAC genomic DNA fragment (nbxb0019M20, accession no. AC087545) located on chromosome 10; a region of this fragment shows perfect match with the RT region of *Tos17*. Further analysis identified the two identical LTRs, the 5-bp targeting-site duplication (CTCCT, Hirochika et al. 1996), and all intact components for a typical *copia*-like retrotransposon, thus indicating that this BAC clone contains the complete sequence of *Tos17*. Based on this sequence information, we identified seven *Hpa*II/*Msp*I restriction sites (5′-CCGG-3′) within *Tos17*, a unique *Xba*I site, and one *Bst*XI site within each of the LTRs (Fig. 1). Thus, genomic Southern analysis with double-enzyme digests with *Bst*XI plus *Hpa*II or *Msp*I and using an internal part of *Tos17* as a probe could possibly generate variable numbers of bands depending on cytosine-methylation status of the seven CCGG sites within *Tos17* (Fig. 1).

Tos17 was mobilized by tissue culture in one of the three rice lines

As there is a unique restriction site for *Xba*I and none for *Eco*RI and *Hin*dIII within *Tos17* (Fig. 1), using an internal fragment of the element, such as the RT region, to probe Southern blots carrying DNA digested with each of these enzymes will give a conservative estimation on copy number of the element. The three rice lines used have different copy numbers of *Tos17*, with the two introgression lines apparently possessing more copies than their parental line (Fig. 2, compare lane 1 across panels a, b, and c). Given that *Z. latifolia*-DNA introgression into rice could cause mobilization of *Tos17* (Liu and Wendel 2000), we assumed that difference in copy number of *Tos17* in the three lines is likely due to *Zizania*-DNA introgression. This assumption was based on the fact that since production, the introgression lines had always been grown together with the parental line under normal conditions; thus, no other stress apart from alien-DNA integration was conceivable to mobilize the element, and that there was no homolog of *Tos17* in *Z. latifolia* detectable under the Southern hybridization stringency used; thus, the extra copies were not from the wild species (Liu and Wendel 2000). Nevertheless, it was demonstrated that the mobilization of *Tos17* by *Zizania*-DNA introgression was rapidly repressed, and copy number of the element is stable in all subsequent generations of the introgression lines (Liu and Wendel 2000).

Interestingly, when the three lines were subjected to tissue culture, copy number of *Tos17* increased in calli and regenerated plants only in introgression line RZ35 (Fig. 2c); for parental line Matsumae and introgression line RZ2, the element copy number remained stable even in calli subcultured for $\geq$12 months and in plants regenerated from them (Fig. 2a, b). The mobilization of *Tos17* in calli of RZ35 was likely rapidly repressed, as prolonged duration (up to 24 months) in subculture did not result in further elevation of element copy number, thus implying efficient silencing of element transposition even at the callus stage (before plant regeneration) following initial mobilization.

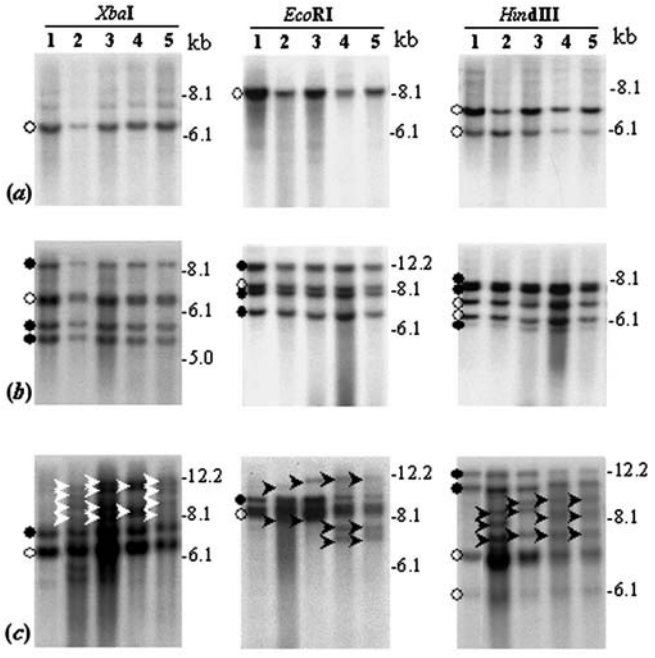

Fig. 2a–c Copy-number estimation of *Tos17* in uncultured and tissue-cultured plant materials from the three rice lines: **a** the parental line cv. Matsumae, **b** introgression line RZ2, and **c** introgression line RZ35. The Southern blotting profiles were obtained by hybridizing the RT probe (Fig. 1) to blots carrying genomic DNA digested with each of the three enzymes, *Xba*I, *Eco*RI, and *Hin*dIII. *Lanes 1* through *5* are, respectively, seed plants, a 6-month-old callus, 6-month-old callus-regenerated plants, a 12–14-month-old callus, and 12–14-month-old callus-regenerated plants. The *empty* and *filled circles* refer, respectively, to the original copy of *Tos17* in parental line Matsumae and its derived introgression lines RZ2 and RZ35 and copies mobilized by alien-DNA integration in the introgression lines, whereas *arrowheads* denote copies mobilized by tissue culture. Sizes are indicated on the *right side* of each profile

Activation and inactivation of *Tos17* in introgression line RZ35 under tissue-culture conditions are accompanied by cytosine hypo- and hypermethylation at the CG sites and hemi- and full methylation at CNG sites within the element

Because transcription is the first step in retrotransposon mobilization, we investigated *Tos17* transcription status in seed plants, calli, and regenerated plants of all three lines by northern-blot analysis and RT-PCR assay. Interestingly, although there was northern-level detectable steady-state transcripts of the element in expanded leaves of seed plants of the parental line Matsumae and introgression line RZ35, no hybridization signal was visible in comparable amounts of RNA isolated from corresponding

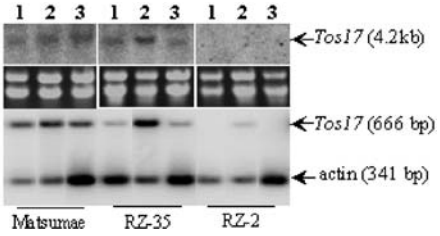

Fig. 3 Detection of *Tos17* steady-state transcript level in seed plants (*lane 1*), a 6-month-old callus (*lane 2*) and regenerated plants (*lane 3*) of the three lines by northern-blot analysis and RT-PCR assay. For northern blotting, about 20 µg total RNA was loaded for each sample. Before blotting, ethidium bromide-stained RNA was visualized under UV light (*middle panel*). For RT-PCR, total RNA was treated by DNase, as specified by the supplier (Invitrogen), and a pair of primers specific to the RT region of *Tos17* was used to amplify a 666-bp fragment (Fig. 1). A pair of rice actin-specific primers amplifying a 341-bp cDNA fragment (or a 424-bp genomic DNA fragment) was multiplexed in the same tube as an internal control. Twenty cycles were performed to ensure that the RT-PCR amplification was within the exponential range. The signal was detected by PhosporImager after resolving amplicons on 2% agarose gel, transferring to nylon filter, and hybridizing to the radiolabeled RT probe of *Tos17*. Similar results were observed in independent samples wherein calli of each line were subcultured for 12–14 months

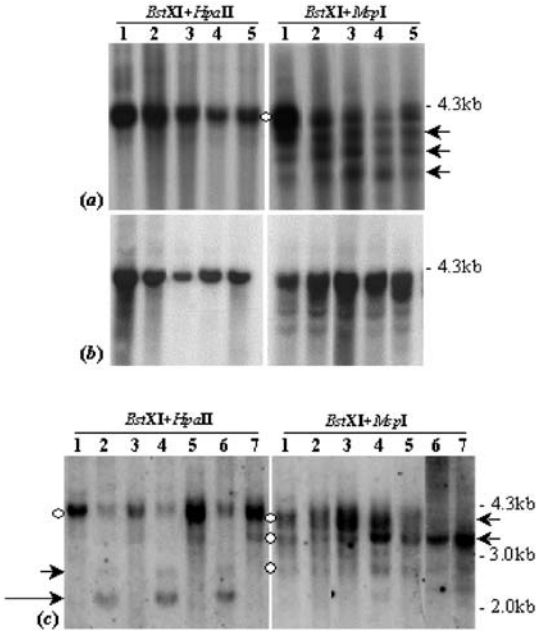

Fig. 4a–c CG- and CNG-methylation states within *Tos17* in uncultured and tissue-cultured plant materials from the three rice lines: **a** the parental line Matsumae, **b** introgression line RZ2, and **c** introgression line RZ35. The Southern blotting profiles were obtained by hybridizing the RT probe (Fig. 1) to blots carrying genomic DNA digested with double enzymes: *Bst*XI plus *Hpa*II or *Msp*I. *Lanes 1* through *5* are, respectively, seed plants, a 6-month-old callus, 6-month-old callus-regenerated plants, a 12–14-month-old callus, and 12–14-month-old callus-regenerated plants. For RZ35, the 24-month-old callus (*lane 6*) and its regenerated plants (*lane 7*) were also included. In the *right panel* of **a**, the *circle* denotes the prominent hybridizing band in uncultured Matsumae, which became apparently less intense in cultured samples, whereas the *arrows* indicate several lower-molecular-weight bands that were significantly intensified in cultured samples, thus indicating double-stranded CNG demethylation at the CCGG sites in calli and regenerated plants. In the *left panel* of **c**, the *circle* denotes the single band in uncultured RZ35, which became significantly less intense in callus samples (hence indicating general demethylation), whereas the *short* and *long arrows* indicate, respectively, double-stranded CG demethylation (the band exists in the corresponding *Msp*I digest, *right panel*) and single-stranded (semi-) CNG demethylation (the band does not exist in the corresponding *Msp*I digest, *right panel*) in callus samples. In the *right panel* of **c**, the *circles* denote bands in uncultured RZ35 that underwent changed intensity in cultured samples, whereas the *arrows* indicate double-stranded CNG demethylation of the CCGG sites. Sizes are indicated on the *right*

material of introgression line RZ2 (compare lane 1 of the uppermost and middle panels in Fig. 3). No discernible increase in transcripts was observed in calli or regenerated plants of parental line Matsumae (the seemingly stronger signal in regenerated plants is likely due to more RNA, as was confirmed by RT-PCR analysis, below), and likewise, no hybridization signal was detectable in calli of introgression line RZ2; in contrast, a marked increase in transcripts occurred in calli of introgression line RZ35, and that remained high even in prolonged cultures (data not shown), but underwent a sharp decrease in regenerated plants (uppermost panel of Fig. 3). RT-PCR largely confirmed the northern-blot results; although by this more sensitive approach, trace amounts of transcript could be detected in calli of RZ2, indicating its weak transcriptional induction (lowermost panel in Fig. 3).

To investigate cytosine-methylation status of the CCGG sites within *Tos17* in seed plants, calli, and regenerated plants of the three lines, genomic DNA was digested to completion by *Bst*XI (to delineate the element, Fig. 1) plus *Hpa*II or *Msp*I and hybridized with the RT probe described above. *Hpa*II and *Msp*I are isoschizomers that recognize the same site (5′-CCGG-3′), but have different sensitivity to methylation at the cytosines. *Hpa*II will not cut if either C is fully (both strands) methylated, but will cut if the outer C is hemimethylated (one strand), whereas *Msp*I will not cut if the outer C is fully or hemimethylated (McClelland et al. 1994). Based on this information, the following results are generalized: (1) *Tos17* was completely methylated at CG positions and heavily methylated at CNG positions in all three lines, as being reflected by a single (in *Bst*XI +*Hpa*II digest) or a prominent (in *Bst*XI +*Msp*I digest) ~4.1-kb hybridizing band (delineated by *Bst*XI) (lane 1 in Fig. 4a, b, c); (2) for parental line Matsumae, no change in the completely methylated status at the CG position of any of the seven CCGG sites occurred in tissue culture, as a single ~4.1-kb monomorphic band was detected in all materials (seed plants, calli, and regenerated plants), but there was noticeable double-stranded demethylation at the CNG sites in calli (shift of band intensity downward in *Bst*XI + *Msp*I digest) that was not restored after plant regeneration (Fig. 4a); (3) for introgression line RZ2, the completely or heavily methylated states at CG and CNG, respectively, of all CCGG sites remain highly stable in all materials (seed plants, calli, and regenerated plants) studied, as a virtually identical banding pattern was observed for all the mate-

rials in each digest (Fig. 4b); and (4) for introgression line RZ35, a pattern of hypo- and hypermethylation at the CG sites (presence and absence of a ~2.7-kb band, indicated by a short arrow) and hemi- and full methylation at the CNG sites (presence and absence of a ~2.2 kb band, Fig. 4c, indicated by a long arrow) was observed for calli and plants (seed derived or callus regenerated), respectively, indicating demethylation in tissue culture, followed by rapid and complete remethylation upon plant regeneration (Fig. 4c, left panel), while a general trend of progressive, double-stranded demethylation was detected at the CNG sites with the prolongation in callus culture (shift of the prominent band downward) that was not restored following plant regeneration (Fig. 4c, right panel).

Thus, the hypo- and hypermethylation status at CG sites and hemi- and full methylation at the CNG sites of *Tos17*, respectively, in calli and plants of introgression line RZ35 is accompanied by the presence and absence of the element transcripts. On the other hand, the static hypermethylation state at CG sites and double-stranded methylation status at the CNG sites in parental line Matsumae and introgression line RZ2 is associated with the absent or constant low level of the element transcripts. This, together with the observation described above (that copy number of *Tos17* was increased only in calli and regenerated plants of introgression line RZ35), suggests that demethylation at CG and hemimethylation at CNG positions of *Tos17* CCGG sites correlates with transcriptional activation of the element, and that only when the transcripts accumulated to a certain threshold could transposition result, which, however, was rapidly repressed while still in callus culture, as prolonged culture duration did not result in more copies of the element, as previously described. In contrast, transcript level of the element was decreased only after plant regeneration (Fig. 3).

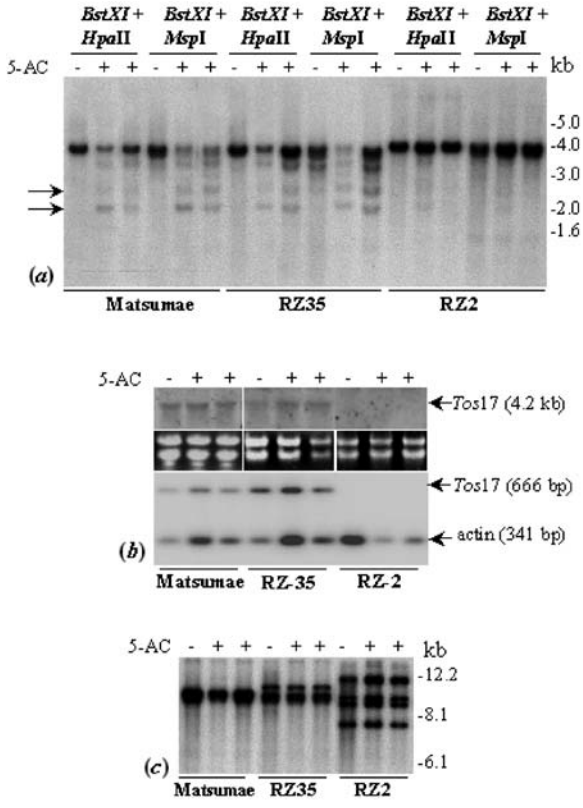

Fig. 5a–c Effect of 5-azacytidine treatment at two concentrations (for each lane, 100 μg/ml concentration is marked by the *first plus sign*, 50 μg/ml concentration is marked by the *second plus sign*, and control is marked by the *minus sign*) on methylation status, possible transcriptional activation, and mobilization of *Tos17* in the three rice lines. **a** Southern hybridization of the RT probe to blot carrying double-enzyme (*Bst*XI plus *Hpa*II or *Msp*I)-digested genomic DNA from control and 5-azacytidine-treated plants. Sizes are indicated on the *right*. **b** Detection of *Tos17* steady-state transcript level in control and 5-azacytidine-treated plants by northern-blot analysis and RT-PCR assay, as was detailed in Fig. 3. **c** Copy-number estimation of *Tos17* in control and 5-azacytidine-treated plants by Southern blot analysis. Genomic DNA was digested with *Eco*RI and hybridized against the RT probe of *Tos17*, as was detailed in Fig. 2

5-azacytidine-induced partial demethylation is not accompanied by *Tos17* activation

The foregoing results indicate that callus formation in introgression line RZ35 activates transcription and transposition of *Tos17*, which was associated with double-stranded demethylation at CG and semi-demethylation at CNG positions of the CCGG sites within the element. To investigate if genome-wide demethylation was sufficient for *Tos17* activation, we subjected all three lines to 5-azacytidine treatments at two concentrations (see Materials and methods). It was found that 5-azacytidine effectively induced double-stranded demethylation at both CG and CNG positions of *Tos17* CCGG sites in parental line Matsumae and introgression line RZ35, but not in introgression line RZ2, wherein demethylated bands are negligible and the element remained hypermethylated at both CG and CNG positions (Fig. 5a). We then examined transcript level and copy number of *Tos17* in control and 5-azacytidine-treated plants of all three lines and found static, low-level element transcripts in parental line Matsumae and introgression line RZ35 and no transcript in introgression line RZ2 (Fig. 5b). Consistent with this lack of transcriptional activation of *Tos17* by the drug treatment, no new transposition was detected (Fig. 5c). It thus is evident that double-stranded partial demethylation is not accompanied by *Tos17* activation in rice.

Activation of *Tos17* causes heritable cytosine-methylation changes in flanking genomic regions

To address the question of whether transcriptional and transpositional activation of *Tos17* might impact structural and/or methylation status of flanking genomic regions, we attempted to isolate a set of *Tos17*-flanking sequences by TAIL-PCR (Liu et al.1995) from uncultured and cultured plants of the three lines. By sequencing multiple clones from the third-round TAIL-PCR products of each sample, we totally isolated 12 distinct sequences:

four from seed plants of RZ35, three (new ones) from a callus plant of RZ35, one from seed plants of Matsumae, and four from seed plants of RZ2. Consistent with immobilization of *Tos17* in Matsumae and RZ2 in tissue culture (Fig. 2), we failed to obtain new sequences from callus-regenerated plants of these two lines even after exhaustive sequencing of more than 60 different clones. Identity of all the isolated sequences as bona fide *Tos17*-flanking regions was verified by the identification of a complete 138-bp LTR at 5′ termini of each of the sequences (Yamazaki et al. 2001). To authenticate that some of the clones are indeed newly transposed, long-range PCR amplification was performed with Ex *Taq* (TaKaRa Biotech, Dalian) by sequence-specific primers designed upon sequence information obtained from the TAIL-PCR products (for the 3′ part) and the public *japonica* rice genome sequence information (for the 5′ part) at the RGP Web site (http://rgp.dna.affrc.go.jp). By this way, all three genomic regions isolated from a callus-regenerated plant of RZ35 that were allegedly flanking newly transposed *Tos17* copies (according to the sequence analysis of TAIL-PCR products) were verified (e.g., Fig. 6d). BlastX results showed that 9 of the 12 *Tos17*-flanking sequences bear significant similarity to known-function genes, mainly those encoding for metabolic enzymes and disease resistance (our unpublished data).

Southern blot analysis of each of the 12 probes on double-enzyme digests (see Materials and methods) indicated that none of the five sequences from Matsumae and RZ2 showed structural or methylation changes in calli and regenerated plants (in comparison with seed plants) of the respective lines (data not shown). In contrast, three of the seven sequences from RZ35 detected apparent cytosine-methylation alterations at both CG and CNG sites of the flanking regions, although no structural change could be deduced; this was being reflected by monomorphic banding patterns between seed plants and calli or regenerated plants in digests by single enzymes (methylation insensitive), but polymorphic patterns in digests by double enzymes (one being methylation sensitive), for each probe (Fig. 6a, b; data not shown). To test if the altered methylation patterns were heritable, progenies of the regenerated plants were subjected to the same Southern blot analysis. It was found that in all three cases, the altered methylation patterns in calli and calli-regenerated plants were stably transmitted to the next generation (Fig. 6c; data not shown).

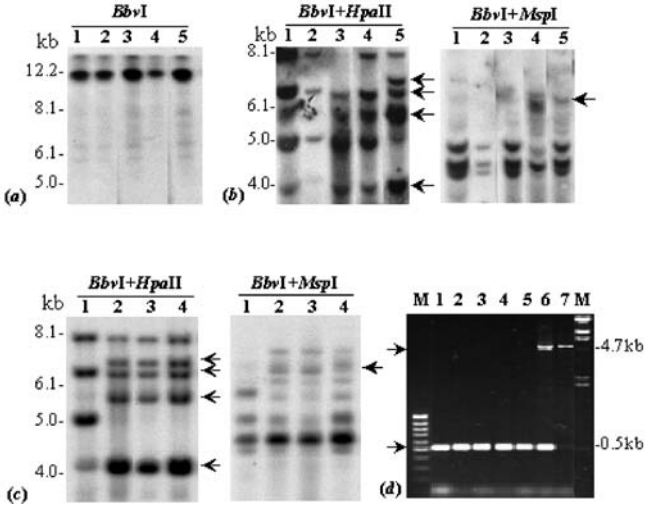

Fig. 6 Alteration of cytosine-methylation patterns in a genomic region (Ti35-28-1) flanking a newly transposed *Tos17* in cultured plants of introgression line RZ35 and its inheritance. The Southern blotting profile was obtained by hybridizing Ti35-28-1 to cultured and uncultured plant materials of introgression line RZ35 digested with *Bbv*I and *BbV*I plus *Hpa*II or *Msp*I. *BbV*I was chosen because it locates at the 5′ terminus of Ti35-28-1, i.e., immediately downstream of the 3′ LTR of *Tos17*, thus serving to delineate this flanking genomic region (see Materials and methods). *Lanes 1* through *5* in **a** and **b** are, respectively, seed plants of RZ35, a 6-month-old callus, a 6-month-old callus-regenerated plant, a 12-month-old callus, and a 12-month-old callus-regenerated plant. *Arrows* refer to changed bands due to alterations of cytosine methylation at the CCGG sites of sequence Ti35-28-1. *Lanes 1* through *4* in **c** are, respectively, seed plants of RZ35, a 12-month-old callus-regenerated plant (the same as in **a** and **b**), and two progeny individuals of this plant. *Arrows* refer to changed bands being stably inherited. Sizes are indicated on the *left*. **d** Authentication of the Ti35-28-1-flanking *Tos17* as a newly transposed copy induced by tissue culture in introgression line RZ35. Sequence-specific primers (forward 5′-CTCTGCAACCATCTTCATACCG and reverse 5′-GCTATTCGTGGCATGGTCAATA) and Ex *Taq* (TaKaRa Biotech, Dalian) were used for the amplification. A 4.7-kb or 0.5-kb band should be amplified in this genomic region with or without a *Tos17* insertion (marked by *arrows*). *Lanes 1*, *2*, *3*, *4*, *5*, *6*, and *7* are, respectively, seed-plant DNA of Matsumae, callus DNA of Matsumae, seed-plant DNA of RZ2, callus DNA of RZ2, seed-plant DNA of RZ35, callus DNA of RZ35, and a regenerated-plant DNA of RZ35. The *leftmost* and *rightmost lanes* are molecular size markers of 100 bp (Fermentas, Maryland) and lambda-*Hin*dII digest (TaKaRa Biotech)

Discussion

Possible mechanism for *Tos17* repression

LTR retrotransposons are prominent components of plant genomes and usually accumulate to highly reiterated copy numbers (Bennetzen 1996 2000; Kumar and Bennetzen 1999). For example, a single retrotransposon family in barley, *BARE1*, makes up 3–5% of the nuclear genome in different natural accessions of wild barley (Kalendar et al. 2000). In this respect, the rice endogenous retrotransposon *Tos17* provides a sharp contrast, as it has extremely low copy numbers (ranging from one to four) in various rice cultivars across two subspecies, *japonica* and *indica* (Hirochika 1997; our unpublished data). Nevertheless, copy number of *Tos17* could be significantly increased by tissue culture (Hirochika et al. 1996). This, together with the propensity of the element to insert into low-copy genic regions throughout the genome, renders it an efficient tool for gene tagging in rice (Hirochika 2001; Yamazaki et al. 2001). *Tos17* is found to be active only during callus culture and becomes immediately silent upon plant regeneration (Hirochika et al. 1996; Hirochika

2001). However, mechanism for the element control remains obscure.

We showed in this paper that the likelihood of *Tos17* to be activated by tissue culture is genotype dependent, as only one (RZ35) of the three rice lines exhibits increased element copy numbers in calli and regenerated plants (Fig. 2). It is worth mentioning that the three lines are genetically akin to each other, being different only by containing none to various minute amounts of alien DNA from wild rice, *Z. latifolia* (Liu et al. 1999; unpublished data); yet, they showed such a difference with regard to activity of *Tos17* under tissue-culture conditions. It is particularly interesting that although *Tos17* in parental line Matsumae was mobilized by alien-DNA introgression (Liu and Wendel 2000), it is not activated by tissue culture (Fig. 2a), suggesting that alien-DNA integration and tissue culture might constitute different stress or "genome shock" (sensu McClintock 1984). Because the "original" copy of *Tos17* in parental line Matsumae was not activated in tissue culture, the activated copy or copies in introgression line RZ35 (Fig. 2c) must be those transposed by alien-DNA introgression. The difference for being activated or not of *Tos17* between the two introgression lines RZ35 and RZ2 might have been due to transposition of the activated *Tos17* into a different chromatin environment following alien-DNA introgression, as will be discussed in the following sections. In introgression line RZ35, activity and inactivity of *Tos17* in calli and plants is accompanied, respectively, by hypo- and hyper-CG methylations and hemi- and full CNG methylations within the element, whereas inactivity of the element in the other two lines is associated with constant hyper-CG methylation and double-stranded CNG-methylation status (Fig. 3c, left panel). It is interesting to note that double-stranded demethylation at the CNG sites did not activate *Tos17* transcription (Fig. 3a, right panel). Although occurrence of strand-specific or semi-cytosine demethylation has been described in cultured plant cells (Zhou et al. 1998) and has been demonstrated as a controlling mechanism for chromatid selectivity of the *Ac*/*Ds* transposons in maize (Ros and Kunze 2001), to our knowledge, our results are the first indication that semi-cytosine demethylation at CNG sites could be correlated with activation of an endogenous retrotransposon. Thus, our results suggest that both CG methylation and CNG methylation might play a regulatory or reinforcing role in repressing *Tos17* in rice. However, the observation that 5-azacytidine treatment did not activate *Tos17* in any line, despite marked CG and CNG demethylation in two lines (Fig. 5), implies that either demethylation alone is insufficient for the element activation, or points to the existence of other cellular controlling factor(s) for activity of *Tos17*, that might act in trans but might also be influenced by 5-azacytidine treatment. This possibility is consistent with previous observations that different transposons or retroelements often respond, in terms of transpositional activation, quite differently to drug-induced demethylation. For example, whereas the *Tad* element in *Neurospora* could be mobilized by 5-azacytidine-induced demethylation (Zhou et al. 2001), the *MAGGY* element of *Magnaporthe grisea* is only transcriptionally responsive to the drug treatment (Nakayashiki et al. 2001).

Notwithstanding strong correlative evidence implicating an association between DNA methylation and a transposon's activity in several well-characterized transposons, such as the *Ac*/*Ds* and *En*/*Spm* of maize (Chandler and Walbot 1986; Schwartz and Dennis 1986; Banks et al. 1988; Ros and Kunze 2001) and the *Tam3* of *Antirrhinum majus* (Kitamura et al. 2001), it was not until recently that a causal connection between the two phenomena was established in *Arabidopsis* (Kato et al. 2003). Specifically, it was found that mutation in a DNA methyltransferase, *METHYLTRANSFERASE 1* (*MET1*) (mainly resulting in CG demethylation, Finnegan and Dennis 1993) causes transcriptional, but not transpositional, activation of a transposon family (*CACTA*), whereas mutation in *CHROMOMETHYLASE 3* (*CMT3*) (specifically causing non-CG demethylation, Lindroth et al. 2001) results in both transcriptional and transpositional activation of the transposon family; moreover, double mutants of both methyltransferases have synergistically increased the element-mobilization frequency, thus indicating that loss of cytosine methylation is sufficient for transposon mobilization, and that non-CG methylation plays a greater role in element repression than CG methylation (Kato et al. 2003). It is not yet known whether retrotransposons could be mobilized in a mutant defective in either or both of the DNA methyltransferases, but transcriptional activation of a retrotransposon family (*Ta3*) was detected in *cmt3* plants (Lindroth et al. 2001). In addition, in *ddm1* plants (with genome-wide hypomethylation), a silenced foreign retrotransposon *Tto1* was reactivated and mobilized, and an endogenous retrotransposon *Tar17* was also transcriptionally activated (Hirochika et al. 2000). This shows that in *Arabidopsis*, cytosine methylation also plays a role in retrotransposon silencing. Nevertheless, because *DDM1* encodes a *SWI2/SNF2*-like chromatin-remodeling factor (Vongs et al. 1993), andhence, its mutation also causes alterations in chromatin structure (Jeddeloh et al. 1999; Singer et al. 2001), it is possible that the role of cytosine methylation in retrotransposon repression is secondary or downstream to that of chromatin remodeling. This possibility is reinforced by the finding that a subset of retrotransposons was also transcriptionally activated, without any detectable demethylation, in *Arabidopsis* plants defective in *MORPHEUS' MOLECULE 1* (*MOM1*, also encoding a putative chromatin-remodeling factor), thus suggesting that in wild-type *Arabidopsis* plants, at least some retrotransposons are repressed by chromatin structure that is independent of DNA methylation (Amedeo et al. 2000).

It is tempting to speculate that activity of *Tos17* might also be primarily controlled by chromatin structure, with CG and CNG methylation serving as a secondary and reinforcing force that might be superimposed on, and further fortify, the repressive chromatin state. According to this scenario, *Tos17*'s differential activity in the three lines could be explained as follows: In introgression line

RZ35, tissue culture could presumably induce both chromatin conformational change [(wherein a copy or copies of the element reside(s)] and cytosine-methylation alteration within the element; the collaborative or synergetic effect of these two epigenetic modifications is sufficient to activate *Tos17* (transcript accumulation to a certain threshold and followed by transposition, Fig. 3). In contrast, 5-azacytidine treatments resulted in demethylation but did not alter chromatin structure and thus, could not activate *Tos17*. The ineffectiveness of tissue culture in activating *Tos17* in the other two lines is likely due to residing of all copies of the element in an "unfavorable" chromatin environment that is incapable of remodeling and/or undergoing cytosine-methylation alterations under tissue-culture conditions. This proposed explanation is consistent with the hypothesis stating that plant tissue culture disrupts default epigenetic controls (Phillips et al. 1994), thus implying a compromised, intrinsic repressive-chromatin state, possibly including cytosine demethylation (Kaeppler et al. 1994) in callus cultures. Consequently, cryptic transposons and retrotransposons, like *Tos17*, could be activated in tissue culture.

Potential impact of *Tos17* mobilization on flanking genomic regions

The most obvious effect of transposon and retrotransposon mobilization is genetic, i.e., insertional mutagenesis on targeted sequences. Thus, it is conceivable that most mobilization events are intrinsically deleterious to the host, particularly if the targeting sites are genic; hence, the opinion that mobile elements are merely genomic parasites, i.e., being selfish or junk components, pervaded before the late 1980s (reviewed in Kidwell and Lisch 2000, 2001). Nevertheless, accumulated evidence over the last two decades strongly suggests that mobile elements could be a major source of genetic diversity in plants and animals (Kidwell and Lisch 1997, 2000), implying that at least some elements' activity could be beneficial to the host. In this regard, the LTR retrotransposons might be particularly noteworthy because, apart from their abundance, many LTR retrotransposons are found to reside in or near normal plant genes, thus underscoring possible roles that LTR retrotransposons might have played in plant-genome evolution (White et al. 1994; Wendel 2000; Wendel and Wesseler 2000). Among the myriad paradigms that an activated retrotransposon might benefit the host is its potential impact on the epigenetic states, such as DNA-methylation patterns of flanking sequences. Moreover, because LTR retrotransposons often contain potent regulatory sequences, their transcriptional activation might alter the activity of nearby genes via read-through or readout transcription, as demonstrated in maize over a decade ago (Barkan and Martienssen 1991) and recently in both animals (Whitelaw and Martin 2001) and plants (Kashkush et al. 2003).

We found in this study that activated (transcriptionally or transpositionally) *Tos17* in introgression line RZ35 has caused heritable alterations in cytosine-methylation patterns of several flanking genomic regions in regenerated plants (e.g., Fig. 6), whereas regions flanking quiescent copies of the element in parental line Matsumae and introgression line RZ2 did not show such changes. Given that the majority of the *Tos17*-flanking regions contain cellular genes, it is possible that in some cases their expression could be altered as a result of heritable cytosine-methylation changes. In this respect, we note that regenerated plants from introgression line RZ35 exhibited multiple phenotypic novelties, including changes in morphology, yield-component traits, and disease resistance, that far exceeded the scope of somaclonal variation in other rice lines where *Tos17* was not activated (unpublished data). It will be interesting to investigate whether any of the phenotypic variations in RZ35 could be attributed to alteration in DNA-methylation patterns caused by activity of *Tos17*.

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