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**Complex genetic nature of sex-independent transmission ratio distortion in Asian rice species: the involvement of unlinked modifiers and sex-specific mechanisms**

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1 **Complex genetic nature of sex-independent transmission ratio distortion in Asian**  
2 **rice species: the involvement of unlinked modifiers and sex-specific mechanisms**

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1 **Abstract**

2 Transmission ratio distortion (TRD), in which one allele is transmitted more frequently  
3 than the opposite allele, is presumed to act as a driving force in the emergence of a  
4 reproductive barrier. TRD acting in a sex-specific manner has been frequently observed  
5 in interspecific and intraspecific hybrids across a broad range of organisms. In contrast,  
6 sex-independent transmission ratio distortion (*si*TRD), which results from preferential  
7 transmission of one of the two alleles in the heterozygote through both sexes, has been  
8 detected in only a few plant species. We previously reported  $S_6$  locus-mediated *si*TRD, in  
9 which the  $S_6$  allele from an Asian wild rice strain (*Oryza rufipogon*) was transmitted  
10 more frequently than the  $S_6^a$  allele from an Asian cultivated rice strain (*O. sativa*) through  
11 both male and female gametes in heterozygous plants. Here, we report on the effect of a  
12 difference in genetic background on  $S_6$  locus-mediated *si*TRD based on the analysis using  
13 near-isogenic lines and the original wild strain as a parental strain for crossing. We found  
14 that the degree of TRD through the male gametes varied depending on the genetic  
15 background of the female (pistil) plants. Despite the occurrence of TRD through both  
16 male and female gametes, abnormality was detected in ovules, but not in pollen grains, in  
17 the heterozygote. These results suggest the involvement of unlinked modifiers and  
18 developmentally distinct, sex-specific genetic mechanisms in  $S_6$  locus-mediated *si*TRD,  
19 raising the possibility that *si*TRD driven by a single locus may be affected by multiple  
20 genetic factors harbored in natural populations.

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1 **Introduction**

2 Transmission ratio distortion (TRD) refers to a naturally occurring phenomenon in which  
3 the two alleles at a heterozygous locus are not transmitted equally to the progeny, and this  
4 leads to a deviation in the genotype frequencies from the expected Mendelian ratios. TRD  
5 is induced by a variety of mechanisms, such as non-random chromosome segregation  
6 during meiosis (Birchler *et al.*, 2003; Fishman and Saunders, 2008), preferential gamete  
7 dysfunction in hybrids (Lyttle, 1991; Moyle and Graham, 2006; Long *et al.*, 2008; Chen  
8 *et al.*, 2008; Tao *et al.*, 2009a and b; Phadnis and Orr, 2009), and preferential gamete  
9 success during fertilization (Price, 1997; Fishman *et al.*, 2008). Because TRD can  
10 dramatically alter the frequency of alleles in a population by disrupting proper Mendelian  
11 segregation, it has been hypothesized that TRD is a driving force in the emergence of a  
12 reproductive barrier (Frank, 1991; Hurst and Pomiankowski, 1991). With regard to the  
13 process of TRD-mediated reproductive barrier formation, Frank (1991) and Hurst and  
14 Pomiankowski (1991) independently proposed that the genes responsible for gamete  
15 dysfunction in hybrids and consequently induce TRD are fixed rapidly in a population  
16 due to their “selfish nature,” but that they may easily become suppressed within a  
17 population to alleviate their deleterious effects on fertility. As a result, two allopatric  
18 populations might evolve different TRD systems. If these populations later hybridize,  
19 normally suppressed TRD within one population will be re-expressed in hybrids of  
20 individuals from each population, leading to hybrid sterility, which acts as a reproductive  
21 barrier between the two allopatric populations (Frank, 1991; Hurst and Pomiankowski,  
22 1991).

1           In plants, TRD has been detected many times in interspecific and intraspecific  
2 hybrids (Morishima *et al.*, 1992; Koide *et al.* 2008b; and references therein). Among  
3 them, TRD occurred in either the male (*m*TRD) or female (*f*TRD) gametes has been  
4 frequently reported and some of the genes causing sex-specific TRD have been cloned  
5 (Chen *et al.*, 2008; Long *et al.*, 2008). On the other hand, there are few reports on sex-  
6 independent TRD (*si*TRD), which results from preferential transmission of both male and  
7 female gametes carrying one of the two alleles in the heterozygote (Rick, 1966; Koide *et*  
8 *al.*, 2008c). Little is known about the genetic basis and evolutionary history of *si*TRD,  
9 although *si*TRD exerts the strongest effect on segregation distortion among these types of  
10 TRD.

11           We previously reported  $S_6$  locus-mediated *si*TRD in a hybrid of Asian cultivated  
12 rice (*Oryza sativa*) and wild rice (*Oryza rufipogon*) (Sano, 1992; Koide *et al.*, 2008a).  
13 Asian cultivated rice and wild rice belong to the same biological species, forming a  
14 primary gene pool (*O. sativa*-*O. rufipogon* complex) according to the classification  
15 system for gene pools (Harlan 1975). Thus, this provides an opportunity to examine the  
16 genetic basis of intraspecific TRD. We observed a reduction in seed setting among the  $F_1$   
17 plants derived from a cross between T65wx (*O. sativa* ssp. *japonica*) and a near-isogenic  
18 line (NIL; designated as NIL- $S_6$  in this study) carrying a segment of chromosome 6  
19 derived from a strain of *O. rufipogon* (Ruf- $S_6$  in this study) (Sano, 1992). When the  $F_1$   
20 hybrids were reciprocally crossed with T65wx, the resultant  $BC_1F_1$  progeny plants  
21 exhibited a reduced seed-setting rate, while the  $F_2$  progeny plants derived from self-  
22 pollination of the  $F_1$  hybrid plants exhibited a normal seed-setting rate (Sano, 1992).

1           This phenomenon is due to an interaction between a gene designated  $S_6$  in the  
2 chromosomal segment derived from Ruf- $S_6$ , and its opposing allele ( $S_6^a$ ) in T65wx. The  
3  $S_6$  allele acted as a “gamete eliminator,” and was transmitted more frequently than  $S_6^a$   
4 through both the male and female gametes in heterozygotes ( $S_6/S_6^a$ ). Female gametes  
5 possessing the  $S_6^a$  allele were aborted in the heterozygotes, causing a reduced seed-setting  
6 rate (Sano, 1992; Koide *et al.*, 2008a). In contrast, no defect was observed in the pollen  
7 grains of the heterozygotes, although male gametes possessing the  $S_6^a$  allele were rarely  
8 transmitted to the next generation (Sano, 1992; Koide *et al.*, 2008a). We have also  
9 revealed that Asian rice strains frequently harbor an additional allele ( $S_6^n$ ), which  
10 however, does not induce any preferential abortion in heterozygotes ( $S_6/S_6^n$  and  $S_6^a/S_6^n$ ) at  
11 the  $S_6$  locus (Koide *et al.*, 2008a), as shown by test-cross experiments and subsequent  
12 genetic mapping using NILs that carry the genetic background of T65wx. The presence of  
13 the  $S_6^n$  allele, which modifies the effect of the  $S_6$  allele in heterozygotic state at the  $S_6$   
14 locus, suggested that  $S_6$  locus-mediated *si*TRD was caused by the allelic differentiation at  
15 the  $S_6$  locus occurred during the evolution of Asian rice.

16           It is conceivable that changes in genetic factors that positively or negatively  
17 control  $S_6$  locus-mediated *si*TRD occurred during the evolution of Asian rice and such  
18 changes might have affected the presence or absence of reproductive barrier between  
19 constituents of the Asian rice population. With such possibilities in mind, in this study,  
20 we compared the effect of  $S_6$  locus-mediated TRD between two  $F_2$  populations that were  
21 produced using a NIL and its original wild strain as respective parental strains for  
22 crossing and examined whether there are genes which modify the effect of  $S_6$  locus-  
23 mediated *si*TRD that exist in the genetic background of Asian rice strain. We also

1 examined the extent of male- and female-specific TRD by reciprocal backcross  
2 experiments. Based on the results, together with those of subsequent genetic and  
3 cytological analyses, we report the involvement of unlinked modifiers and sex-specific  
4 mechanisms in this phenomenon.

## 6 **Materials and Methods**

### 7 **Genetic stocks**

8 Three lines, T65 $wx$ , Ruf- $S_6$ , and NIL- $S_6$  were used. T65 $wx$  carries  $wx$  (*waxy*) gene as a  
9 genetic marker in the genetic background of Taichung 65 (*O. sativa* ssp. *japonica*). Ruf-  
10  $S_6$  is a perennial type strain of *O. rufipogon*, W593. NIL- $S_6$  carries the short arm and a  
11 portion of the long arm of chromosome 6 from Ruf- $S_6$  in the genetic background of  
12 T65 $wx$  (Sano, 1992; Matsubara *et al.*, 2003; Koide *et al.*, 2008a; formally named as  
13 T65 $S_6$ [W593]). T65 $wx$  harbors the  $S_6^a$  allele at the  $S_6$  locus (near the centromeric region  
14 of chromosome 6), while Ruf- $S_6$  and NIL- $S_6$  harbor the  $S_6$  allele at the  $S_6$  locus (Koide *et*  
15 *al.*, 2008a). Although T65 $wx$  harbors  $wx$  gene from Kinoshita-mochi (Oka, 1974; derived  
16 from BC<sub>12</sub>),  $wx$  gene does not affect  $S_6$  locus-mediated TRD.

### 17 **Genetic crosses and genotyping to detect $S_6$ locus-mediated TRD**

18 To examine the effect of  $S_6$  locus-mediated TRD on linked loci on chromosome 6, a total  
19 of 98 F<sub>2</sub> segregating plants derived from T65 $wx$  × NIL- $S_6$  were genotyped using 15 DNA  
20 markers from chromosome 6 ( $Wx$ , E12, R1962, RM204, RM314, *OsC1*, RM276, RM539,  
21 *Hdl*, R538, R111C, R32, RM3498, G2028, and RM1340). Additionally, to examine the  
22 effect of  $S_6$  locus-mediated TRD in the hybrids between *O. sativa* and the original wild  
23 strain of *O. rufipogon*, a total of 103 F<sub>2</sub> segregating plants derived from T65 $wx$  × Ruf- $S_6$

1 were genotyped using eight DNA markers from chromosome 6 (E12, RM204, RM276,  
2 *Hdl*, R111C, RM3, RM3498, and RM1340).

3 To further characterize the  $S_6$  locus-mediated TRD in the cross of  $T65_{wx} \times Ruf-S_6$ ,  
4 transmission of the  $S_6$  allele through males (i.e., *m*TRD) and females (i.e., *f*TRD) was  
5 assessed by reciprocal backcross experiments. To estimate the degree of *m*TRD,  $F_1$  plants  
6 ( $T65_{wx} \times Ruf-S_6$ ) were used as the pollen parents and pollinated to female  $T65_{wx}$  and  
7  $Ruf-S_6$  plants. On the other hand, to estimate the degree of *f*TRD,  $F_1$  plants ( $T65_{wx} \times$   
8  $Ruf-S_6$ ) were used as the female parents and pollinated with male  $T65_{wx}$  and  $Ruf-S_6$   
9 plants. The segregation ratio at the  $S_6$  locus was estimated from that of the tightly linked  
10 DNA marker R111C.

11 For genotyping, genomic DNA was isolated from a small piece of frozen leaf  
12 according to the method of Monna *et al.* (2002) with slight modifications. Three Indel  
13 markers (*Wx*, *OsCl1*, and *Hdl*), three restriction fragment length polymorphism (RFLP)  
14 markers (R538, R32, and G2028), and a cleaved amplified polymorphic sequence  
15 (CAPS) marker, E12, from chromosome 6 were used for genotyping according to the  
16 method of Matsubara *et al.* (2003). A CAPS marker, R111C, was used according to the  
17 method of Koide *et al.* (2008a). Seven microsatellite markers (RM204, RM314, RM276,  
18 RM539, RM3498, RM3, and RM1340) were selected from a public database  
19 (<http://www.gramene.org>). Additionally, one CAPS marker, R1962, was designed based  
20 on a sequence from the public database (acc. no. AP006554). The sequences of the  
21 primers used for a CAPS marker, R1962, were 5'-gct tgg att atg aca ttt ag-3' and 5'-tga  
22 agc aag gaa caa aca-3'. To detect the polymorphism, the amplified products were digested  
23 with *TaqI*. The recombination values were estimated based on the maximum likelihood

1 method (Allard, 1956).

## 2 **Cytological observations and pollen tissue PCR**

3 Spikelets were sampled from the panicles before heading. The samples were fixed in  
4 FAA (formalin: glacial acetic acid: 70% ethanol, 1:1:18) and stored in 70% ethanol. The  
5 ovaries were dehydrated in a graded ethanol-butanol series, embedded in Paraplast Plus  
6 (Oxford Labware, St. Louis, MO, USA), and then cut into 10- $\mu$ m thick sections. The  
7 sections were stained with safranin and Fast Green (Sylvester and Ruzin, 1993) and  
8 observed by light microscopy (BH-2, Olympus, Tokyo, Japan).

9 To examine whether the  $S_6$  locus-mediated  $m$ TRD occurred before or after pollen  
10 grain production, pollen grains from heterozygous plants were genotyped according to  
11 the method of Petersen *et al.* (1996) with modifications. A total of 2-3  $\mu$ g of pollen grains  
12 were collected from  $F_1$  plants derived from  $T65_{wx} \times NIL-S_6$  at the flowering stage and  
13 transferred to tubes containing 32.7  $\mu$ L of  $H_2O$ , 5  $\mu$ L of 10 $\times$  *Takara Ex Taq* buffer, 5  $\mu$ L  
14 of 50% dimethyl sulfoxide, 2.5 mM each dNTP, 1  $\mu$ L of a 20 pM solution of each primer,  
15 and 0.3  $\mu$ L of *Takara Ex Taq* DNA polymerase (5 U  $\mu$ L<sup>-1</sup>). The CAPS marker R111C was  
16 used for genotyping. PCR was performed for 30 cycles (1 min at 96°C, 1 min at 56°C,  
17 and 1 min at 72°C), followed by 10 min at 72°C. For polymorphism detection, the  
18 amplified products were separated electrophoretically on a 2.5% agarose gel in 1 $\times$  TAE  
19 buffer and the DNA fragments were detected by staining with ethidium bromide.

20

## 21 **Results**

### 22 **Effects of the genetic background on $S_6$ locus-mediated TRD**

23 To examine the effect of genetic background on the strength of  $S_6$  locus-mediated  $si$ TRD,

1 we analyzed the difference in TRD at the  $S_6$  locus between two  $F_2$  populations derived  
2 from crosses of  $T65wx \times NIL-S_6$  and  $T65wx \times Ruf-S_6$ . To compare the effect of  $S_6$  locus-  
3 mediated TRD, we used the DNA marker R111C, which is tightly linked with the  $S_6$  locus  
4 (Koide *et al.*, 2008a).

5         Although TRD was detected in both crosses, the effect was different. In the  $F_2$   
6 population derived from  $T65wx \times NIL-S_6$ , almost all of the plants (84/98) were  
7 homozygous for the *O. rufipogon*-derived allele ( $S_6$ ). No homozygote for the *O. sativa*-  
8 derived allele ( $S_6^a$ ) was detected (Table 1), indicating that transmission of the  $S_6^a$  allele  
9 was reduced in both the female and male gametes (i.e., *si*TRD), consistent with previous  
10 data (Sano, 1992; Koide *et al.*, 2008a). However, in the  $F_2$  population derived from  
11  $T65wx \times Ruf-S_6$ , the numbers of homozygotes for the *O. rufipogon*-derived allele ( $S_6$ ),  
12 heterozygotes, and homozygotes for the *O. sativa*-derived allele ( $S_6^a$ ) were 48, 49, and 6,  
13 respectively (Table 1). The segregation ratio of the  $F_2$  plants was close to 1:1:0 in this  
14 cross.

15         Such a difference in the segregation ratio between the two cross combinations can  
16 be explained by either of the following models: (1) the degree of  $S_6$  locus-mediated TRD  
17 was changed by unlinked genes when the original wild strain of *O. rufipogon* ( $Ruf-S_6$ )  
18 was used; (2) a novel TRD which tends to transmit the *O. sativa*-derived allele ( $S_6^a$ ) and  
19 counteracts the over-transmission of the  $S_6$  allele occurred at a locus linked to  $S_6$  when the  
20 original wild strain of *O. rufipogon* ( $Ruf-S_6$ ) was used. To examine these two possibilities,  
21 the segregation ratio at markers on chromosome 6 was analyzed using two  $F_2$  populations  
22 derived from crosses of  $T65wx \times NIL-S_6$  and  $T65wx \times Ruf-S_6$  (Figure 1). In both cases,  
23 strong TRD was detected only near the centromeric region where  $S_6$  is located. Moreover,

1 with an increase in the genetic distance from the centromeric region the degree of TRD  
2 decreased. If other loci on chromosome 6 were to affect the segregation pattern, the  
3 pattern of reduction in TRD should be affected near the causative loci. Thus, these results  
4 suggest that no novel TRD occurred on chromosome 6, but the degree of the  $S_6$  locus-  
5 mediated TRD was changed by unlinked genes when the original wild strain of *O.*  
6 *rufipogon* (Ruf- $S_6$ ) was used as one of the parents. In addition, in both populations, TRD  
7 was detected even at distal DNA marker loci 50 cM distant from R111C, indicating that  
8 the  $S_6$  locus-mediated TRD affected most of this chromosomal region irrespective of the  
9 genetic background.

#### 10 **The degree of $S_6$ locus-mediated *m*TRD depends on the female parent**

11 The segregation ratio of homozygotes for the *O. rufipogon*-derived allele ( $S_6$ ),  
12 heterozygotes, and homozygotes for the *O. sativa*-derived allele ( $S_6^a$ ) at R111C was close  
13 to 1:1:0 in the  $F_2$  plants derived from T65wx  $\times$  Ruf- $S_6$ , as mentioned above (Table 1). This  
14 result suggests that the transmission of the  $S_6^a$  allele was reduced through female or male  
15 gametes (*f*TRD or *m*TRD), or that transmission of the  $S_6^a$  allele was partially reduced  
16 through both female and male gametes. To examine which type of TRD occurred in the  
17 progeny of the cross between *O. sativa* (T65wx) and *O. rufipogon* (Ruf- $S_6$ ), we carried  
18 out backcrossing experiments. Using  $F_1$  plants as the female parents, the degree of *f*TRD  
19 was estimated from the segregation ratio of  $BC_1F_1$  plants. In contrast, the degree of  
20 *m*TRD was estimated using  $F_1$  plants as the male parents.

21 All of the  $BC_1F_1$  plants were heterozygous or homozygous for the *O. rufipogon* -  
22 derived allele ( $S_6$ ) at R111C when  $F_1$  plants were used as the female parents and crossed  
23 with T65wx or Ruf- $S_6$ , respectively (Table 1). Thus, the proportion of the transmission of

1  $S_6$  through female gametes was 100%, indicating complete  $f$ TRD. Similarly, when T65wx  
2 plants were used as the female parents and crossed with  $F_1$  plants, almost all of the  $BC_1F_1$   
3 plants (25/26) were heterozygous (Table 1), indicating  $m$ TRD. In contrast, when Ruf- $S_6$   
4 plants were used as the female parents and crossed with  $F_1$  plants, the transmission ratio  
5 of  $S_6$  through male gametes was 70% (19/26; Table 1), indicating incomplete  $m$ TRD.  
6 There was a significant difference in the transmission ratios of  $S_6$  through male gametes  
7 between the two  $BC_1F_1$  populations ( $P=0.049$  by Fisher's exact test), indicating that the  
8 degree of  $S_6$  locus-mediated  $m$ TRD varied depending on the background genotype of the  
9 female (pistil) parent. These results suggest that the degree of  $S_6$  locus-mediated  $m$ TRD  
10 was partly suppressed by unlinked modifier(s) in the progeny of the cross between *O.*  
11 *sativa* (T65wx) and *O. rufipogon* (Ruf- $S_6$ ), while that of  $f$ TRD was not suppressed.  
12 Moreover, these results also suggest that heterozygotes ( $S_6/S_6^a$ ) produced both  $S_6$  and  $S_6^a$   
13 pollen grains of normal fertilization potential.

14 **Abortion occurs *after* meiosis in female gametogenesis, but not in male**  
15 **gametogenesis**

16 Our backcross experiments suggested that  $S_6$  locus-mediated preferential abortion  
17 occurred in female gametes, while it did not occur in pollen grains in the heterozygotes  
18 ( $S_6/S_6^a$ ). To test this possibility, cytological observations were performed and the specific  
19 developmental stage at which the abnormality occurred was determined (Figure 2).  
20 Abnormal ovules were detected in the heterozygotes: bi-nucleate embryo sacs with a  
21 single enlarged nucleus (Figure 2a), tri-nucleate (Figure 2b), and penta-nucleate embryo  
22 sacs (Figure 2c) were observed in the abnormal ovules. This indicates that a defect in the  
23  $S_6^a$  female gametophyte in the heterozygotes occurred during the mitotic stage; thus, the

1  $S_6$  locus-mediated *f*TRD occurred *after* meiosis.

2         On the other hand, no developmental defect was observed in the mono-, bi-, and  
3 tri-nucleate stages of pollen development in the heterozygotes ( $S_6/S_6^a$ ). To examine the  
4 genotype of mature pollen grains produced in the heterozygotes ( $S_6/S_6^a$ ), pollen tissue  
5 PCR was carried out. DNA fragments that corresponded to both genotypes were  
6 amplified by PCR from pollen grains, as were amplified from leaf DNA (Figure 3),  
7 indicating that the heterozygotes ( $S_6/S_6^a$ ) produced both  $S_6$  and  $S_6^a$  pollen grains. Taken  
8 together, these results indicate that the preferential abortion of gametes occurred after  
9 meiosis in the  $S_6$  locus-mediated *f*TRD, while no detectable abnormality occurred in the  
10  $S_6$  locus-mediated *m*TRD.

11

## 12 **Discussion**

### 13 **Chromosomal regions affected by the TRD caused by allelic interactions at the $S_6$** 14 **locus**

15 The  $S_6$  locus has been mapped to a region including the centromere of chromosome 6  
16 (Koide *et al.*, 2008a). In the present study, we found that the degree of TRD caused by the  
17  $S_6$  locus decreased along with the genetic distance from the centromeric region in the  $F_2$   
18 population derived from the cross between T65 $wx$  and NIL- $S_6$  (Figure 1). If other hybrid  
19 sterility loci on chromosome 6 were to affect the segregation pattern in this cross  
20 combination, the pattern of the reduction in TRD should be affected near the causative  
21 loci. A clear reduction pattern in TRD towards the distal end of chromosome 6 was  
22 observed, indicating that the segregation distortion caused by the  $S_6$  locus was  
23 independent of that caused by other hybrid sterility loci, as had been previously suggested

1 (Koide *et al.*, 2008a). Moreover, a similar pattern of reduction in TRD was observed in  
2 the F<sub>2</sub> population derived from the cross between T65<sub>wx</sub> and Ruf-*S*<sub>6</sub> (Figure 1). These  
3 results suggest that the *S*<sub>6</sub> locus is the causal factor of TRD on DNA marker loci on  
4 chromosome 6 in both of the F<sub>2</sub> populations derived from T65<sub>wx</sub> × NIL-*S*<sub>6</sub> and T65<sub>wx</sub> ×  
5 Ruf-*S*<sub>6</sub>.

6 In *Mimulus*, Fishman and Willis (2005) examined the pattern of the reduction in  
7 TRD by developing NILs with a meiotic drive locus, *D*, from *M. guttatus*. The *D* allele  
8 exhibited a nearly 100% transmission advantage via female meiosis in hybrids with  
9 *M. nasutus* (Fishman and Willis, 2005). The effect of the TRD caused by the *D* locus was  
10 observed even at a locus 55 cM away. Similarly, the effect of the strong TRD induced by  
11 an alien 5B chromosome was observed at a locus 50 cM from the most distorted locus in  
12 wheat (Kumar *et al.*, 2007). The chromosomal ranges affected by the *S*<sub>6</sub> locus were  
13 comparable to those affected by the most distorted locus in *Mimulus* and wheat,  
14 suggesting that strong TRD often affects a locus 50 cM distant.

#### 15 ***f*TRD, governed by the centromeric region, occurred *after* meiosis**

16 In this study, the most severe TRD was observed at R111C near the centromere. This  
17 result is comparable with that from genetic mapping using a segregating population  
18 consisting of a large number of individual plants (Koide *et al.*, 2008a). Several examples  
19 of TRD near centromeric or neocentromeric regions have been reported in *Mimulus* and  
20 maize (Dawe and Cande, 1996; Yu *et al.*, 1997; Fishman and Willis, 2005; Fishman and  
21 Saunders, 2008). In *Mimulus*, because the *D* locus near the centromere caused significant  
22 *f*TRD without an increase in ovule or seed mortality, it was suggested that *f*TRD is a  
23 consequence of the preferential transmission of chromosomes with a centromere

1 containing the *D* allele during asymmetric female meiotic division processes (Fishman  
2 and Willis, 2005; Malik, 2005). The Ab10/knob system in maize involves the genetic  
3 activation of neocentromeric knob regions that competitively bind microtubules and  
4 orient the carrier chromatids toward the outer spindle poles at meiosis II (Dawe and  
5 Cande, 1996; Yu *et al.*, 1997). In both cases, the *f*TRD which is governed by the  
6 centromeric or neocentromeric region occurs *during* meiosis, with no deleterious effect  
7 on female gametes.

8         In the *S<sub>6</sub>* locus-mediated *f*TRD system, approximately half of the ovules exhibited  
9 an abnormality in embryo sac structure during female gametogenesis, and the seed-  
10 setting rate was reduced in heterozygotes (*S<sub>6</sub>/S<sub>6</sub><sup>a</sup>*) (Koide *et al.*, 2008a), indicating that  
11 *f*TRD occurred post-meiosis, which is different from that mediated by the *D* locus in  
12 *Mimulus* or the Ab10/knob system in maize. By cytological observation, bi-nucleate  
13 embryo sacs with a single enlarged nucleus, tri-nucleate embryo sacs, and penta-nucleate  
14 embryo sacs were found in the abnormal embryo sacs produced by the heterozygotes  
15 (*S<sub>6</sub>/S<sub>6</sub><sup>a</sup>*; Figure 2), indicating that an abnormality in nuclear division or migration occurred  
16 during the second or third round of mitosis after meiosis.

17         Mutations affecting female gametogenesis after the mono-nucleate stage have  
18 been reported in *Arabidopsis* and maize (Sheridan and Huang, 1997; Drews *et al.*, 1998).  
19 In *Arabidopsis hdd* (*hadad*) mutants, female gametophytes are arrested at the bi-, tetra-,  
20 or octa-nucleate stage (Drews *et al.*, 1998). In *lo2* (*lethal ovule2*) mutants in maize,  
21 nuclear division is affected and embryo sacs are arrested at the mono-, bi-, or tetra-  
22 nucleate stage, and, in some cases, the nuclei enlarge dramatically, suggesting a failure of  
23 entry into the prophase (Sheridan and Huang, 1997). In the embryo sacs of the *lo2*

1 mutants, abnormal behavior of the tubulin cytoskeleton was also observed. The failure to  
2 display a normal pattern of cytoskeleton behavior in the mutant embryo sacs was  
3 suggested to be an indirect result of abnormal interactions between defective nuclei  
4 lacking normal nuclear surface features and microtubule components of the microtubular  
5 cytoskeleton that are required for normal spindle orientation and nuclear migration  
6 (Huang and Sheridan, 1994; Sheridan and Huang, 1997).

7         The phenotype observed in the  $S_6$  locus-mediated *f*TRD system is similar to the  
8 *hdd* mutants in *Arabidopsis* and *lo2* mutants in maize. In all cases, embryo sacs are  
9 arrested during mitotic division. Moreover, in the cases of  $S_6$  and *lo2*, enlarged nuclei in  
10 the abnormal embryo sacs were observed. Based on the fact that the abnormalities in the  
11 embryo sacs of the  $S_6/S_6^a$  heterozygotes were similar to those in the *hdd* and *lo2* mutants,  
12 and given that  $S_6$  was mapped to a region including the centromere where the attachment  
13 of microtubules to the kinetochore occurs during mitosis, it appears likely that  $S_6$  is  
14 located close to the centromere and that its location and/or function disrupts the normal  
15 relationship between microtubules and the centromeric region. Detailed analyses of the  
16 behavior of the chromosomes or cytoskeleton during mitosis will help advance our  
17 understanding of the molecular mechanisms underlying the  $S_6$  locus-mediated  
18 preferential abortion of female gametes.

### 19 **Genetic mechanisms controlling the degree of *m*TRD**

20 In this study, differences in the degree of TRD at the  $S_6$  locus were observed between two  
21  $F_2$  populations derived from crosses between T65 $wx$  and a NIL (NIL- $S_6$ ) and between  
22 T65 $wx$  and the original wild strain (Ruf- $S_6$ ). *si*TRD was observed in the  $F_2$  population  
23 derived from T65 $wx$   $\times$  NIL- $S_6$ , while the degree of TRD was reduced in the  $F_2$  population

1 derived from T65<sub>wx</sub> × Ruf-*S*<sub>6</sub>. The segregation ratio of homozygotes for the *O. rufipogon*-  
2 derived allele (*S*<sub>6</sub>), heterozygotes, and homozygotes for the *O. sativa*-derived allele (*S*<sub>6</sub><sup>a</sup>)  
3 was close to 1:1:0 in this latter population (Table 1). Because NIL-*S*<sub>6</sub> and Ruf-*S*<sub>6</sub> are of  
4 different genetic backgrounds, the effect of *S*<sub>6</sub> locus-mediated *si*TRD may be due to  
5 differences in the genes in the respective genetic backgrounds. Moreover, backcrossing  
6 experiments revealed that the degree of *m*TRD was reduced only when Ruf-*S*<sub>6</sub> was used  
7 as the female (pistil) parent, whereas transmission of the *S*<sub>6</sub> allele through the female  
8 parent (*f*TRD) was 100% when T65<sub>wx</sub> or Ruf-*S*<sub>6</sub> was used as the male (pollen) parent  
9 (Table 1). Transmission of the *S*<sub>6</sub><sup>a</sup> allele from male T65<sub>wx</sub> × Ruf-*S*<sub>6</sub> plants was observed  
10 following crosses with female Ruf-*S*<sub>6</sub> pistils (Table 1), and pollen grains carrying the *S*<sub>6</sub><sup>a</sup>  
11 allele were detected by tissue PCR in the heterozygotes (Figure 3). Thus, the  
12 heterozygotes produced not only *S*<sub>6</sub>, but also *S*<sub>6</sub><sup>a</sup> pollen grains with normal fertilization  
13 potential, consistent with previous cytological observations of normal mature pollen  
14 grains in *S*<sub>6</sub>/*S*<sub>6</sub><sup>a</sup> heterozygotes (Koide *et al.*, 2008a). This suggests that the *m*TRD  
15 observed in the cross between the T65<sub>wx</sub> × Ruf-*S*<sub>6</sub> male and T65<sub>wx</sub> female was not due to  
16 the dysfunction of pollen grains carrying the *S*<sub>6</sub><sup>a</sup> allele, and occurred after pollen grain  
17 production.

18 A plausible mechanism for the *m*TRD which occurred after pollen grain  
19 production is difference in pollen performance, such as the ability of germination or the  
20 rate of pollen tube elongation, between the two types of pollen grains (i.e., those carrying  
21 the *S*<sub>6</sub> and *S*<sub>6</sub><sup>a</sup> alleles). Further experiments on the ability of pollen germination or the rate  
22 of pollen tube elongation might reveal a difference between pollen grains carrying the *S*<sub>6</sub>  
23 and *S*<sub>6</sub><sup>a</sup> alleles. Pollen tube competition has been observed in diverse plant taxa (e.g.,

1 Nelson, 1993; Ramsey *et al.*, 2003; Rahme *et al.*, 2009). In maize and rice, numerous loci  
2 for gametophyte factor (*ga*) have been reported. The *Ga* allele is known to confer a  
3 pronounced advantage on fertilization as the result of competition among pollen grains,  
4 leading to *mTRD* in later generations. In the extreme case of pollen competition caused  
5 by the maize *gal* locus, the growth of *gal* pollen tubes is retarded or arrested, depending  
6 on the genotype of the female parent (Nelson, 1993). In the *Silene* genus, the effect of  
7 competition between the pollen grains from *S. latifolia* and *S. dioica* is also related to the  
8 genotype of the female parent (Rahme *et al.*, 2009).

9         The degree of *S<sub>6</sub>* locus-mediated *mTRD* was reduced only when plants with a  
10 Ruf-*S<sub>6</sub>* genetic background were used as the female (pistil) parent in the backcross  
11 experiments (Table 1), suggesting that the difference in pollen performance is controlled  
12 by an interaction between the pollen (*S<sub>6</sub>* or *S<sub>6</sub><sup>a</sup>*) and pistil genotypes, and that the effects  
13 of the difference in pollen performance were weakened or partly suppressed by modifiers  
14 in the genetic background of the female Ruf-*S<sub>6</sub>*. To identify the modifier(s) involved in  
15 the suppression of *mTRD*, the development of recombinant inbred lines, each with  
16 different chromosomal segments in the genetic background, will be needed. A question  
17 arises as to how such a pattern of the difference in pollen performance and its modifier  
18 evolved in Asian rice population. It is tempting to speculate that *O. rufipogon*, which has  
19 a relatively higher outcrossing rate than *O. sativa*, might have traits suitable for  
20 outcrossing, such as a high pollen competition ability and a capacity of stigmas to receive  
21 alien pollen. On the other hand, *O. sativa*, which is predominantly selfing plants, might  
22 have lost such traits during the evolutionary process. Further analysis of the causative  
23 genes will help shed light on the evolution of *mTRD* and its modifier(s) in Asian rice.

1           We note that the result of our backcrossing experiments is not fully consistent  
2 with the segregation pattern observed in the F<sub>2</sub> population derived from T65wx × Ruf-S<sub>6</sub>.  
3 In our experiments, approximately 27% of the S<sub>6</sub><sup>a</sup> allele was transmitted to the progeny  
4 through male gametes when Ruf-S<sub>6</sub> was used as the female (pistil) parent, whereas no S<sub>6</sub><sup>a</sup>  
5 allele was transmitted to the progeny when T65wx or Ruf-S<sub>6</sub> was used as the male  
6 (pollen) parent (Table 1). On the other hand, the segregation ratio of homozygotes for the  
7 *O. rufipogon*-derived allele (S<sub>6</sub>), heterozygotes, and homozygotes for the *O. sativa*-  
8 derived allele (S<sub>6</sub><sup>a</sup>) in the F<sub>2</sub> population, was close to 1:1:0 (Table 1), suggesting that  
9 approximately 50% of S<sub>6</sub><sup>a</sup> allele was transmitted to the F<sub>2</sub> plants through male gametes.  
10 Moreover, a few homozygotes for S<sub>6</sub><sup>a</sup> were observed in the F<sub>2</sub> population, suggesting that  
11 the S<sub>6</sub><sup>a</sup> allele was transmitted through both male and female parents, even though the  
12 transmission frequency was very low (Table 1). Although it is still unclear why the  
13 transmission ratio of the S<sub>6</sub><sup>a</sup> allele in backcrossing was different from that in selfing, there  
14 are several possibilities that may explain the result. One simple explanation is that the  
15 number of samples in the backcross experiments might have not been large enough to  
16 detect transmission of S<sub>6</sub><sup>a</sup> allele through the female parent. Alternatively, abnormalities  
17 which induce failure in seed development and segregation ratio distortion in the  
18 subsequent generation might have occurred after backcrossing. Another possibility is that  
19 a complex mechanism involving unknown factors in the genetic background, such as an  
20 epistatic interaction or a heterospecific gene interaction between male (pollen) and  
21 female (pistil) parents, might have reduced the degree of TRD in the F<sub>2</sub> plants derived  
22 from T65wx × Ruf-S<sub>6</sub>.

23           Although the underlying mechanisms are unknown, these results show that the

1 transmission of the  $S_6$  allele through female gametes ( $f$ TRD) was nearly complete, while  
2 the transmission of the  $S_6$  allele through male gametes ( $m$ TRD) changed depending on  
3 the genotype of the female (pistil) plants, suggesting the presence of unlinked modifiers  
4 in this phenomenon. Furthermore, the results suggest that two different genetic  
5 mechanisms controlling  $m$ TRD and  $f$ TRD are involved in  $S_6$  locus-mediated  $si$ TRD  
6 though it is unknown whether these two phenomena are governed by two tightly linked  
7 genetic components or the pleiotropic effects of a single gene. In combination with the  
8 observation that the degree of  $S_6$  locus-mediated TRD differed between different  
9 combinations of cultivated and wild rice strains (Koide *et al.*, 2008a; Table 2), the finding  
10 of a modifier(s) and sex-specific mechanisms in this study raises the possibility that  
11 multiple genetic factors affect the degree of  $si$ TRD mediated by the  $S_6$  locus apart from  
12 the  $S_6^n$  allele. TRD of various degrees could have been established by different  
13 combinations of genes in Asian rice.

14

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19

#### 20 **Conflict of interest**

21 The authors declare no conflict of interest.

22

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3 values in heredity. *Hilgardia* **24**: 235-278.
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18

1 **Titles and legends to figures**

2 Figure 1. Map position and transmission ratio distortion of markers on chromosome 6 in  
3 the F<sub>2</sub> populations. (a) Physical map of the DNA markers on chromosome 6 based on  
4 Rice Genome Research Program data (<http://rgp.dna.affrc.go.jp>). The solid circle  
5 represents the centromere. (b) Frequency of each allele of the DNA markers along the  
6 genetic linkage map of chromosome 6 in F<sub>2</sub> populations derived from T65<sub>wx</sub> × NIL-*S*<sub>6</sub>  
7 (*n* = 98) and T65<sub>wx</sub> × Ruf-*S*<sub>6</sub> (*n* = 103). The position of each marker was determined  
8 based on the genetic distance (in cM) from R111C. The frequencies of the *O. rufipogon*  
9 homozygous genotype (solid squares), heterozygous genotype (open circles), and *O.*  
10 *sativa* homozygous genotype (open squares) are plotted at the marker positions.

11

12 Figure 2. Embryo sacs at different developmental stages in the *S*<sub>6</sub>/*S*<sub>6</sub><sup>a</sup> heterozygotes and  
13 *S*<sub>6</sub><sup>a</sup>/*S*<sub>6</sub><sup>a</sup> homozygotes. (a-c) Abnormal embryo sacs in the *S*<sub>6</sub>/*S*<sub>6</sub><sup>a</sup> heterozygotes. (a)  
14 Abnormal bi-nucleate embryo sac with enlarged nuclei (arrowhead). (b) Abnormal tri-  
15 nucleate embryo sac. (c) Abnormal penta-nucleate embryo sac. (d-g) Normal embryo sac  
16 development in the *S*<sub>6</sub><sup>a</sup>/*S*<sub>6</sub><sup>a</sup> homozygotes. (d) A functional megaspore. (e) A bi-nucleate  
17 embryo sac. (f) A tetra-nucleate embryo sac. (g) An embryo sac after the third division.  
18 EN, egg nucleus; SY, synergid; PN, polar nuclei; AN, antipodal cell nuclei. Bar = 20 μm.

19

20 Figure 3. Genotype of pollen grains from a heterozygote as determined using the marker  
21 R111C. *S*<sub>6</sub><sup>a</sup>/*S*<sub>6</sub><sup>a</sup>, *S*<sub>6</sub>/*S*<sub>6</sub>, and *S*<sub>6</sub>/*S*<sub>6</sub><sup>a</sup> indicate homozygotes for the *O. sativa*-derived allele,  
22 homozygotes for the *O. rufipogon*-derived allele, and heterozygotes, respectively.

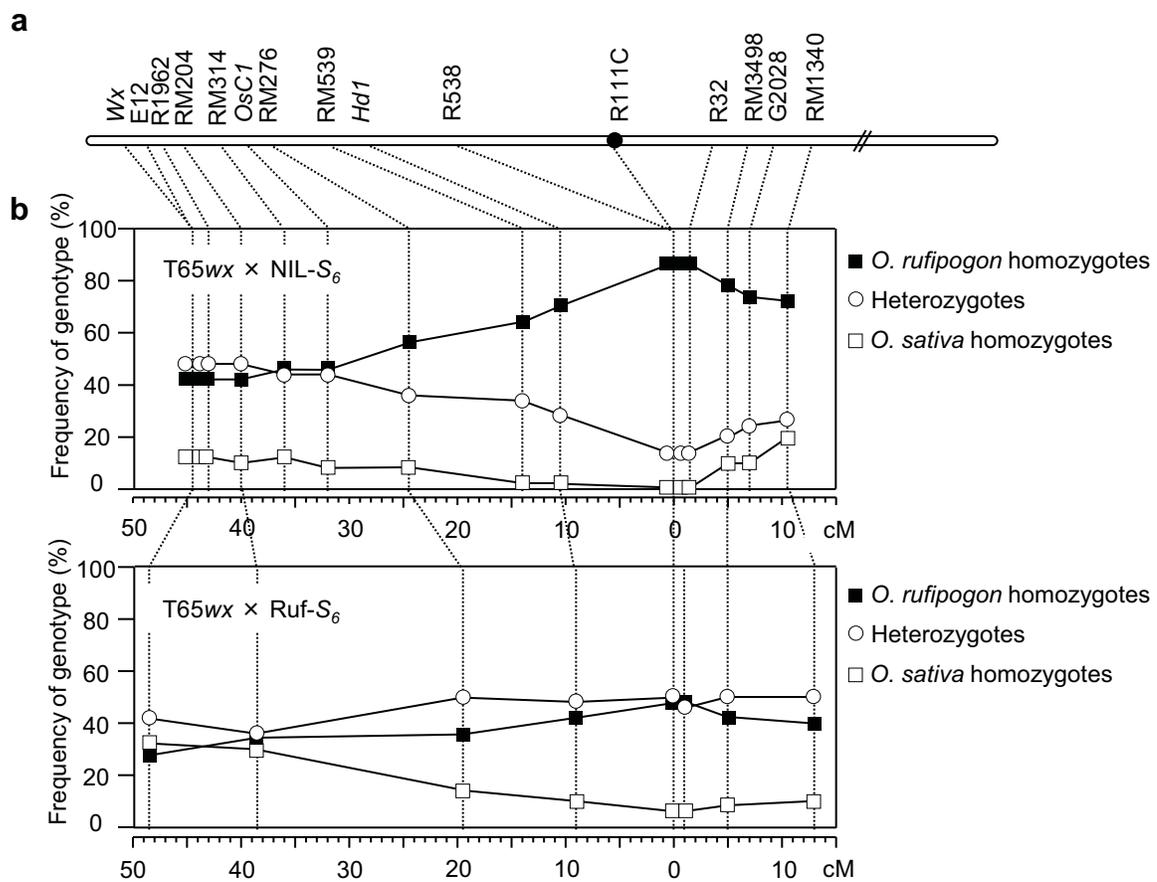


Figure 1

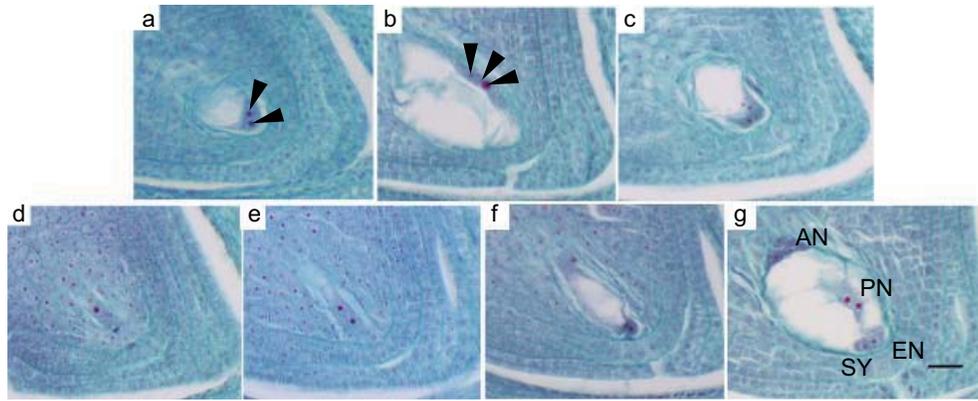


Figure 2

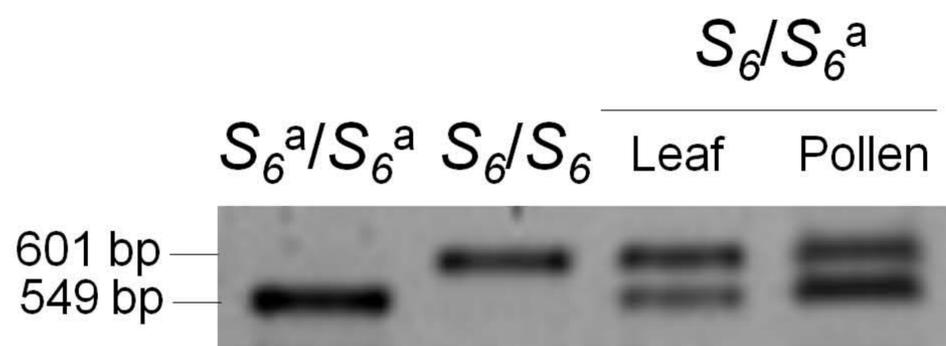


Figure 3

**Table 1** Frequencies of each allele of a DNA marker (R111C) in the F<sub>2</sub> plants from the crosses of T65wx × NIL-S<sub>6</sub>, T65wx × Ruf-S<sub>6</sub>, and BC<sub>1</sub>F<sub>1</sub>

Generation and cross		No. of florets pollinated	No. of seeds obtained	No. of each genotype at R111C*			Total
				S <sub>6</sub> /S <sub>6</sub>	S <sub>6</sub> /S <sub>6</sub> <sup>a</sup>	S <sub>6</sub> <sup>a</sup> /S <sub>6</sub> <sup>a</sup>	
T65wx × NIL-S <sub>6</sub> F <sub>2</sub>		-	-	84	14	0	98
T65wx × Ruf-S <sub>6</sub> F <sub>2</sub>		-	-	48	49	6	103
Female		Male					
T65wx × Ruf-S <sub>6</sub> F <sub>1</sub>	T65wx	72	50	0	50	0	50
T65wx × Ruf-S <sub>6</sub> F <sub>1</sub>	Ruf-S <sub>6</sub>	63	21	17	0	0	17
T65wx	T65wx × Ruf-S <sub>6</sub> F <sub>1</sub>	68	36	0	25	1	26
Ruf-S <sub>6</sub>	T65wx × Ruf-S <sub>6</sub> F <sub>1</sub>	83	32	19	7	0	26

\* S<sub>6</sub> and S<sub>6</sub><sup>a</sup> represent the alleles carried by *O. rufipogon* and *O. sativa*, respectively.