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ORIGINAL PAPER

Identification and fine mapping of *S-d*, a new locus conferring the partial pollen sterility of intersubspecific F_1 hybrids in rice (*Oryza sativa* L.)

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Abstract The partial pollen abortion of hybrids between the *indica* and *japonica* subspecies of Asian cultivated rice is one of the major barriers in utilizing intersubspecific heterosis in hybrid rice breeding. Although a single hybrid pollen sterility locus may have little impact on spikelet fertility, the cumulative effect of several loci usually leads to a serious decrease in spikelet fertility. Isolating of the genes conferring hybrid pollen sterility is necessary to understand this phenomenon and to overcome the resulting genetic barrier. In this study, a new locus for F_1 pollen sterility, *S-d*, was identified on the short arm of chromosome 1 by analyzing the genetic effect of substituted segments of the near-isogenic line E11-5 derived from the *japonica* variety Taichung 65 (recurrent parent) and the *indica* variety Dee-geo-woo-gen (donor parent). The *S-d* locus was first mapped to a 0.8 cM interval between SSR markers PSM46 and PSM80 using a F_2 population of 125 individuals. The flanking markers were then used to identify recombinants from a population of 2,160 plants derived from heterozygotes of the primary F_2 population. Simultaneously, additional markers were developed from genomic sequence divergence in this region. Analysis of the recombinants in the region resulted in the successful mapping of the *S-d* locus to a 67-kb fragment, containing 17 predicted genes. Positional cloning of this gene will contribute to our understanding of the molecular basis for partial pollen sterility of intersubspecific F_1 hybrids in rice.

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Introduction

Rice (*Oryza sativa* L.) is one of the most important staple crops in the world, serving as the primary food supply for almost half of the world's population. With world population rapidly increasing and the cultivable land sharply decreasing, more productive rice varieties are needed. Strong heterosis between *indica* and *japonica* subspecies of Asian cultivated rice provides an appealing possible solution to this problem. However, the partial sterility that frequently occurs in *indica-japonica* hybrids is a major obstacle (Kato et al. 1928; Oka 1957, 1974).

Ikehashi and Araki (1986) proposed an allelic interaction model for explaining the mechanism of hybrid sterility. According to the model, there are three alleles at the wide compatibility locus (S_5). The S_5^i, S_5^j, and S_5^n alleles are present in *indica*, *japonica* and wide compatibility varieties, respectively. The *indica/japonica* heterozygotes (S_5^i/S_5^j) produce semi-sterile panicles, resulting from partial abortion of female gametes carrying S_5^j, whereas heterozygote with the S_5^n allele and either of the other two alleles, e.g., $S_5^n\ S_5^i$ or $S_5^n\ S_5^j$ are fully fertile. In the subsequent studies, a series of female sterility loci including major genes and QTLs were identified and mapped (Wan and Ikehashi 1995; Wan et al. 1993, 1996; Liu et al. 1997; Wang et al. 1998; Zhang et al. 1998; Zhu et al. 1998; Yan et al. 2000; Liu et al. 2001; Song et al. 2005).

Male gamete abortion also plays a key role in *indica-japonica* hybrid sterility (Zhang and Lu 1989, 1993; Zhang et al. 1994; Zhuang et al. 1999, 2002; Li et al. 2002; Song et al. 2005; Wang et al. 2006). Although a single hybrid pollen sterility locus may not seriously reduce spikelet fertility, several loci working together normally will lead to a notable decrease in spikelet fertility (Zhang et al. 1994). Therefore, there is a crucial need to investigate hybrid

pollen sterility and understand the molecular basis for this phenomenon in order to overcome the resulting genetic barrier. In a previous study, Zhang and Lu (1989) identified three loci (*S-a*, *S-b*, and *S-c*) for F_1 pollen sterility from diallel crosses between Taichung 65, a *japonica* variety, and five near-isogenic lines, which were derived from five *indica* donors by successive backcrosses (Oka 1974). Three additional sterility loci, namely *S-d*, *S-e*, and *S-f*, were found by testcrosses between the near-isogenic lines of Taichung 65 and a number of other varieties (Zhang et al. 1994). Of the six F_1 pollen sterility loci identified to date, only the *S-a* (Zhuang et al. 1999), *S-b* (Li et al. 2002) and *S-c* (Zhang and Zhang 2001; Zhuang et al. 2002) loci have been mapped and the latter two, *S-b* (Li et al. 2006) and *S-c* (Yang et al. 2004), have been fine mapped.

In order to map the remaining F_1 pollen sterilty loci, three *indica* varieties (Dee-geo-woo-gen, Zhai-ye-qing and Xiao-bai-dao) known to carry S^i alleles at the other sterility loci (Zhang et al. 1994) were used as donor parents in crosses with Taichung 65 to develop a set of near-isogenic lines. Li et al. (2003) reported the results of a survey of the substituted segments of 50 near-isogenic lines based on whole genome polymorphism screening. In addition, hundreds of testcrosses between 50 near-isogenic lines, recurrent parent and other near-isogenic lines with known alleles were made and pollen fertility was examined as well (W. T. Li et al., unpublished results). These preliminary results have facilitated the identification of genes with minor effect on substituted segments of the near-isogenic lines.

In this study, we report the successful fine mapping of the *S-d* locus to a 67-kb region on the short arm of chromosome 1 by meticulously analyzing the genetic effect of substituted segments of the near-isogenic lines E11-5. These results provide the foundation for cloning the *S-d* gene as the first step in understanding the molecular basis of partial pollen sterility of intersubspecific F_1 hybrids in rice.

Materials and methods

Plant materials

The *japonica* variety Taichung 65 (T65) and its near-isogenic lines E11-5 and TISL2 were used in this study. E11-5 is a BC_2 line derived from a cross between the *indica* donor, Dee-geo-woo-gen and T65. Li et al. (2003) have surveyed the substituted segments of 50 near-isogenic lines derived from three donors (Dee-geo-woo-gen, Zhai-ye-qing and Xiao-bai-dao) using 158 evenly distributed SSR markers. In line E11-5 (NIL5), five chromosomal segments from the donors were identified on chromosomes 1, 5, 7, and 8 (two segments), respectively (Fig. 1). Meanwhile, one substituted segment was identified in the region of the *S-b* locus on chromosome 5 (Li et al. 2002), while no substituted segments were found in the region of the *S-a* on chromosome 1 (Zhuang et al. 1999) and the *S-c* loci on chromosome 3 (Zhang and Zhang 2001; Zhuang et al. 2002). The near-isogenic line TISL2 was also selected as the testcross parent in order to determine the alleles of E11-5 at the six known loci for F_1 pollen sterility. Zhang and Lu (1993, 1996) previously reported that the alleles of T65 are S^j/S^j at the six loci whereas the alleles of TISL2 are the same as T65 except at the *S-b* locus, which is S^i/S^i.

In the early season of 2003, testcrosses were made between E11-5 and the lines T65 and TISL2. Pollen fertility of the F_1 progeny was examined in the late season of 2003. In the early season of 2004, a F_2 population of 125 plants derived from the testcross combination E11-5 and TISL2 was used to map the newly identified sterility locus. This was followed by fine mapping of the locus using a larger mapping population of 2,160 individuals derived from eight heterozygous F_2 plants in the two successive planting seasons. All materials were planted and maintained in the experimental farm of South China Agricultural University, Guangzhou, China.

Pollen fertility examination

Pollen fertility was assayed according to the method described by Zhang and Lu (1989). Ten florets per panicle

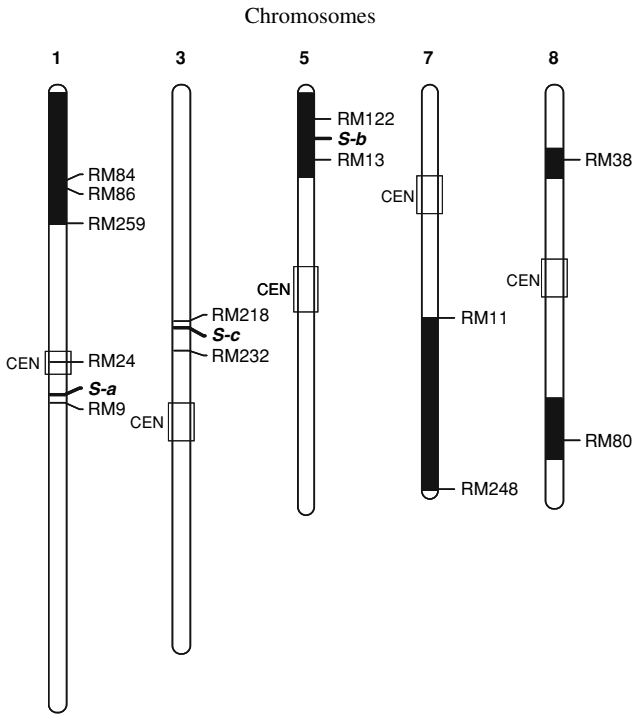

Fig. 1 Distribution of substituted segments on the chromosomes of near-isogenic line E11-5 (adapted from Li et al. 2003). Substitute segments from the donor Dee-geo-woo-gen are shown in *black*. The recurrent parent is T65

were collected from the upper one-third portion of the panicle and fixed in FAA solution [89% (v/v) ethanol, 6% (v/v) formaldehyde and 5% (v/v) acetic acid]. Six anthers from the floret were mixed and spread on a microscope slide. Pollen was stained with an I_2-KI solution containing 0.1% (w/v) iodine and 1% (w/v) potassium iodide. Pollen samples were classified into three groups based on their staining and shape: fertile (round and full dark), staining abortive (round and partial dark) and empty abortive (irregular and yellow).

Molecular marker development and linkage analysis

SSR markers were developed by using the sequence of the delimited region from the International Rice Genome Sequencing Project (IRGSP) database (http://rgp.dna.affrc.go.jp/IRGSP/index.html). Suitable SSR sequences were identified using the online SSR identification tool SSRIT (http://www.gramene.org/microsat/). Primers for the amplification of target SSRs were designed using software Primer Premier 5.0 (Premier Biosoft International, http://www.premierbiosoft.com). Additionally, Insertion-Deletion (InDel) markers were developed by selecting suitable InDels (insertion or delection of 5–100 bp) in the delimited region from the genome-wide DNA polymorphism database of rice (Shen et al. 2004; http://shenghuan.shnu.edu.cn/ricemarker). Sequences of ~400 bp around the InDels were then chosen and PCR primers were designed which amplified products ranged from 80 to 250 bp for analysis by polyacrylamide gel electrophoresis.

A mini-scale DNA extraction method (Zheng et al. 1995) was used to prepare DNA samples of the mapping population. DNA from the parental plants and recombinants were isolated using the CTAB method (Murray and Thompson 1980). The PCR profile used for SSR amplification was according to the protocol described by Panaud et al. (1996), and InDel marker amplification was performed as described by Li et al. (2006). PCR products were resolved on 6% non-denaturing polyacrylamide gel and subjected to the silver staining procedure as described by Li et al. (2002).

Data was analyzed with Mapmaker/EXP 3.0 program (Lander et al. 1987) to determine the linkage relationship between the sterility locus and molecular markers. A LOD threshold of 3.0 was adopted for constructing the local genetic map.

Results

Identifying the *S-d* locus

The alleles of recurrent parent T65 and near-isogenic line TISL2 at the six known loci for F_1 pollen sterility have been previously reported (Zhang and Lu 1993, 1996). In this study, the alleles of the near-isogenic line E11-5 at these loci were determined by performing two testcrosses, T65 × E11-5 and TISL2 × E11-5. The percentage of fertile pollen was very low, only 24.51%, in the testcross combination of E11-5 and T65 while the testcross combination of E11-5 and TISL2 exhibited 66.32% fertile pollen (Table 1). From this, it was possible to conclude that E11-5 and TISL2 shared the same allele, namely S^i, at the *S-b* locus since the two test lines T65 and TISL2 differ only at that locus (Zhang and Lu 1993, 1996). The pollen fertility results also agreed well with results of the marker survey of substituted segments, which showed that E11-5 has one substituted segment in the region of the *S-b* locus (Fig. 1). Although pollen fertility of the E11-5/TISL2 hybrid was much higher compared with that of the E11-5/T65 hybrid, 31.97% of pollen produced by the E11-5/TISL2 hybrid was classified as staining abortive (Table 1). Interestingly, since no substituted segments were detected at either the *S-a* or *S-c* loci (Fig. 1), it can be further inferred that the other four substituted segments where the alleles of E11-5 and T65 differ may harbor additional sterility loci.

Since E11-5 and TISL2 shared the S^i allele at the *S-b* locus, a F_2 mapping population of 125 plants derived from the TISL2 × E11-5 cross was constructed to eliminate the influence of the *S-b* locus. Our results from analysis of pollen fertility of this F_2 population showed a bimodal distribution with an apparent valley at 85–95% (Fig. 2). Furthermore, individuals could be distinctly divided into fertile and partially sterile plants using 90% pollen fertility as the criterion (Fig. 2). Pollen fertility of fertile plants ranged between 93.37 and 100.00%, with an average of 98.80%, while the distribution for fertility of partially sterile plants was between 59.34 and 82.62%, with an average of 70.20%. The number of fertile and partially sterile plants was 65 and 60, respectively. A chi-square test showed that the segregation ratio of fertile to sterile plants exhibited a good fit with a 1:1 ratio ($\chi^2 = 0.2$, $P > 0.90$), indicating that the segregation of pollen fertility in this population was controlled by one sterility locus.

Table 1 Pollen fertility (%) of the test lines (T65 and TISL2), E11-5 and F_1 hybrids derived from E11-5 and test lines

Test line or cross combination	Fertile pollen	Stainable abortive pollen	Empty abortive pollen
T65	98.29 ± 2.10	0.14 ± 1.87	1.57 ± 1.78
TISL2	99.28 ± 2.29	0.19 ± 1.67	0.53 ± 1.56
E11-5	98.19 ± 2.14	1.06 ± 1.77	0.82 ± 1.44
T65 × E11-5	24.51 ± 8.18	75.48 ± 8.18	0.00 ± 0.00
TISL2 × E11-5	66.32 ± 2.29	31.97 ± 1.85	1.71 ± 1.07

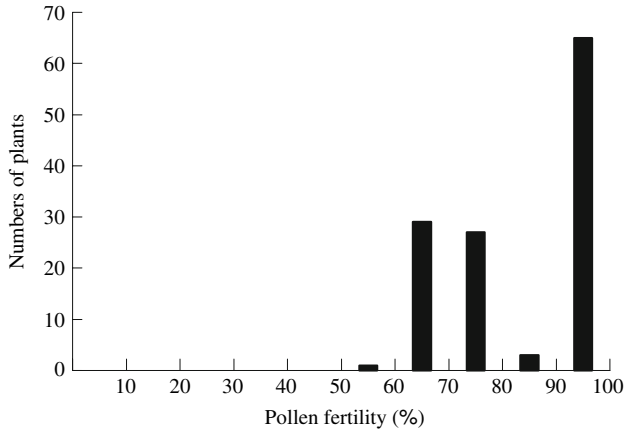

Fig. 2 Distribution of pollen fertility (%) of the F_2 plants derived from the testcross TISL2×E11-5

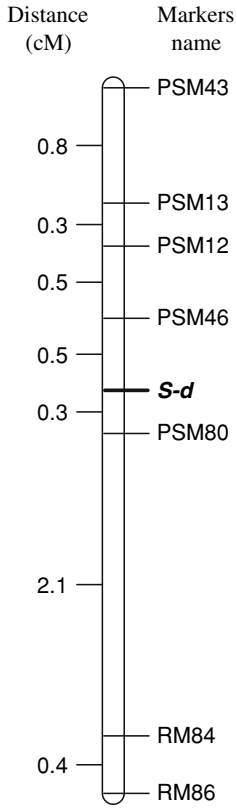

Fig. 3 The linkage map of the *S-d* locus on rice chromosome 1. SSR markers are indicated on the *right* and genetic distance (cM) is indicated on the *left*

To determine the position of the locus, linkage analysis between pollen fertility and the SSR markers from the substituted segments was conducted using the F_2 population derived from E11-5 × TISL2. As mentioned earlier, the alleles of the *S-b* locus were homozygous S^i/S^i in the population, thus only markers from the other four substituted segments were examined. The analysis indicated that the SSR markers RM84 and RM86 on the substituted segment of chromosome 1 are closely linked to this new sterility locus, which was designated as *S-d* (Fig. 3).

Fine mapping the *S-d* locus

In order to fine map the *S-d* locus, additional SSR markers were developed using publicly available rice genome sequence (International Rice Genome Sequencing Project 2005). Primers for 14 SSR markers in the *S-d* region were designed, five of which revealed polymorphism between the parents (Table 2). Analysis of the F_2 population indicated that the *S-d* locus resides in the interval between PSM46 and PSM80 (Fig. 3).

Subsequently, a total of 2,160 plants derived from the heterozygous plants of the primary mapping population were genotyped using flanking markers PSM46 and PSM80 to further reduce the genomic region containing the *S-d* locus. Twenty-five recombinants were identified and subjected to pollen fertility examination, which was reconfirmed in the F_3 populations (data not shown).

At the PSM46-PSM80 interval of interest, a series of SSR and InDel markers were developed from genomic sequence. Twenty-three SSR primer pairs were screened and only four (PSM74, PSM91, PSM93, and PSM96) detected polymorphisms between parents (Table 2). In addition, ten InDel (IND) markers in this region were selected from the genome-wide DNA polymorphism database (Shen et al. 2004) and five showed polymorphism between parents (Table 2). Analysis of the recombinants using the nine new markers indicated that the *S-d* locus is located between PSM93 and IND10 (Fig. 4). In addition, four markers (IND3, IND4, IND8, and PSM96) were found to co-segregate with the locus (Fig. 4). Based on the sequence of Nipponbare (http://rgp.dna.affrc.go.jp/E/IRGSP/index.html), the genomic region containing the *S-d* locus is about 67-kb in length.

The genetic distance between PSM93 and IND10, the two markers flanking the locus, is 0.046 cM (Fig. 4a), while the corresponding physical size of this region is about 67 kb. Therefore, the average physical/genetic distance ratio in the interval is 1.46 Mb/cM, which is much higher than the estimated average ratio of 0.28 Mb/cM for the whole rice genome (Arumuganathan and Earle 1991).

Putative genes at the *S-d* locus

Gene prediction analysis of the 67-kb region from Nipponbare using the online RiceGAAS system (http://ricegaas.rgp.dna.affrc.go.jp) identified 17 putative open reading frames (ORFs) (Table 3). The functions of two predicted ORFs are unknown, whereas the remaining fifteen ORFs have diverse putative functions. In addition, the BLAST results of RiceGAAS system showed that all the ORFs share high similarity with rice cDNA sequences (Table 3), which suggests that the annotated ORFs are actually expressed in the rice genome. According to the RNA

Table 2 Markers developed for fine mapping the *S-d* locus

Marker	Marker type	Motif	Primer sequence	Product size in Nipponbare (bp)
PSM12	SSR	$(CT)_{15}$	5′-GTCAGGAGACTTGGTTTTGAA-3′	185
			5′-AGGTGATGCTGGAAGAATAGA-3′	
PSM13	SSR	$(CTT)_{16}$	5′-CGTTGGCGTAGTGGACGAT-3′	143
			5′-GAGGGTTCTTGCTTTGCTTA-3′	
PSM43	SSR	$(CTC)_{8}$	5′-CGTAGTGGTCCATCGGAGGC-3′	108
			5′-TGAGCTGAGCTGCGGCAAG-3′	
PSM46	SSR	$(CT)_{15}$	5′-CCCGTCACCAACTCACCATA-3′	131
			5′-TCCCAACTCTAAGCCACCCT-3′	
PSM74	SSR	$(TC)_{10}$	5′-GGGGAGAAGATAGGAGGA-3′	104
			5′-ACACCGCACAGACAAATA-3′	
PSM80	SSR	$(AG)_{12}$	5′-CCACCGAAACAGGAAAGG-3′	128
			5′-TCGTAGCACCCGAGCAG-3′	
PSM91	SSR	$(GAG)_{6}$	5′-CAAAGCCAAGAAAGAAACC-3′	135
			5′-CAACCTCGCCAACTGTAC-3′	
PSM93	SSR	$(TC)_{20}$	5′-GAGGACACCGAACAGCC-3′	271
			5′-CATTGGGTAGACGCAAGTA-3′	
PSM96	SSR	$(AG)_{7}$	5′-TACAGGTATCATCGGCTTCA-3′	140
			5′-TGCGTAGGCTCGTCGTCT-3′	
IND1	InDel		5′-ACGAGACCTTCCTTCCGC-3′	175
			5′-GCCCTCCATTGACGCAGA-3′	
IND3	InDel		5′-CGGTCAGGTTTGAGTAGTTGC-3′	165
			5′-ATTGTTGGCTAAGCCATGCTA-3′	
IND4	InDel		5′-GGGAGGGAGAGGAGGGAC-3′	115
			5′-ACGGCGAATCGGGTAGAT-3′	
IND8	InDel		5′-CATTGGGAATTCTACAGTGGA-3′	176
			5′-CGTTGTTGATTTGAATGTTTG-3′	
IND10	InDel		5′-CAAAAAATAAAATCTGCACCA-3′	230
			5′-TGATATATGAGGGAGGAGGAA-3′	

source of the highly similar cDNAs, we can infer that ORF3, ORF6 and ORF10 are spatially expressed in flowers while ORF1 and ORF16 are expressed in the panicles. The expression patterns of these five ORFs suggest they are good candidates for the *S-d* gene. Furthermore, predicted proteins encoded by three ORFs (ORF4, 10, and 17) contain conserved domains that mediate protein–protein interactions.

Discussion

In this study, we have successfully identified a novel F_1 hybrid pollen sterility loucs, *S-d*, on the short arm of chromosome 1 by analyzing the genetic effect of substituted segments of the near-isogenic line E11-5. Near-isogenic lines are great resources in helping to identify genes with minor genetic effect (Monforte and Tanksley 2000). Although the genetic effect of the *S-d* locus is relatively modest, accounting for about 29.80% of sterile pollen, we were able to successfully isolate the locus by developing and utilizing a set of near-isogenic lines. It would have been extremely difficult to discern this locus if a routine mapping population derived from the cross combination of T65 and Dee-geo-woo-gen, the donor parent of E11-5, was directly used for gene identification because alleles of the *indica* variety Dee-geo-woo-gen differed from T65 at four sterility loci (Zhang et al. 1994).

Results of the fine mapping of the *S-d* locus suggest that this region shows reduced recombination. The same phenomenon has been observed in regions containing the rice blast resistance locus *Pib* (Wang et al. 1999) and the lesion mimic gene *Spl11* (Zeng et al. 2002), where ratios of 1.34 and 2.46 Mb/cM, respectively, were reported. Several genetic and molecular studies have found direct evidence that genomic repetitive sequences probably have a negative influence on the chromosomal recombination rate in plants (Arabidopsis Genome Initiative 2000; Fu et al. 2002). We used the sequence delimited by PSM93 and IND10 to conduct BLAST against TIGR *Oryza* Repeat Database

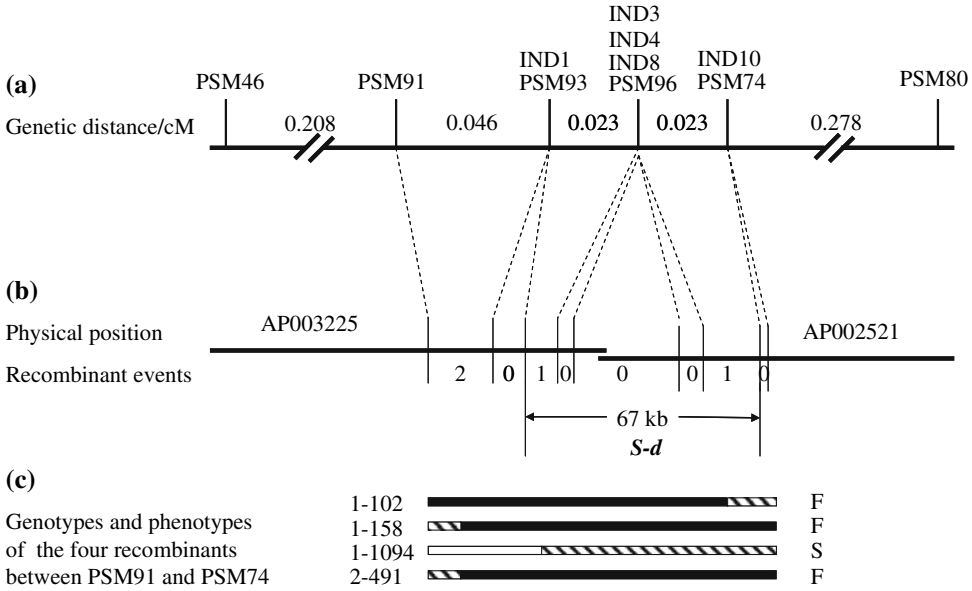

Fig. 4 The genetic and physical map of the *S-d* locus on rice chromosome 1. The values between markers in **a** indicate the genetic distances calculated by genotyping the recombinants while the numbers between markers in **b** indicate the recombination events detected between the *S-d* locus and the respective markers. The long horizontal line indicates the genomic region encompassing the *S-d* locus. The short horizontal lines represent BAC/PAC clones of Nipponbare with the accession numbers as indicated. The *double-arrow line* indicates the candidate region of the *S-d* locus. The genotypes and phenotypes of the four recombinants (1-102, 1-158, 1-1,094, and 2-491) between PSM 91 and PSM 74 are shown in **c**. The *white* and *black regions* indicate the segments from T65 and Dee-geo-woo-gen, respectively, while the hatched regions indicate heterozygous. PSM = SSR markers, *IND* InDel markers, *F* fertility, and *S* sterility

Table 3 Predicted genes at the *S-d* locus based on analysis using the online RiceGAAS system

Predicted gene	Predicted function	Similar EST
ORF 1	Putative AsmA	AK106766
ORF 2	Putative Beta-1,3-glucuronyltransferase	CL957339
ORF 3	Putative Mucin-5B precursor	CL957340; AK072658
ORF 4	Putative crystal structure of the exportin cse1p complexed with its cargo and rangtp	CL957341; AK103127; D78504
ORF 5	Putative Tha8	AK109233
ORF 6	Putative Serine carboxypeptidase 2 precursor	AK071183
ORF 7	Unknown protein	CL956154
ORF 8	Putative ATPP2-A1	AK102322; AK063812
ORF 9	Putative arginyl-tRNA synthetase	CB665677; CB665676
ORF 10	Putative leucine rich repeat family protein	AK070173
ORF 11	Putative RNA-directed DNA polymerase	AU075566; AK105414
ORF 12	Putative DNA binding/protein binding/zinc ion binding	CL957345
ORF 13	Putative transcriptional repressor NF-X1	AK066765
ORF 14	Putative transcription factor HBP-1b	AK106334; CL957346
ORF 15	Unknown protein similar to flagellar biosynthetic protein	CL953223
ORF 16	Putative Nmr ensemble of fasciclin-like protein from Rhodobacter Sphaeroides	AK108308
ORF 17	Putative solution structure of ring finger	CL957347; AK073728

(http://www.tigr.org/tdb/e2k1/osa1/) and only found one miniature inverted repeats transposable elements (MITE) in the region, therefore repetitive sequences may not account for the reduction of chromosomal recombination rate in this region since MITEs are the most common and randomly distributed element in the rice genome. However, investigations into the sequence divergence between *japonica* variety Nipponbare and *indica* variety 93-11 of this region

using BLAST found a large gap of about 20 kb in length (data not shown). This could be responsible for the suppressed recombination we observed.

Analysis of the genomic sequence of the *S-d* locus in the variety Nipponbare suggests that there are several ORFs in the region. Based on the available gene expression data and the annotation data of RiceGAAS, the most likely candidate for the *S-d* gene is ORF10. The predicted protein encoded by ORF10 contains a conserved LRR domain. LRRs are 20-29 residue sequence motifs, which have been found in over 60 different proteins with diverse functions and cellular locations that all appear to be involved in protein-protein interactions (Kobe and Deisenhofer 1995). In the case of hybrid sterility, partial gametes will abort only when the alleles on the sterility loci are heterozygous which implies an interaction between the alleles at these loci. So genes encoding proteins, which contain domains mediating protein–protein interactions represent good candidates for hybrid sterility genes. Interestingly, fine mapping of the *S-b* locus (Li et al. 2006) also resulted in the identification of a candidate gene encoding a protein with a conserved domain involved in mediating interactions between proteins.

The genetic behavior of hybrid sterility between subspecies *indica* and *japonica* is clear (i.e., the fertility of gametes produced from homozygous sporophytes is normal while partial gametes derived from heterozygotes are abortive); however, the underlying molecular mechanism is still ill-defined. At present, most studies still focus on gene identification, primary mapping and fine mapping (Ji et al. 2005; Qiu et al. 2005; Li et al. 2006; Wang et al. 2006). Molecular isolation and characterization of hybrid sterility genes remains challenging. The major difficulty lies in confirming the candidate gene using current techniques.

For example, in most positional cloning efforts, confirmation that the target gene has been cloned is achieved by genetic transformation and recovery of function (i.e., functional complementation). This approach is dependent on the dominant-recessive nature of alleles. In the case of inter-subspecific hybrid sterility, however, there is no such dominant-recessive relationship between the *indica* and *japonica* alleles. Instead, hybrid sterility appears to be caused by the interactions between the *indica* and *japonica* alleles, as observed in this and a number of previous studies (Ikehashi and Araki 1986; Liu et al. 1997; Zhuang et al. 1999, 2002; Zhang and Zhang 2001; Li et al. 2002, 2006; Wang et al. 2006). Therefore, investigating hybrid sterility genes by the functional complementation approach is especially difficult due to this limitation. An alternative approach would be to transform heterozygous plants with the neutral allele S^n in order to recover fertility. RNAi might also be useful as several candidate ORFs were identified in this and a previous study (Li et al. 2006), which have domains that mediate the interaction of proteins.

Our results, together with the mapping of *S-a* (Zhuang et al. 1999), *S-b* (Li et al. 2002, 2006) and *S-c* (Zhang and Zhang 2001; Zhuang et al. 2002), will greatly facilitate the isolation of these genes and understanding the molecular mechanism underlying F_1 pollen partial sterility.

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