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The Evolution of Sex-Independent Transmission Ratio Distortion Involving Multiple Allelic Interactions at a Single Locus in Rice

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ABSTRACT

Transmission ratio distortion (TRD) is frequently observed in inter- and intraspecific hybrids of plants, leading to a violation of Mendelian inheritance. Sex-independent TRD (*si*TRD) was detected in a hybrid between Asian cultivated rice and its wild ancestor. Here we examined how *si*TRD caused by an allelic interaction at a specific locus arose in Asian rice species. The *si*TRD is controlled by the S_6 locus via a mechanism in which the S_6 allele acts as a gamete eliminator, and both the male and female gametes possessing the opposite allele (S_6^a) are aborted only in heterozygotes (S_6/S_6^a). Fine mapping revealed that the S_6 locus is located near the centromere of chromosome 6. Testcross experiments using near-isogenic lines (NILs) carrying either the S_6 or S_6^a alleles revealed that Asian rice strains frequently harbor an additional allele (S_6^n) the presence of which, in heterozygotic states (S_6/S_6^n and S_6^a/S_6^n), does not result in *si*TRD. A prominent reduction in the nucleotide diversity of S_6 or S_6^a carriers relative to that of S_6^n carriers was detected in the chromosomal region. These results suggest that the two incompatible alleles (S_6 and S_6^a) arose independently from S_6^n and established genetically discontinuous relationships between limited constituents of the Asian rice population.

TRANSMISSION ratio distortion (TRD) refers to a naturally occurring phenomenon in which the two alleles at a heterozygous locus do not transmit equally to the progeny (Crow 1988; Lyttle 1991; Temin *et al.* 1991). TRD is induced by a variety of mechanisms, including the nonrandom segregation of chromosomes during meiosis (Pardo-Manuel de Villena and Sapienza 2001; Birchler *et al.* 2003; Fishman and Willis 2005), preferential dysfunction of gametes in hybrids (Lyttle 1991; Temin *et al.* 1991; Silver 1993; Moyle and Graham 2006; Úbeda and Haig 2005), and preferential success of gametes in fertilization (Price 1997; Diaz and MacNair 1999). TRD is detected not only within a given population but also between populations and/or species. Genomewide surveys have frequently revealed significant TRD in intra- and interspecific hybrids (Moyle and Graham 2006). Because TRD dramatically alters the frequencies of alleles in a population by disrupting Mendelian segregation, it has been hypothesized that TRD is the driving force that contributes to the rise of reproductive barriers (Frank 1991; Hurst and Pomiankowski 1991; Orr and Irving 2004).

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB433361–AB433506, AB433508–AB433511, and AB433513–AB433529.

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In plants, preferential dysfunction of gametes can take place in either the male (Cameron and Moav 1957; Loegering and Sears 1963; Sano 1983) or the female (Maguire 1963; Scoles and Kibirge-Sebnya 1983), or in both (Rick 1966; Endo and Tsunewaki 1975; Sano *et al.* 1979; Finch *et al.* 1984). Among these types, sex-independent TRD (*si*TRD) exerts the strongest effect on segregation distortion, since the abortion of both male and female gametes carrying one of the two alleles is induced by the presence of the opposite allele in the heterozygote (Rick 1966; Endo and Tsunewaki 1975; Sano *et al.* 1979; Finch *et al.* 1984).

In rice, extensive studies have been carried out on the genetic basis of isolating barriers in intra- and interspecific hybrids, since barriers often present a serious problem in hybridization efforts in breeding programs. A variety of genetic mechanisms have been proposed to explain hybrid sterility (reviewed by Koide *et al.* 2008a), including those involving combinations of chromosomes bearing cryptic structural differences (Li *et al.* 1997), nuclear and cytoplasmic genomes of different origin (Shinjo 1984), recessive alleles of duplicate genes (Oka 1974, 1988), two complementary dominant genes (Chu and Oka 1972; Li *et al.* 1997), and different alleles in a single gene (Ikehashi and Araki 1986; Morishima *et al.* 1992). Among these, the interaction between alleles is most frequently observed as the cause of preferential dysfunction of gametes leading to TRD

(Ikehashi and Araki 1986; Morishima *et al.* 1992; Koide *et al.* 2008a). Recent genomewide surveys have revealed the presence of a number of chromosomal regions that are involved in TRD in inter- and intraspecific crosses in rice (Xu *et al.* 1997; Harushima *et al.* 2001). However, there has been no report to date that addresses how each TRD system has arisen and become fixed in the rice populations.

We have previously reported that a gamete eliminator (S_6) functions in a hybridization between *Oryza sativa* and *O. rufipogon* (Sano 1992). The hybrid plants between T65*wx* (*O. sativa* ssp. *japonica*) and near-isogenic lines (NILs) carrying a segment of chromosome 6 derived from W593 (*O. rufipogon*) in the genetic background of T65*wx* exhibited a reduced rate of seed setting. When the hybrids were reciprocally crossed with T65*wx*, all the resultant progeny exhibited a reduced seed-setting rate, while the progeny derived from self-pollination of the hybrid plants exhibited a normal seed-setting rate (Sano 1992). This phenomenon was due to an interaction between the gene designated S_6 in the chromosomal segment derived from W593 and its opposite allele (S_6^a) in T65*wx*. The S_6 allele acts as a "gamete eliminator," and both male and female gametes possessing the S_6^a allele are aborted only in the heterozygote (S_6/S_6^a) (Sano 1992). This locus thus affords an opportunity to examine the genetic basis and evolution of the *si*TRD system. In the present study, we focus on *si*TRD caused by the allelic interaction of the S_6 locus and report the first detailed characterization of the *si*TRD system. We describe the presence of an additional allele (S_6^n) that induces no preferential abortion in heterozygotes with either the S_6 or S_6^a allele, which was revealed by testcrosses examining allelic distribution in wild and cultivated rice accessions. We also report the histological analysis of gametogenesis, genetic mapping of the S_6 gene, and the analysis of nucleotide diversity around the S_6 locus. On the basis of the findings, the involvement of the *si*TRD system in the formation of isolating barriers in the evolution of the Asian rice population is discussed.

MATERIALS AND METHODS

Genetic stocks: Three NILs, T65*wx*, T65S_6 (W593), and T65*WxSe1* (Pat), were made and used for genetic mapping (Table 1). T65S_6 (W593) and T65*WxSe1* (Pat) harbor the short arm of chromosome 6 from W593 (*O. rufipogon* from Malaysia) and Patpaku (ssp. *indica* of *O. sativa* from Taiwan), respectively, in the background of T65*wx*. The detailed genotypes of these three NILs are described in Matsubara *et al.* (2003). To examine the allelic distribution at the S_6 locus, two other NILs, T65$st_1S_6^a$ and T65st_1S_6, were also established and used for the analysis (Table 1). These two NILs carried *stripe1* (*st1*) as a visible marker, which is linked with the S_6 locus ($P = 0.13$, Sano 1992). T65$st1S_6^a$ carried the S_6^a allele from T65*wx*. T65st_1S_6 was selected from an F_2 population of T65$st_1S_6^a$ × T65S_6 (W593). To fine-map the S_6 gene, another NIL, A58S_6 was established using A58 (ssp. *japonica* of *O. sativa*) as the recurrent parent because of the short life cycle of A58 (Table 1). A58S_6 carries the S_6 allele introduced from T65S_6 (W593).

Seeds were germinated in petri dishes at 30° in late April, and each of the seedlings was transplanted into plastic pots and grown in a greenhouse. The plants were placed in short-day fields (10.5 hr) 8 weeks after sowing due to photoperiod sensitivity, as needed.

Cytological observations: Spikelets were sampled from panicles before heading. Samples were fixed in FAA (formalin:glacial acetic acid:70% ethanol = 1:1:18) and stored in 70% ethanol until use. Pollen fertility was estimated from the percentages of pollen grains stainable with potassium iodine solution (I_2-KI). Ovaries were dehydrated in a graded ethanol-butanol series, embedded in Paraplast Plus (Oxford Labware, St. Louis), and then cut into 10-μm thick sections. Sections were stained with safranin and fast green (Sylvester and Ruzin 1993) and observed under light microscopy (BH-2, Olympus, Tokyo).

Mapping of the S_6 gene: To map the S_6 locus, a total of 1886 segregating plants of A58 × A58S_6 were genotyped. Since the S_6 allele kills female gametes possessing the S_6^a allele in heterozygotes (S_6/S_6^a), the seed-setting rate was analyzed to detect the heterozygotes of S_6/S_6^a according to the method described below. In addition, three recombinant lines (P-1, P-2, and P-3) were obtained from the derivatives of T65*wx* × T65*WxSe1* (Pat) to determine the allelic state at the S_6 locus of Patpaku.

For genotyping the alien segments with molecular markers, genomic DNA was isolated from a small piece of frozen leaf according to the method of Monna *et al.* (2002) with slight modifications. Six markers on chromosome 6 (*Wx*, *OsC1*, *Hd1*, RG264, RM3498, and G2028) were used according to the method of Matsubara *et al.* (2003). Two microsatellite markers (RM3183 and RM3498) were selected from the public database (http://www.gramene.org/). In addition, five cleaved amplified polymorphic sequence (CAPS) markers (S14439, P139, R111C, G05, and C133A) were designed on the basis of sequences in the public database (accession nos. AP003763, AP003574, AP005656, AP005967, and AP005450). The primers for PCR amplification were as follows: 5′-ccg aaa aga gtc ctc cga ag-3′ and 5′-cca cct aag aag cca gca cc -3′ for S14439; 5′-gaa atg cca ctg gcc tac at-3′ and 5′-ttc agg cga gca att tag gt-3′ for P139; 5′-tca ggg cta atc aat ggc gaa g-3′ and 5′-tta gtg gat gcc tgg acg atg a-3′ for R111C; 5′-cca ttc ctc cgt cca aac aca t-3′ and 5′-ccc aaa tca cac aca tcg tgc t-3′ for G05; and 5′-cct aaa cgc aag cca ctg tc-3′ and 5′-gca ttg cat gtt cag ttt tc-3′ for C133A. To detect the polymorphisms in the CAPS markers (S14439, P139, R111C, G05, and C133A), the amplified products were digested with *Hpy*CH4, *Alu*I, *Hin*fI, *Sau*3A, and *Msp*I, respectively. The recombination values were estimated on the basis of the maximum likelihood method.

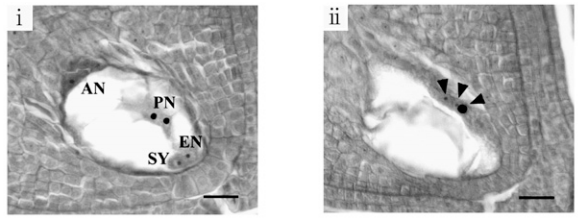
Survey for allelic distribution at the S_6 locus: To examine the distribution of the S_6 allele in Asian rice strains, 9 strains of *O. sativa* and 14 strains of *O. rufipogon* were surveyed (Table 2). The strains of *O. sativa* included 3 strains of ssp. *japonica* and 6 ssp. *indica*. Since intervarietal crossings frequently produce semi-sterility as well as TRD in rice (Oka 1988), the *si*TRD specific to the S_6 locus was evaluated on the basis of the level of seed setting of F_1 plants and distorted segregation for a marker (st_1) linked to S_6. The rate of seed setting was determined by counting the number of fertile and sterile spikelets of two panicles for each plant. Strains carrying the S_6 allele are expected to yield a low seed-setting rate in F_1 hybrids and a segregation distortion for st_1 in F_2 when crossed with T65$st_1S_6^a$, while strains carrying the S_6^a allele are expected to exhibit these abnormalities when crossed with T65st_1S_6. Furthermore, when no difference is found between the crosses with both T65$st_1S_6^a$ and T65st_1S_6, the strain is expected to carry an additional allele (S_6^n), the presence of which in heterozygotic states (S_6/S_6^n and S_6^a/S_6^n) does not result in *si*TRD, as reported in the tomato by

Rick (1966, 1971). To determine the allelic state of W1807 (*O. rufipogon*), segregation distortion was examined for a molecular marker, P139, closely linked to S_6 (Figure 2).

Diversity survey: Nine strains of *O. sativa* and 14 strains of *O. rufipogon* were used for the analysis of the DNA polymorphisms (Table 2). The nucleotide sequences of five regions (P139, F06, D11, R111C, and C133A) around the S_6 gene were determined by direct sequencing of the polymerase chain reaction (PCR) products. The following primers were used for the PCR: 5′-ccg aaa aga gtc ctc cga ag-3′ and 5′-cca cct aag aag cca gca cc-3′ for F06; and 5′-gaa atg cca ctg gcc tac at-3′ and 5′-ttc agg cga gca att tag gt-3′ for D11. These primers were designed on the basis of sequences in the public database (accession nos. AP003545 and AP003512). One loosely linked locus, *Hd3a*, which is located on the short arm of chromosome 6, and two unlinked loci, *sd1* (*semi-dwarf1*), and *qSH4*, which are located on chromosome 1 and chromosome 4, respectively, were used as references. The nucleotide sequences of *sd1* and *qSH4* were determined according to the methods of Nagano *et al.* (2005) and Onishi *et al.* (2007), respectively. For *qSH4*, the combined sequences of the two regions (43A12-161k and 04A17-9k) were used. For *Hd3a*, the primers used were 5′-agc tag ata gct gcc tct atc aca gta t-3′ and 5′-cta gct tca tga gag acc tta gcc-3′, 5′-ccc tgc acc aca cac agt tc-3′ and 5′-tgt ctg aac ctg caa tgt at-3′ or 5′-agc tag ata gct gcc tct atc aca gta t-3′ and 5′-tat ata tgt tgt gtg tcg aga atc att tc-3′. These primers were designed on the basis of sequences in the public database (accession no. AP007223). Each accession was sequenced in both directions using a Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI310 automatic sequencer (Applied Biosystems). The DNA sequences determined in this study are available from DDBJ/GenBank/EMBL (accession nos. AB433361–AB433506, AB433508–AB433511, and AB433513–AB433529). The sequence alignment was done using the CLUSTAL W computer program (Thompson *et al.* 1994) with minor modifications by visual inspection. Molecular population genetic analysis was conducted using DnaSP version 3.14 (Rozas and Rozas 1999). We compared the level of nucleotide diversity per silent site based on π (Nei 1987) and θ_w (Watterson 1975) and calculated Tajima's *D* statistic for testing neutrality (Tajima 1989). Among the five regions around the S_6 gene, D11 and C133A had portions of protein coding regions predicted from the public database (Rice Genome Research Project, http://rgp.dna.affrc.go.jp). D11 contained a part of the first exon and all of the second and third exons of a putative 2′-hydroxyisoflavone reductase. C133A contained the fifth exon of the putative mlo2 protein. To rule out selection acting on the functional regions, the silent sites were used to compare nucleotide diversity.

RESULTS

Effects of allelic interaction at the S_6 locus on gametogenesis: A previous study indicated that the S_6 gene, derived from the wild rice (*O. rufipogon*) strain W593, induced the preferential abortion of both male and female gametes possessing its allelic alternative (S_6^a) from cultivated rice (*O. sativa*) strain T65 only in the heterozygote, and as a result, no S_6^a allele was transmitted to the progeny (Sano 1992). To examine the female gametogenesis in the heterozygote (S_6/S_6^a) which was causing the *si*TRD, histological investigations were carried out (Figure 1). If dysfunction of the female gametes carrying the S_6^a allele is induced in the presence of the S_6 allele in the heterozygote, half of the embryo sacs should degenerate. Of 42 ovules, 23 in fact did exhibit an abnormality in the embryo sac structure, while the remaining 19 ovules had a mature seven-celled structure like that found in the parental strain (T65*wx*) (Figure 1). The frequency of arrested embryo sacs was 0.55 (23/42), indicating that the embryo sacs carrying the S_6^a allele were aborted, which is consistent with the notion that transmission of the S_6^a allele is extremely reduced in the heterozygote (S_6/S_6^a). On the other hand, no abnormalities were detected in mature pollen grains in the heterozygote (S_6/S_6^a), as also in T65*wx*. To confirm the TRD through male gametes, the segregation of F_2 plants derived from T65*wx* × T65S_6 (W593) was analyzed by using a molecular marker, R111C, tightly linked with the S_6 locus (see below). Almost all F_2 plants (84/98) were homozygotes for the W593-derived allele and no homozygotes for the T65*wx*-derived allele were detected, indicating that transmission of the S_6^a allele is reduced not only through female but also male gametes, as has been inferred from backcrosses (Sano 1992).



B

No. of ovules observed			χ^2
Normal	Abnormal	Total	(1:1)
19	23	42	0.381^{ns}

Figure 1.—Embryo sac abnormality in the heterozygotes of S_6^a/S_6. (A) Normal (i) and abnormal (ii) embryo sacs in T65*wx*/T65S_6 (W593). Arrow heads indicate nuclei. EN, egg nucleus; SY, synergid cell; PN, polar nuclei; AN, antipodal cell nuclei. Bar, 20 μm. (B) Frequencies of normal and abnormal embryo sacs in T65*wx*/T65S_6 (W593). ns indicates nonsignificant deviation from 1:1 ratio.

Fine mapping: To roughly map the S_6 gene, 216 segregating plants from A58 × A58S_6 were genotyped using four molecular markers (*Hd1*, RM3183, C133A, and RM3498) (Figure 2A). A58 and A58S_6 carry the S_6^a and S_6 alleles, respectively (Table 1). The rate of seed setting of each F_2 plant was scored to examine the phenotypic segregation of the S_6 locus, since the dysfunction of female gametes in the heterozygote (S_6/S_6^a) is reflected in the productivity of the F_2 plants. The F_2 plants were clearly segregated into two distinct groups: one exhibiting <50% (32.8 ± 4.4%) seed-setting rate and the other exhibiting >70% (80.2 ± 0.4%), and no F_2 plant exhibited a seed-setting rate between these values. Plants of the former and the latter

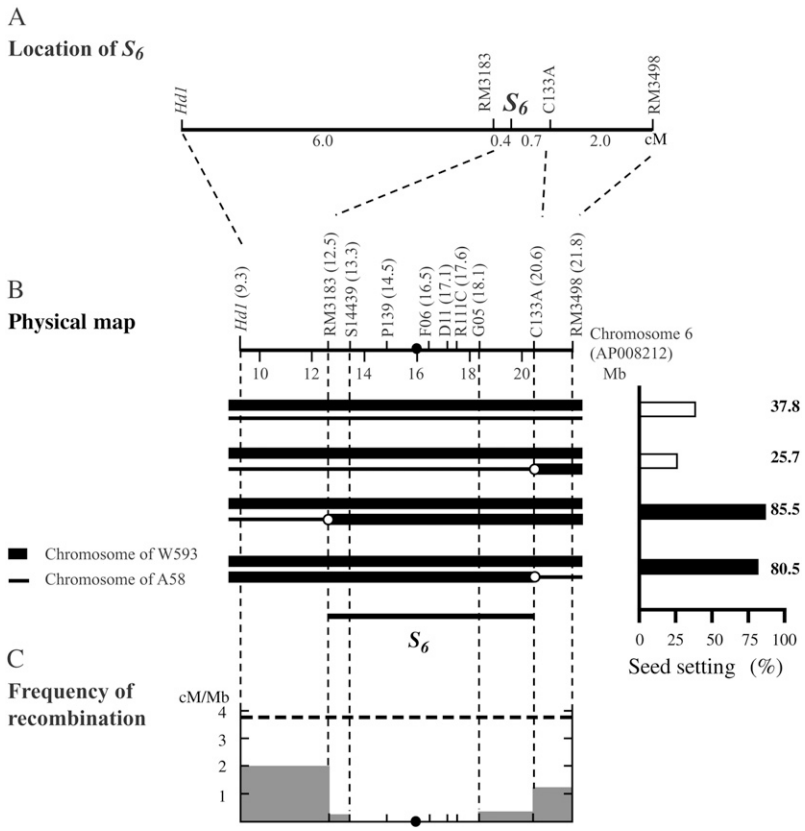


FIGURE 2.—Localization of the S_6 gene on the rice linkage map. (A) Location of the S_6 gene on choromosome 6. Genetic distance was estimated on the basis of 1886 segregating plants of A58 × A58S_6 (BC4 and BC5) except for the genetic distance between *Hd1* and RM3183, which was estimated using 216 BC4 F_2 plants from the same population. (B) Graphical genotypes of chromosome 6 of recombinant plants and the rates of seed setting of the respective plants through self-fertilization. The physical map of chromosome 6 is based on the Rice Genome Research Program (http://rgp.dna.affrc.go.jp). The map position of each marker along chromosome 6 (AP008212) is shown in parentheses (in megabases). A solid circle represents the centromere. Chromosomal regions derived from W593 (carrying the S_6 allele) and A58 (carrying the S_6^a allele) are indicated by thick and thin lines, respectively. Open circles represent the A58-derived alleles. The estimated range of the position of the S_6 gene is shown by a bar below the graphical genotypes. The rate of seed setting was determined by counting the number of fertile and sterile spikelets of two panicles for each plant. The data represent the average values obtained from at least two individual plants for each genotype. The rate of seed setting is shown as a percentage. Solid and open bars indicate seed fertility above and below 50%, respectively. (C) Regional recombination frequency on chromosome 6. The frequency of recombination (in centimorgans/megabases) was estimated using the mapping populations. The average recombination value of chromosome 6 (3.87 cM/Mb; WU *et al.* 2003) is shown by a dashed line.

groups were classified as heterozygotes and homozygotes, respectively. The allelic states of the above markers indicated that all plants classified as homozygotes were those of the S_6 allele. As a consequence of this analysis, the estimated recombination values of RM3183-S_6, S_6-C133A, and C133A-RM3498 were calculated to be 0.0040 ± 0.0010, 0.0070 ± 0.0014, and 0.0201 ± 0.0023, respectively (Figure 2A). The frequencies of the W593-derived alleles were significantly higher than those expected from Mendelian inheritance, especially at RM3183 (208/216, 96.3%) and C133A (206/216, 95.4%), which confirmed the notion that the S_6 allele is preferentially transmitted through both male and female gametes.

For fine mapping, 1670 additional segregating plants were surveyed with four additional markers (S14439, P139, R111C, and G05). However, no recombination was detected between S14439 and G05, indicating that the frequency of recombination is extremely low near the centromere. The ratio of genetic distance to physi-

TABLE 1

Genetic stocks of NILs and recurrent parents used in this study

Lines	Genotype *st1*	Genotype S_6	Donor (species)	Backcross generation	Reference
T65S_6 (W593)[a]	+	S_6	W593 (*O. rufipogon*)	BC8	SANO (1992)
T65 *WxSe1* (Pat)[b]	+	S_6^{n}[c]	Patpaku (*O. sativa* ssp. *indica*)	BC8	DUNG *et al.* (1998)
A58S_6	+	S_6	W593 (*O. rufipogon*)	BC4 and BC5	This study
T65*st1*S_6^a	*st1*	S_6^a	F1176 (*O. sativa* ssp. *japonica*)	BC7	This study
T65*st 1*S_6	*st1*	S_6	F1176 (*O. sativa* ssp. *japonica*) and W593 (*O. rufipogon*)	BC7	This study
T65*wx*	+	S_6^a	Kinoshita-mochi (*O. sativa* ssp. *japonica*)	BC12	OKA (1974)
A58	+	S_6^a	Kokusyokuto-2 (*O. sativa* ssp. *japonica*)		

[a] Formerly named W593A (SANO 1992).
[b] Formely named T65 (*Wx*-pat) (DUNG *et al.* 1998).
[c] The allelic state was determined in this study.

TABLE 2

Seed setting and segregation patterns of the marker *st1* observed in testcrosses for allelic identification in the S_6 locus

Species	Strain	Subspecies or type	Origin	Crossing with T65*st1*S_6		Crossing with T65*st1*S_6^a		A/B	Putative allele
				F_1 seed setting (A)[a]	F_2 *st1* (%)	F_1 seed setting (B)[a]	F_2 *st1* (%)		
O. sativa	T65	ssp. *japonica*	Taiwan	34.1	58.4[b]**	72.3	23.9	0.47	S_6^a
	Koshihikari	ssp. *japonica*	Japan	41.5	60.2[b]**	88.0	22.6	0.47	S_6^a
	A58	ssp. *japonica*	Japan	46.2	57.3[b]**	86.0	22.8	0.53	S_6^a
	PTB10	ssp. *indica*	India	16.7	20.3	14.0	17.8	1.19	S_6^n
	IR36	ssp. *indica*	Philippines	80.2	17.1**	76.7	17.2	1.05	S_6^n
	Patpaku	ssp. *indica*	Taiwan	72.7	23.3	67.0	21.3	1.09	S_6^n
	Acc27590	ssp. *indica*	Bangladesh	82.1	—	79.0	—	1.04	S_6^n
	Acc27591	ssp. *indica*	Bangladesh	31.7	9.2**	44.1	24.1	0.72	S_6^n
	444	ssp. *indica*	India	65.2	17.8	54.9	26.5	1.19	S_6^n
O. rufipogon	W107	Annual	India	42.1	29.7	55.1	20.5	0.76	S_6^n
	W2002	Annual	Myanmar	80.3	21.6	74.4	22.2	1.08	S_6^n
	W630	Annual	Myanmar	47.6	12.5**	42.7	—	1.11	S_6^n
	W2048	Perennial	China	20.4	45.1**	25.7	23.5	0.79	S_6^a
	W1718	Perennial	China	52.5	—	40.8	—	1.29	S_6^n
	W1943	Perennial	China	71.0	—	73.3	—	0.97	S_6^n
	W1944	Perennial	China	31.1	18.4	23.5	26.3	1.32	S_6^n
	W1945	Perennial	China	54.6	20.9	47.6	20.5	1.15	S_6^n
	W1952	Perennial	China	47.6	13.6*	42.3	—	1.13	S_6^n
	W1681	Perennial	India	82.1	—	74.9	—	1.10	S_6^n
	W593	Perennial	Malaysia	81.4	15.0*	39.1	2.7**	2.08	S_6
	W172	Perennial	Thailand	67.2	11.2	25.5	3.6**	2.64	S_6
	W1294	Perennial	Philippines	67.9	20.6	28.0	2.6**	2.43	S_6
	W1807[c]	Perennial	Sri Lanka	77.1	26.7	26.6	0.1**	2.90	S_6

* and ** indicate significant deviation from Mendelian inheritance at 5% and 1% levels, respectively.

[a] The rate of seed setting was determined by counting fertile and sterile spikelets of two panicles for each plant.

[b] When more tightly linked marker, R111C, was used, the frequency of the W593-derived allele was close to 100% in the F_2 populations derived from A58 × A58S_6 and T65*wx* × T65S_6 (W593) (see text).

[c] The allelic state of W1807 was analyzed by crossings the line with T65*Wx*S_6 (W593) and T65*wx*. The frequency of the alleles in the progeny was determined by the marker P139 linked with the S_6 locus.

cal distance (cM/Mb) greatly decreased with decreasing distance from the centromere, confirming the restriction of recombination near the centromere (Figure 2C). Due to a deficit of recombinants in this region, the S_6 locus was delimited to a region of >8000 kb between RM3183 and C133A, even with a large mapping population (Figure 2B).

Distribution of the S_6 and S_6^a alleles in Asian rice accessions: To examine the allelic distribution at the S_6 locus, two NILs carrying the S_6 or S_6^a allele, namely, the T65st_1S_6 or T65$st_1S_6^a$ lines, respectively, were established (Table 1). The allelic distribution at the S_6 locus in 23 strains of Asian cultivated rice and wild rice were investigated by testcrosses with these NILs (Table 2). Because *st1* is linked with the S_6 locus, and its phenotype in homozygotes is manifested at the young seedling stage, it was used as a visible marker of the segregation distortions caused by the S_6 locus.

The F_1 hybrids between three strains of *O. sativa* ssp. *japonica* and T65st_1S_6 all exhibited a low seed-setting rate, but the F_1 hybrids between these strains and T65$st_1S_6^a$ did not, but rather, exhibited a normal rate (Table 2). In addition, a marked excess of st_1 homozygotes in the F_2 populations was observed when the three strains were crossed with T65st_1S_6. These results indicate that the three strains carry the S_6^a allele at the S_6 locus. In contrast, the F_1 hybrids between the four wild strains (W593, W172, W1294, and W1807) and either T65$st_1S_6^a$ or T65*wx* exhibited a low seed-setting rate but the F_1 hybrids between these strains and T65st_1S_6 or T65S_6 (W593) did not, indicating that the four wild strains carry the S_6 allele at the S_6 locus. A marked distortion of the linked *st1* marker in the F_2 populations supported the conclusion that the four wild strains harbor the S_6 allele.

The other six *O. sativa* ssp. *indica* and nine *O. rufipogon* strains (W107, W2002, W630, W1718, W1943, W1944, W1945, W1952, and W1681) produced a pattern different from that described above (Table 2). There was no substantial difference in the seed-setting rate in the F_1 hybrids derived from the two testcrosses, although the seed-setting rate varied depending on the crossing parents. A marked excess of *st1* homozygotes was detected in neither of the F_2 populations derived from the two testcrosses. Therefore, it was suspected that these strains carry an additional allele (S_6^n; "n" refers to

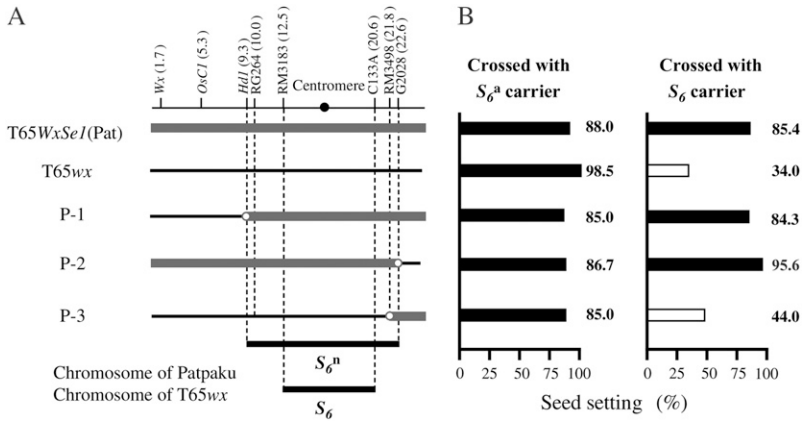


FIGURE 3.—Localization of the S_6^n gene on the rice linkage map. (A) Location of the S_6^n gene on chromosome 6. The physical map of chromosome 6 is based on the Rice Genome Research Program (http://rgp.dna.affrc.go.jp). The map position of each marker along chromosome 6 (AP008212) is shown in parentheses (in megabases). Graphical genotypes of NILs used for mapping the S_6^n gene are shown below the linkage map. Chromosomal regions derived from Patpaku (carrying the S_6^n allele) and T65*wx* (carrying the S_6^a allele) are indicated by thick shaded lines and thin solid lines, respectively. A solid circle represents the centromere. Open circles represent T65*wx*-derived alleles. The estimated ranges of the positions of the S_6^n and S_6 genes are shown by solid bars below the graphical genotypes. (B) The rate of seed setting in F_1 plants derived from crosses between NILs for the S_6^n gene and S_6 or S_6^a carriers. For the analysis of seed setting, see legend to Figure 2. The rate of seed setting is shown as a percentage (average of two individual plants). Solid and open bars indicate rates of seed setting above and below 50%, respectively.

"neutral"; RICK 1966, 1971), which induces no preferential abortion in the heterozygotes (S_6/S_6^n and S_6^a/S_6^n). Subsequent analysis indicated that the estimated chromosomal location of the S_6^n gene overlapped with that of the S_6 gene, suggesting that the S_6^n gene is allelic to the S_6 gene (see Figure 3).

The F_1 hybrids between the remaining strain W2048 and the two tester lines exhibited a low seed-setting rate. When the seed-setting rate of a hybrid is low, TRD would be expected to be a more reliable indicator for determining the allelic state at the S_6 locus. Unlike the other wild rice strains, W2048 displayed the segregation distortion: a marked excess of *st1* homozygotes was detected in the F_2 population derived from a cross between W2048 and T65st_1S_6, but not that from a cross between W2048 and T65$st_1S_6^a$. These results indicate that W2048 carries the S_6^a allele. Taken together, these results reveal that plants carrying the S_6^n allele are present at a high frequency and are widely distributed in Asia. The S_6 allele was detected in four strains from South to Southeast Asia, and the S_6^a allele was detected in three cultivated (*O. sativa* ssp. *japonica*) and one wild (*O. rufipogon*) rice strain from East Asia.

The additional allele (S_6^n) at the S_6 locus: As mentioned above, of the 23 Asian wild rice and cultivated rice strains examined, 15 strains exhibited no TRD, as evaluated by the st_1 phenotype in the crosses with either T65st_1 S_6 or T65st_1 S_6^a (Table 2), suggesting the presence of an additional allele (S_6^n) at the S_6 locus, which induces no preferential abortion in the heterozygotes (S_6/S_6^n and S_6^a/S_6^n), as proposed in the tomato (RICK 1966, 1971). To test this possibility, a NIL, T65*WxSe1* (Pat), was characterized. Because the testcross indicated that Patpaku carried the S_6^n gene (Table 2), the chromosomal fragment transgressed from Patpaku should contain the S_6^n gene, if S_6^n is allelic to the S_6 gene (Figure 3). Hybrid plants between T65*WxSe1* (Pat) and T65*wx* (S_6^a carrier), and those between T65*WxSe1* (Pat) and T65S_6 (W593), both exhibited a high seed-setting rate (88.0 and 85.4%, respectively). This shows that neither the S_6 nor the S_6^a alleles induces abortion of gametes carrying the Patpaku-derived allele in the heterozygotes. The fragment introduced from Patpaku was further segmented by selfing the heterozygote plants. Three F_3 lines with distinct homozygous introgressions (P-1, P-2, and P-3; Figure 3) in the S_6 region were selected on the basis of the six markers (*Wx*, *OsC1*, *Hd1*, RG264, RM3498, and G2028) and were used to map the S_6^n gene. When the three lines were crossed with both the S_6^a and S_6 carriers, only the hybrid plants between P-3 and T65S_6 (W593) exhibited a low seed-setting rate (44.0%) (Figure 3). This result indicates that if a plant harbors the chromosomal region between *Hd1* and G2028 from Patpaku, the plant induces no preferential abortion in response to the S_6 and S_6^a alleles. Since the region between *Hd1* and G2028 contained the region in which the S_6 gene was located, we concluded that Patpaku possesses the S_6^n allele at the S_6 locus.

Survey of nucleotide diversity in chromosomal regions around the S_6 locus: Owing to the presence of the additional allele, association between genetic diversity and allelic state at the S_6 locus was analyzed on the basis of the relative nucleotide diversity between plants carrying the incompatible alleles (S_6 or S_6^a) and those carrying the additional allele (S_6^n). We analyzed the nucleotide sequences at the five loci (P139, F06, D11, R111C, and C133A) encompassing the centromeric region (Figure 4), and compared Watterson's θ (θ_w) and π between these loci (Table 3). Silent sites were used for comparing nucleotide diversity to rule out selection acting on the functional regions. Tajima's *D* statistic revealed no significant deviations from neutrality at these loci (Table 3). The S_6^n carriers showed values ranging from 7.78×10^{-3} (D11) to 10.50×10^{-3} (C133A) for θ_w, and from 6.75×10^{-3} (P139) to 8.66×10^{-3} (F06) for π, which is comparable to the values for the nucleotide diversity previously reported in nuclear genes of rice (YOSHIDA *et al.* 2004; YOSHIDA and

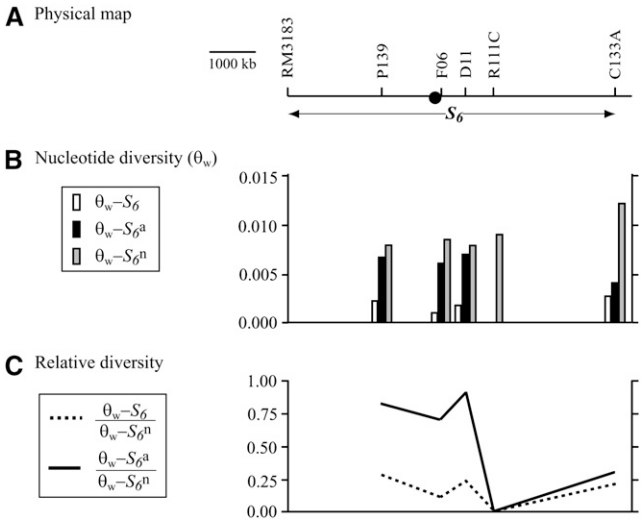


FIGURE 4.—Survey of the nucleotide diversity around the centromere on chromosome 6. (A) A physical map of the candidate region for the S_6 gene. The position of the centoromere is indicated by a solid circle. (B) Levels of nucleotide diversity (θ_w) for the S_6, S_6^a, and S_6^n carriers at five sites in the chromosomal region. (C) The relative diversities of the S_6 and S_6^a carriers to the S_6^n carriers at five sites in the chromosomal region.

MIYASHITA 2005; RAKSHIT *et al.* 2007; ZHU *et al.* 2007). At these five loci, the estimates of θ_w and π were lower in the S_6 and S_6^a carriers than those in the S_6^n carriers (Table 3 and Figure 4B). In particular, the estimates were zero at the R111C locus in the S_6 and S_6^a carriers. Since the F06 and D11 loci were physically closer to the centromere than the R111C locus, but a higher level of diversity was detected at these loci compared to the diversity at the R111C locus, the low nucleotide diversities of the S_6 and S_6^a carriers in the R111C locus is evidently not due solely to the effect of low recombination near the centromere. The relative diversities (θ_w-S_6/θ_w-S_6^n and θ_w–S_6^a/θ_w-S_6^n) increased depending on the physical distance from the R111C locus (Figure 4C). The levels of nucleotide diversity at the P139 and C133A loci were similar to those at the unlinked loci *sd1* and *qSH4* and the loosely linked locus *Hd3a* (Table 3), suggesting that the reduction in nucleotide diversity is specific to the chromosomal region around the R111C locus, and this is not accounted for by either demographic effects or selection acting on the sites. These results indicate an association between nucleotide diversity in the chromosomal region and the allelic state at the S_6 locus, and are consistent with the notion that the S_6 and S_6^a alleles arose independently from the S_6^n allele (discussed below).

DISCUSSION

The *si*TRD caused by the S_6 locus and its independence from the effects of other loci associated with sexual affinity on the same chromosome: TRD violates Mendel's rules by the preferential transmission of a particular chromosome or allele at the expense of its partner. Preferential gamete dysfunction has been frequently reported as a mechanism of TRD in a range of organisms (LYTTLE 1991; TEMIN *et al.* 1991; SILVER 1993; ÚBEDA and HAIG 2005; MOYLE and GRAHAM 2006). Cytological observations revealed that about half of the female gametes degenerated in the S_6/S_6^a heterozygote (Figure 1). However, no defect was detected in the pollen grains by this method, although genetic analysis indicated that the transmission of both male and female gametes carrying the S_6^a allele to the next generation was very low (SANO 1992; this study). This probably indicates that functional difference(s) between the male gametes with different alleles, which accounts for the TRD, occurs after the production of pollen grains. No reduction in the seed-setting rate was observed when the S_6/S_6^a heterozygote was used as a male parent for crosses, which indicates that no aberration occurred after fertilization, although almost no S_6^a alleles were transmitted to the progeny (SANO 1992). Taken together, these observations suggest that the primary event(s) resulting in the TRD phenomenon through the male gametes in the S_6/S_6^a heterozygote occurs during a prezygotic process after the production of pollen grains. A plausible explanation for these observations is that TRD through the male gametes is caused by pollen tube competition (certation). Alternatively, it is possible that the dysfunction of the male gametes occurred at an early stage of gametogenesis and the observed pollen grains consist only of carriers of the S_6 allele. In any event, the genetic effects of the S_6 locus on the *si*TRD were altered in neither the female nor male gametes, even after repeated backcrosses, which indicates the stability of the phenomenon.

Genetic mapping revealed that the S_6 locus is located near the centromere of chromosome 6. On the same chromosome, genes that affect the fertility of F_1 hybrids have been identified. Among these are the *cim* (the cross-incompatibility reaction in the male) gene and the two *Cif* (the cross-incompatibility reaction in the female) genes responsible for the abortion of hybrid seeds, especially in endosperm, observed in a cross between W593 and T65*wx* (MATSUBARA *et al.* 2003; KOIDE *et al.* 2008c), which are the same parental strains of the materials used in the present study. The *cim* and one of the *Cif* genes, Cif_2, have been mapped in a chromosomal region that overlaps with the S_6 locus (MATSUBARA *et al.* 2003; KOIDE *et al.* 2008c). Another *Cif* gene, Cif_1 is located on the short arm of chromosome 6. The plants carrying *cim* derived from T65*wx* exhibit cross-incompatibility when pollinated to the female plants carrying both Cif_1 and Cif_2 derived from W593. The genotypes of T65*wx* and W593 are cif_1 cif_2 *cim*/cif_1 cif_2 *cim* and Cif_1 Cif_2 *Cim*/Cif_1 Cif_2 *Cim*, respectively. These three genes (*cim*, Cif_1, and Cif_2) act sporophytically, so that the heterozygotes (Cif_1 Cif_2 *Cim*/cif_1 cif_2 *cim*) produce only gametes that are cross-compatible. As a

TABLE 3

Levels of nucleotide diversity

		S_6 ($n = 4$)				$S_6{}^a$ ($n = 4$)				$S_6{}^n$ ($n = 15$)			
Loci	Length[a]	No. of polymorphic positions	Nucleotide diversity[b] ($\theta_w \times 10^3$)	Nucleotide diversity[b] ($\pi \times 10^3$)	Tajima's *D*	No. of polymorphic positions	Nucleotide diversity[b] ($\theta_w \times 10^3$)	Nucleotide diversity[b] ($\pi \times 10^3$)	Tajima's *D*	No. of polymorphic positions	Nucleotide diversity[b] ($\theta_w \times 10^3$)	Nucleotide diversity[b] ($\pi \times 10^3$)	Tajima's *D*
						Chromosome 6							
P139	748	3	2.18 ± 1.58	2.23 ± 0.59	0.16766	9	6.55 ± 3.97	6.01 ± 3.19	−0.82943	19	7.81 ± 3.27	6.75 ± 0.95	−0.55548
F06	530	1	0.98 ± 0.98	0.90 ± 0.48	−0.61237	6	5.95 ± 3.80	5.45 ± 2.89	−0.80861	15	8.37 ± 3.42	8.66 ± 0.99	0.13696
D11	688	1	1.72 ± 1.72	1.58 ± 0.84	−0.75455	4	6.89 ± 4.70	6.31 ± 3.35	−0.79684	8	7.78 ± 3.79	7.33 ± 1.52	−0.25041
R111C	590	0	0.00 ± 0.00	0.00 ± 0.00	—	0	0.00 ± 0.00	0.00 ± 0.00	—	17	8.86 ± 3.77	6.68 ± 0.64	−0.99328
C133A	892	7	4.59 ± 2.87	4.21 ± 1.88	−0.81734	6	2.65 ± 1.81	2.43 ± 1.29	−0.80861	32	10.50 ± 4.21	8.14 ± 1.87	−1.03951
Hd3a	2448	18	4.59 ± 2.63	4.45 ± 1.94	−0.31310	15	3.82 ± 2.22	3.50 ± 1.72	−0.84729	43	6.30 ± 2.44	5.88 ± 0.51	−0.28539
						Chromosome 1							
sd1	1883	9	3.92 ± 2.37	3.59 ± 1.90	−0.82943	7	3.04 ± 1.90	2.79 ± 1.48	−0.82407	36	11.05 ± 4.45	8.55 ± 1.48	−1.16932
						Chromosome 4							
qSH4	1734	18	5.75 ± 3.29	5.46 ± 1.76	−0.50485	18	5.72 ± 3.28	5.24 ± 2.78	−0.85194	45	8.10 ± 3.13	8.23 ± 0.99	−0.11812

Indels were ignored for computation.

[a] No. of nucleotide positions analyzed.

[b] Silent substitutions including synonymous substitutions and changes in the noncoding positions were used for the analysis.

consequence, the cross-incompatibility system controlled by the *cim* and *Cif* genes acts on the formation of hybrid seeds after fertilization (Matsubara *et al.* 2003; Koide *et al.* 2008c), whereas that by the S_6 locus acts on gametes produced in the F_1 plants. Therefore, the developmental phase of the S_6 locus-mediated abortion and consequent segregation pattern are both unequivocally distinguished from those mediated by the *Cif/cim* genes, although the possibility cannot be ruled out that abortions at different developmental phases are caused by pleiotropic effects of a single gene, which would resemble a phenomenon caused by the *Tcb1* locus in maize (Kermicle 2006).

The hybrid sterility loci, S_1 (Sano 1990), S_5 (Yanagihara *et al.* 1995), S_8 (Singh *et al.* 2006), S_{10} (Sano *et al.* 1994), and S_{26} (Kubo and Yoshimura 2001), have also been mapped to chromosome 6, a mapping procedure carried out using different pairs of crossing parents. These genes were mapped in chromosomal regions apart from the S_6 locus, and segregation analyses of marker genes tightly linked with these loci also indicated that the segregation distortion caused by these loci was independent of that by the S_6 locus (our unpublished data). These results indicate that the *si*TRD phenomenon caused by the S_6 locus is not affected by the allelic state at the other hybrid sterility loci on the same chromosome. The independence of the S_6 locus-mediated *si*TRD ensures that the pattern of nucleotide diversity around the S_6 locus (Table 3) depends exclusively on the allelic state at the S_6 locus.

The S_6^n allele is widely distributed in Asian rice and functions in a locus-specific manner: We examined the allelic distribution at the S_6 locus in 23 strains of Asian rice (Table 2). In addition to the S_6 locus, we have also analyzed the allelic distribution at the S_1 locus that causes *si*TRD in the hybrids between the Asian and African rice species (Koide *et al.* 2008b). Although many loci causing TRD have been identified in the rice genome (see Koide *et al.* 2008a), no other attempts that analyze allelic distribution of a single locus in various rice accessions have been reported. A notable difference between the *si*TRD caused by the S_6 and S_1 loci is the presence or absence of an additional allele the presence of which, in heterozygotic states, does not result in *si*TRD; both the testcross experiments and subsequent genetic mapping indicated the presence of the S_6^n allele at the S_6 locus (Table2; Figure 3), whereas no such additional allele has been detected regarding the S_1 locus (Koide *et al.* 2008b). The testcross experiments also indicated that the S_6^n allele was distributed widely, and was predominant in Asian rice (Table 2). A phylogenetic analysis using the nucleotide sequence data from the five loci (P139, F06, D11, R111C, and C133A) around the S_6 locus showed that both the S_6 and S_6^a carriers clustered, and these clusters were separated from each other on the phylogenetic tree (data not shown). These results suggest that the S_6^n allele could be the ancestral state of the locus and the S_6 and S_6^a alleles evolved from the ancestral S_6^n allele independently.

It has been frequently reported in varietal crosses that hybrid sterility in rice is caused by a single locus (Kitamura 1962; Oka 1964; Ikehashi and Araki 1986; Yanagihara *et al.* 1995; Liu *et al.* 1997). In these loci, a wide array of compatible alleles conferring compatibility to a wide range of strains has been proposed, and some have actually been mapped (Ji *et al.* 2005; Qiu *et al.* 2005; Wang *et al.* 2006). However, regarding the wide compatibility proposed in rice, whether these compatible alleles act specifically on the alleles at their own locus or on sterility genes at different loci, as reported in wheat (Tsujimoto and Tsunewaki 1985; Friebe *et al.* 2003) and Drosophila (Lyttle 1991; Temin *et al.* 1991), is not known. In the testcross experiments, the hybrids between the S_6^n carriers and the tester lines did not necessarily exhibit a higher seed-setting rate than the hybrids between S_6 or S_6^a carriers and the tester lines (Table 2). These results suggest that, unlike alleles conferring wide compatibility, the S_6^n allele does not reduce the level of hybrid sterility that might be caused by other sterility loci, but rather interacts specifically with the S_6 and S_6^a alleles.

Chromosomal region-specific reduction in genetic diversity associated with allelic state: The presence of the S_6^n allele prompted us to survey the chromosomal region around the S_6 locus in terms of nucleotide diversity and examine the association between the nucleotide diversity and allelic state at the S_6 locus. Association-based mapping could identify regions that are associated with a particular phenotype (Thornsberry *et al.* 2001; Palaisa *et al.* 2003). If a new sequence variant underlying a given phenotype has emerged, then there would have been less time for recombination, and thus a significant association would be expected between phenotype and genotype in the vicinity of the causative mutation. As a result, a reduction in nucleotide diversity should occur around the causative mutation within a population of individuals exhibiting the same phenotype. If the S_6 and S_6^a alleles have arisen independently from the S_6^n allele, a reduction in nucleotide diversities of the S_6 and S_6^a carriers relative to the diversity of the S_6^n carriers should be observed near the locus, as the relative nucleotide diversities would tend to increase with physical distance from it due to the shuffling into different genetic backgrounds through recombination. In fact, the nucleotide diversities of the S_6 and S_6^a carriers were zero at the R111C locus and the relative diversities (θ_w-S_6/θ_w-S_6^n and θ_w-S_6^a/θ_w-S_6^n) increased depending on the physical distance from the R111C locus (Figure 4B). Thus, a clear association between nucleotide diversity and allelic state at the S_6 locus was detected by comparing nucleotide diversity of the S_6 or S_6^a carriers with that of the S_6^n carriers, which most probably reflects independent lineages of the S_6 and S_6^a

carriers. These results also suggest that the causative alleles are most likely located in a region close to the R111C locus among the loci examined.

The resolution of association analysis depends on the structure of the linkage disequilibrium (LD) across the entire genome. Garris *et al.* (2003) reported that significant LD was detected at sites which were at most 100 kb apart around the *xa5* locus in cultivated rice strains. However, it has not established how far the LD extends in the region near the centromere where recombination rates are low. The results of the present study consequently suggest that association mapping is potentially useful even for chromosomal regions where recombination is restricted. Further analyses using more accessions and loci would reveal whether the local nucleotide diversity is directly associated with the S_6 locus-mediated phenotype.

Allelic differentiation at the S_6 locus and evolution of the *si*TRD system that serves as an isolating barrier: The Dobzhansky–Muller model proposes that hybrid incompatibilities are generally caused by an interaction between genes that have functionally diverged in each population (Coyne and Orr 2004). This model assumes two or more interacting loci. TRD systems often involve alleles at a minimum of two closely linked loci, a distorter and its *cis*-acting target (Lyttle 1991). Examples of the TRD system of this type include the mouse *t*-haplotype (Silver 1993), *segregation distorter* (*SD*) of Drosophila (Temin *et al.* 1991), and *Gametocidal 2* (*Gc2*) in wheat (Friebe *et al.* 2003).

Alternatively, the development of hybrid incompatibility might be explained by a simple one-locus stepwise mutation model (Nei *et al.* 1983). This model assumes changes in allelic state in two different populations: A_0 mutates to A_1 in one population and to A_{-1} in another population. If the heterozygotes for alleles that are two or more steps apart from each other are infertile, hybrid incompatibility appears in A_1A_{-1} despite the fact that A_1A_0 and $A_{-1}A_0$ are completely fertile. This model was adopted for explaining allelic differentiation at the *Gamete eliminater* (*Ge*) locus in tomato (Rick 1971). Rick (1966, 1971) reported that there are three alleles (Ge^p, Ge^c, and Ge^n) at the locus. Ge^p induces abortion of the male and female gametes carrying Ge^c in the heterozygote (Ge^p/Ge^c). On the basis of their geographical distribution, it was suggested that Ge^p and Ge^c have arisen from the neutral allele Ge^n. Likewise, because of the presence of the $S_6{}^n$ allele at the S_6 locus in rice, the evolutionary process at the S_6 locus can be explained by the one-locus stepwise mutation model. The results obtained so far are also consistent with the notion that the $S_6{}^n$ allele is the ancestral state of the locus, as described above.

Recent studies have suggested that TRD, which is normally masked within a species, plays a role in establishing the sterility barriers between species (Dermitzakis *et al.* 2000; Tao *et al.* 2001; Orr and Irving 2004). There are at least two possible models for the formation of isolating barriers induced by normally masked TRD. Frank (1991) along with Hurst and Pomiankowski (1991) proposed that genes causing TRD are rapidly fixed in a population due to their "selfish nature," but may often become suppressed within each population to alleviate their deleterious effects on fertility. These genes can become reexpressed in hybrids between individuals from allopatric populations and cause the sterility of hybrids.

Another possible explanation is that a transmission ratio distorter never did appear in the history of the two allopatric populations and TRD instead appeared as a consequence of an inappropriate interaction between genes that had diverged in these populations. These genes have no evident selfish behavior in so far as they are present in each population. In this model, genes of neither lineage pass through the adaptive valley represented by the driving genotype that results in TRD (Orr and Presgraves 2000). Assuming that the S_6 and $S_6{}^a$ alleles have arisen independently from the $S_6{}^n$ allele, the incompatible alleles (S_6 and $S_6{}^a$) could have become fixed in different populations without carrying any selective disadvantage, which would favor the latter explanation. The predominance of the $S_6{}^n$ allele is consistent with the fact that the Asian wild rice population consists primarily of plants with interfertile relationships (Morishima *et al.* 1963; Chu *et al.* 1969). The *si*TRD system mediated by the allelic interaction between the S_6 and $S_6{}^a$ alleles established genetically discontinuous relationships between limited constituents (*e.g.*, between *O. sativa* ssp. *japonica* and *O. rufipogon* in Southeast Asia) of the existing rice population.

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