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Original Paper

Inactivation of retrotransposon *Tos17*$^{\mathrm{Chr.7}}$ in rice cultivar Nipponbare through CRISPR/Cas9-mediated gene editing

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Abstract Retrotransposons are mobile genetic elements capable of transposition via reverse transcription of RNA intermediates. Rice cultivar Nipponbare contains two nearly identical genomic copies of *Tos17*, an endogenous *copia*-like LTR retrotransposon, on chromosomes 7 (*Tos17*$^{\mathrm{Chr.7}}$) and 10 (*Tos17*$^{\mathrm{Chr.10}}$), respectively. Previous studies demonstrated that only *Tos17*$^{\mathrm{Chr.7}}$ is active in transposition during tissue culture. *Tos17*$^{\mathrm{Chr.7}}$ has been extensively used for insertional mutagenesis as a tool for functional analysis of rice genes. However, *Tos17*$^{\mathrm{Chr.7}}$ transposition might generate somaclonal mutagenesis with undesirable traits during rice transformation, which would affect the evaluation or application of transgenes. In this study, we generated a *Tos17*$^{\mathrm{Chr.7}}$ knockout mutant D873 by using CRISPR/Cas9 gene editing system. The gene-edited allele of *Tos17*$^{\mathrm{Chr.7}}$ in D873, designated as *Tos17*$^{\mathrm{D873}}$, has an 873-bp DNA deletion in the *pol* gene of *Tos17*$^{\mathrm{Chr.7}}$, which caused the deletion of the GAG-pre-integrase domain and the integrase core domain. Although the transcription of *Tos17*$^{\mathrm{D873}}$ was activated in D873 calli, no transposition of *Tos17*$^{\mathrm{D873}}$ was detected in the regenerated D873 plants. The results demonstrate that the GAG-pre-integrase domain and the integrase core domain are essential for *Tos17*$^{\mathrm{Chr.7}}$ transposition and the deletion of the two domains could be not complemented by other LTR retrotransposons in rice genome. As the *Tos17*$^{\mathrm{Chr.7}}$-derived somaclonal mutagenesis is blocked in the D873 plants, the generation of the *Tos17*$^{\mathrm{D873}}$ allele will be helpful in production of transgenic rice plants for gene function study and genetic engineering. Similar approach can be used to inactivate other retrotransposons in crop breeding.

Key words: CRISPR/Cas9, gene editing, retrotransposon, rice, *Tos17*.

Introduction

Retrotransposons are mobile genetic elements capable of transposition via reverse transcription of RNA intermediates and are abundant in the genomes of both plants and animals (Kumar and Bennetzen 1999). They consist of two subclasses, the long terminal repeat (LTR) retrotransposons and the non-LTR retrotransposons (Kumar and Bennetzen 1999). The LTRs contain the promoters and terminators associated with the transcription of LTR retrotransposons. The LTR retrotransposons can be further divided into two major groups, *Ty1-copia*-like and *Ty3-gypsy*-like, based on the order of the internal domains (Kumar and Bennetzen 1999). Both *Ty1-copia*-like and *Ty3-gypsy*-like carry *gag* and *pol* in their coding regions. The proteins encoded by the *gag* and *pol* genes are synthesized as a polyprotein that is cleaved into functional peptides by *pol*-encoded protease (PR). The capsid-like protein (CP) encoded by the *gag* gene is involved in the maturation and packaging of the retrotransposon RNA and proteins into a form suitable for integration into the genome, whereas the integrase (INT), reverse transcriptase (RT) and RNase-H (RH) encoded by the *pol* gene are required for replication and transposition of the retrotransposon.

Rice (*Oryza sativa* L.) is the staple food for nearly half of the world's population. *Tos17* is an endogenous *copia*-like LTR retrotransposon present in only one to five copies in rice genome, depending on the cultivar (Cheng et al. 2006; Hirochika et al. 1996). Two nearly identical genomic copies of *Tos17* reside in chromosomes 7 (*Tos17*$^{\mathrm{Chr.7}}$) and 10 (*Tos17*$^{\mathrm{Chr.10}}$) of Nipponbare, a reference cultivar whose genome has been completely sequenced and annotated. Interestingly, only *Tos17*$^{\mathrm{Chr.7}}$ is active in transposition during tissue culture, whereas *Tos17*$^{\mathrm{Chr.10}}$ is inactive possibly due to the presence of an

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upstream promoter which may cause transcriptional interference (Cheng et al. 2006; Hirochika et al. 1996). The activity of *Tos17* in rice is epigenetically regulated (Cheng et al. 2006; Liu et al. 2004; Hirochika et al. 1996). *Tos17* is suppressed by heavy DNA methylation in seed plants grown under normal conditions (Cheng et al. 2006). Transcription of *Tos17* is activated due to DNA demethylation in prolonged tissue culture and transposition occurs (Cheng et al. 2006; Hirochika et al. 1996). The transcription of newly transposed and original *Tos17* copies is re-suppressed by de novo DNA methylation during the plant regeneration from cultured cells and the growth and development of regenerated plants (Cheng et al. 2006). To support these findings, SDG714, a histone H3K9 methyltransferase in rice, was found to be involved in *Tos17* DNA methylation and its transposition suppression (Ding et al. 2007), whereas DNG701, a 5-methylcytosine DNA glycosylase/lyase in rice, was identified to be able to demethylate *Tos17* DNA and promote its transposition (La et al. 2011).

Due to its favourable features, together with its preferential insertion into gene-rich regions, *Tos17* has been extensively used for insertional mutagenesis as a tool for functional analysis of rice genes (Hirochika et al. 1996, 1997; Miyao et al. 2003; Piffanelli et al. 2007). However, tissue culture-induced activation of *Tos17* might generate somaclonal mutagenesis with undesirable traits during rice transformation, which would affect the evaluation or application of transgenes. To prevent the interference of *Tos17* in rice genome during transgenic study, we intended to generate *Tos17*$^{\text{Chr.7}}$ deletion mutant in Nipponbare background by using CRISPR/Cas9-mediated gene editing system and test the activity of the mutated *Tos17*$^{\text{Chr.7}}$ through tissue culture.

Materials and methods

Construct and A. tumefaciens strains

Two sgRNA sequences, Target 1 (5′-ATT GTG TAA GGT TAC TCA TCA G-3′) and Target 2 (5′-GTT CCA GCT CAT TTC TGG GCA G-3′), which specifically bind to an upstream site of the GAG-pre-integrase domain (Pre-INT) and a downstream site of the integrase core domain (INT) in the coding region of *Tos17*, were designed for CRISPR/Cas9-mediated gene editing (Figure 1A). Two gRNA expression cassettes driven by rice U3 and U6 small nuclear RNA (snRNA) promoters, respectively, were assembled into the binary CRISPR/Cas9 vector pYLCRISPR/Cas9P$_{\text{ubi}}$-H to generate binary construct pYLCas9-Tos17D, according to the method described previously (Ma et al. 2015). pYLCas9-Tos17D was introduced into *Agrobacterium tumefaciens* stain AGL1 by electroporation (Lessard 2013).

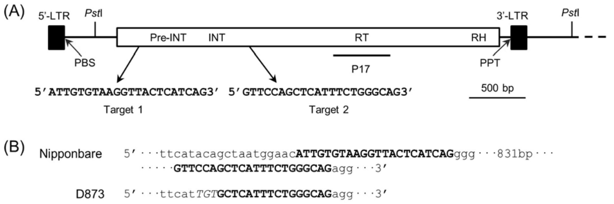

Figure 1. Gene editing of *Tos17*$^{\text{Chr.7}}$. (A) Structural features of *Tos17*$^{\text{Chr.7}}$ and target sequences for gene editing. The Pre-INT and INT domains are flanked by two targets, Target 1 and Target 2, for CRISPR/Cas9-mediated gene editing. Only *Pst*I digestion sites are shown. Scale bar is indicated. Abbreviations: INT, integrase core domain; LTR, long terminal repeat; Pre-INT, GAG-pre-integrase domain; P17, DNA probe for *Tos17*; PBS, tRNA primer binding site; PPT, polypurine tract; RH, RNase H; RT, Reverse transcriptase. (B) DNA sequences at the target sites of *Tos17*$^{\text{Chr.7}}$ in Nipponbare and *Tos17*$^{\text{D873}}$ in D873. The three nucleotide sequences derived from gene editing in D873 are shown in italic uppercase letters. D873, gene-edited plant carrying the *Tos17*$^{\text{D873}}$ allele derived from T0-24.

Rice tissue culture and Agrobacterium-mediated transformation

Rice tissue culture and *Agrobacterium*-mediated transformation were carried out according to the method described previously (Hiei et al. 1994) with slight modification. Rice callus was induced from mature rice seeds of Nipponbare on NB$_0$ medium (N6 major salts, B5 minor salts and B5 vitamins, 300 mg/l casein enzymatic hydrolysate, 500 mg/l proline, 30 g/l sucrose, 3.2 g/l phytagel, pH 5.8) containing 2 mg/l 2,4-D at 25°C in darkness. Vigorously growing embryogenic callus derived from the scutellum of mature embryos was co-cultivated with *A.* tumefaciens strain AGL1 containing binary construct pYLCas9-Tos17D. The co-cultivated callus was washed thoroughly with sterile distilled water and cultured on NB$_0$ medium containing 2 mg/l 2,4-D, 250 mg/l cefotaxime and 50 mg/l hygromycin at 25°C in darkness. After 4 to 6 weeks selection, putative hygromycin-resistant callus derived from the co-cultivated callus was excised and sub-cultured on NB$_0$ medium containing 2.0 mg/l KT, 0.02 mg/l NAA and 50 mg/l hygromycin at 25°C with 16-h light and 8-h darkness. The regenerated hygromycin-resistant plantlets were transferred to half-strength MS medium (Murashige and Skoog 1962) containing 50 mg/l hygromycin for shoot and root elongation. Tissue culture plants were also regenerated from 6-month-old calli derived from mature seeds of Nipponbare or gene-edited *Tos17* mutants. Transgenic plants and tissue culture plants were transplanted to soil in pots and grown in a rice greenhouse at temperatures ranging from 32°C during the daytime to 26°C at night, with average percentage of humidity at 84% and a photoperiod of 12 to 13 h.

PCR analysis

Plant genomic DNA was extracted using a protocol published previously (Dellaporta et al. 1983). PCR reaction was performed on a PTC-100 programmable thermal controller (MJ Research., Mass.). The PCR reaction mixture of 20 μl consisted of 10 ng of rice genomic DNA, 0.15 mM each of dNTPs, 0.15 mM of each primer, 2 μl of 10x PCR buffer and 0.5 unit of Taq polymerase (QIAGEN, Germany). Thermal cycling was done at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s. After the final cycle, the reactions were maintained at 72°C for 5 min before completion. The amplified product was resolved by electrophoresis on a 1.5% agarose gel in 1×TAE buffer. The DNA primer pair for detection of gene editing in either *Tos17*$^{\mathrm{Chr.7}}$ or *Tos17*$^{\mathrm{Chr.10}}$ was TosDS-F (5′CAA CTG TTA CCC CAA TTG CC3′)/TosDS-R (5′GTG CAG AAG GAT CAT AAC AG3′). The DNA primer pair for specific detection of gene editing in *Tos17*$^{\mathrm{Chr.7}}$ was Tos17-7F2 (5′CTA TCA GAT AAA AGA TCA GAC TC3′)/TosDS-R. The DNA primer pair for specific detection of gene editing in *Tos17*$^{\mathrm{Chr.10}}$ was Tos17-10F1 (5′TAA GCC TGC ACT TTC TTG GTG3′)/TosDS-R. The DNA primer pair for the hygromycin B phosphotransferase gene (*Hpt*) was Hpt-F (5′CGT CTG TCG AGA AGT TTC TGA TCG3′)/Hpt-R (5′CTA TTT CTT TGC CCT CGG ACG AGT3′). The primer pair for the *Cas9* gene was pYLCas9-F1 (5′TCT CTT CGA GGA TAG GGA GAT G3′)/pYLCas9-R1 (5′GTC GAA CTT CCT CTG GGT AAT G3′).

RT-PCR analysis

The expression of *Tos17*$^{\mathrm{Chr.7}}$ in rice calli was detected by RT-PCR with the eukaryotic elongation factor 1-alpha gene in rice (*eEF-1a*, Os03g0178000) as the internal control. Briefly, total RNAs were isolated from 6-month-old calli of Nipponbare and D873, respectively. The samples of total RNAs were treated with DNaseI to remove genomic DNA. The DNaseI-treated total RNAs were aliquoted into two parts. Part I was used as the RNA templates for the synthesis of the first-strand cDNA of *Tos17* using *Tos17*-specific primer Tos17-Rev-R (5′GCC TCC TGA TAA GTA GAT GGC3′). Part II was used as the RNA templates for the synthesis of the first-strand cDNA of protein-coding genes in rice using DNA primer Oligo $(dT)_{25}$ [TTT TTT TTT TTT TTT TTT TTT TTT T(A/C/G)(A/T/C/G)]. The subsequent PCR amplification was conducted according to the method mentioned above. The DNA primer pair for the *Tos17*$^{\mathrm{Chr.7}}$ and *Tos17*$^{\mathrm{D873}}$ alleles was Tos17-7F2/TosDS-R. The primer pair for the *eEF-1a* gene was EF1F2 (5′GCA CGC TCT TCT TGC TTT C3′)/EF1R2 (5′AGG GAA TCT TGT CAG GGT TG3′).

Southern blot analysis

About 2–4 μg of DNA was digested with appropriate restriction enzymes and fractionated on 0.8% agarose gel. After electrophoresis, DNA was blotted to HybondTM-N+ nylon membrane (Amersham Biosciences). The DNA blots were hybridized with DNA probes in a hybridization solution (DIG Easy Hyb Granules, Cat. No. 11796895001, Roche). DNA probes were labelled using a DIG DNA Labeling Kit (DIG Luminescent Detection Kit, Cat. No. 11363514910, Roche). The DNA probe for *Tos17* (P17) was PCR products amplified with DNA primers Tos17-F3 (5′ GGA AAT TGG CTA TGT CAG AGG AGC 3′) and Tos17-R (5′TAA CAA CAA AGT ACG ACC ACG GGA G 3′). The DNA probe for the *Hpt* gene was PCR products amplified with DNA primers Hpt-F and Hpt-R.

Results

Generation of *Tos17*$^{\mathrm{Chr.7}}$mutant lines using CRISPR/Cas9-mediated gene editing system

CRISPR/Cas9-mediated gene editing system was employed to create DNA deletion in the coding region of *Tos17*. Two sgRNA sequences were designed, which specifically target to an upstream site of the GAG-pre-integrase domain and a downstream site of the integrase core domain in the coding region of the *pol* gene in *Tos17*$^{\mathrm{Chr.7}}$ or *Tos17*$^{\mathrm{Chr.10}}$ (Figure 1A). The physical distance between the two targets is 834 bp. Twenty-six independent transgenic T0 plants were obtained in Nipponbare background after *Agrobacterium*-mediated rice transformation. Primer pair TosDS-F/TosDS-R, which could detect gene editing in either *Tos17*$^{\mathrm{Chr.7}}$ or *Tos17*$^{\mathrm{Chr.10}}$, was used to screen for gene editing in the *Tos17* genes. Six T0 plants (T0-1, T0-2, T0-6, T0-9, T0-24 and T0-26) were identified to have undergone gene editing either on *Tos17*$^{\mathrm{Chr.7}}$ or *Tos17*$^{\mathrm{Chr.10}}$ or both (Figure 2A). Southern blot analysis with restriction enzyme digestion by *Pst*I and *Tos17* probe P17 demonstrated that 1-3 additional hybridization bands of *Tos17* were detected in the 6 T0 plants (Figure 2B). Plants T0-6, T0-9, T0-24 and T0-26 produced *Pst*I bands with molecular size smaller than the 4389-bp band of wild-type *Tos17*$^{\mathrm{Chr.7}}$, indicating that these plants might carry gene-edited *Tos17*$^{\mathrm{Chr.7}}$ alleles (Figure 2B). T0-6, T0-24 and T0-26 were selected for further characterization, while T0-9 was infertile and abandoned.

Identifying gene-edited *Tos17*$^{\mathrm{Chr.7}}$ allele *Tos17*$^{\mathrm{D873}}$ through molecular and genetic characterization

In addition to the 4389-bp *Pst*I band of *Tos17*$^{\mathrm{Chr.7}}$ and

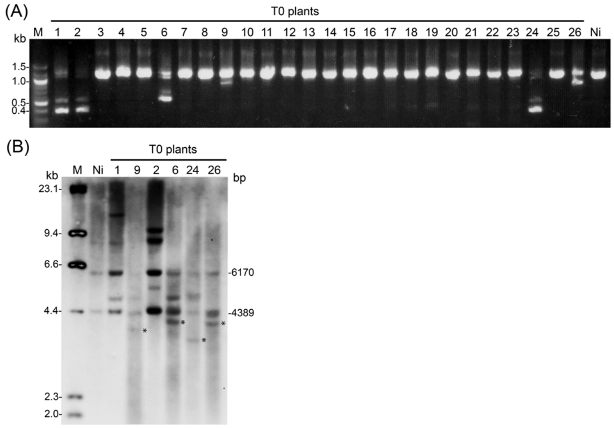

Figure 2. Molecular characterization of transgenic plants. (A) Screening for DNA deletion in *Tos17* in transgenic T0 plants. Image shows PCR products of wild-type and gene edited *Tos17* amplified with primers TosDS-F and TosDS-R. (B) Southern blot analysis of transgenic T0 plants. Rice genomic DNA was digested by *Pst*I. The Southern blot was hybridized with the *Tos17* probe P17. The *Pst*I bands smaller than 4389 bp are marked with "*". bp, base pair; kb, kilobase; M, molecular marker; Ni, Nipponbare.

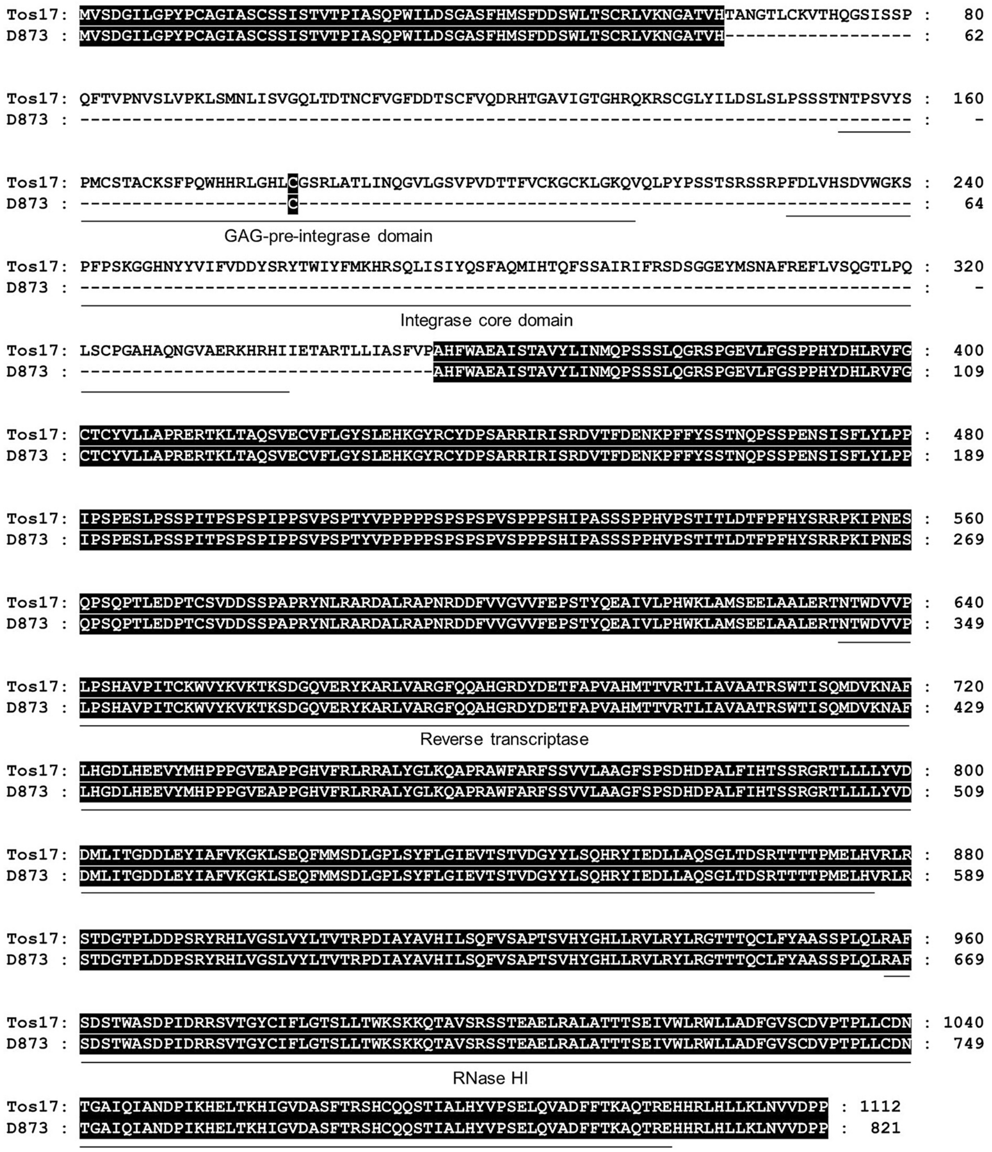

Figure 3. Alignment of the deduced amino acid sequences of the predicted polyprotein encoded by *Tos17*$^{\mathrm{Chr.7}}$ and *Tos17*$^{\mathrm{D873}}$. The amino acid sequences were aligned using the program Clustal W (Higgins and Sharp 1988) and shaded with GENEDOC software (https://genedoc.software.informer.com). The identical amino acids between the two predicted polyproteins are highlighted in black. Tos17, polyprotein encoded by *Tos17*$^{\mathrm{Chr.7}}$; D873, polyprotein encoded by *Tos17*$^{\mathrm{D873}}$.

the 6170-bp *Pst*I band of *Tos17*$^{\mathrm{Chr.10}}$, T0-26 produced only one extra *Pst*I band with molecular size smaller than 4389 bp. However, both T0-6 and T0-24 produced extra *Pst*I bands with molecular size larger than 4389 bp (Figure 2B). These extra bands could be newly transposed *Tos17*$^{\mathrm{Chr.7}}$, gene-edited *Tos17*$^{\mathrm{Chr.10}}$ or newly transposed *Tos17*$^{\mathrm{Chr.7}}$ followed by gene editing. To eliminate these undesirable *Tos17* copies, T0-24 and T0-26 as well as T0-6 were used as males to cross with Nipponbare followed by backcrossing for one generation and self-pollination of BC1F1 plants. The F1, BC1F1 and BC1F2 plants derived from these crosses were screened for gene editing on *Tos17*$^{\mathrm{Chr.7}}$ using *Tos17*$^{\mathrm{Chr.7}}$-specific primer pair Tos17-7F2/TosDS-R. The PCR products were sequenced to identify exact DNA mutation in the gene-edited *Tos17*$^{\mathrm{Chr.7}}$ alleles. In the meanwhile, they were also screened for the presence or absence of T-DNA harbouring the *Hpt* gene and the CRISPR-Cas9 gene editing system.

The gene-edited *Tos17*$^{\mathrm{Chr.7}}$ allele derived from T0-6 had 706-bp DNA deletion flanking the Target 1, which starts from the position at −22 bp upstream of Target 1 to the position at −173 bp upstream of Target 2. Based on the size of DNA deletion, the *Pst*I fragment derived from this gene-edited *Tos17*$^{\mathrm{Chr.7}}$ allele should be 3683 bp, which was smaller than the smallest *Pst*I band detected in T0-6 by Southern blot analysis (Figure 2B). By comparing with another *Pst*I band above the 4389-bp *Pst*I band, which was most likely derived from a newly transposed *Tos17*$^{\mathrm{Chr.7}}$, the *Tos17*$^{\mathrm{Chr.7}}$ allele with 706-

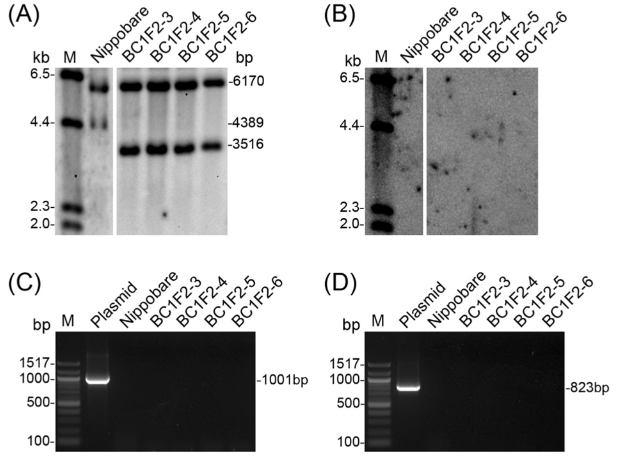

Figure 4. Molecular analysis of Nipponbare and D873 plants. (A), (B) Detection of *Tos17* (A) and *Hpt* gene (B) in rice plants by Southern blot analysis. (C), (D) Detection of the *Hpt* gene (C) and the *Cas9* gene (D) in rice plants by PCR analysis.Genomic DNA isolated from Nipponbare and D873 plants (BC1F2-3 to BC1F2-6) was digested by *Pst*I and subjected to Southern blot analysis. Southern blots were hybridized with the *Tos17* probe (A) and the *Hpt* gene probe (B), respectively. The binary construct pYLCas9-Tos17D (Plasmid in C and D) was used as the positive control for the *Hpt* gene and the *Cas9* gene. BC1F2-3 to BC1F2-6, individual D873 plants; bp, base pair; kb, kilobase; M, molecular marker.

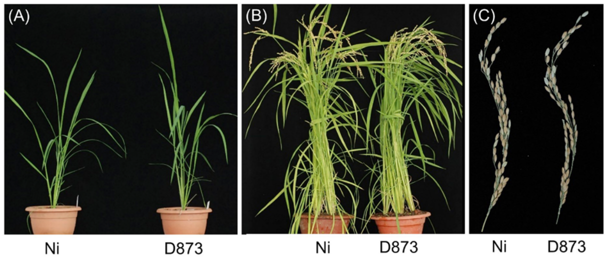

Figure 5. Morphological phenotypes of Nipponbare and D873 plants. (A), (B) Morphological phenotypes of Nipponbare and D873 plants at 5 weeks (A) and 13 weeks (B) after sowing. (C) Panicles of Nipponbare and D873 Ni, Nipponbare.

bp deletion in T0-6 should be derived from the newly transposed *Tos17*$^{\text{Chr.7}}$ rather than the original *Tos17*$^{\text{Chr.7}}$. Indeed, molecular and genetic analysis failed to identify a homozygous plant that contains this *Tos17*$^{\text{Chr.7}}$ allele with 706-bp deletion only in the absence of wild-type *Tos17*$^{\text{Chr.7}}$ allele (data not shown). T0-26 and its derived plants only carried a mutated *Tos17*$^{\text{Chr.7}}$ allele with gene editing at the Target 1 site. The gene-edited *Tos17*$^{\text{Chr.7}}$ allele had 435-bp DNA deletion, staring from the 14th position of Target 1 to the +421 bp position to its downstream followed by DNA insertion of 41 bp, which caused the deletion of the GAG-pre-integrase domain and frameshift of open reading frame. As T0-6 and T0-26 and its derived plants carried mutated alleles of *Tos17*$^{\text{Chr.7}}$ either derived from a newly transposed *Tos17*$^{\text{Chr.7}}$ or undergone partial gene editing, they were not included in additional study.

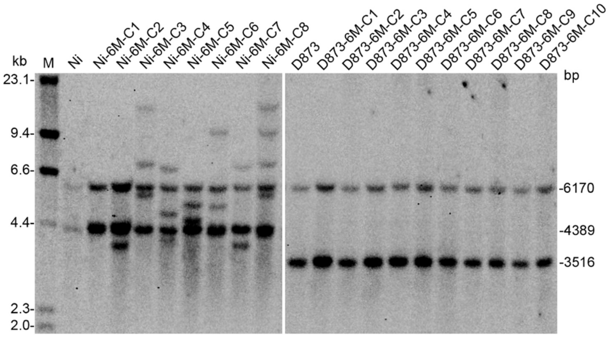

Figure 6. Detection of *Tos17* in tissue culture plants regenerated from 6-month-old calli. Rice genomic DNA was digested by *Pst*I and then subjected to Southern blot analysis. Southern blots were hybridized with the *Tos17* probe P17. bp, base pair; kb, kilobase; M, molecular; Ni-6M-C1 to Ni-6M-C8, individual plants regenerated from 6-month-old calli of Nipponbare; D873-6M-C1 to D873-6M-C10, individual plants regenerated from 6-month-old calli of D873.

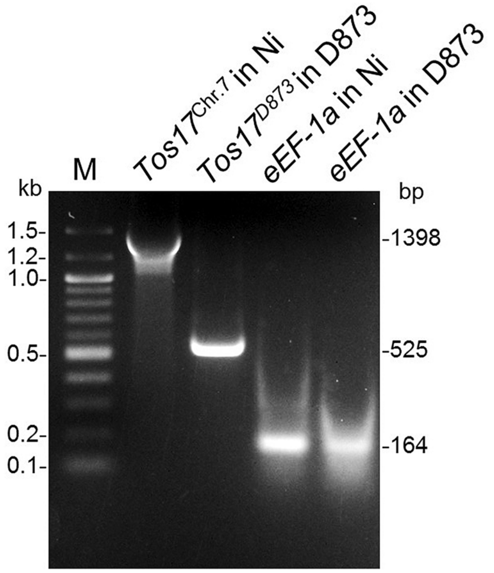

Figure 7. Detection of *Tos17* transcripts in the calli of Nipponbare and D873. Transcripts of *Tos17*$^{\text{Chr.7}}$ and *Tos17*$^{\text{D873}}$ were detected by RT-PCR in 6-month-old calli of Nipponbare and D873, respectively. The expression of the *eEF-1α* gene was set as the internal control. M, Molecular marker; Ni, Nipponbare.

The mutated allele of *Tos17*$^{\text{Chr.7}}$ derived from T0-24 has undergone relatively precise gene editing. It had 876-bp DNA deletion starting from the −14 bp position upstream of Target 1 to the 6 bp position of Target 2 followed by 3-bp DNA insertion (Figure 1B). Both GAG-pre-integrase domain and integrase core domain of *Tos17*$^{\text{Chr.7}}$ were deleted in the mutated allele (Figure 3). The mutated allele of *Tos17*$^{\text{Chr.7}}$ was designated as *Tos17*$^{\text{D873}}$, while the T0-24/BC1F2 plants that contained homozygous *Tos17*$^{\text{D873}}$ alleles were designated as D873 plants. A 3516-bp *Pst*I band derived from the *Tos17*$^{\text{D873}}$ allele was detected in the D873 plants (Figure 4A). As the control, a 4389-bp *Pst*I band derived from the wild-type *Tos17*$^{\text{Chr.7}}$ allele was only detected in Nipponbare (Figure 4A). Like Nipponbare, D873 plants produced a 6170-bp *Pst*I band derived from the wild-type *Tos17*$^{\text{Chr.10}}$ allele (Figure 4A). *Tos17*$^{\text{Chr.10}}$ could also be edited as it carries both Target 1 and Target 2 in its genomic sequence. Genomic DNA covering Target 1 and Target 2 on *Tos17*$^{\text{Chr.10}}$ was amplified from D873 using *Tos17*$^{\text{Chr.10}}$-specific primer pair Tos17-10F1/TosDS-R. DNA sequencing of the PCR products indicated that *Tos17*$^{\text{Chr.10}}$ in the D873 plants was not edited (data not shown). No *Hpt* or *Cas9* gene was detected in the D873 plants by Southern blot hybridization (Figure 4B) and PCR analysis (Figure 4C, D), indicating that the T-DNA in T0-24 has been eliminated from the D873 plants through crossing and backcrossing with Nipponbare and/or subsequent genetic segregation. The D873 plants displayed similar morphological phenotypes at different developmental stages and had similar growth duration to that of Nipponbare (Figures 5A, C). In addition, the seed setting rate of D873 was 77.9±3.6%, similar to that of Nipponbare at 78.7±6.9%. D873, as a *Tos17*$^{\text{Chr.7}}$ knockout mutant in Nipponbare background, was selected for additional study.

Test of Tos17$^{\text{D873}}$ *activity through tissue culture*

Calli were induced from mature seeds of D873 and Nipponbare and sub-cultured monthly on fresh medium. Tissue culture plants were regenerated from calli that had been cultured for 6 months after callus induction. The copy number of *Tos17* in the regenerated plants was detected by Southern blot analysis. In control experiments, besides for the 4389-bp *Pst*I band derived from the wild-type *Tos17*$^{\text{Chr.7}}$ allele and the 6170-bp *Pst*I band derived from the wild-type *Tos17*$^{\text{Chr.10}}$ allele, additional *Tos17* bands were detected in 7 of the 8 regenerated plants of Nipponbare genetic background, indicating that the transposition of *Tos17*$^{\text{Chr.7}}$ had occurred after prolonged tissue culture (Figure 6). As expected, no additional *Tos17* band was detected in the 10 regenerated plants in D873 genetic background tested (Figure 6). Interestingly, the deletion in the *pol* gene of *Tos17*$^{\text{Chr.7}}$ did not affect the transcription of the mutated *Tos17*$^{\text{Chr.7}}$allele during tissue culture, both *Tos17*$^{\text{D873}}$ and *Tos17*$^{\text{Chr.7}}$ transcripts were detected by RT-PCR in 6-month-old calli of D873 and Nipponbare, respectively (Figure 7). The results demonstrated that the deletion of GAG-pre-integrase domain and integrase core domain in *Tos17*$^{\text{Chr.7}}$ had abolished its activity for transposition even though the transcription of *Tos17*$^{\text{D873}}$ was activated during tissue culture. In addition, no transposition of *Tos17*$^{\text{Chr.10}}$ was detected in the regenerated D873 plants, which confirms the previous finding that *Tos17*$^{\text{Chr.10}}$ is not activated during tissue culture (Cheng et al. 2006; Hirochika et al. 1996).

Discussion

Previous studies demonstrated that Nipponbare harbours two nearly identical genomic copies of *Tos17*, *Tos17*$^{\text{Chr.7}}$ and *Tos17*$^{\text{Chr.10}}$, in its genome with only *Tos17*$^{\text{Chr.7}}$ showing transposon activity during tissue culture (Cheng et al. 2006; Hirochika et al. 1996). Our main objective in this study was to inactivate*Tos17*$^{\text{Chr.7}}$ through internal gene deletion mediated by CRISPR/Cas9 gene editing system. Two sgRNAs, Target 1 and Target 2, were designed to target to positions at the upstream of the GAG-pre-integrase domain and at the downstream of integrase core domain of *Tos17*, respectively (Figure 1A). Firstly, as *Tos17*$^{\text{Chr.7}}$ and *Tos17*$^{\text{Chr.10}}$ share identical coding region for the *pol* gene, both *Tos17* loci could be edited in this study. Secondly, the newly transposed *Tos17*$^{\text{Chr.7}}$ generated during rice transformation could be further edited by the CRISPR/Cas9 gene editing system. Finally, gene editing might occur at only one target site that causes point mutation or partial DNA deletion. Taking together, there might be gene editing at *Tos17*$^{\text{Chr.7}}$, *Tos17*$^{\text{Chr.10}}$ or both loci, gene editing at the transposed *Tos17*$^{\text{Chr.7}}$, partial gene editing either at Target 1 or Target 2 only, or the different combinations of the gene editing events. Therefore, both molecular and genetic analyses were conducted to obtain mutant plants that contain complete internal gene deletion at the *Tos17*$^{\text{Chr.7}}$ locus by eliminating undesirable gene-edited alleles and T-DNA in the gene-edited plants. In this study, twenty-six independent transgenic T0 plants were obtained, but only one T0 plant (T0-24) produced progeny that carried an expected gene edited allele of *Tos17*$^{\text{Chr.7}}$. The efficiency of obtaining desirable gene editing line was low, but the identification of the *Tos17*$^{\text{D873}}$ allele was sufficient for the functional study of *Tos17*$^{\text{Chr.7}}$ with knock-out mutation.

The transposition of LTR retrotransposons depend on intact *cis*-acting elements, including two LTRs at both ends, a potential tRNA primer binding site (PBS) and a polypurine tract (PPT), and *trans*-acting proteins encoded by the *gag* and *pol* genes with some having a third gene equivalent to a retroviral *env* gene (Finnegan 2012). In this study, all *cis*-acting elements, including the two LTRs, PBS and PPT are intact in *Tos17*$^{\text{D873}}$. The CRISPR/Cas9-mediated gene editing in the coding region of *Tos17*$^{\text{Chr.7}}$ caused the deletion of the GAG-pre-integrase domain and the integrase core domain in *Tos17*$^{\text{D873}}$. Although RT-PCR analysis indicated that, like *Tos17*$^{\text{Chr.7}}$, the transcription of *Tos17*$^{\text{D873}}$ was activated during tissue culture, the deletion of two domains completely abolished its transposition activity. Previously, bioinformatics analysis indicated that both *Tos17*$^{\text{Chr.7}}$ and *Tos17*$^{\text{Chr.10}}$ lack a *gag* open reading frame and the authors claimed that *Tos17* is a non-autonomous LTR retrotransposon requiring an active one in order to ensure its transposition (Sabot 2014). While it remains to be determined experimentally whether *Tos17* needs an autonomous partner to provide GAG for its transposition, our results demonstrate that the GAG-pre-integrase domain and the integrase core domain are essential for *Tos17*$^{\mathrm{Chr.7}}$ transposition and the deletion of the two domains could be not complemented by *trans*-acting proteins encoded by other LTR retrotransposons in rice genome (Hirochika et al. 1996).

Saika et al. (2019) recently reported the production of *Tos17*-deficient plants by CRISPR/Cas9-mediated targeted mutagenesis. According to the report, gene editing with a sgRNA targeting to both 5′ and 3′ LTRs of *Tos17* (*Tos17*$^{\mathrm{Chr.7}}$ and *Tos17*$^{\mathrm{Chr.10}}$) could generate *Tos17*-deficient plants (Saika et al. 2019). In this study, *Tos17*$^{\mathrm{Chr.7}}$ was mutated with deletion in the coding region of the *pol* gene by CRISPR/Cas9-mediated gene editing with two sgRNAs. The generation and characterization of the *Tos17*$^{\mathrm{D873}}$ allele indicated that *Tos17* requires the GAG-pre-integrase domain and the integrase core domain encoded by the *pol* gene for its transposition. In addition, as *Tos17*$^{\mathrm{Chr.10}}$ did not show any transposition activity in *Tos17*$^{\mathrm{Chr.7}}$ mutant line D873, confirming the previous report that only *Tos17*$^{\mathrm{Chr.7}}$ is active during tissue culture (Cheng et al. 2006). As the *Tos17*$^{\mathrm{Chr.7}}$-derived somaclonal mutagenesis is blocked in the D873 plants, the generation of the *Tos17*$^{\mathrm{D873}}$ allele will be helpful in production of transgenic rice plants for gene function study and genetic engineering. . Similar approach can be used to inactivate other transposons in crop breeding.

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