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Epigenetic regulation of the rice retrotransposon *Tos17*

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Abstract Transposable elements are major components of plant genomes. Their activity seems to be epigenetically regulated by gene silencing systems. Here we report epigenetic variation in the retrotransposon *Tos17* activity in rice varieties. Of the two copies of *Tos17* present in chromosome 7 (*Tos17*$^{chr.7}$) and chromosome 10 (*Tos17*$^{chr.10}$), *Tos17*$^{chr.7}$ is strongly activated by tissue culture in most varieties including Nipponbare except for Moritawase, despite the identity of the DNA sequences in Moritawase and Nipponbare. *Tos17*$^{chr.7}$ activity correlated with its methylation status, and *Tos17*$^{chr.7}$ in Moritawase was heavily methylated and activated by treatment of 5-azacytidine (5-azaC), a DNA methylation inhibitor. Although the original copies of *Tos17* are methylated to some extent in all varieties examined, the transposed copies in calli mostly are not methylated. When plants were regenerated from calli, the degree of methylation of the *Tos17* DNA increased gradually with the growth of plants, and a significant progress of DNA methylation occurred in the next generation after a completed reproductive cycle. With increasing DNA methylation, the transcription of transposed and original *Tos17* copies driven by its own as well as by a flanking gene promoter were suppressed. We conclude that *Tos17* DNA methylation controls the transpositional activity of *Tos17*, and modulates the activity of neighboring genes. Based on the analysis of the inactive *Tos17*$^{chr.10}$, we propose that another mechanism, called transcriptional interference, is involved in the control of *Tos17* activity.

Keywords Retrotransposon · Rice · Epigenetic regulation · DNA methylation · Tissue culture

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Introduction

Transposable elements (TEs), including DNA-type transposons (also called class II elements) and retrotransposons (class I elements), are widespread constituents of eukaryote genomes. DNA-type transposons carry terminal inverted repeats (TIRs), and are transposed by a transposase encoded by the transposon itself that catalyzes excision and re-insertion ("cut-and-paste" mode) (for reviews, see Feschotte et al. 2002). In contrast, retrotransposons are transposed via an RNA intermediate, and either have long terminal repeats (LTR retrotransposons) or not (LINE or SINE elements). The transposition mechanism of LTR retrotransposons resembles that of retroviruses, where an RNA intermediate is transcribed from the enhancer-

promoter LTRs. The resulting RNA is copied into a double-stranded DNA molecule and subsequently re-integrated into the genome (Kumar and Bennetzen 1999; Hirochika et al. 1996a). The LTR retrotransposons amplify themselves by this "copy-and-paste" mechanism successfully and became major components of some plant genomes (Meyers et al. 2001; Mccarthy et al. 2002). Unlike in plants, the LINEs and SINEs are the predominant transposable elements in mammals including human (International human genome sequencing consortium 2001). Considering their wide distribution and the types of induced mutations, the TEs are thought to have contributed significantly to the evolution of eukaryotic genes and genomes (for reviews, see Feschotte et al. 2002; Kazazian 2004).

However, the immediate consequences of TE transposition are often deleterious, because their insertion into genes will disrupt gene function, and their insertion in the vicinity of genes is likely to interfere with the regulation of the genes. Therefore, it is crucial for the host organisms to control TE activity. First insights into the regulation of TEs have been obtained through the analysis of the natural epigenetic variation of TE activity. For example, *MuDR* elements (autonomous *Mu* elements) in maize can cycle between active and inactive states (Martienssen 1996). The inactive form of *Spm* elements in maize can be reactivated by exposure to active elements (Fedoroff 1996). To date, several lines of evidence suggest that the gene-silencing machinery, including transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), plays an important role in controlling TE activity (for review, see Okamoto and Hirochika 2001; Feschotte et al. 2002). The TGS is caused by a blockage of transcription and is often associated with DNA methylation and chromatin modification. Genome-wide analyses have shown that the TEs are specifically methylated in plant genomes, in contrast to mammals where most exons are methylated as well (Rabinowicz et al. 2003). In methylation-defective mutants of *Arabidopsis thaliana* such as *met1*, *cmt3* and *ddm1*, silenced TEs were transcriptionally and/or transpositionally reactivated (Hirochika et al. 2000; Miura et al. 2001; Kato et al. 2003). These results indicate a key role of DNA methylation in the control of TE activity. PTGS is caused by a post-transcriptional RNA degradation, and involves a double-stranded (ds) RNA that is dissected by an RNase III-like enzyme called Dicer into short interfering RNAs (siRNA), 21–25 nt in length. Suppression of the TEs by PTGS was demonstrated in the *Tc1* transposon of *Caenorhabditis elegans* (Sijen and Plasterk 2003) and *MuDR* of maize recently (Slotkin et al. 2005). In the two cases, a readthrough transcript from an adjacent host gene produces a dsRNA which is subsequently cleaved to produce siRNA, leading to destruction of the *Tc1* or *MuDR* RNA. Presence of siRNA corresponding to TE sequences has been detected in many TEs (Hamilton et al. 2002; Lippman et al. 2004), suggesting the suppression of TEs by PTGS may be a general phenomenon. Recent evidence suggests that the siRNA plays an important role in directing chromatin modification and DNA methylation, suggesting that TGS and PTGS are linked by a common mechanism (Hamilton et al. 2002; Zilberman et al. 2003; Chan et al. 2004).

An interesting feature of the TEs is their responsiveness to genetic and environmental cues. Tissue culture-induced activation has been found in two plant LTR retrotransposons, *Tto 1* in tobacco (Hirochika 1993; Hirochika et al. 2000) and *Tos17* in rice (Hirochika et al. 1996b), and in the MITE element *mPing* in rice (anther culture, Kikuchi et al. 2003). A LINE element in rice, *Karma*, was transcriptionally activated in cultured cell and transposition occurred in the second and later generations of regenerated plants (Komatsu et al. 2003). In addition to tissue culture, *Tto1* and the tobacco retrotransposon *Tnt1* are activated transcriptionally by wounding and by treatments with jasmonate and fungal elicitors which induce defense responses (Melayah et al. 2001; Takeda et al. 1998, 1999). Moreover, gamma-ray irradiation has been suggested to activate *mPing* (Jiang et al. 2003; Nakazaki et al. 2003). Similarly, transposition of the human SINE element *Alu* was induced in mouse cells by exposure to the topoisomerase II inhibitor etoposide, a toxic agent that inhibits religation of topoisomerase II cleavage products which results in double strand breaks (Hagan et al. 2003). In *Saccharomyces cerevisiae*, transposition of the LTR-retrotransposon *Ty1* was stimulated at the level of *Ty1* cDNA synthesis when DNA lesions were created by a loss of telomere function (Scholes et al. 2003). These examples support the genome shock theory proposed first by McClintock (1984), according to which the activation of transposable elements could be a response to challenges to the genome. However, while it is mostly unclear how the organisms sense the stress and activate TEs, the mechanisms probably are different for different TEs (Capy et al. 2000). For *Tto1*, the MYB-related transcription factor NtMYB2, which is induced by wounding and elicitors, has been identified as a positive regulator (Sugimoto et al. 2000).

As another feature reflecting the symbiosis of TEs and host genome, the TEs will regulate the expression of nearby genes. The regulation could be due to *cis*-elements carried by the TEs. For example, the human *L1* (LINE element) inhibited transcriptional elongation when inserted into a gene, which may be due to its

unusual AT-rich composition (Han et al. 2004). In Drosophila, insertions of the transposon *P*-element into the 5′-UTR of the *nup154* gene were lethal or viable, depending on the orientation of the insert. An outward-directed promoter within the *P*-element's 3′ end was found to be responsible for expression of *nup154* in viable animals (Kiger et al. 1999). Similarly, LTRs of retrotransposons under transcriptional activation can drive the expression of adjacent genes, as has been demonstrated in wheat (Kashkush et al. 2003). However, regulation of genes by nearby TEs is frequently not due to direct effects of special *cis*-elements carried by the TEs, but to an epigenetic regulation (Martienssen 1998, 2001). For example, the *Mu* element controls the transcription of neighboring genes, probably via its own methylation status (Barkan and Martienssen 1991). Recently, the TEs were shown to play a general role in DNA methylation and/or chromatin modification in *A. thaliana* (Lippman et al. 2004), which is probably directed by siRNA of TEs. Since siRNA-directed DNA methylation and/or chromatin modification seem not to require that the TEs affected are intact or carry any special *cis*-elements, epigenetic regulation by TEs may be a common phenomenon, given the large number of defective TEs in eukaryotic genomes (Lippman et al. 2004).

The rice *Tos17* is one of the few active LTR-retrotransposons in plants. It is normally inactive, but becomes activated under tissue culture conditions (Hirochika et al. 1996b). Unlike most other plant TEs, *Tos17* is present in only one to five copies in the rice genome, depending on the cultivar. Nipponbare, the standard cultivar for the international rice sequencing project, carries two copies. After 5 months of tissue culture, ten transposed copies on average are observed in regenerated plants. Analysis of insertion sites showed that insertion events are three times more frequent in genic than in intergenic regions (Miyao et al. 2003). These features have turned *Tos17* into a powerful tool for the functional analysis of rice genes by gene disruption (Miyao et al. 2003; Hirochika et al. 2004). However, little is known about the regulation of *Tos17* activity except that its transcription and transposition appear to be correlated (Hirochika et al. 1996b).

In this study, we investigated the presence and mobilization of *Tos17* in 11 *japonica* varieties including Nipponbare. All varieties examined carried at least one copy of *Tos17*. Epigenetic variation of *Tos17* activity was found among varieties. Transposed copies were only weakly methylated in calli. When plants were regenerated from the calli, *Tos17* methylation progressed gradually with growth of plants, and a significant progress of DNA methylation was observed after a passage of a reproductive process. With the progress of DNA methylation, the transcription of *Tos17* driven by its own promoter or its flanking gene promoter were suppressed to a variable extent. Here, we discuss the role of DNA methylation in activity control and insertion target-site preference of *Tos17*, as well as the regulation of neighboring genes.

Materials and methods

Plant material

The seeds of the varieties used for callus induction were supplied by the Gene Bank of the National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan. Regenerated R_1 seeds used for callus induction and DNA methylation analysis were supplied by Dr. Akio Miyao, NIAS.

Tissue culture

Callus induction and culture were performed essentially as described previously (Miyao et al. 2003). R_0 plants of Nipponbare were regenerated from 4 months cultured calli. Treatment of Moritawase calli by 5-azaC is done as follows: 2 months cultured calli were transferred to the media containing 5-azaC and cultured for 3 (120 mg/l 5-azaC) to 6 (24 mg/l 5-azaC) weeks, then cultured for total 5 months in 5-azaC-free media. Except for the prolonged culture for Koshihikari (24 months), all other calli described in this work are cultured for 8 months.

Southern and Northern blot analysis

Total DNA was extracted as described before (Miyao et al. 2003). DNA samples quantifying 500 ng from calli or plant tissues were digested with the restriction enzymes *Pst*I and/or *Xba*I, and then separated on 0.8% (w/v) agarose gels, and transferred to Hybond N+ membranes (Amersham, Little Chalfont, UK) using the MULTIBLOTTER MB24 system (Labimap, Plaisir, France).

Total RNA of the calli was isolated with the Isogen kit (Nippon Gene, Toyama, Japan), and 10 μg of total RNA was electrophoresed on 1.2% (w/v) agarose gels and transferred to Hybond N+ membranes.

Probes were made by PCR amplification and purified with the QIAquick Gel Extraction Kit (QIAGEN, USA). Transferred membranes described earlier were hybridized with the ^{32}P-labeled probe as described previously (Miyao et al. 2003).

DNA methylation analysis

The methylation status of the genomic DNA was determined by Southern analysis using the methylation-sensitive restriction enzyme *Hpa*II. For the methylation-sensitive PCR, 25 ng samples of either digested or undigested DNA in 20 µl reaction mixture were amplified with LA Taq polymerase (TAKARA, Kyoto, Japan), and 25 cycles (96°C for 20 s, 60°C for 30 s, 72°C for 2 min) were run. Primers used for PCR (Figs. 2b, 3a) are: P1: GATGAACATGAGGCAATACC; P2: TAGTTGGCCCATGTCCAGC; P3: AAGTAGGAGTACTTGGCACC; P4: GATTGGCAAGTCCACAGCAC; P5: CATCCCTTGCTGCTTACTGTC; P6: GATGCTTGGTGAGAACGGAAC; P7: ATGCTCCAACTATCGAGTGC.

For methylation-sensitive PCR for newly amplified *Tos17* copies**,** flanking sequences of newly transposed *Tos17* copies were first isolated from a callus clone by TAIL-PCR as detailed before (Miyao et al. 2003). The same DNA samples were then digested with *Hpa*II, and PCR was carried out using a *Tos17* primer (TCGACATCTTGTGGGTAGCC) and a flanking primer of the particular copy of *Tos17*. These flanking primers were TGTTCGTGCTGCATGCCGTGT for *Tos17*$^{chr.7}$, AGGCTGTACAGAGGGGAGT for *Tos17*$^{chr.10}$, TCTGCCTTCCTGCAGCGAG for new copy 1, AGCACCACTGTTCGCTGCC for new copy 2, and CATGCTGCATACCATCTGCC for new copy 3 (see the diagram on Fig. 2c). DNA methylation-sensitive PCR for *Tos17* in the 5AzaC-treated Moritawase calli was done similarly except the primers P1, P3 and P4 were used.

Results

Numbers of *Tos17* copies in *japonica* varieties

The rice varieties Koshihikari, Nipponbare, and C5924 carry one, two, and three copies of *Tos17*, respectively (Hirochika et al. 1996b). The first two varieties belong to the subspecies japonica, whereas the latter one belongs to the subspecies *indica*, suggesting that *Tos17* is distributed throughout the species *Oryza sativa*. To study *Tos17* activity in the varieties other than Nipponbare, we first investigated the copy number of *Tos17* in eleven japonica varieties including Nipponbare as a control. Database search using the published *Tos17* reverse transcriptase (RT) as a query revealed that the two copies of *Tos17* in Nipponbare are located on chromosomes 7 [*Tos17*$^{chr.7}$, AP005292 (nt 186486–190599) in GenBank] and 10 [*Tos17*$^{chr.10}$, AE017097 (nt 118778–122981)], respectively. Both copies include one open reading frame (ORF) and two identical LTRs, suggesting that both of them are intact (Fig. 1a). However, the two copies differ in a 90 bp insertion present only in *Tos17*$^{chr.10}$, and in seven base changes. Two of the changed bases are located in the LTR regions, while the others are in the coding region. The base changes in nt 1799 and nt 3398 cause an amino acid change (Fig. 1a).

Southern blotting revealed that one to four copies of *Tos17* were present in other varieties (Fig. 1b, upper panel). Because the sequences of the two *Tos17* copies and their flanking regions from Nipponbare were known, the two corresponding bands in the Southern blot images could be identified (Fig. 1b, upper panel). Accordingly, four varieties were found to carry only the *Tos17*$^{chr.10}$ copy, while four others carried both *Tos17*$^{chr.10}$ and *Tos17*$^{chr.7}$. In one of the latter, Moritawase, the *Tos17*$^{chr.7}$ signal was much stronger than the *Tos17*$^{chr.10}$ signal. Using a different restriction endonuclease *Eco*RI, we found that Moritawase carries three copies of *Tos17* (data not shown). The varieties Norin6 and Rikuu132 carried one or two additional copies, which was clearly visible in the standard experiment (Fig. 1b). The presence or absence of *Tos17*$^{chr.7}$ and *Tos17*$^{chr.10}$ in all varieties was further confirmed by polymerase chain reaction (PCR; data not shown).

Variation of *Tos17* activity in rice varieties

Although it has been demonstrated that tissue culture activates *Tos17* in Nipponbare, it is unclear whether both copies are active or not. It is also not known whether the variation in copy number between rice varieties leads to a variation in *Tos17* activity, similarly as in the copy number-dependent silencing of the tobacco retrotransposon *Tto1* (Hirochika et al. 2000). To address these questions, we carried out Northern blot analysis of calli after 8 months of culture, to survey the transcription of *Tos17* in the 11 varieties. Consistent with previous results, a *Tos17* probe (probe 2; Fig. 1a) identified two species (4 and 7 kb) of transcripts in Nipponbare (Fig. 1c, upper panel). The 4 kb RNA had been shown previously to be the full length transcript of *Tos17* (Hirochika et al. 1996b). To clarify the nature of the 7 kb transcript, we searched the rice full-length cDNA database (http://www.cdna01.dna.affrc.go.jp/cDNA/) using *Tos17* as a query. One clone, AK106572, had the *Tos17* sequence in its 5′ part and the flanking sequence of *Tos17*$^{chr.10}$ in its 3′ region (Fig. 1c). Hybridization with a probe corresponding to the flanking region of *Tos17*$^{chr.10}$ (probe 3, Fig. 1c) identified a transcript with the same size as the 7 kb RNA detected by the *Tos17* probe (Fig. 1c, middle

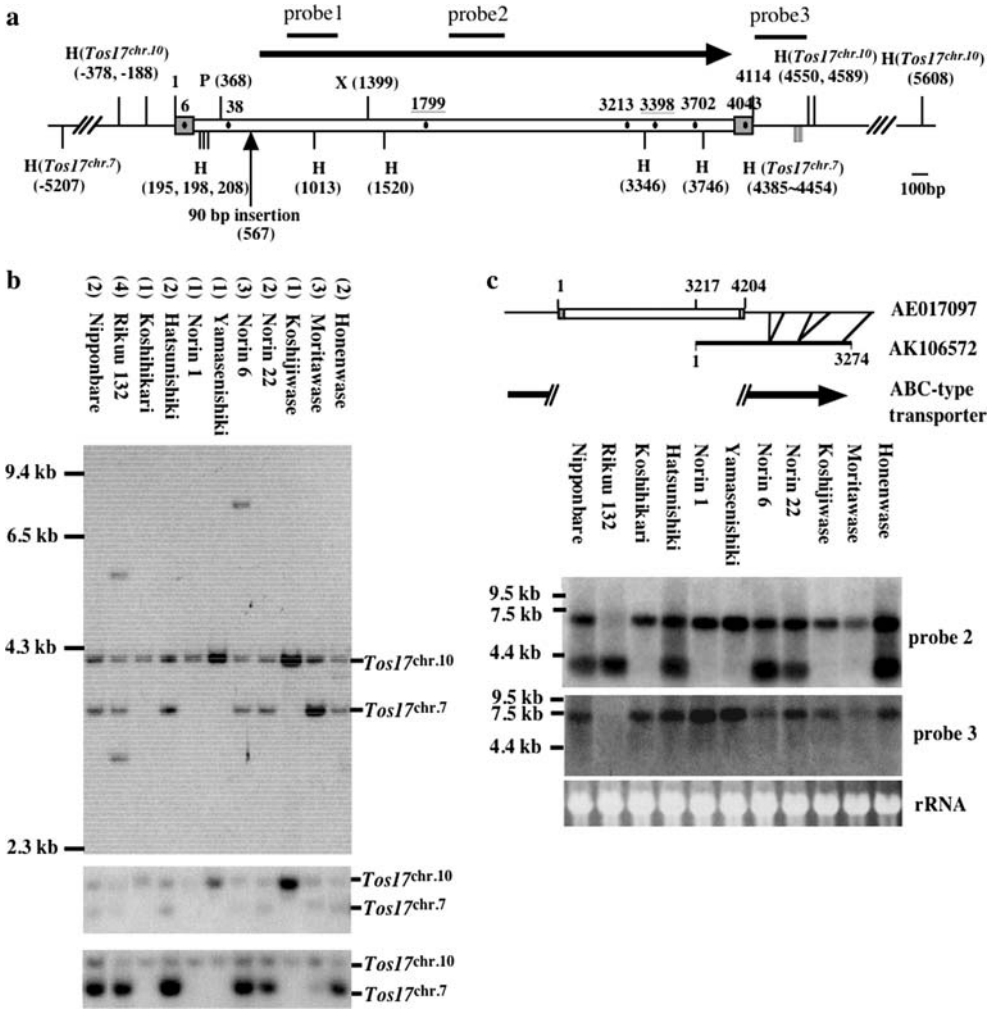

Fig. 1 Structue and activity of different copies of *Tos17* in different rice varieties. **a** *Tos17* structure. *Boxes* represent the *Tos17* sequence and *lines* represent the flanking regions. The LTRs are shown by *two grey boxes* at the borders of *Tos17*. *Tos17* is depicted based on the structure of *Tos17*$^{chr.7}$, and the bases that differ between *Tos17*$^{chr.7}$ and *Tos17*$^{chr.10}$ are indicated by *closed circles* and the corresponding *base number*. The base changes at positions 1799 and 3398 that alter amino acids are *underlined*. The 90 bp insertion in *Tos17*$^{chr.10}$ is indicated by an *arrow*. The *long bold arrow* represents the ORF of *Tos17*. Restriction sites of *Hpa*II (H), *Pst*I (P), and *Xba*I (X), and positions of probes 1, 2, and 3 are also shown. *Hpa*II sites on flanking regions of *Tos17* are shown on the *top* (*Tos17*$^{chr.10}$) or *bottom* (*Tos17*$^{chr.7}$) of the diagram. **b** Southern blot analysis of *Tos17* copy number and transposition in 11 rice varieties. DNA samples from plant tissues (*upper and middle panel*) or calli (*lower panel*) were digested with *Pst*I and *Xba*I, and were hybridized with probe 1 (*middle and lower panel*) or probe 2 (*upper panel*). Copy numbers of *Tos17* are indicated with the variety names. Positions of signals from *Tos17*$^{chr.7}$ or *Tos17*$^{chr.10}$ are indicated on the right. **c** Northern blot analysis of *Tos17* transcription in 11 rice varieties. RNA samples were prepared from calli, and hybridized with probe 2 (*upper panel*) or probe 3 (*lower panel*). Equal loading of RNA samples is shown by the quantity of rRNA stained with ethidium bromide (*lower panel*). The diagram on *top* shows the full-length cDNA clone AK106572 with the *Tos17* sequence in the 5′ part and the flanking sequence of *Tos17*$^{chr.10}$ in the 3′ region, and the neighboring putative ABC-type transporter gene

panel). Thus, the 7 kb transcript represented the readthrough transcript of *Tos17*$^{chr.10}$. Homology search showed that the flanking sequence of *Tos17*$^{chr.10}$ encodes an ABC transporter-like protein, suggesting that the promoter of this gene may be responsible for the readthrough transcription (Fig. 1c).

Although at least one copy of *Tos17* exists in each of the 11 varieties examined, the 4 kb RNA corresponding to the *Tos17* transcript was found only in varieties carrying the *Tos17*$^{chr.7}$ copy (Fig. 1c, upper panel). Therefore, it appears likely that *Tos17*$^{chr.7}$ is activated by tissue culture, while *Tos17*$^{chr.10}$ is transcribed constantly by the promoter of the ABC transporter-like gene. In fact, the readthrough transcript of *Tos17*$^{chr.10}$ had been found previously in rice leaf tissue (Hirochika et al. 1996b). Intriguingly, the transcription of *Tos17*$^{chr.7}$ in Moritawase and the readthrough transcription of *Tos17*$^{chr.10}$ in Rikuu132 are not identified.

To further characterize the transposition of *Tos17*, we carried out Southern blot analysis using two endo-

nucleases that cut *Tos17* (*Pst*I and *Xba*I) and a probe (probe 1) hybridizing to the cut fragments (see Fig. 1a). Transposition of *Tos17* can be monitored using the signal strength in Southern blot images, since transposition leads to the increased copy number. The signals derived from *Tos17*$^{chr.7}$ and *Tos17*$^{chr.10}$ can be distinguished by the size difference of 90 bp, because the segments cut by the two endonucleases span the 90 bp insertion. For all varieties carrying both copies of *Tos17* except Moritawase, the signal strength of *Tos17*$^{chr.7}$ was greatly increased after 8 months of tissue culture, while that of *Tos17*$^{chr.10}$ was not significantly changed compared to samples from plant tissue (Fig. 1c; compare the middle and lower panel). Similarly, no tissue culture-dependent effects were detected in varieties carrying only *Tos17*$^{chr.10}$. Together with the Northern blot analysis, these results indicate that *Tos17*$^{chr.7}$ was activated by tissue culture in all varieties except Moritawase, while *Tos17*$^{chr.10}$ remained unaffected. Resequencing of *Tos17*$^{chr.7}$ in Moritawase showed that it was identical to its counterpart in Nipponbare (data not shown), suggesting that epigenetic variation rather than sequence variation led to the silencing of *Tos17*$^{chr.7}$ in Moritawase.

Newly transposed copies are unmethylated in calli

The perfect correlation observed between transcriptional and transpositional activities suggested that *Tos17* activity was controlled at the transcription level. Because DNA methylation is often associated with transcriptional silencing of TEs, we studied the methylation status of *Tos17* in all varieties by Southern blot analysis using the methylation-sensitive endonuclease *Hpa*II. *Hpa*II cuts the CCGG site only when both Cs are not methylated, so that if *Tos17* is not methylated and cut by *Hpa*II, a band of 1.8 kb should appear. In plant tissues, only larger bands (over 4 kb) appeared in all varieties, indicating *Tos17* copies in plant tissues are methylated (Fig. 2a). From the location of *Hpa*II sites (see Fig. 1a) and by a methylation sensitive PCR (Fig. 2b), the ~4 kb band in varieties, such as Nipponbare, was speculated to be derived from *Hpa*II cut at nt (195~208) (3 sites) and at a downstream site of *Tos17*$^{chr.7}$ [nt (4385~4454), 8 sites], the ~5.5 kb band was from *Hpa*II cut at nt (195~208) and at a downstream site (nt 5608) of *Tos17*$^{chr.10}$, while the ~6.5 kb band in the varieties such as Koshihikari must be from *Hpa*II cut at nt (195~208) and at a site further downstream of *Tos17*$^{chr.10}$. Rikuu132 and Moritawase, which have higher copy numbers (4 and 3 copies, respectively) than other varieties, produced bands with larger size (Fig. 2a). Corresponding to this observation, methylation sensitive PCR showed that the site at nt (195~208), which was cut by *Hpa*II in most of varieties, was not cut in the two varieties (Fig. 2b, note the same quantity of PCR product in the *Hpa*II digested and undigested samples). These results showed that although *Tos17* copies in plant tissues are methylated in all varieties, they are more densely methylated in Rikuu132 and Moritawase. In calli, Nipponbare, and five other varieties which had been shown to carry the active *Tos17*$^{chr.7}$ copy produced a pronounced band of 1.8 kb besides these large size bands. This 1.8 kb band corresponds to the fragment between the two *Hpa*II sites located at nt 1520 and nt 3346, respectively (Fig. 1a).

A possible explanation for the occurrence of the 1.8 kb band was that the original copies of *Tos17* were demethylated during tissue culture, causing an activation of *Tos17*. If this would be the case, the *Tos17* bands should become weaker in those varieties that accumulated the 1.8 kb band, because similar quantities of DNA were loaded onto all gels for Southern blotting. However, the density of the main *Tos17* bands did not change in accordance with the occurrence of the 1.8 kb band (Fig. 2a). Therefore, the 1.8 kb band probably was derived from newly transposed copies and not from the original copies of *Tos17*. Extrachromosomal copies of *Tos17* were not detected by Southern blotting of undigested DNA samples, indicating that all the *Tos17* copies are integrated into the genome.

To determine whether newly transposed copies of *Tos17* were not methylated in calli, we isolated the flanking sequences of several new copies from a Nipponbare callus clone. The methylation status of individual copies was studied by the methylation sensitive PCR (Fig. 2c). Because *Hpa*II sites are present only in *Tos17*, the quantity of PCR product should correlate with the degree of methylation of the *Tos17* sequence. For the original two copies, similar quantities of PCR products were found in *Hpa*II-digested and -undigested samples, irrespective of the origin of the DNA samples. However, for the newly transposed copies, the PCR yielded no products at all after *Hpa*II digestion. These results confirmed that the original copies, whether in plants or in callus cultures, were methylated, whereas newly transposed copies were not. In the process of transposition of the retrotransposons, the newly synthesized cDNA must be unmethylated, and DNA methylation occurs some time after the integration of the cDNA into the genome. In consequence, the low methylation status of new copies of *Tos17* indicates that *Tos17*-directed methylation activity must be very low in callus cells.

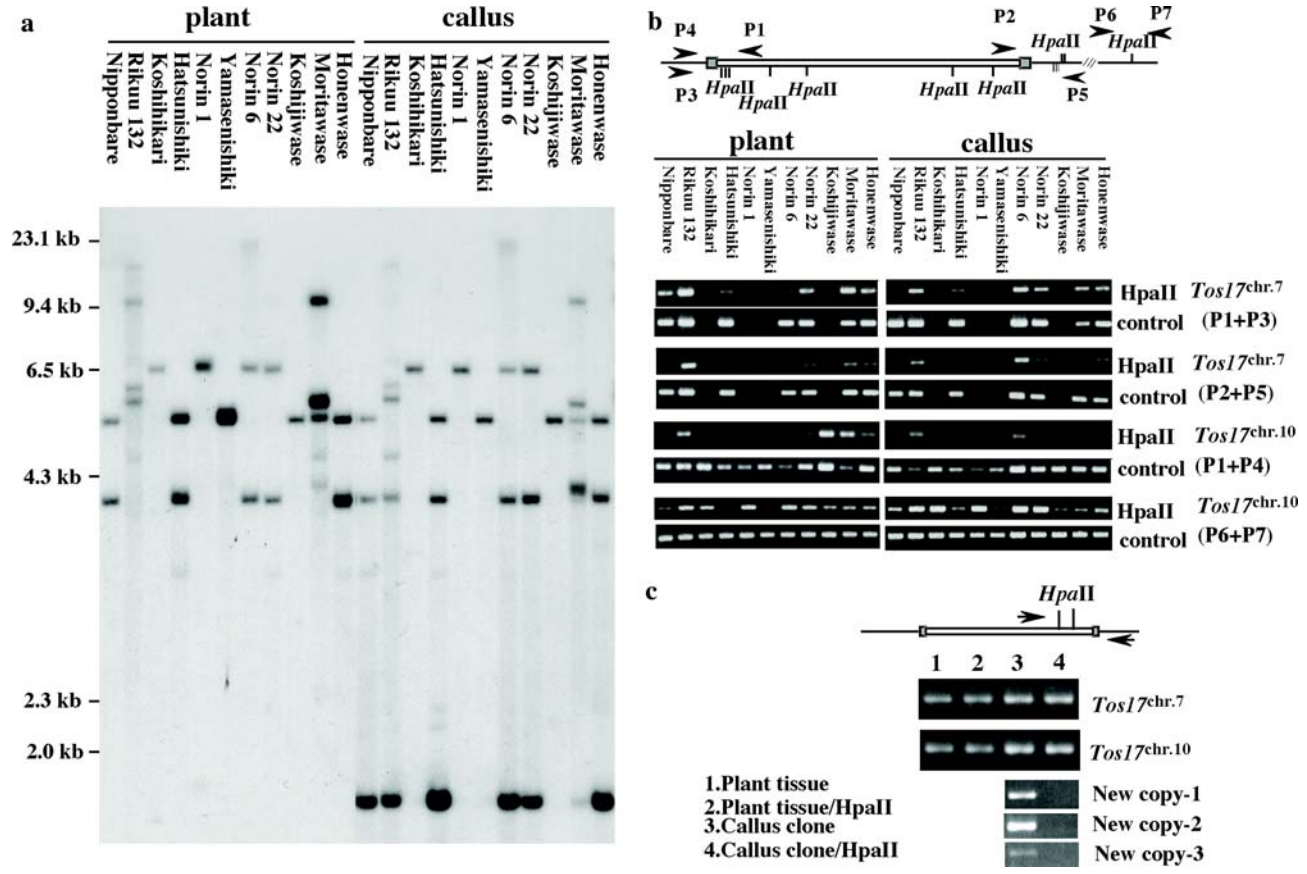

Fig. 2 DNA methylation in plant tissues and calli from 11 rice varieties. **a** Southern blot analysis of DNA methylation of *Tos17*. DNA samples from plant tissues or calli were digested with *Hpa*II and were hybridized with probe 2. **b** Methylation-sensitive PCR for 11 varieties. DNA samples from plant tissues or calli were digested or undigested (control) with *Hpa*II before PCR. P1 and P2 are *Tos17* primers, P3 and P5 are primers hybridizing to the *Tos17*$^{chr.7}$ flanking region, P4, P6 and P7 are primers hybridizing to the *Tos17*$^{chr.10}$ flanking region. *Hpa*II sites between P1and P3 (P4) correspond to nt 195∼198 in Fig. 1a, and that between P2 and P5 are at nt 4385∼4454, p6 and P7 are at nt5608. Note less PCR products in *Hpa*II digested samples for most of the varieties. **c** Methylation-sensitive PCR for newly transposed copies of *Tos17* in calli. DNA samples from plant tissues or calli of Nipponbare were digested with *Hpa*II (*lane 2 and 4*) or not (*lane 1 and 3*) before PCR. *Hpa*II sites and primer positions are shown on the *diagram*

Except for the 1.8 kb fragment derived from newly transposed *Tos17* copies, no significant differences in the band patterns were found between callus and plant samples from most varieties. Only in calli of Rikuu132 and Moritawase, which seem to have more densely methylated DNA *in planta*, a band shift to lower molecular weights was observed, indicating that tissue culture induced demethylation of the original *Tos17* copies (Fig. 2a). The effect was more pronounced in Rikuu132 than in Moritawase. Notably, a ∼4.0 kb band, which was derived from a *Hpa*II cut at nt (195∼208) in *Tos17*$^{chr.7}$ in varieties, such as Nipponbare, appeared in calli of Rikuu132. Methylation sensitive PCR showed that *Hpa*II sites at nt (195∼208) in *Tos17*$^{chr.7}$ was cut in Rikuu132 calli (Fig. 2b, note the decrease of PCR product in calli after *Hpa*II digestion), indicating demethylation occurred in Rikku132 under tissue culture. Calli of Moritawase, in which *Tos17*$^{chr.7}$ has been shown to be inactive (see Fig. 1c), produced only a very weak 4 kb band and a corresponding very weak signal of the suggestive 1.8 kb band from transposed copies.

Activation of *Tos17* by 5-azacytidine treatment and prolonged culture

The inactive *Tos17*$^{chr.7}$ in Moritawase has identical sequence to its active counterpart in Nipponbare, but has higher level of DNA methylation than those in other varieties, suggesting that *Tos17*$^{chr.7}$ in Moritawase is subjected to DNA methylation-related suppression. To confirm this hypothesis, we treated the Moritawase calli with 5-azaC, a DNA methylation inhibitor, with different concentrations and durations. DNA methylation sensitive PCR showed that the methylation in both the *Tos17*$^{chr.7}$ and *Tos17*$^{chr.10}$ is

decreased (Fig. 3a). Northern blot showed that both of the *Tos17* transcripts and the readthrough transcripts were increased in all the treated samples, indicating that both *bona fide* transcription and the readthrough transcription of *Tos17* are activated (Fig. 3b). In order to examine whether *Tos17* did transpose in the 5-azaC treated samples, the change of *Tos17* copy number was monitored by simply comparing *Tos17* signal strength in Southern image. As shown in Fig. 3c, the signal corresponding to the *Tos17*$^{chr.7}$ is increased in the 5-azaC treated samples, but that of *Tos17*$^{chr.10}$ did not change, indicating that *Tos17*$^{chr.7}$ but not *Tos17*$^{chr.10}$ transposed after 5-azaC treatment. These results indicated that *Tos17*$^{chr.7}$ in Moritawase is subjected to the DNA methylation-related epigenetic suppression, but *Tos17*$^{chr.10}$ is not.

All the above analysis indicated that *Tos17*$^{chr.10}$ is an inactive copy. Sequence variation of *Tos17*$^{chr.10}$ from *Tos17*$^{chr.7}$ suggests that *Tos17*$^{chr.10}$ may be a structurally dead copy. However, it is also possible that *Tos17*$^{chr.10}$ may be under an epigenetic control, which is different from DNA methylation. To distinguish the two possibilities, 24-month-old cultures of Koshihikari were investigated to assess the activity of *Tos17*$^{chr.10}$. As newly transposed *Tos17* copies are not methylated, *Tos17* activity can be evaluated simply by *Hpa*II digestion and subsequent Southern blot analysis. As shown in Fig. 3d (2nd lane from the left), an unmethylated DNA band absent from plant tissue preparations was detected, indicating that *Tos17*$^{chr.10}$ had low activity. Investigation of individual clones derived from the 24-month-old culture revealed that strong activity of *Tos17*$^{chr.10}$ is observed in some clones, but no activity in others, indicating that *Tos17*$^{chr.10}$ activity is not consistently increased under the prolonged culture. In contrast, this variation pattern among clones suggested

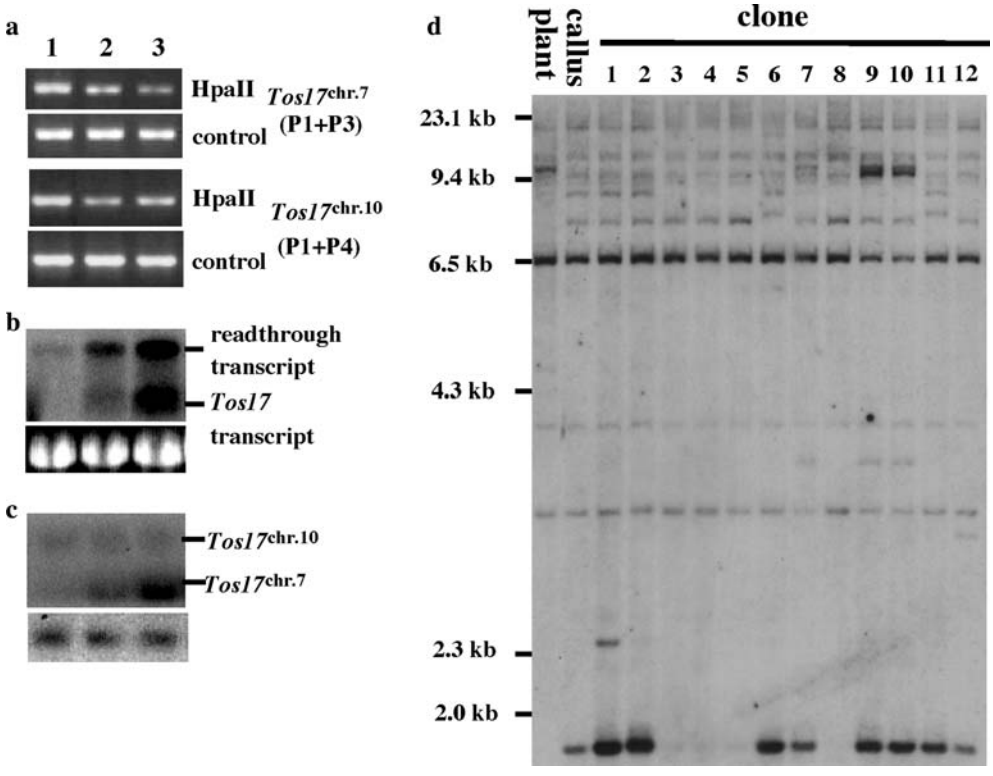

Fig. 3 Activation of *Tos17*$^{chr.7}$ by 5-azaC treatment and *Tos17*$^{chr.10}$ by prolonged culture. **a** Effect of 5-azaC treatment on DNA demethylation of *Tos17*. Genomic DNA was prepared from 5-azaC- treated or -untreated Moritawase calli and digested with *Hpa*II followed by PCR amplification. *Hpa*II sites and primers for PCR were same as Fig. 2b. *Lanes 1-3* Untreated, treated with 120 mg/l for 3 weeks and 24 mg/l of 5-azaC for 6 weeks, respectively. **b** Transcription of *Tos17* in 5-azaC treated calli of Moritawase. Total RNA from 5-azaC treated or untreated calli were hybridized with probe 2. Positions of the *Tos17*$^{chr.7}$ transcript or the readthrough transcript of *Tos17*$^{chr.10}$ are indicated on the *right*. Equal loading of RNA sample is shown by the quantity of rRNA stained with ethidium bromide (*lower panel*). *Lanes 1-3* RNA samples from calli described in **a** were used. **c** Southern blot analysis to show transposition of *Tos17*$^{chr.7}$ in 5-azaC treated calli of Moritawase. DNA was digested with *Pst*I and *Xba*I, and hybridized with probe 1. Equal loading of DNA samples is shown by hybridizing the same membrane with probe 2 (*lower panel*). Positions of the signal from *Tos17*$^{chr.7}$ or *Tos17*$^{chr.10}$ are indicated on the *right*. *Lanes 1-3* DNA samples from calli described in **a** were used. **d** Transposition of *Tos17*$^{chr.10}$ in Koshihikari calli cultured for 24 months. DNA samples from plant tissues, total calli, and cloned calli (1~12) were digested with *Hpa*II and were hybridized with probe 2

that the low activity of *Tos17*$^{chr.10}$ is due to an epigenetic suppression: once a new copy is transposed from the original *Tos17*$^{chr.10}$ in one cell stochastically, the epigenetic suppression is released, leading to the burst of transposition. Therefore, we proposed that *Tos17*$^{chr.10}$ is under an epigenetic suppression different from DNA methylation.

Progressive DNA methylation and the suppression of *Tos17*

Having established that newly transposed copies of *Tos17* in calli are unmethylated, we were interested in the methylation status of these copies in plants regenerated from calli. We compared the methylation status of *Tos17* in calli, regenerated plants (R_0), and the progeny of regenerated plants (R_1) of Nipponbare by Southern blot analysis using the methylation sensitive *Hpa*II. The copy number of *Tos17* in each sample was examined by using the methylation insensitive endonuclease *Xba*I. To detect transposed copies individually, cloned calli were used (Hirochika et al. 2000). Over ten newly transposed copies were detected in each clone; most were unmethylated as indicated by the strong 1.8 kb bands (Fig. 4a, *Hpa*II treatment). In R_0 seedling plants, the number of bands of higher molecular weights increased compared to the callus clones, although the 1.8 kb band corresponding to unmethylated *Tos17* was still present in a strong signal (Fig. 4b). The unmethylated bands became weaker when the plant growth went into reproductive stage (Fig. 4b, flag leaf and young panicle). To investigate whether DNA methylation further progresses during prolonged vegetative growth, the rice stem was cut after the R_0 plant matured, ratoon plants induced were grown for 2 months, and then leaves were sampled for DNA methylation analysis. As shown in Fig. 4b, the 1.8 kb band corresponding to unmethylated *Tos17* became further weaker in the ratoon plants. These results indicate that during the regeneration process, transposed copies became methylated, and the methylation progressed with vegetative growth of plants. However, most of the bands derived from new copies in all the R_0 plant tissue are smaller than original *Tos17*$^{chr.7}$ copies, indicating that the new copies of *Tos17* are not densely methylated.

In the seven R_1 plants derived from two R_0 parental lines (Lines 1 and 2), the 1.8 kb bands corresponding to unmethylated *Tos17* copies disappeared completely, and many bands larger than the original *Tos17*$^{chr.7}$ copies appeared, indicating that the new *Tos17* copies became more densely methylated (Fig. 4c, *Hpa*II treatment). Furthermore, of the two original copies, *Tos17*$^{chr.7}$ in one R_1 plant (No.2 of Line 1) and *Tos17*$^{chr.10}$ in other two R_1 plants (No. 1 and No. 2 of Line 2) disappeared or was reduced in intensity, suggesting that the methylation of the original copies also had progressed. Thus, the germ-line passage dramatically increased the degree of DNA methylation in both new and original copies of *Tos17*.

Increased DNA methylation of *Tto1* is correlated with a silencing of the transposon when it is transformed into the *A. thaliana* genome (Hirochika et al. 2000). We investigated the relationship between the progress of DNA methylation and the activity of the endogenous *Tos17*. Because *Tos17* became more densely methylated in the R_1 generation, the transcriptional and transpositional activity of *Tos17* was assessed in R_1 seed-derived calli. The transposon was transcribed in both of the R_1 cultures derived from Line 1 (Fig. 4d), but no readthrough transcription could be detected in one of them. One of the two R_1 cultures that originated from Line 2 showed a strong *Tos17* signal but no readthrough transcription while the other showed no signal at all. Southern blot analysis revealed that *Tos17* was transposed in those R_1 lines in which *Tos17* was transcribed, but not in the line in which the transcription was suppressed (Fig. 4e). These results indicate that both the *bona fide* and the readthrough transcription of *Tos17* were suppressed to a variable extent in the R_1 generation, which probably was a consequence of progressive DNA methylation (see Discussion).

Discussion

We characterized the transcriptional and transpositional activity of *Tos17* in 11 genetically-related *japonica* varieties, a very small sample given the wide genetic variation of the numerous rice varieties (Cheng et al. 2003). Considerable variation was found in the varieties tested. The variation of the activity is mainly due to the distribution of the "active" *Tos17*$^{chr.7}$ and the "inactive" *Tos17*$^{chr.10}$. Although *Tos17*$^{chr.7}$ is highly active in most varieties, it is inactive in Moritawase, despite the fact that the copy in Moritawase has the same sequence as its active counterpart in Nipponbare. In contrast, the "inactive" *Tos17*$^{chr.10}$ becomes active in some, but not all cells in Koshihikari under prolonged culture. Obviously, the *Tos17* retrotransposon in rice is epigenetically regulated, similar to the DNA-type transposons *MuDR* and *Spm* in maize.

The DNA methylation is often associated with epigenetic regulation of TEs. The DNA methylation of *Tos17* is identified in all varieties examined. Activation

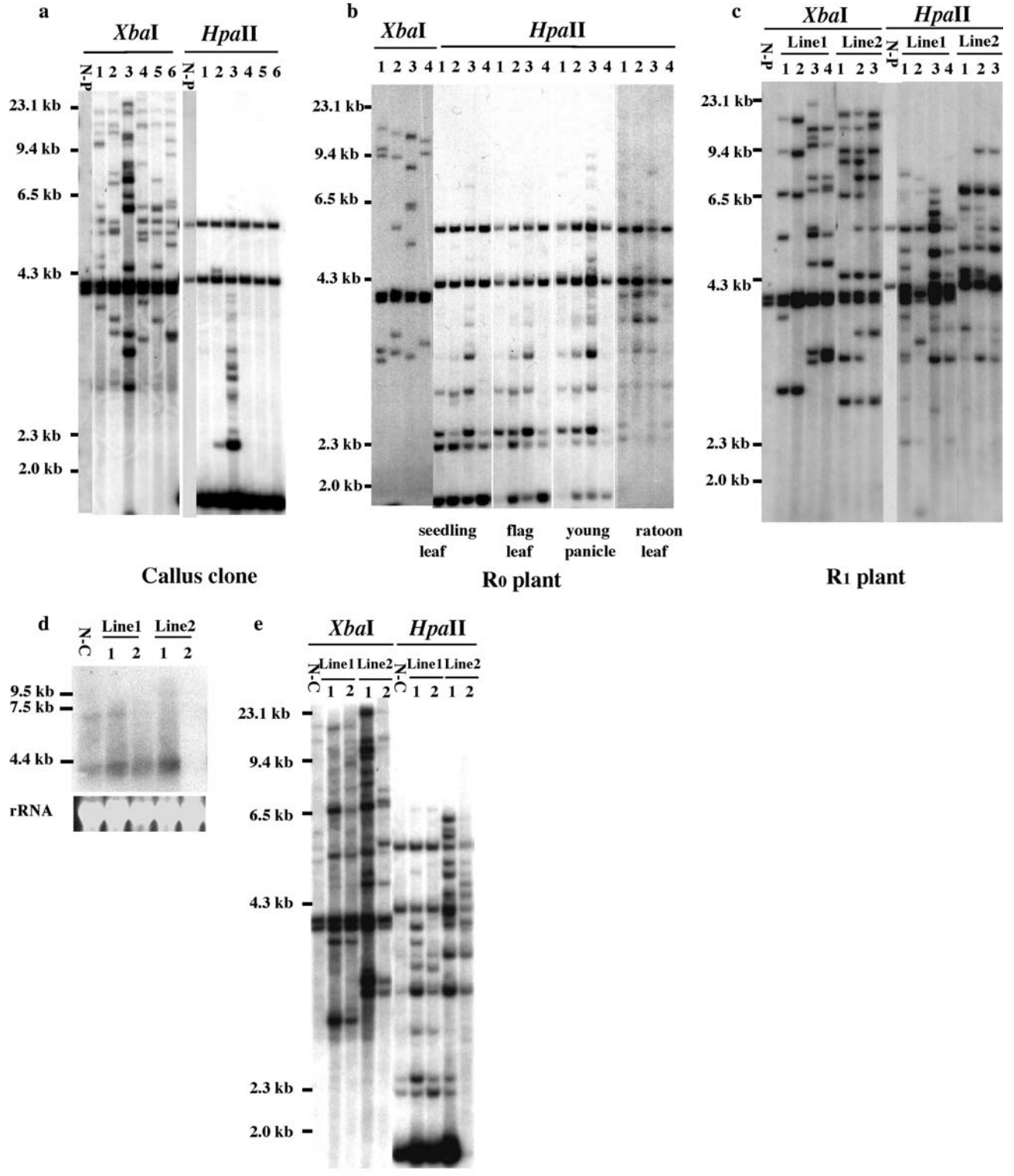

Fig. 4 Progressive methylation and transcriptional silencing of *Tos17*. All Southern or Northern blots were analyzed with probe 2. "N–P" indicates normal plant tissues of the original Nipponbare. "N–C" indicates RNA samples from the original Nipponbare calli. **a** Methylation of *Tos17* in six callus clones. **b** Methylation of *Tos17* in R_0 plants. DNA samples were extracted from seedling leaf, flag leaf, young panicle, and ratoon leaf from the same plants. **c** Methylation of *Tos17* in R_1 plants. Seven R_1 lines derived from the two R_0 lines (*Line 1 and Line 2*) are shown.**d** Transcription analysis of *Tos17* in R_1 calli. Four R_1 lines derived from two R_0 lines (*Line1 and Line2*) are shown. Equal loading of RNA is demonstrated by the rRNA control shown below. **e** Transposition analysis of *Tos17* in R_1 calli. DNA samples were from the calli used in **d**

of the methylated *Tos17* by tissue culture suggests a model that tissue culture induces a general demethylation and a subsequent activition of *Tos17*. However, no significant demethylation of *Tos17* is induced by tissue culture for most of the varieties studied here. It is possible the demethyaltion of *Tos17* occurred in a very small fraction of cells, but such a demethylation is unable to explain the fact that *Tos17* activation by tissue culture occurred almost in every cell (Hirochika et al. 1996; Miyao et al. 2003). These observations indicate that activation of *Tos17* by tissue culture does not proceed via the induction of *Tos17* demethylation, and the methylation level of *Tos17* in most of varieties is not high enough to suppress *Tos17* activity. Actually, in Moritawase and some regenerated progenies of Nipponbare which showed a particularly high level of DNA methylation, *Tos17* is not activated by tissue culture, indicating DNA methylation become a key factor controlling *Tos17* activity in these cases. Therefore, we proposed that the tissue-culture-induced *Tos17* activity and DNA-methylation-controlled *Tos17* activity are independent. The former is general and innate (thus genetic), but the latter is sporadic and reversible (thus epigenetic).

The callus specific activity of *Tos17* is probably due to the promoter character of *Tos17*. Actually, when the enhancer-promoter LTR of *Tos17* was fused to the GUS reporter gene and transformed into rice, GUS was expressed only in callus culture but not in plant tissues, indicating that callus-specific expression is an intrinsic character of the *Tos17* promoter (K. Sugimoto and H. Hirochika, unpublished data). The stress-induced MYB-related transcription factor NtMYB2 has been shown to be involved in the activation of the retrotransposon *Tto1* (Sugimoto et al. 2000). It is probable that the activation of *Tos17* by tissue culture proceeds also via the induction of some transcription factors related to stress responses.

Suppression of *Tos17*$^{chr.10}$ may not depend on a homology-dependent gene silencing mechanism, such as TGS or PTGS. If *Tos17*$^{chr.10}$ would be suppressed by PTGS or TGS, *Tos17*$^{chr.7}$ should also be suppressed in the same way in varieties carrying both copies. However, in many varieties carrying both copies, *Tos17*$^{chr.7}$ is active but *Tos17*$^{chr.10}$ is suppressed. Furthermore, although the DNA methylation level is decreased by 5-azaC treatment, *Tos17*$^{chr.10}$ in Moritawase was not mobilized. It has been demonstrated in prokaryotic systems and mammalian cells that if one gene nests in another, readthrough transcription from the upstream gene into the downstream one will reduce the expression of the latter, a phenomenon called transcriptional interference (Adhya and Gottesman 1982; Proudfoot 1986; Bateman and Paule 1988). The readthrough transcription of *Tos17*$^{chr.10}$ by an upstream promoter is reminiscent of the transcriptional interference phenomenon, suggesting that suppression of *Tos17*$^{chr.10}$ is due to the presence of an upstream promoter.

We found that the newly transposed copies in calli are not methylated. Methylation of new copies of *Tos17* progressed gradually in regenerated plants with the plant growth, and prolonged vegetative growth can further increase methylation degree. However, the methylation degree of new copies is still relatively low even under prolonged vegetative growth condition. In contrast, a reproduction cycle seemed to promote the methylation of these copies significantly and strengthened the methylation status of the original copies in the next plant generation. Our primary result suggested that germline passage can establish de novo methylation more effectively than the somatic progress.

Although *Tos17* becomes highly methylated during reproduction process, its activity is not suppressed in many R_1 lines (Fig. 4d), in contrast to *Tto1* transformed into *A. thaliana*. With the increase of copy numbers due to transposition, *Tto1* copies become highly methylated and completely silenced (Hirochika et al. 2000). Therefore, the DNA-methylation-related silencing of *Tos17* seemed not to work as efficiently as in *Tto1*. One possibility is that the gene silencing machinery works more efficiently on introduced elements (*Tto1* in *A. thaliana*) than on endogenous elements (*Tos17* in rice). Moreover, rice with its relatively large genome may be able to tolerate higher transposon activity than *A. thaliana* (Okamoto and Hirochika 2001).

The role of TEs in the epigenetic regulation of neighboring genes has been demonstrated in some cases. In the present study, the readthrough transcription of *Tos17*$^{chr.10}$ was suppressed in 3 of 4 R_1 lines (see Fig. 4d). Interestingly, the suppression was also found in the variety Rikuu132 and Moritawase, which carries more copies and a denser DNA methylation than other varieties (see Figs. 1c, 3b). Given that *Tos17* becomes progressively methylated and transcriptionally suppressed with increasing copy number, it appears likely that the silencing signal, probably DNA methylation, spreads from *Tos17*$^{chr.10}$ to its upstream region and thus suppressed the readthrough transcription. As the readthrough transcription of *Tos17*$^{chr.10}$ seemed to be initiated from the promoter of an ABC transporter-like gene, the co-suppression of the *bona fide* and readthrough transcription of *Tos17* provides an example of epigenetic regulation of a gene by a neighboring TE in rice. We hypothesize that some tissue-culture-induced mutations in rice

(Hirochika et al. 1996b) may be caused by the epigenetic regulation of genes by *Tos17*.

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