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Comparing Genetic Characteristics of Retrotransposon *TOS17* During Different Tissue Culture Processes in the Rice Cultivars Nipponbare and Shishoubaimao

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Abstract

As a retrotransposon, *TOS17* was a useful tool for rice genetic and functional genomic research. To ascertain the feasibility of constructing a *TOS17* insertion mutation library in the rice cultivar Shishoubaimao, the genetic and expression characteristics of *TOS17* were analyzed. We made solid and suspension tissue cultures and confirmed the copy numbers of *TOS17* at different time points in both tissue culture processes by real-time quantitative PCR (RT-qPCR). Three primary copies of *TOS17* were detected in naturally grown Shishoubaimao. *TOS17* was activated by tissue culture, and the copy numbers of *TOS17* increased along with a prolonged tissue culture time in both the Nipponbare and the Shishoubaimao cultivars. Therefore, Shishoubaimao is a potential candidate for constructing a *TOS17* insertion mutant library. Compared with Nipponbare, *TOS17* was more active in Shishoubaimao during tissue culture. Higher copy numbers of *TOS17* were obtained with the suspension tissue culture process than with the solid tissue culture process over the same time courses. We concluded that 3-4 months of suspension tissue culture time is suitable for constructing a *TOS17* insertion mutant library in Shishoubaimao.

Key words: Shishoubaimao, tissue culture, *TOS17*, copy number, insertion mutant library

INTRODUCTION

Rice is the most important cereal crop, feeding more than half of the world's population. The entire genomic sequence of rice was determined via a worldwide collaborative effort, and the next great challenge is the functional characterisation of its genes (Miyao *et al*. 2007; International Rice Genome Sequencing Project 2005). Mutational analysis through gene disruption is one of the most efficient methods for identifying gene function. Other tools for functional analysis of the rice genome, such as T-DNA and transposon insertion lines, have been created and are publicly available (Miyao *et al*. 2003; Zhang *et al*. 2006; Hsing *et al*. 2007; Piffanelli *et al*. 2007; Larmande *et al*. 2008).

Retrotransposons are a class of mobile genetic elements that propagate via reverse transcription of RNA intermediates (Gao *et al*. 2007). In 1996, Hirochika *et al*. (1996) isolated twenty rice retrotransposons (*TOS1*-*TOS20*) using conserved domains of reverse transcriptase. Copy numbers of the retrotransposons *TOS10*, *TOS17* and *TOS19*, which were inactive in the normal growth of plants, increased during tissue culture. As one of the most active retrotransposons, copies of *TOS17* accumulated with extended tissue culture time (Hirochika *et al*. 1996). Recent research revealed that transposition of *TOS17* was controlled by methylation modification (Ding *et al*. 2007; Liu M *et al*. 2004). Further analysis found that the copy number of *TOS17* was extremely low, ranging from one to four depending on the cultivar (Cheng *et al*. 2006; Liu M *et al*. 2004). At

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the same time, the target site-specificity of *TOS17* is low and spread throughout the chromosomes. The *TOS17* retrotransposon preferentially integrates into low-copy-number sequences that often are gene-rich regions (Cheng *et al*. 2006; Miyao *et al*. 2003). With these features, *TOS17* insertion lines have great advantages for the functional analysis of rice genes (Hirochika 2001).

Although many advances have been made in rice genetic research, the most widely used cultivar, Nipponbare, has a long life cycle compared with *Arabidopsis* that often delays the progress of research. The japonica cultivar Shishoubaimao, which was first grown in China in the 1950s, has an extremely short life cycle (Guo *et al*. 2004). When grown in a greenhouse, Shishoubaimao flowers in 45 days and matures in 70-80 days. As a japonica variety, Shishoubaimao is easy to grow in tissue culture and to transform (Liu Z L *et al*. 2004). Therefore, Shishoubaimao is suitable for rice genetic research. In this study, we investigated the characteristics of *TOS17* in Shishoubaimao with Nipponbare as the control. Through measurement of the changes in *TOS17* copy numbers under different conditions, we optimised a method for producing a *TOS17* insertion mutant library in Shishoubaimao.

MATERIALS AND METHODS

Plant materials and tissue culture

Two japonica varieties, Nipponbare and Shishoubaimao, were used in this study. Callus induction was performed as described previously (Herve and Kayano 2006). One-month-old cultured calli were transformed to N6 solid medium and cultured for a total of 2, 3 and 4 months. In parallel, other one-month-old cultured calli were transformed to R2S liquid medium and cultured for a total of 1, 2 and 3 months. R_0 plants were regenerated from the different cultured calli as described by Nishimura *et al*. (2006). Four independent natural growth plants of Shishoubaimao and Nipponbare were selected for Southern blot analysis. Another transgenic plant was selected from our T-DNA insertion mutant library, which obtained by an *Agrobacterium*-mediated transformation as described by Cheng *et al*. (2003).

Southern blot analysis

Genomic DNA was extracted using the CTAB method (Murray and Thompson 1980). DNA samples of 5 mg each were digested with the restriction enzyme *Eco*R I and separated on 0.8% (w/v) agarose gel. After electrophoresis, the digested DNA was transferred to a Hybond N^+ nylon membrane (Amersham Pharmacy Biotech, USA) and hybridised with a ^{32}P-dCTP-labeled *TOS17* gene probe. The blot was washed at 65°C under stringent conditions. The hybridised membrane was exposed to a phospho-rimaging screen and was screened using a Typhoon-8600 (Amersham Biosciences, USA). The *TOS17* probe was made by PCR amplification using the primers listed in Table 1. The PCR product of *TOS17* used for probe was inserted in the T-vector, which was used as plasmid control in Southern blot.

Semiquantitative reverse transcription PCR (RT-PCR)

Total RNA of calli and plant tissue was isolated using the Trizol D0410 reagent, according to the manufacturer's instructions (Invitrogen, USA). The first-strand cDNA was synthesised from 3 μg of the total RNA using moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA). Semiquantitative RT-PCR was performed using the primers listed in Table 1. The PCR products were loaded on 1.2% agarose gels and imaged by a CCD camera. The gene expression levels were compared by the band density after normalisation of initial variation in the sample concentration by the density of the housekeeping gene *ACTIN* (GenBank accession no. X63830).

Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using the LightCycler480 Probes Master Kit on the LightCycler480 machine (Roche Diagnostics, Basel, Switzerland), following the manufacturer's instructions. The amplification program for RT-qPCR was performed as follows: one cycle of 5 min at 95°C followed by 45 cycles of 10 s at 95°C, 20 s at 65°C, 1 s at 72°C. Triplicate quantitative assays were performed on each DNA sample. To generate standard

curves for the *TOS17* and sucrose phosphate synthase (SPS) genes, genomic DNA were serially diluted to final concentrations of 500, 100, 20, 4, and 0.8 ng μL^{-1} and subjected to RT-qPCR assay in triplicate. The gene-specific primers and the corresponding UPL (Universal Probe Library) probe were designed using the online analysis center from the Roche Web site (http://www.universalprobelibrary.com) and given in Table 1.

Statistical analysis of data

Statistical significances were analyzed using SAS program (SAS Institute Inc. Cary, NC).

RESULTS

Copy numbers of *TOS17* in Nipponbare and Shishoubaimao

To study the activity of *TOS17* in Shishoubaimao, we first investigated the original copy number of *TOS17*. A 666-bp fragment of *TOS17* that has been used as a probe for Southern blots by others (Zhu *et al*. 2004) was amplified by PCR and purified. Southern blotting revealed that two copies of *TOS17* were present in Nipponbare, which is in accordance with previous reports (Cheng *et al*. 2006; Hirochika *et al*. 1996). We found that Shishoubaimao has three copies of *TOS17*, two of which correspond to the copies in Nipponbare and one additional copy (Fig.1). To exclude inaccurate results, we used a plasmid containing *TOS17* and a transgenic plant of the Nipponbare background as controls. The results indicated that a single band was found in the plasmid and three *TOS17* copies were discovered in the transgenic plant (Fig.1). The increased *TOS17* copy number in the transgenic plant must be due to transposition of *TOS17* during tissue culture process.

TOS17 is activated during tissue culture and re-silenced in regenerated plants

Although there were two *TOS17* copies in Nipponbare, only the *TOS17* located on chromosome 7 ($TOS17^{chr.7}$) was activated by tissue culture, whereas the other copy ($TOS17^{chr.10}$) remained unaffected (Cheng *et al*. 2006). In the rice cultivar Moritawase, neither copy of *TOS17* was affected by tissue culture (Cheng *et al*. 2006). To investigate the activity of *TOS17* in Shishoubaimao, we checked the expression of *TOS17* in different tissue culture processes. As expected, *TOS17* was activated during the callus culture period, and decreased expression in differentiating callus and was finally re-silenced in regenerated plants of Nipponbare (Fig.2). The expression pattern of *TOS17* in Shishoubaimao was very similar to that in Nipponbare (Fig.2). Therefore, *TOS17* can be used in Shishoubaimao for constructing an insertion mutant library. A much weaker band was detected in regenerated plants that may be due to the constant expression of $TOS17^{chr.10}$ (Fig.2).

Table 1 Primers used for RT-PCR and RT-qPCR

Gene	Purpose	Orientation	Sequence (5′→3′)	UPL number
TOS17	Southern blot and RT-PCR	Forward	gctacccgttcttggactat	-
		Reverse	ctgaaatcggagcactgaca	-
ACTIN	RT-PCR	Forward	tcagcaactgggatgatatggag	-
		Reverse	gccgttgtggtgaatgagtaac	-
TOS17	RT-qPCR	Forward	gataaaagatcagtaagcctgcact	119
		Reverse	aaatccgtgtcccagttgaa	
SPS	RT-qPCR	Forward	ggcaatttctcgtaaattcaggt	57
		Reverse	tccccgacgatcagatacat	

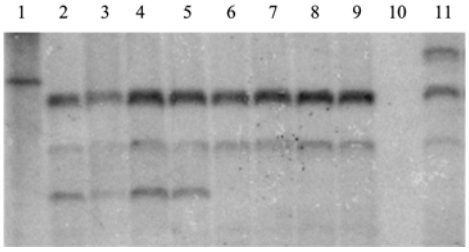

Fig. 1 Southern blot of *TOS17*. Lane 1, plasmid control; lanes 2-5, independent natural growth Shishoubaimao plants; lanes 6-9, independent natural growth Nipponbare plants; lane 10, blank control; lane 11, transgenic plant control in the Nipponbare background with nearly two months tissue culture before regeneration.

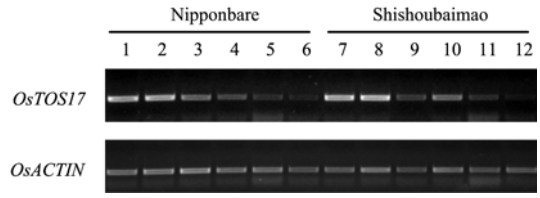

Fig. 2 The expression levels of *TOS17* in different tissue culture stages. RNA was extracted from different samples of Nipponbare and Shishoubaimao. 1, 2, 7, and 8, 5-wk-old calli; 3, 4, 9, and 10, calli after two weeks of differentiation; 5, 6, 11, and 12, leaves from regenerated plants. The rice *ACTIN* gene was used as an internal control.

The transposition of *TOS17* in Nipponbare and Shishoubaimao

We used qPCR to study the copy numbers of *TOS17* during tissue culture in this research. The endogenous *SPS* gene was employed as an internal standard as previously described (Ding *et al*. 2004). Standard curves were prepared for *TOS17* and the *SPS* gene, and they were compared with the experimentally determined levels in each sample to calculate the copy numbers of *TOS17*. The correlation coefficients of the standard curves were 0.9901 (*TOS17*) and 0.9927 (*SPS*) (Fig.3). The reaction efficiencies for *TOS17* and *SPS* were 0.9164 and 0.9389, both of which were above 0.90 and very similar (Fig.3). According the criteria proposed by other researches, our standard curve was applicable to the estimation of copy numbers (Yang *et al*. 2005).

Our study confirmed that *TOS17* was activated by prolonged culture time (Hirochika *et al*. 1996). The copies of *TOS17* were significantly increased in both solid and suspension tissue culture after 2 and 3 months, respectively (Table 2). Although the number of copies of *TOS17* increased during solid and suspension tissue culture, a greater increase was obtained in suspension tissue culture over the same culture time. Similar numbers of transposed copies were observed in suspension tissue cultures performed for 1, 2 or 3 months as in solid cultures performed for 2, 3 or 4 months, respectively (Table 2). These differences in the two tissue culture methods were independent of the rice cultivar. This phenomenon can be explained by the faster growth of calli in suspension tissue culture than in solid culture.

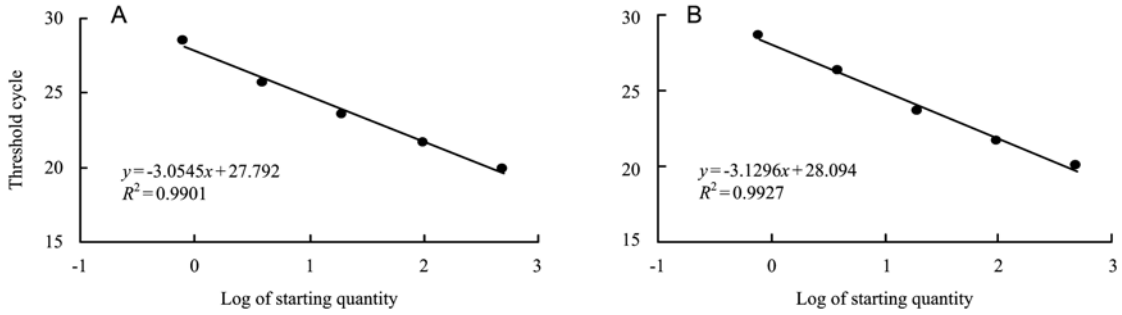

Fig. 3 RT-qPCR standard curves of *TOS17* (A) and *SPS* (B).

Table 2 Copy numbers of *TOS17* in regenerated plants from different tissue conditions

Regenerated plants	Copy numbers of *TOS17*											
	Nipponbare						Shishoubaimao					
	Solid culture time (mon)			Suspension culture time (mon)			Solid culture time (mon)			Suspension culture time (mon)		
	2	3	4	1	2	3	2	3	4	1	2	3
1	2	3	3	2	5	5	3	5	6	4	6	5
2	2	3	3	3	2	6	3	4	5	3	4	6
3	2	3	3	3	5	5	3	5	5	3	5	7
4	2	3	4	4	3	4	4	6	5	3	5	6
5	3	2	5	3	4	4	4	4	5	4	4	7
6	2	3	5	4	4	3	3	6	6	3	7	7
Average	2.16 a	2.83 ab	3.83 cd	3.17 bc	3.83 cd	4.50 de	3.33 bc	5.00 e	5.33 e	3.33 bc	5.17 e	6.33 f
New copy	0.16	0.83	1.83	1.17	1.83	2.5	0.33	2	2.33	0.33	2.17	3.33

Different letters are significantly different at $P<0.05$ (LSD test).

On average, Shishoubaimao had more newly transposed copies than Nipponbare under the same tissue culture conditions (Table 2). We speculate that the additional *TOS17* copy in the genome of Shishoubaimao may transpose during tissue culture.

DISCUSSION

Many insertion mutant libraries have recently been established in rice using T-DNA (Zhang *et al*. 2006; Hsing *et al*. 2007; Larmande *et al*. 2008; Piffanelli *et al*. 2007). However, transgenic plants with selected markers, such as antibiotic or herbicide resistance, may not be distributed widely because of environmental concern. Use of endogenous insertion mutagens avoids this problem (Hirochika 2001). The rice retrotransposon *TOS17* has previously been used in Nipponbare for making an insertion mutant library (Miyao *et al*. 2003). The long life cycle of Nipponbare, which is over 100 days, is problematic for rice genetic research. In contrast, Shishoubaimao matures in 70 days after germination when grown in a greenhouse (Guo *et al*. 2004). Therefore, Shishoubaimao is more suitable for rice genetic research. In this study, we compared the characteristics of *TOS17* in Nipponbare and Shishoubaimao. The similar genetic characteristics of *TOS17* were observed between Shishoubaimao and Nipponbare. The results suggested that Shishoubaimao is a suitable rice cultivar for constructing a *TOS17* insertion mutant library.

To further explore the conditions for constructing a *TOS17* insertion mutant library in Shishoubaimao, we measured the variation of *TOS17* copy numbers under different culture conditions. More newly transposed *TOS17* can be obtained by suspension tissue culture than by solid culture over the same culture time (Table 2). To make an insertion mutant library, a prolonged tissue culture period is needed for more transposition of *TOS17*, which will lead to more gene mutation. However, prolonged culture leads to a reduction of the heterogeneity of mutation, possibly as a result of the selection of specific cell types (Hirochika *et al*. 1996). In this scenario, the suspension tissue culture is better for constructing an insertion mutant library because a similar number of *TOS17* copies can be produced by this method in a shorter tissue culture time. A 5-month suspension tissue culture period has been adopted for the production of *TOS17* mutant lines in Nipponbare with nearly 10 *TOS17* copies on average (Miyao *et al*. 2003). Here, we observed that Shishoubaimao accumulated more *TOS17* copies than Nipponbare under the same culture conditions (Table 2). Considering the difference between Shishoubaimao and Nipponbare, more than 10 copies of *TOS17* will be got in a 5-month suspension tissue culture period for Shishoubaimao. A 3-month suspension tissue culture time in Shishoubaimao led to 6.33 *TOS17* copies with 3.33 newly transposed copies on average. Because the *TOS17* has low target site specificity and preference to insert in genes (Miyao *et al*. 2003), 3.33 copies insertion is almost enough to construct mutant library. Therefore, we suggested a 3- to 4-month suspension tissue culture period for producing a *TOS17* insertion mutant library in Shishoubaimao.

In this research, we employed RT-qPCR to determine the copy numbers of *TOS17* in regenerated seedlings. More and more publications have employed this method in place of Southern blot, which is labour-intensive and requires larger DNA samples (Shou *et al*. 2004; Yang *et al*. 2005). A critical point for RT-qPCR is the reaction efficiency of a gene, which should be above 0.90. Here, we showed that the reaction efficiencies of the *TOS17* and SPS genes were 0.9164 and 0.9389, suggesting the two genes are suitable for RT-qPCR. Meanwhile, the standard curves of *TOS17* and *SPS* exhibit good correlation coefficients, indicating that our method was applicable to the estimation of *TOS17* copy numbers.

CONCLUSION

By Southern blotting, we detected three *TOS17* copies in Shishoubaimao, and a *TOS17* expression pattern similar to that of Nipponbare was observed. This indicates that *TOS17* can be used for constructing an insertion mutant library in Shishoubaimao by tissue culture. Furthermore, we proposed that a 3- to 4-month suspension tissue culture period may be the optimal condition for producing a *TOS17* insertion mutant library.

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References

Cheng C, Daigen M, Hirochika H. 2006. Epigenetic regulation of the rice retrotransposon Tos17. *Molecular Genetics and Genomics*, **276**, 378-390.

Cheng S, Jin W, Wang M, Zhang F, Zhou J, Jia Q, Wu Y, Liu F, Wu P. 2003. Distribution and characterization of over 1000 T-DNA tags in rice genome. *The Plant Journal*, **36**, 105-113.

Ding J, Jia J, Yang L, Wen H, Zhang C, Liu W, Zhang D. 2004. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *Journal o f Agriculture Food Chemistry*, **52**, 3372-3377.

Ding Y, Wang X, Su L, Zhai J, Cao S, Zhang D, Liu C, Bi Y, Qian Q, Cheng Z, Chu C, Cao X. 2007. SDG714, a histone H3K9 methyltransferase, is involved in Tos17 DNA methylation and transposition in rice. *The Plant Cell*, **19**, 9-22.

Gao D, He B, Sun L. 2007. Research advances on transposable elements in rice. *Chinese Bulletin of Botany*, **24**, 667-676. (in Chinese)

Guo J, Chen Z, Liu Y. 2004. Identification of QTLs controlling rice heading date and analysis of the genetic effects. *Molecular Plant Breeding*, **2**, 788-794.

Herve P, Kayano T. 2006. Japonica rice varieties (*Oryza sativa* Nipponbare, and others). *Methods in Molecular Biology*, **343**, 213-222.

Hirochika H. 2001. Contribution of the *Tos17* retrotransposon to rice functional genomics. *Current Opinion of Plant Biology*, **4**, 118-122.

Hirochika H, Sugimoto K, Otsuki Y, Tsugawa H, Kanda M. 1996. Retrotransposons of rice involved in mutations induced by tissue culture. *Proceedings of the National Academy of Sciences of the USA*, **93**, 7783-7788.

Hsing Y I, Chern C G, Fan M J, Lu P C, Chen K T, Lo S F, Sun P K, Ho S L, Lee K W, Wang Y C, *et al*. 2007. A rice gene activation/knockout mutant resource for high throughput functional genomics. *Plant Molecular Biology*, **63**, 351-364.

International Rice Genome Sequencing Project. 2005. The map-based sequence of the rice genome. *Nature*, **436**, 793-800.

Larmande P, Gay C, Lorieux M, Perin C, Bouniol M, Droc G, Sallaud C, Perez P, Barnola I, Biderre-Petit C, *et al*. 2008. *Oryza* Tag Line, a phenotypic mutant database for the genoplante rice insertion line library. *Nucleic Acids Research* (Database Issue), **36**, D1022-1027.

Liu M, Sun Z, Xu T. 2004. Construction of several plant expression vectors using multiple cell wall degrading enzyme genes from *Trichoderma harzianum* and rice transformation. *Journal of Zhejiang University* (Agriculture & Life Science), **30**, 596-602.

Liu Z L, Han F P, Tan M, Shan X H, Dong Y Z, Wang X Z, Fedak G, Hao S, Liu B. 2004. 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. *Theoretical and Applied Genetics*, **109**, 200-209.

Miyao A, Iwasaki Y, Kitano H, Itoh J, Maekawa M, Murata K, Yatou O, Nagato Y, Hirochika H. 2007. A large-scale collection of phenotypic data describing an insertional mutant population to facilitate functional analysis of rice genes. *Plant Molecular Biology*, **63**, 625-635.

Miyao A, Tanaka K, Murata K, Sawaki H, Takeda S, Abe K, Shinozuka Y, Onosato K, Hirochika H. 2003. Target site specificity of the *Tos17* retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome. *The Plant Cell*, **15**, 1771-1780.

Murray M G, Thompson W F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, **8**, 4321-4325.

Nishimura A, Aichi I, Matsuoka M. 2006. A protocol for *Agrobacterium*-mediated transformation in rice. *Nature Protocol*, **1**, 2796-2802.

Piffanelli P, Droc G, Mieulet D, Lanau N, Bes M, Bourgeois E, Rouviere C, Gavory F, Cruaud C, Ghesquiere A, Guiderdoni E. 2007. Large-scale characterization of *Tos17* insertion sites in a rice T-DNA mutant library. *Plant Molecular Biology*, **65**, 587-601.

Shou H, Frame B R, Whitham S A, Wang K. 2004. Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. *Molecular Breeding*, **13**, 201-202, 208.

Yang L, Ding J, Zhang C, Jia J, Weng H, Liu W, Zhang D. 2005. Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. *Plant Cell Reports*, **23**, 759-763.

Zhang J, Li C, Wu C, Xiong L, Chen G, Zhang Q, Wang S. 2006. RMD: a rice mutant database for functional analysis of the rice genome. *Nucleic Acids Research* (Database Issue), **34**, D745-748.

Zhu DY, Lan L Z, Shan DY , Pu H F, Yuan H M, Hao S, Liu B. 2004. Alien DNA introgression into rice causes heritable alterations in DNA methylation patterns in an active retrotransposon *Tos17*. *Acta Botanica Sinica*, **46**, 100-109.

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