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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

Yohei Koide¹, Yuhei Shinya¹, Mitsunobu Ikenaga¹, Noriko Sawamura¹,
Kazuki Matsubara¹, Kazumitsu Onishi¹, Akira Kanazawa², Yoshio Sano¹

¹*Plant Breeding Laboratory and* ²*Laboratory of Cell Biology and Manipulation, Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan*

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

Yohei Koide¹, Yuhei Shinya¹, Mitsunobu Ikenaga¹, Noriko Sawamura¹, Kazuki Matsubara¹, Kazumitsu Onishi¹, Akira Kanazawa², Yoshio Sano¹

¹*Plant Breeding Laboratory and* ²*Laboratory of Cell Biology and Manipulation, Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan*

Correspondence: *Y. Koide, Plant Breeding Laboratory, Research Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo, 060-8589 Japan, Tel: +81-90-2876-5932, E-mail: yoheikoide@gmail.com*

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Abstract

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

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 F₁
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 F₁
20 hybrids were reciprocally crossed with T65wx, the resultant BC₁F₁ progeny plants
21 exhibited a reduced seed-setting rate, while the F₂ progeny plants derived from self-
22 pollination of the F₁ 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 BC₁₂), 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 F₂ 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 F₂ 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 $T65_{wx} \times \text{Ruf-}S_6$,
4 transmission of the S_6 allele through males (i.e., $m\text{TRD}$) and females (i.e., $f\text{TRD}$) was
5 assessed by reciprocal backcross experiments. To estimate the degree of $m\text{TRD}$, F_1 plants
6 ($T65_{wx} \times \text{Ruf-}S_6$) were used as the pollen parents and pollinated to female $T65_{wx}$ and
7 $\text{Ruf-}S_6$ plants. On the other hand, to estimate the degree of $f\text{TRD}$, F_1 plants ($T65_{wx} \times$
8 $\text{Ruf-}S_6$) were used as the female parents and pollinated with male $T65_{wx}$ and $\text{Ruf-}S_6$
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- μ 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 μ 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 μ L of H_2O , 5 μ L of 10 $\times$ *Takara Ex Taq* buffer, 5 μ L
14 of 50% dimethyl sulfoxide, 2.5 mM each dNTP, 1 μ L of a 20 pM solution of each primer,
15 and 0.3 μ L of *Takara Ex Taq* DNA polymerase (5 U μ L⁻¹). 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 $T65_{wx} \times NIL-S_6$ and $T65_{wx} \times Ruf-S_6$. 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 $T65_{wx} \times NIL-S_6$, 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 $T65_{wx} \times Ruf-S_6$, 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 $T65_{wx} \times NIL-S_6$ and $T65_{wx} \times Ruf-S_6$ (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

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

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

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

(Koide *et al.*, 2008a). Moreover, a similar pattern of reduction in TRD was observed in the F₂ population derived from the cross between T65_{wx} and Ruf-*S*₆ (Figure 1). These results suggest that the *S*₆ locus is the causal factor of TRD on DNA marker loci on chromosome 6 in both of the F₂ populations derived from T65_{wx} × NIL-*S*₆ and T65_{wx} × Ruf-*S*₆.

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

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

In this study, the most severe TRD was observed at R111C near the centromere. This result is comparable with that from genetic mapping using a segregating population consisting of a large number of individual plants (Koide *et al.*, 2008a). Several examples of TRD near centromeric or neocentromeric regions have been reported in *Mimulus* and maize (Dawe and Cande, 1996; Yu *et al.*, 1997; Fishman and Willis, 2005; Fishman and Saunders, 2008). In *Mimulus*, because the *D* locus near the centromere caused significant *f*TRD without an increase in ovule or seed mortality, it was suggested that *f*TRD is a 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 *S₆* 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 (*S₆/S₆^a*) (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 (*S₆/S₆^a*; 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_{wx} × Ruf-*S*₆. The segregation ratio of homozygotes for the *O. rufipogon*-
2 derived allele (*S*₆), heterozygotes, and homozygotes for the *O. sativa*-derived allele (*S*₆^a)
3 was close to 1:1:0 in this latter population (Table 1). Because NIL-*S*₆ and Ruf-*S*₆ are of
4 different genetic backgrounds, the effect of *S*₆ 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*₆ was used
7 as the female (pistil) parent, whereas transmission of the *S*₆ allele through the female
8 parent (*f*TRD) was 100% when T65_{wx} or Ruf-*S*₆ was used as the male (pollen) parent
9 (Table 1). Transmission of the *S*₆^a allele from male T65_{wx} × Ruf-*S*₆ plants was observed
10 following crosses with female Ruf-*S*₆ pistils (Table 1), and pollen grains carrying the *S*₆^a
11 allele were detected by tissue PCR in the heterozygotes (Figure 3). Thus, the
12 heterozygotes produced not only *S*₆, but also *S*₆^a pollen grains with normal fertilization
13 potential, consistent with previous cytological observations of normal mature pollen
14 grains in *S*₆/*S*₆^a heterozygotes (Koide *et al.*, 2008a). This suggests that the *m*TRD
15 observed in the cross between the T65_{wx} × Ruf-*S*₆ male and T65_{wx} female was not due to
16 the dysfunction of pollen grains carrying the *S*₆^a 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*₆ and *S*₆^a 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*₆
23 and *S*₆^a 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 *S₆* locus-mediated *mTRD* was reduced only when plants with a
10 Ruf-*S₆* 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 (*S₆* or *S₆^a*) 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-*S₆*. 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.

We note that the result of our backcrossing experiments is not fully consistent with the segregation pattern observed in the F₂ population derived from T65wx × Ruf-S₆. In our experiments, approximately 27% of the S₆^a allele was transmitted to the progeny through male gametes when Ruf-S₆ was used as the female (pistil) parent, whereas no S₆^a allele was transmitted to the progeny when T65wx or Ruf-S₆ was used as the male (pollen) parent (Table 1). On the other hand, the segregation ratio of homozygotes for the *O. rufipogon*-derived allele (S₆), heterozygotes, and homozygotes for the *O. sativa*-derived allele (S₆^a) in the F₂ population, was close to 1:1:0 (Table 1), suggesting that approximately 50% of S₆^a allele was transmitted to the F₂ plants through male gametes. Moreover, a few homozygotes for S₆^a were observed in the F₂ population, suggesting that the S₆^a allele was transmitted through both male and female parents, even though the transmission frequency was very low (Table 1). Although it is still unclear why the transmission ratio of the S₆^a allele in backcrossing was different from that in selfing, there are several possibilities that may explain the result. One simple explanation is that the number of samples in the backcross experiments might have not been large enough to detect transmission of S₆^a allele through the female parent. Alternatively, abnormalities which induce failure in seed development and segregation ratio distortion in the subsequent generation might have occurred after backcrossing. Another possibility is that a complex mechanism involving unknown factors in the genetic background, such as an epistatic interaction or a heterospecific gene interaction between male (pollen) and female (pistil) parents, might have reduced the degree of TRD in the F₂ plants derived from T65wx × Ruf-S₆.

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

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

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Conflict of interest

The authors declare no conflict of interest.

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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 F₂ 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 F₂ populations derived from T65wx × NIL-*S₆*
7 (*n* = 98) and T65wx × Ruf-*S₆* (*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 *S₆/S₆^a* heterozygotes and
13 *S₆^a/S₆^a* homozygotes. (a-c) Abnormal embryo sacs in the *S₆/S₆^a* 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 *S₆^a/S₆^a* 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. *S₆^a/S₆^a*, *S₆/S₆*, and *S₆/S₆^a* indicate homozygotes for the *O. sativa*-derived allele,
22 homozygotes for the *O. rufipogon*-derived allele, and heterozygotes, respectively.

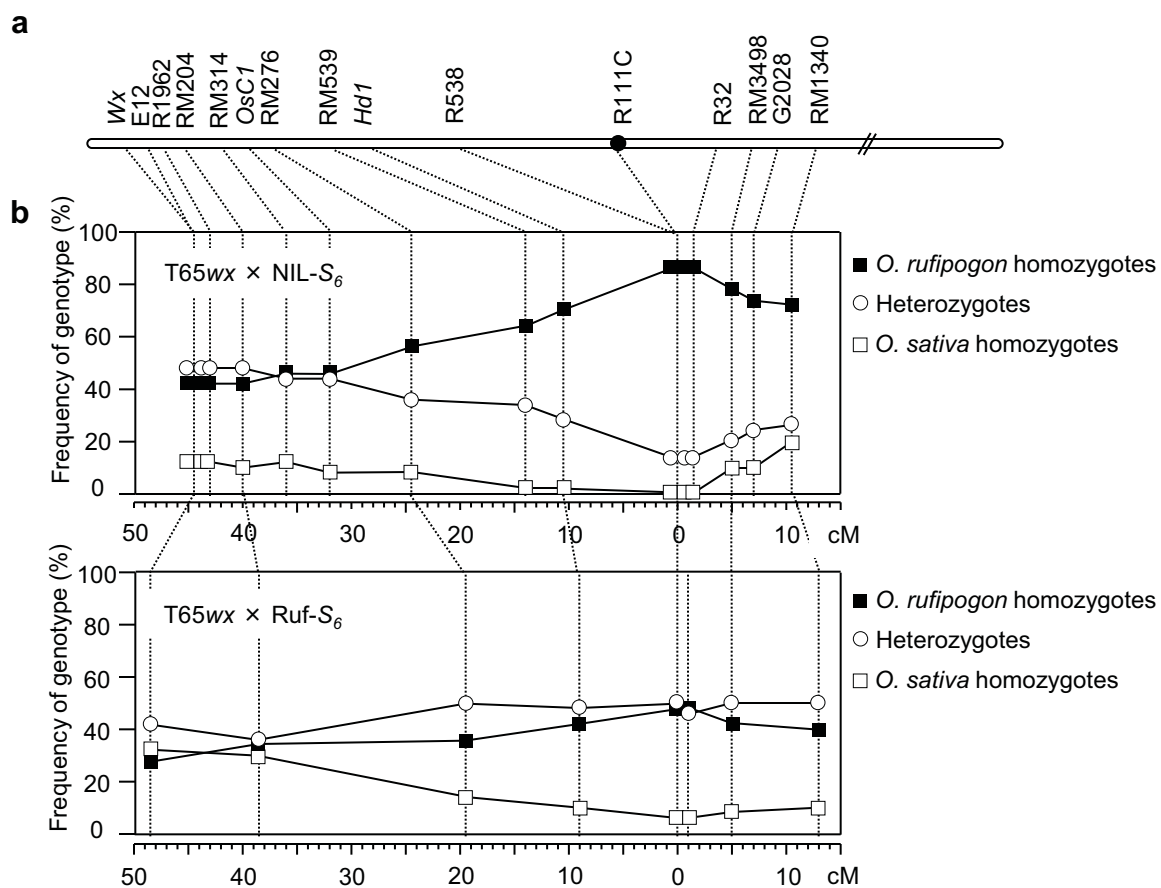


Figure 1

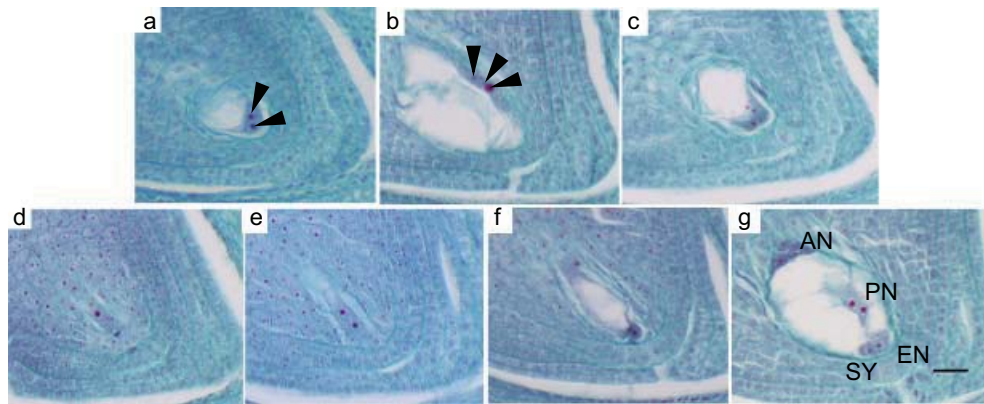


Figure 2

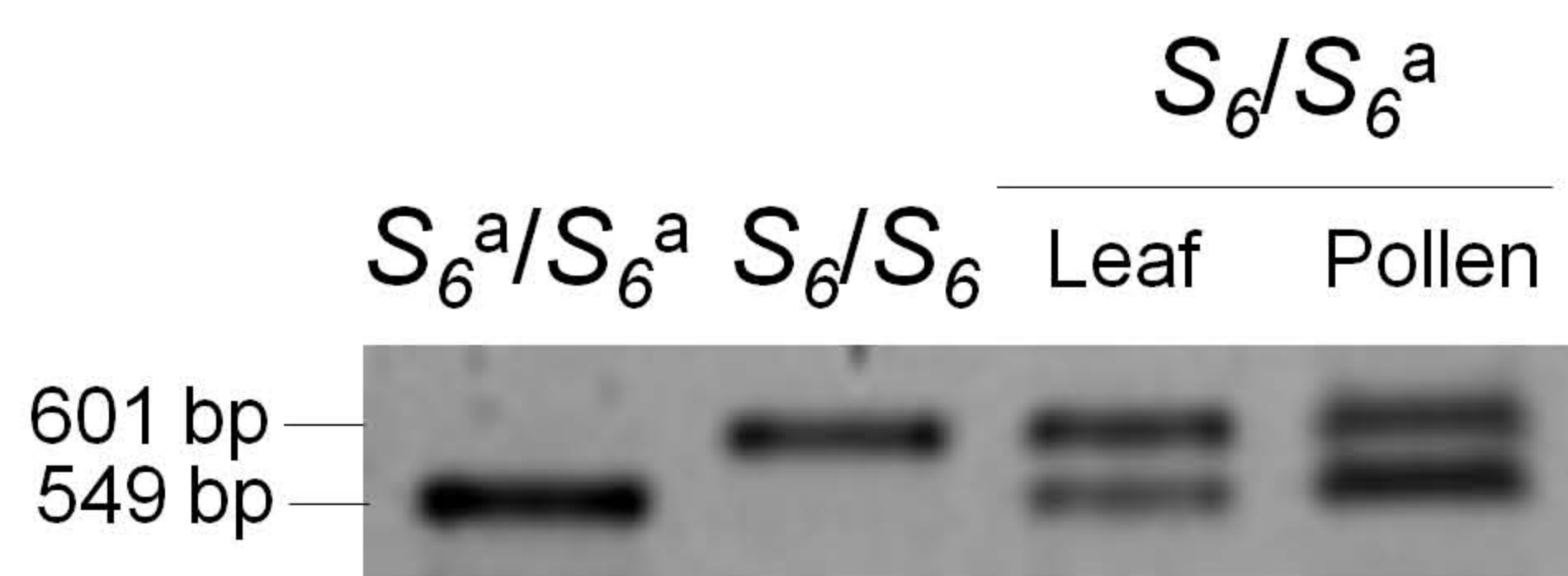


Figure 3

Table 1 Frequencies of each allele of a DNA marker (R111C) in the F₂ plants from the crosses of T65wx × NIL-S₆, T65wx × Ruf-S₆, and BC₁F₁

Generation and cross		No. of florets <i>pollinated</i>	No. of seeds <i>obtained</i>	No. of each genotype at R111C [*]			Total
				<i>S₆/S₆</i>	<i>S₆/S₆^a</i>	<i>S₆^a/S₆^a</i>	
T65wx × NIL-S ₆ F ₂		-	-	84	14	0	98
T65wx × Ruf-S ₆ F ₂		-	-	48	49	6	103
Female	Male						
T65wx × Ruf-S ₆ F ₁	T65wx	72	50	0	50	0	50
T65wx × Ruf-S ₆ F ₁	Ruf-S ₆	63	21	17	0	0	17
T65wx	T65wx × Ruf-S ₆ F ₁	68	36	0	25	1	26
Ruf-S ₆	T65wx × Ruf-S ₆ F ₁	83	32	19	7	0	26

^{*} S₆ and S₆^a represent the alleles carried by *O. rufipogon* and *O. sativa*, respectively.