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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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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 TRD (*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 an 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.
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INTRODUCTION

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

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

We previously reported S_6 locus-mediated *si*TRD in a hybrid of Asian cultivated rice (*Oryza sativa*) and wild rice (*Oryza rufipogon*; Sano, 1992; Koide *et al.*, 2008a). Asian cultivated rice and wild rice belong to the same biological species, forming a primary gene pool (*O. sativa-O. rufipogon* complex) according to the classification system for gene pools (Harlan 1975). Thus, this provides an opportunity to examine the genetic basis of intraspecific TRD. We observed a reduction in seed setting among the F_1 plants derived from a cross between T65*wx* (*O. sativa* ssp. *japonica*) and a near-isogenic

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line (NIL; designated as NIL-S_6 in this study) carrying a segment of chromosome 6 derived from a strain of *O. rufipogon* (Ruf-S_6 in this study; Sano, 1992). When the F_1 hybrids were reciprocally crossed with T65*wx*, the resultant BC_1F_1 progeny plants exhibited a reduced seed-setting rate, whereas the F_2 progeny plants derived from self-pollination of the F_1 hybrid plants exhibited a normal seed-setting rate (Sano, 1992).

This phenomenon is due to an interaction between a gene designated S_6 in the chromosomal segment derived from Ruf-S_6, and its opposing allele (S_6^a) in T65*wx*. The S_6 allele acted as a 'gamete eliminator,' and was transmitted more frequently than S_6^a through both the male and female gametes in heterozygotes (S_6/S_6^a). Female gametes possessing the S_6^a allele were aborted in the heterozygotes, causing a reduced seed-setting rate (Sano, 1992; Koide *et al.*, 2008a). In contrast, no defect was observed in the pollen grains of the heterozygotes, although male gametes possessing the S_6^a allele were rarely transmitted to the next generation (Sano, 1992; Koide *et al.*, 2008a). We have also revealed that Asian rice strains frequently harbor an additional allele (S_6^n), which however, does not induce any preferential abortion in heterozygotes (S_6/S_6^n and S_6^a/S_6^n) at the S_6 locus (Koide *et al.*, 2008a), as shown by test-cross experiments and subsequent genetic mapping using NILs that carry the genetic background of T65*wx*. The presence of the S_6^n allele, which modifies the effect of the S_6 allele in heterozygotic state at the S_6 locus, suggested that S_6 locus-mediated *si*TRD was caused by the allelic differentiation at the S_6 locus occurred during the evolution of Asian rice.

It is conceivable that changes in genetic factors that positively or negatively control S_6 locus-mediated *si*TRD occurred during the evolution of Asian rice, and such changes might have affected the presence or absence of reproductive barrier between constituents of the Asian rice population. With such possibilities in mind, in this study, we compared the effect of S_6 locus-mediated TRD between two F_2 populations that were produced using a NIL and its original wild strain as respective parental strains for crossing, and examined whether there are genes which modify the effect of S_6 locus-mediated *si*TRD that exist in the genetic background of Asian rice strain. We also examined the extent of male- and female-specific TRD by reciprocal backcross experiments. Based on the results, together with those of subsequent genetic and cytological analyses, we report the involvement of unlinked modifiers and sex-specific mechanisms in this phenomenon.

MATERIALS AND METHODS

Genetic stocks

Three lines, T65*wx*, Ruf-S_6 and NIL-S_6 were used. T65*wx* carries *wx* (*waxy*) gene as a genetic marker in the genetic background of Taichung 65 (*O. sativa* ssp. *japonica*). Ruf-S_6 is a perennial type strain of *O. rufipogon*, W593. NIL-S_6 carries the short arm and a portion of the long arm of chromosome 6 from Ruf-S_6 in the genetic background of T65*wx* (Sano, 1992; Matsubara *et al.*, 2003; Koide *et al.*, 2008a formally named as T65S_6 [W593]). T65*wx* harbors the S_6^a allele at the S_6 locus (near the centromeric region of chromosome 6), whereas Ruf-S_6 and NIL-S_6 harbor the S_6 allele at the S_6 locus (Koide *et al.*, 2008a). Although T65*wx* harbors *wx* gene from Kinoshita-mochi (Oka, 1974; derived from BC_{12}), *wx* gene does not affect S_6 locus-mediated TRD.

Genetic crosses and genotyping to detect S_6 locus-mediated TRD

To examine the effect of S_6 locus-mediated TRD on linked loci on chromosome 6, a total of 98 F_2 segregating plants derived from T65*wx*×NIL-S_6 were genotyped using 15 DNA markers from chromosome 6 (*Wx*, E12, R1962, RM204, RM314, *OsC1*, RM276, RM539, *Hd1*, R538, R111C, R32, RM3498, G2028, and RM1340). Additionally, to examine the effect of S_6 locus-mediated TRD in the hybrids between *O. sativa* and the original wild strain of *O. rufipogon*, a total of 103 F_2 segregating plants derived from T65*wx*×Ruf-S_6 were genotyped using eight DNA markers from chromosome 6 (E12, RM204, RM276, *Hd1*, R111C, RM3, RM3498, and RM1340).

To further characterize the S_6 locus-mediated TRD in the cross of T65*wx*×Ruf-S_6, transmission of the S_6 allele through males (that is, *m*TRD) and females (that is, *f*TRD) was assessed by reciprocal backcross experiments. To estimate the degree of *m*TRD, F_1 plants (T65*wx*×Ruf-S_6) were used as the pollen parents and pollinated to female T65*wx* and Ruf-S_6 plants. On the other hand, to estimate the degree of *f*TRD, F_1 plants (T65*wx*×Ruf-S_6) were used as the female parents and pollinated with male T65*wx* and Ruf-S_6 plants. The segregation ratio at the S_6 locus was estimated from that of the tightly linked DNA marker R111C.

For genotyping, genomic DNA was isolated from a small piece of frozen leaf according to the method of Monna *et al.* (2002), with slight modifications. Three Indel markers (*Wx*, *OsC1* and *Hd1*), three restriction fragment length polymorphism markers (R538, R32 and G2028) and a cleaved amplified polymorphic sequence (CAPS) marker, E12, from chromosome 6 were used for genotyping according to the method of Matsubara *et al.* (2003). A CAPS marker, R111C, was used according to the method of Koide *et al.* (2008a). Seven microsatellite markers (RM204, RM314, RM276, RM539, RM3498, RM3 and RM1340) were selected from a public database (http://www.gramene.org). Additionally, one CAPS marker, R1962, was designed based on a sequence from the public database (acc. no. AP006554). The sequences of the primers used for a CAPS marker, R1962, were 5′-gct tgg att atg aca ttt ag-3′ and 5′-tga agc aag gaa caa aca-3′. To detect the polymorphism, the amplified products were digested with *Taq*I. The recombination values were estimated, based on the maximum likelihood method (Allard, 1956).

Cytological observations and pollen tissue PCR

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

To examine whether the S_6 locus-mediated *m*TRD occurred before or after pollen grain production, pollen grains from heterozygous plants were genotyped according to the method of Petersen *et al.* (1996) with modifications. A total of 2–3 μg of pollen grains were collected from F_1 plants derived from T65*wx*×NIL-S_6 at the flowering stage and transferred to tubes containing 32.7 μl of H_2O, 5 μl of 10×Takara Ex Taq buffer (Takara Bio, Otsu, Japan), 5 μl of 50% dimethyl sulfoxide, 2.5 mM of each deoxyribonucleotide triphosphate, 1 μl of a 20 pM solution of each primer and 0.3 μl of Takara Ex Taq DNA polymerase (5 U μl^{-1}). The CAPS marker R111C was used for genotyping. PCR was performed for 30 cycles (1 min at 96 °C, 1 min at 56 °C and 1 min at 72 °C), followed by 10 min at 72 °C. For polymorphism detection, the amplified products were separated electrophoretically on a 2.5% agarose gel in 1×TAE buffer and the DNA fragments were detected by staining with ethidium bromide.

RESULTS

Effects of the genetic background on S_6 locus-mediated TRD

To examine the effect of genetic background on the strength of S_6 locus-mediated *si*TRD, we analyzed the difference in TRD at the S_6 locus between two F_2 populations derived from crosses of T65*wx*×NIL-S_6 and T65*wx*×Ruf-S_6. To compare the effect of S_6 locus-mediated TRD, we used the DNA marker R111C, which is tightly linked with the S_6 locus (Koide *et al.*, 2008a).

Although TRD was detected in both crosses, the effect was different. In the F_2 population derived from T65*wx*×NIL-S_6, almost all of the plants (84/98) were homozygous for the *O. rufipogon*-derived allele (S_6). No homozygote for the *O. sativa*-derived allele (S_6^a) was detected (Table 1), indicating that transmission of the S_6^a allele was reduced in both the female and male gametes (that is, *si*TRD), consistent with previous data (Sano, 1992; Koide *et al.*, 2008a). However, in the F_2

Table 1 Frequencies of each allele of a DNA marker (R111C) in the F_2 plants from the crosses of T65*wx*×NIL-S_6 and T65*wx*×Ruf-S_6, and the BC_1F_1 plants

Generation and cross		*Number of florets pollinated*	*Number of seeds obtained*	*Number of each genotype at R111C**			
				S_6/S_6	S_6/S_6^a	S_6^a/S_6^a	Total
T65*wx*×NIL-S_6 F_2		—	—	84	14	0	98
T65*wx*×Ruf-S_6 F_2		—	—	48	49	6	103
Female	*Male*						
T65*wx*×Ruf-S_6 F_1	T65*wx*	72	50	0	50	0	50
T65*wx*×Ruf-S_6 F_1	Ruf-S_6	63	21	17	0	0	17
T65*wx*	T65*wx*×Ruf-S_6 F_1	68	36	0	25	1	26
Ruf-S_6	T65*wx*×Ruf-S_6 F_1	83	32	19	7	0	26

* S_6 and S_6^a represent the alleles carried by *O. rufipogon* and *O. sativa*, respectively.

population derived from T65*wx*×Ruf-S_6, the numbers of homozygotes for the *O. rufipogon*-derived allele (S_6), heterozygotes and homozygotes for the *O. sativa*-derived allele (S_6^a) were 48, 49, and 6 respectively (Table 1). The segregation ratio of the F_2 plants was close to 1:1:0 in this cross.

Such a difference in the segregation ratio between the two cross combinations can be explained by either of the following models: (1) the degree of S_6 locus-mediated TRD was changed by unlinked genes when the original wild strain of *O. rufipogon* (Ruf-S_6) was used; (2) a novel TRD which tends to transmit the *O. sativa*-derived allele (S_6^a) and counteracts the over-transmission of the S_6 allele occurred at a locus linked to S_6 when the original wild strain of *O. rufipogon* (Ruf-S_6) was used. To examine these two possibilities, the segregation ratio at markers on chromosome 6 was analyzed using two F_2 populations derived from crosses of T65*wx*×NIL-S_6 and T65*wx*×Ruf-S_6 (Figure 1). In both cases, strong TRD was detected only near the centromeric region where S_6 is located. Moreover, with an increase in the genetic distance from the centromeric region, the degree of TRD decreased. If other loci on chromosome 6 were to affect the segregation pattern, the pattern of reduction in TRD should be affected near the causative loci. Thus, these results suggest that no novel TRD occurred on chromosome 6, but the degree of the S_6 locus-mediated TRD was changed by unlinked genes when the original wild strain of *O. rufipogon* (Ruf-S_6) was used as one of the parents. In addition, in both populations, TRD was detected even at distal DNA marker loci 50 cM distant from R111C, indicating that the S_6 locus-mediated TRD affected most of this chromosomal region, irrespective of the genetic background.

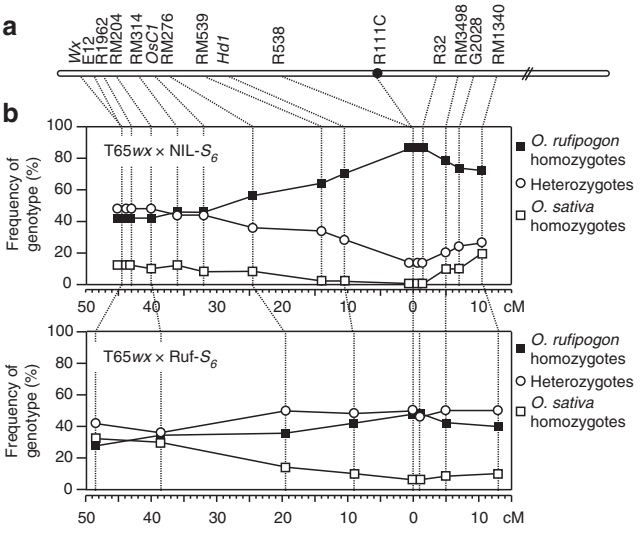

Figure 1 Map position and TRD of markers on chromosome 6 in the F_2 populations. (**a**) Physical map of the DNA markers on chromosome 6, based on Rice Genome Research Program data (http://rgp.dna.affrc.go.jp). The solid circle represents the centromere. (**b**) Frequency of each allele of the DNA markers along the genetic linkage map of chromosome 6 in F_2 populations derived from T65*wx*×NIL-S_6 (n=98) and T65*wx*×Ruf-S_6 (n=103). The position of each marker was determined, based on the genetic distance (in cM) from R111C. The frequencies of the *O. rufipogon* homozygous genotype (solid squares), heterozygous genotype (open circles) and *O. sativa* homozygous genotype (open squares) are plotted at the marker positions.

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

The segregation ratio of homozygotes for the *O. rufipogon*-derived allele (S_6), heterozygotes and homozygotes for the *O. sativa*-derived allele (S_6^a) at R111C was close to 1:1:0 in the F_2 plants derived from T65*wx*×Ruf-S_6, as mentioned above (Table 1). This result suggests that the transmission of the S_6^a allele was reduced through female or male gametes (*f*TRD or *m*TRD), or that transmission of the S_6^a allele was partially reduced through both female and male gametes. To examine which type of TRD occurred in the progeny of the cross between *O. sativa* (T65*wx*) and *O. rufipogon* (Ruf-S_6), we carried out backcrossing experiments. Using F_1 plants as the female parents, the degree of *f*TRD was estimated from the segregation ratio of BC_1F_1 plants. In contrast, the degree of *m*TRD was estimated using F_1 plants as the male parents.

All of the BC_1F_1 plants were heterozygous or homozygous for the *O. rufipogon*-derived allele (S_6) at R111C, when F_1 plants were used as the female parents and crossed with T65*wx* 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 T65*wx* 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

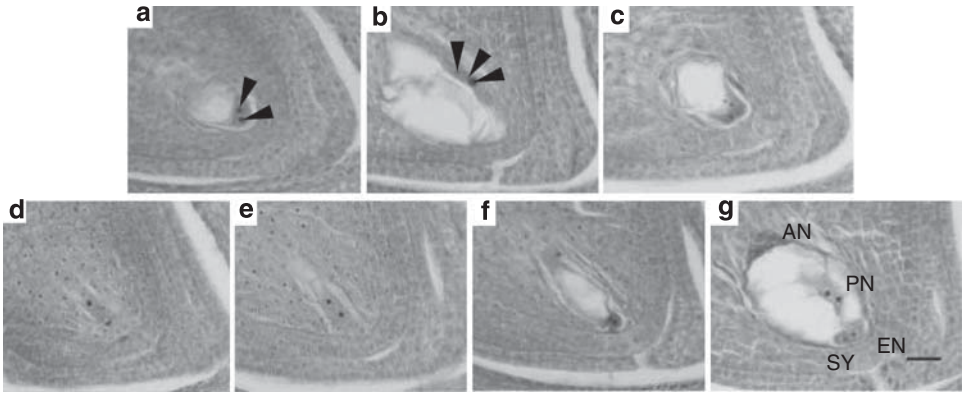

Figure 2 Embryo sacs at different developmental stages in the S_6/S_6^a heterozygotes and S_6^a/S_6^a homozygotes. (**a**–**c**) Abnormal embryo sacs in the S_6/S_6^a heterozygotes. (**a**) Abnormal bi-nucleate embryo sac with enlarged nuclei (arrowhead). (**b**) Abnormal tri-nucleate embryo sac. (**c**) Abnormal penta-nucleate embryo sac. (**d**–**g**) Normal embryo sac development in the S_6^a/S_6^a homozygotes. (**d**) A functional megaspore. (**e**) A bi-nucleate embryo sac. (**f**) A tetra-nucleate embryo sac. (**g**) An embryo sac after the third division. EN, egg nucleus; SY, synergid; PN, polar nuclei; AN, antipodal cell nuclei. Bar=20 μm.

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* (T65*wx*) and *O. rufipogon* (Ruf-S_6), whereas 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, whereas 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 S_6 locus-mediated *f*TRD occurred after meiosis.

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

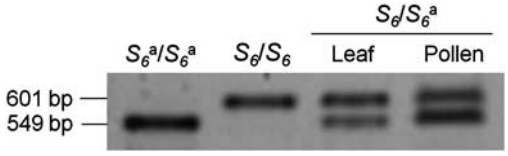

Figure 3 Genotype of pollen grains from a heterozygote as determined using the marker R111C. S_6^a/S_6^a, S_6/S_6, and S_6/S_6^a indicate homozygotes for the *O. sativa*-derived allele, homozygotes for the *O. rufipogon*-derived allele and heterozygotes, respectively.

DISCUSSION

Chromosomal regions affected by the TRD caused by allelic interactions at the S_6 locus

The S_6 locus has been mapped to a region including the centromere of chromosome 6 (Koide *et al*., 2008a). In the present study, we found that the degree of TRD caused by the S_6 locus decreased along with the genetic distance from the centromeric region in the F_2 population derived from the cross between T65*wx* and NIL-S_6 (Figure 1). If other hybrid sterility loci on chromosome 6 were to affect the segregation pattern in this cross combination, the pattern of the reduction in TRD should be affected near the causative loci. A clear reduction pattern in TRD towards the distal end of chromosome 6 was observed, indicating that the segregation distortion caused by the S_6 locus was 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 F_2 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 F_2 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 to 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 containing the *D* allele during asymmetric female meiotic division processes (Fishman and Willis, 2005; Malik, 2005). The Ab10/knob system in maize involves the genetic activation of neocentromeric knob regions that competitively bind microtubules and orient the carrier chromatids toward the outer spindle poles at meiosis II (Dawe and Cande, 1996; Yu *et al*., 1997). In both cases, the *f*TRD, which is governed by the centromeric or neocentromeric region, occurs during meiosis, with no deleterious effect on female gametes.

In the S_6 locus-mediated *f*TRD system, approximately half of the ovules exhibited an abnormality in embryo sac structure during female gametogenesis, and the seed-setting rate was reduced in heterozygotes (S_6/S_6^a; Koide *et al*., 2008a), indicating that *f*TRD occurred post-meiosis, which is different from that mediated by the *D* locus in *Mimulus* or the Ab10/knob system in maize. By cytological observation, bi-nucleate embryo sacs with a single-enlarged nucleus, tri-nucleate embryo sacs and penta-nucleate embryo sacs were found in the abnormal embryo sacs produced by the heterozygotes (S_6/S_6^a; Figure 2), indicating that an abnormality in nuclear division or migration occurred during the second or third round of mitosis after meiosis.

Mutations affecting female gametogenesis after the mono-nucleate stage have been reported in *Arabidopsis* and maize (Sheridan and Huang, 1997; Drews *et al*., 1998). In *Arabidopsis hdd* (*hadad*) mutants, female gametophytes are arrested at the bi-, tetra- or octa-nucleate stage (Drews *et al*., 1998). In *lo2* (*lethal ovule2*) mutants in maize, nuclear division is affected and embryo sacs are arrested at the mono-, bi- or tetra-nucleate stage, and in some cases, the nuclei enlarge dramatically, suggesting a failure of entry into the prophase (Sheridan and Huang, 1997). In the embryo sacs of the *lo2* mutants, abnormal behavior of the tubulin cytoskeleton was also observed. The failure to display a normal pattern of cytoskeleton behavior in the mutant embryo sacs was suggested to be an indirect result of abnormal interactions between defective nuclei lacking normal nuclear surface features and microtubule components of the microtubular cytoskeleton that are required for normal spindle orientation and nuclear migration (Huang and Sheridan, 1994; Sheridan and Huang, 1997).

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

Genetic mechanisms controlling the degree of *m*TRD

In this study, differences in the degree of TRD at the S_6 locus were observed between two F_2 populations derived from crosses between T65*wx* and a NIL (NIL-S_6), and between T65*wx* and the original wild strain (Ruf-S_6). *si*TRD was observed in the F_2 population derived from T65*wx*×NIL-S_6, whereas the degree of TRD was reduced in the F_2 population derived from T65*wx*×Ruf-S_6. The segregation ratio of homozygotes for the *O. rufipogon*-derived allele (S_6), heterozygotes and homozygotes for the *O. sativa*-derived allele (S_6^a) was close to 1:1:0 in this latter population (Table 1). Because NIL-S_6 and Ruf-S_6 are of different genetic backgrounds, the effect of S_6 locus-mediated *si*TRD may be due to differences in genes in the respective genetic backgrounds. Moreover, backcrossing experiments revealed that the degree of *m*TRD was reduced only when Ruf-S_6 was used as the female (pistil) parent, whereas transmission of the S_6 allele through the female parent (*f*TRD) was 100% when T65*wx* or Ruf-S_6 was used as the male (pollen) parent (Table 1). Transmission of the S_6^a allele from male T65*wx*×Ruf-S_6 plants was observed following crosses with female Ruf-S_6 pistils (Table 1), and pollen grains carrying the S_6^a allele were detected by tissue PCR in the heterozygotes (Figure 3). Thus, the heterozygotes produced not only S_6, but also S_6^a pollen grains with normal fertilization potential, consistent with previous cytological observations of normal mature pollen grains in S_6/S_6^a heterozygotes (Koide *et al*., 2008a). This suggests that the *m*TRD observed in the cross between the T65*wx*×Ruf-S_6 male and T65*wx* female was not due to the dysfunction of pollen grains carrying the S_6^a allele, and occurred after pollen grain production.

A plausible mechanism for the *m*TRD, which occurred after pollen grain production, is the difference in pollen performance, such as the ability of germination or the rate of pollen tube elongation, between the two types of pollen grains (that is, those carrying the S_6 and S_6^a alleles). Further experiments on the ability of pollen germination or the rate of pollen tube elongation might reveal a difference between pollen grains carrying the S_6 and S_6^a alleles. Pollen tube competition has been observed in diverse plant taxa (for example, Nelson, 1993; Ramsey *et al*., 2003; Rahme *et al*., 2009). In maize and rice, numerous loci for gametophyte factor (*ga*) have been reported. The *ga* allele is known to confer a pronounced advantage on fertilization as the result of competition among pollen grains, leading to *m*TRD in later generations. In the extreme case of pollen competition caused by the maize *ga1* locus, the growth of *ga1* pollen tubes is retarded or arrested, depending on the genotype of the female parent (Nelson, 1993). In the *Silene* genus, the effect of competition between the pollen grains from *S. latifolia* and *S. dioica* is also related to the genotype of the female parent (Rahme *et al*., 2009).

The degree of S_6 locus-mediated *m*TRD was reduced only when plants with a Ruf-S_6 genetic background were used as the female (pistil) parent in the backcross experiments (Table 1), suggesting that the difference in pollen performance is controlled by an interaction between the pollen (S_6 or S_6^a) and pistil genotypes, and that the effects of the difference in pollen performance were weakened or partly suppressed by modifiers in the genetic background of the female Ruf-S_6. To identify the modifier(s) involved in the suppression of *m*TRD, the development of recombinant inbred lines, each with different chromosomal segments in the genetic background, will be needed. A question arises as to how such a pattern of the difference in pollen performance and its modifier evolved in Asian rice population. It is tempting to speculate that *O. rufipogon*, which has a relatively higher outcrossing rate than *O. sativa*, might have traits suitable for outcrossing, such as high pollen competition ability and a capacity of stigmas to receive alien pollen. On the other hand, *O. sativa*, which is a predominantly selfing plant, might have lost such traits during the evolutionary process. Further analysis of the causative genes will help shed light on the evolution of *m*TRD 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 F_2 population derived from T65*wx*×Ruf-S_6. In our experiments, approximately 27% of the S_6^a allele was transmitted to the progeny through male gametes when Ruf-S_6 was used as the female (pistil) parent, whereas no S_6^a allele was transmitted to the progeny when T65*wx* or Ruf-S_6 was used as the male (pollen) parent (Table 1). On the other hand, the segregation ratio of homozygotes for the *O. rufipogon*-derived allele (S_6), heterozygotes and homozygotes for the *O. sativa*-derived allele (S_6^a) in the F_2 population, was close to 1:1:0 (Table 1), suggesting that approximately 50% of S_6^a allele was transmitted to the F_2 plants through male gametes. Moreover, a few homozygotes for S_6^a were observed in the F_2 population, suggesting that the S_6^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 S_6^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 S_6^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 F_2 plants derived from T65*wx*×Ruf-S_6.

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, whereas the transmission of the S_6 allele through male gametes (*m*TRD) changed depending on the genotype of the female (pistil) plants, suggesting the involvement 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 (Table 1; Koide *et al*., 2008a), the finding of 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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