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Functional Analysis for Rolling Leaf of Somaclonal Mutants in Rice (*Oryza sativa* L.)

Young-Hie Park[1], Hyun-Suk Lee[1], Gi-Hwan Yi[2], Jae-Keun Sohn[1], Kyung-Min Kim[1*]

[1]School of Plant Biosciences, Kyungpook National University, Daegu, Korea; [2]Department of Functional Crop, National Institute of Crop Science, RDA, Milyang, Korea.
Email: yhpark4026@hanmail.net, ghyi@rda.go.kr, {ddr3031, jhsohn, kkm}@knu.ac.kr

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ABSTRACT

This study was carried out to facilitate the functional analysis of rice genes. Some 297 *insertion plants* (1.7%) *of the entire lines with the endogenous retrotransposon Tos*17 *were produced. Phenotypes of these plants in the* S_2 *generation were observed in the field according to different leaf types. Rolling leaf mutants showed thinner sclerenchymatous cells, defective arrangement of vascular bundles, and well-formed bulliform cells as compared to the parental cultivar. Two new copies of Tos*17 *were detected in the rolling leaf type. In the new leaf type, the copy number and activation of Tos*12, 15 *did not appear as 'Ilpum'. Flanking sequence tag* (*FST*) *analysis of Tos*17 *in the rolling leaf mutant indicated that new copies of Tos*17 *were transposed on chromosomes* 11 *and* 12. *Annotated homologues of the tagging genes on chromosome* 11 *were arabinoxylan rabinofuranohydrolase isoenzyme AXAH-I and II. The tagging gene in chromosome* 12 *was highly correlated with* 6 *kinds of genes including a transcript regulated factor and a rough sheath* 2*-like protein. This rolling leaf and flanking sequence data will stimulate the functional analysis of rice genes.*

***Keywords*:** *Tissue Culture*, *Somaclonal Variation*, *Mutant*, *Opaque Endosperm*, *Tos Element*

1. Introduction

Tissue culture of plants is an important means to propagate genetically identical individuals asexually and to produce transgenic plants. However, undesired genetic and cytogenetic modifications are frequently generating genetic variability, somaclonal variations, induced during tissue culture. These tissue culture-induced mutations were reported in many plant species and seemed to be ubiquitous in plants [1,2]. Although tissue cuture-induced mutations were studied extensively as a source for plant improvement, little is known about their molecular causes. Specifically, somatic variations are becoming the limelight of breeders since they improve both the individual and species of crops [3,4]. Rice is used in genome study model of monocotyledonous plant because of the size of its genome (~430 Mb) being relatively small. Also, an international project had already gathered the functional analysis of gene from DNA sequence [5]. There are various methods to analyze the function of the gene. One method used was a genome tilling array to a specific mutation group while handling chemical mutation materials such as N-methyl-N-nitrosourea (MNU) and ethyl methanesulfonate (EMS) or radiation. This method involved inserting foreign DNA fragments with labeled T-DNA, and using transition factor that exist in plants such as *Ac*/*Ds* and *Tos* derivatives which are a "Knock-out" or "Knock-down" of the gene or activation [6-8]. T-DNA or *Ac*/*Ds* gene trap sys- tems needed a lot of time and effort because it involved breeding large-scale mutation populations through the process of transformation to introduce a marked-gene on a rice genome. On the other hand, retrotransposon such as *Tos* can easily get a somaclonal variant through tissue culture without processing the mutagen. This method was widely used for the rice gene function analysis in Japan's NIAS (National Institute of Agrobiological Science, http://Tos.nias.affrc.go.jp), France's CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement, http://urgi.versailles. inra. fr/OryzaTagline), and Korea's POSTECH (Pohang University of Science and Technology, http://www.postech. ac.kr/life/pfg) [1]. Meanwhile, there are about 1,000 retrotransposons with 32 classes in the rice genome that existed [6] and, LTR (long terminal repeats) retrotransposon with a minimum of 59 classed which composed

17% of the rice entire genome, but research about the evolution or introduced time is insufficient [3]. The reverse transcription modulator (RTs; Reverse Transcriptase) of Tos family with 20 classes caused somaclonal variation because it was activated at the tissue culture which were only found in the japonica variety 'Nipponbare' [9-18]. However, very little is known about the ecotype and copy number of the rice species or kind of *Tos*.

Hence, *japonica* variety, 'Ilpum' was bred domestically for rice gene function analysis. S_1 and S_2 progeny populations were bred from the cultured seed, and the incidence of variant in main agronomic characteristics was investigated. The "Rolling leaf" mutation was analyzed to search for the kind of *Tos* and difference of copy number in rice, and the retrotransposon present in *Tos* family.

2. Materials and Methods

2.1. Plant Materials Growing Conditions

Calli of the *japonica* variety, 'Ilpum' of *Oryza sativa* were induced in germinated dehulled seeds on a Murashige-Skoog solid medium [19] containing 2,4-20 dichlorophenoxyacetic acid at 2 $mg{\cdot}L^{-1}$, casein hydrolysate (2 $g{\cdot}L^{-1}$), sucrose (30 $g{\cdot}L^{-1}$), 21 and gelrite (5 $g{\cdot}L^{-1}$). De-hulled seeds were incubated for 4 weeks at 26 ± 1˚C in permanent darkness. After incubation for 4 weeks, calli on the selection medium with somatic embryo-like structures were transferred to MS medium supplemented with 30 $g{\cdot}L^{-1}$ sucrose, 1 $mg{\cdot}L^{-1}$ 1-naphthaleneacetic acid, 5 $mg{\cdot}L^{-1}$ kinetin, and 5 $g{\cdot}L^{-1}$ gelrite and exposed to intense light (approx. 45-5 $mEm^{-2}\ s^{-1}$) at 26 ± 1˚C. After 3 weeks, the regenerated shoots were transferred to a bottle (6 × 16 cm) containing a fresh medium. When the shoots reached the top of the box, the plantlets were transferred to soil.

2.2. Measurement of Agronomic Traits

Progeny populations (S_1, S_2) grown from the regenerated seed-culture (S_0) were planted 8 at 30 × 15 cm planting distance on the practice field in Kyungpook National University, and the main agronomic characters were investigated. Brown rice's protein local, and starch content were analyzed by NIRS (Near-Infrared Spectroscopy, Foss 6500), and the amylose content used 3 repeated mean values provided by colorimetry of Juliano *et al.* [9].

2.3. Genomic DNA Extraction, PCR, Southern, and RT-PCR Analysis

DNA from the mutants was extracted using the CTAB method [20-25]. Ten-microgram aliquots of DNA were first digested with *Hae* III and then electrophoresed onto a 0.8% 18 agarose gel. PCR was performed in 50 $\mu\varrho$ volume of [1.5 mM $MgCl_2$, Taq DNA polymerase, and a corresponding buffer from GibCo (Invitrogen, Carlsbad, Calif., http://www.invitrogen.com). Primer made amino acid sequences [8] of *Tos* 4 ~ *Tos* 20 by DNAsis 3.0 Programs (Hitachi, Japan) using GenBank's data base confirmed the relationship existence of retrotransposon elements of 'Ilpum' (**Table 1**). The DNA was denatured at 93˚C for 5 min followed by 35 cycles of amplification (1 min at 93˚C; 2 min at 40˚C; 2 min at 72˚C); the final incubation at 72˚C was extended to 5 min, and the reaction was cooled and kept at 4˚C. PCR were performed in a PTC-200 thermal cycler (MJ Research). RT-PCR (reverse transcriptase-PCR) was used to analyze the mutants containing the retrotransposon elements as determined by the PCR and Southern analysis [2]. The total RNAs that were from the putative rice plants (leaves) using guanidine thiocyanate were isolated [11].

2.4. Histology and Microscopy Observation

Fresh hand-cut sections (~20 mm) of rice leaf were fixed in FAA solution [(10 ml Formalin, 5 ml Acetic acid (glacial), 95 ml 50% Ethylalcohol), (16 to 48 h, 48˚C)] and dehydrated through a graded ethanol series. Treated samples were transferred into water. The sections were microscopically examined (Automatic plant microtome, Mt-3, NK system) and photographed. The samples were embedded in paraffin (Fluka) and polymerized at 56~58˚C. Sections (50 μm) were cut and dropped with filtered paraffin, microscopically examined (Olympus, BX50), and photographed. Area measurements of the vascular elements were performed using an Olympus analySIS TS Lite software.

2.5. Amplification of Sequences Flanking Transposed *Tos* l7

Sequences flanking the transposed *Tos* 17 (target-site sequences) were amplified by an adaptor-PCR. Target-site sequences were amplified using the rice total DNA, respectively, as described [24] except that the total DNA was digested with *Hae* III. Two sets of primers were used for the two-step PCR: adaptor primer-1, GCGTAATACGACTCACTATAGCAATTAACC and *Tos* l7 primer-1, TGCTCTCCACTATGTGCCCTCCGAGCTA were used for the first PCR; the adaptor primer-2, GACTCACTATAGCAATTAAC and *Tos* 17 primer-2, ACAAGTCGCTGATTTCTTCAC were used for the second reaction. PCR was performed in a PTC-200 thermal cycler (MJ Research) and conducted as follows: first, the denaturetion step at 95˚C for 5 minutes, 20 cycles of 30 seconds at 94˚C and 1 minutes at 72˚C, followed by a final elongation step at 72˚C for 10 minutes; second the PCR was conducted with 5ul of the first PCR product under the following conditions: denaturation step at 94˚C for 5 min,

Table 1. The primer sequence of *Tos* families for PCR.

Tos families	Primer sequence	
	Forward (5' → 3')	Reverse (3' → 5')
Tos 4	ACATTTTTACATggAgAgCTTgAg	ATTCTACATCCTCATCagTAC
Tos 5	ATATgCATCTCCTgATgCAgACTA	CAAgCACAAAATAACTCCCTC
Tos 6	gCACACTATgATTTAgAgTTgCAT	AgTgTTCCTgTCCTggATTgC
Tos 7	gACAgATATAAAgCTAgATTggTT	AgTTTCTCAgTTggTgACAgA
Tos 8	gCTATgTTACAAgCgAggACATAC	CTTTTgCAACTAAgCgAgCTT
Tos 9	gCTTACTCAgCTTCgCAAgCTCgA	AgTCTTgCCTTgTgCCTAATT
Tos 10	gCAAAATCCTAATggAAAggTggA	AgTTATgTCAggTCgTgTAtg
Tos 11	gCTAgTAgTgATgTCAgTCTACTg	gTTgTgAgTgTACATTACTgC
Tos 12	gCATTTTTACATggggAgTTAggA	gAAgATAATCTgAAATgTgCA
Tos 13	gCgTTTTTgCATggAgAgCTTgAg	ACTgAgCAgCTgACAACTTAA
Tos 14	TACATTgTATACCTCgTggATgAC	TAAgCACAAgATCACCCCTTC
Tos 15	gACggTACTATTgAAAAgTACAag	AgTTgATTCCTAgCAATTCTC
Tos 16	CTCAACCTCATAATggACgTgATA	CATggTTTgTATCTCATgAgC
Tos 17	gCTTTTCTTCATggTgATCTTCAT	gAgTAACAACAAAgTAcgACC
Tos 18	gCATTTTTACATggggAgTTAgAA	AgTCAggACgAgAACATACCA
Tos 19	gTTACTTCTTgggAATTgAggTTT	ggATCATTAgCAATCTgTAtg
Tos 20	ACAgTTTgCCCTCAgAACATgTTC	AgTCTTCACATCTAACTgCTC

40 cycles of 30 seconds at 94˚C, 30 seconds at 60˚C, and 1 minute at 72˚C, followed by a final elongation step at 72˚C for 10min. Amplified products were loaded on a 1% agarose gel. The PCR products were purified by Hi-Yield™ Gel/PCR DNA Extraction Kit (RBC) and sequenced by ABI3730XL (using *Tos* 17 primer-2).

2.6. Sequence Analysis and Database Search

Handling of primary sequences and multiple sequence alignment were carried out using the DNAsis 3.0 software (Hitachi, Japan). Computer-based amino acid similarity searches of the Protein Identification Research, Genome annotation DB (Gramene, http://www.gramene.org/) and NCBI (http://www.ncbi.nlm.nih.gov/) were carried out with the FST (Flanking Sequence Tag).

3. Results

There were 4 lines (1.3%) among the entire 297 lines that showed evidence of "rolling 19 leaf" mutant that occurred at the progeny (S_1) using the brown rice culture of *japonica* type 'Ilpum'. Compared with the "rolling leaf" mutant mutant, the donor plant ''Ilpum', showed normal leaf with equal small veins, protoplasm formations, and conductive tissue of right and left in the midrib (**Figure 1**). The mutant had no equal arrangement of midrib and small veins. Because the bulliform cell was well-developed, it ranged extensively, and the conductive tissue of the midrib was thick. Also, the sclerenchyma was thin (**Figures 1(c)** & **(d)**).

Though the mutant "rolling leaf" plant high and the panicle number was less than the donor plant (**Table 2**), it was believed that the growth change with morphological characteristicsof leaf was secondary. These agronomic characteristics should be studied further to understand deeply the mechanism of such leaves.

The retrotransposon's activation known as one of the main causes of the conversion that occurred in tissue culture of rice, specifically for the 17 set oligonucleotide primers from conserved domain of *Tos* 4 ~ 20 reported in the 'Nipponbare' were analyzed 13 (**Table 1**). The PCR that the primers used for 3 classes (*Tos* 12, 15, 17) of the *Tos* 14 element was amplified in the 'Ilpum' of the mutants (**Figure 2(a)**). *Tos* element's that appeared in the 'Ilpum' were different from the 'Nipponbare' [8]. These differences were believed to be from a genotype difference caused by the cultivar which was used in PCR am-

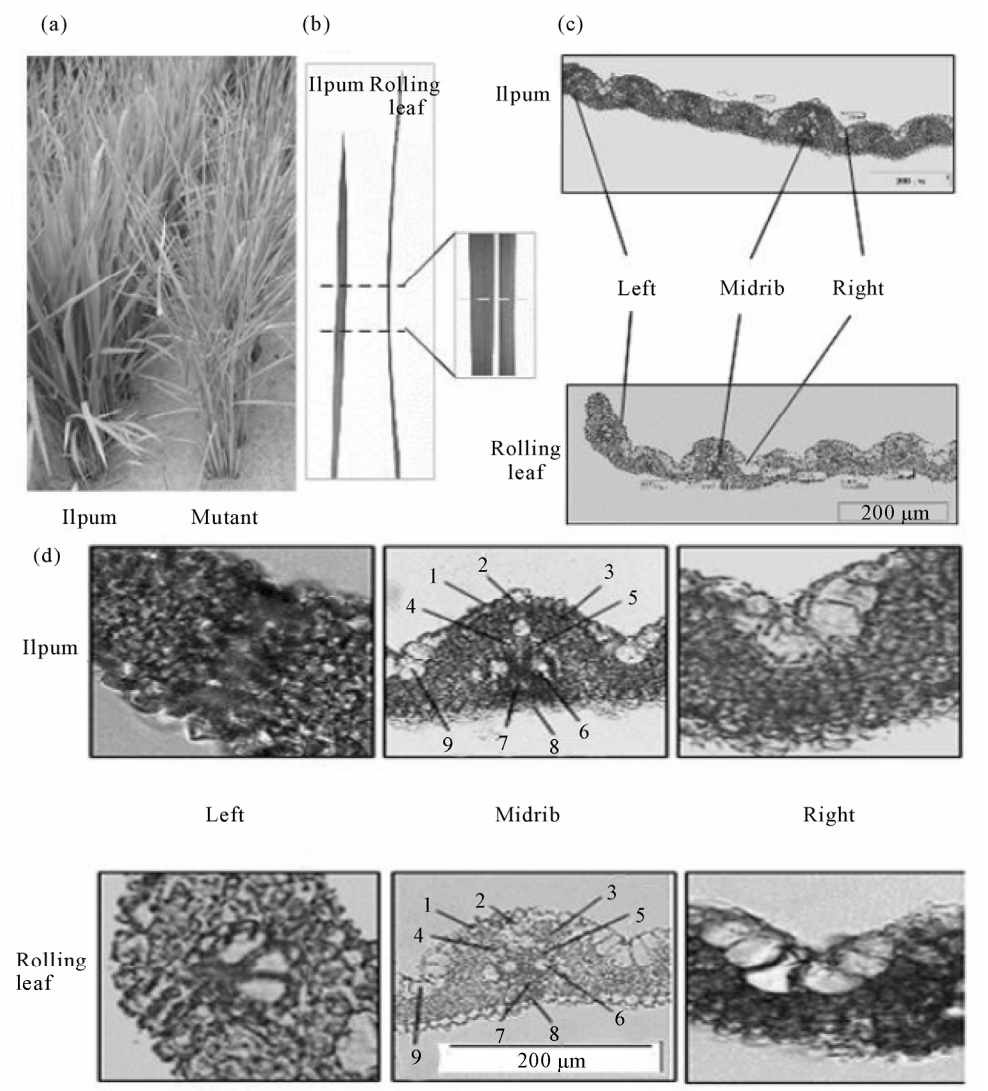

Figure 1. Histological differences between, 'Ilpum' and a mutant with rolling leaf. A: Agronomic traits of the mutants with rolling leaves. B: Young leaf of, 'Ilpum' and the mutant. C: The defective sclerenchymatous cells on the abaxial side of, 'Ilpum' and the mutant. D: Organization of megascopic leaf. 1: upper epidermis, 2: adaxial sclerenchyma, 3: mesophyll cells, 4: vascular bundle sheath, 5: mestome sheath, 6: 11 xylem, 7: phloem, 8: lower epidermis, 9: bulliform cell.

plification. The studied activation and copy numbers confirmed that *Tos* elements were present in 'Ilpum' (**Figure 2(b) and (c)**). There were *Tos* 12 and *Tos* 15 in 19 positions such as the donor plant, and *Tos* 17 was observed in 2 polymorphic bands in #15, #16 mutants, "rolling leaf", respectively. These results could confirm in the Southern analysis by the *Tos* 17 probe, and was activated in the S_2 progeny using the RT-PCR.

Interrelation analysis of the "rolling leaf" mutant and *Tos* 17 activations achieved the FST (Flanking Sequence Tag) analysis in the Genome annotation DB (Gramene, http://www.gramene.org/; NCBI, http://www.ncbi.nlm.nih.gov/) using a construed 1 sequence (**Tables 3 and 4**). *Tos* 17 was situated in chromosome 11 and 12 in the donor 2 plant, 'Ilpum' (**Table 3 and Figure 3**).

The homology gene had about 12 kinds in the area that *Tos* 17 had been inserted into 1 (**Table 4**). They were

Table 2. Agronomic traits of the mutants with rolling leaves.

Lines	No. of lines	Culm length (cm)	Panicle length (cm)	No. of tillers
Rolling leaf	4	81.3 ± 6.8	21.5 ± 2.4	5.9 ± 1.1
Ilpum(Ck)	-	72.0 ± 4.2	21.1 ± 1.2	14.5 ± 2.4

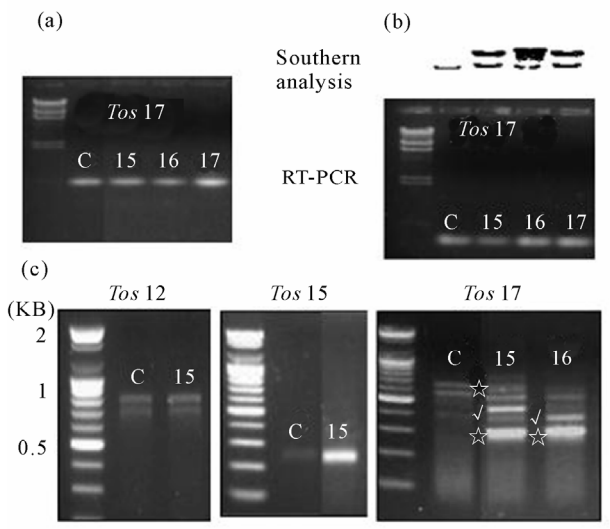

Figure 2. Detection of retrotransposon elements in the mutants and a donor cultivar 'Ilpum' using genomic DNA analysis. A: Detection of retrotransposon *Tos* 17 primer by PCR. m; size marker (λ/HindIII). c; 'Ilpum'. #15~#17; mutants. B: DNA gel blot analysis by S_1 progeny and RT-PCR by S_2 progeny of the mutants, 'rolling leaf'. C: The second PCR products derived from PCR amplification between the first PCR products and adaptor primer-1 in somaclonal mutants and a donor cultivar 'Ilpum'. v; The bands were isolated from agarose gel, and sequenced (pass). ☆; The bands were isolated from agarose gel, and sequenced (fail). c; Ilpum, #15~16; mutants of rolling leaf.

arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II, cytochrome cd1-nitrite reductase-like, RNA-binding region RNP-1, metallothionein-like protein 1, and Cytochrome P450. Also, transcription factor rough sheath 2 like proteins were analyzed and showed high interrelationship in morphogenesis of "rolling leaf".

4. Discussions

The mutant "rolling leaf (rl)" was reported in 11 different kinds of the transposon element which affected the rolling leaf until the present. Also the *rl*1~*rl*6 alelle, recessive gene of 6 kinds, were situated within each chromosome 1, 4, 12, 3, 7 thru formal marker analysis [12,13]. The *rl*7~*rl*10 alelle mapping in the chromosome 5 [16,17, 21,26,27], R*l*(*t*), had an incomplete recessive gene, mapping between the long arms (137 kb area) of chromosome 2 [22]. Yet there was also no report that there was any direct gene cloning. OsAGO7, the "rolling leaf" related gene, was reported to have an upward leaf curling in the overexpressing transformants [23]. Zhang *et al.* [28] reported that the Shallot-like 1 (*SLL* 1), MYB transcription control factor, belonging to the KANADI family was caused by the leaf-rolling cause on the chromosome 9 in the rice. The plant height of the mutant "rolling leaf" was higher than the donor plant. It was reported that there was a photosynthesis difference because of the carbon fixing or the gas exchange according to the three-dimensional structure of leaf [5]. Though the mutant "rolling leaf" was the transposon of the *Tos* 17, it occurred in chromosome 11 and 12, each differed from the studies of Shi *et al.* [23] and Zhang *et al.* [28] and the findings in this research (**Table 3 and Figure 3**). This result maybe

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Rolling leaf chr.11 : 331 QSIQSLIYDDNVSGVCWPDPDFQYYENNRNLYNSTRIGSLDDYGEFFSSDLAKHQARAF 152
                          QSI SLIYDD NVSGV WPDPD+QYYENNRNLYNSTRIGSLDDYGEFFSSDLAKHQAR
Os11g0132900 :        171 QSILSLIYDDTNVSGVYWPDPDYQYYENNRNLYNSTRIGSLDDYGEFFSSDLAKHQAR-- 230

Rolling leaf chr.11 : 151 LHGDLHEEVYMH ~ LFIHTSSRGRTLLLLGLKEGL 230
                                                G+K  L
Os11g0132900 :        231 -----------------------------GIKRRL 241


Rolling leaf chr.12:  447 PNHWIKHDYTRKIFVGGLPPSVGAFLHGDLHEEVYMH ~ LFIHTSSRGRTLLLLAEYLT 626
                          PNHW KHDYTRKIFVGGLPPSVG                          AEYLT
Os12g0572800:         572 PNHWEKHDYTRKIFVGGLPPSVG-------------------------------AEYLT 599

Rolling leaf chr.12:  627 EFFTAEFGPMEEAVVIGIRMGDRVQSRGFGFVKFKREE 740
                          EFFTAEFGP+EEAVVIGIRMGDRVQSRGFGFVKFKREE
Os12g0572800 :        600 EFFTAEFGPVEEAVVIGIRMGDRVQSRGFGFVKFKREE 637


Opaque chr.1 : 628 AMAADGSVLRRLYFSVYNLAFLHGDLHEEVYMH ~ LFIHTSSRGRTLLLLAQVILSMILALLDDGH 820
                   AM   GS +RRLY SVYN                                AQVI   +  LL+ GH
Os01g0150200 : 30  AMTGVGSAVRRLYLSVYNW-------------------------------AQVIYYAVTTLLESGH 71

Opaque chr.1 : 821 EAVYAATERHLLFAQTAAIMEVWF 915
                   EAVYAA+ER L FAQTAA +EVWF
Os01g0150200 : 72  EAVYAAVERPLQFAQTAAFLEVWF 94
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Figure 3. Genes disrupted by insertion of *Tos* 17. Amino acid sequences deduced from target-site sequence (rolling leaf) are aligned with sequenced of GenBank data ('Nipponbare'). Red letters: amino acid sequence of *Tos* 17, Rectangles: no homology.

Table 3. Chromosome locations of *Tos* 17 in a rice mutant with rolling leaf.

Sequenced flanking region of *Tos*-DNA		Matched region with rice chromosomes				*Tos*- DNA end	Adaptor start	Reading length
from	to	length	Chr.	from	to	*Tos*- DNA end	Adaptor start	Reading length
195	306	112	11	1492553	1492664	194	307	340
195	372	178	12	23812926	23812749	194	373	406

Table 4. Annotated genes of *Tos* 17 inserted flanking regions on chromosome in a rice 4 mutant with "rolling leaf".

Chromosome No.	Accession No.	Protein	Origin
11	NM_001072196.1	Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II	Oryza sativa L.
	NM_001072197.1	Cytochrome cd1-nitrite reductase-like	Oryza sativa L.
	NM_001072198.1	Protein kinase family protein	Oryza sativa L.
	NM_001072200.1	TPR-like domain containing protein	Oryza sativa L.
	NM_001072201.1	Resistance protein candidate (Fragment)	Oryza sativa L.
	NM_001072202.1	RNA-binding region RNP-1	Oryza sativa L.
12	FAA00315.1	TPA: metallothionein-like protein	Oryza sativa L.
	ABR25982.1	Metallothionein-like protein 1	Oryza sativa L.
	ABR25363.1	Mitochondrial import inner membrane translocase subunit tim9	Oryza sativa L.
	BAB61618.1	Transcription factor rough sheath 2 like protein	Oryza sativa L.
	BAC78592.1	pre-mRNA splicing factor	Oryza sativa L.
	AAY44413.1	Cytochrome P450	Oryza sativa L.

brought about by the mixing of a lot of gene numbers that caused the "rolling leaf" mutant. The FST analysis of the mutant "rolling leaf" and the cause of the mutation should be studied continuously via genetic and molecular breeding of the S3 progeny to determine the relation of gene function.

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