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Delimitation of the rice wide compatibility gene *S5*^{n} to a 40-kb DNA fragment

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Abstract Wide compatibility varieties (WCVs) are a special class of rice (*Oryza sativa* L.) germplasm that produces hybrids with normal pollen and spikelet fertility when crossed with both *indica* and *japonica* subspecies. The wide compatibility gene *S5*^{n} has been used extensively in intersubspecific hybrid breeding programs. We previously mapped the *S5* locus to a 2.2-cM genomic region between RM253 and R2349 on chromosome 6, using a population of 356 F_1 plants derived from the three-way cross 02428/Nanjing11//Balilla. In this study, a chromosome walking strategy was employed to construct a physical map covering this genomic region using these two closest markers as the starting points. A physical map consisting of six overlapping BAC clones was formed, spanning a genomic region of 540-kb in length. By analyzing recombination events from a population of 8,000 F_1 plants derived from a three-way cross based on near isogenic lines of the *S5* locus, the *S5* locus was localized to a DNA fragment of 40-kb in length, flanked by two shotgun subclones, 7B1 and 15D2. Sequence analysis of this fragment predicted five open reading frames, encoding xyloglucan fucosyltransferases, dnak-type molecular chaperone BiP, a putative eukaryotic aspartyl protease, and a hypothetical protein. This result will be very useful in molecular cloning of the *S5*^{n} allele and marker-assisted transferring of the wide compatibility gene in rice breeding programs.

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Introduction

The Asian cultivated rice (*Oryza sativa* L.) can be classified into two main subspecies, *indica* and *japonica*. F_1 hybrids between these two subspecies usually demonstrate very strong hybrid vigor (Zhang et al. 1996; Zhao et al. 1999), which has attracted considerable research interest with the hope of using such heterosis in hybrid rice production. Development of such intersubspecific hybrids has become a major objective of hybrid rice breeding programs in China and other Asian countries. A major difficulty encountered in the development of such intersubspecific hybrids, however, is the partial sterility frequently observed in most *indica*/*japonica* crosses (Kato et al. 1928). A special class of rice germplasm, known as wide compatibility varieties (WCVs), can produce normal fertility hybrids when crossed with both *indica* and *japonica* (Ikehashi and Araki 1984). Therefore, WCVs are currently the main tools for breaking the fertility barrier between *indica* and *japonica* subspecies.

Due to the importance of WCVs in exploiting the strong heterosis between intersubspecific hybrid rice, studies had been conducted to reveal the genetic basis of wide compatibility in rice and locate the wide compatibility gene with both morphological and molecular marker technologies. Ikehashi and Araki (1986) first proposed a genetic model to account for wide compatibility. According to this model, a single *S5* locus controls the wide compatibility. There are three alleles at the *S5* locus: a neutral allele (wide compatibility allele), *S5*^{n}; an *indica* allele, *S5*^{i}; and a *japonica* allele, *S5*^{j}. *S5*^{n}*S5*^{i} and *S5*^{n}*S5*^{j} plants would be fully fertile, while *S5*^{i}*S5*^{j} plants would be partially sterile. Ikehashi and Araki (1986) also determined, using morphological markers, that the *S5* locus was located on chromosome 6. This chromosomal location has been further confirmed in several studies using isozymes and molecular markers only from chromo-

some 6 (Li et al. 1991; Liu et al. 1992; Zheng et al. 1992; Yanagihara et al. 1995).

Quantitative trait loci (QTL) analysis of the wide compatibility using a population derived from the three-way cross 02428/Nanjing11//Balilla has detected, in addition to the major locus on chromosome 6 (*S5*), two minor loci on chromosomes 2 and 12 conferring significant effect on hybrid sterility (Liu et al. 1997a). The joint effect of the two minor loci could cause an otherwise fertile $S5^nS5^j$ plant to be partially sterile (Liu et al. 1997a). Analysis with another three-way cross population, Dular/Balilla//Nanjing11, has found that wide compatibility was controlled by five QTLs in complicated interactions (Wang et al. 1998). The influences of minor loci and interactions among these loci made it difficult to classify individual plants into discrete fertile versus sterile classes and therefore greatly affected the accuracy of genetic mapping of the *S5* locus.

All the work on wide compatibility and the majority of studies on *indica*/*japonica* hybrid sterility have been based only on spikelet fertility, which is obviously a function of male gamete fertility, female gamete fertility, and affinity between the uniting male and female gametes. Cytological investigation of the intersubspecific sterility revealed that the male gamete abortions (Wang et al. 1991; He et al. 1994; Teng et al. 1996; Zhu et al. 1996; Liu et al. 2004), female gamete abortions (Yokoo 1984; Li 1988; Ling et al. 1991; Li and Ouyang 1992; Wang et al. 1992; Zhu et al. 1996; Liu et al. 1993, 1997b, 2004), and reduced dehiscence of the anthers (Maeka et al. 1991; Liu et al. 1993) could cause hybrid sterility. Recent QTL analysis using a population from the same cross as described by Liu et al. (1997a) identified two QTLs for pollen fertility, one QTL for embryo-sac fertility, and three other QTLs, including the major locus on chromosome 5 (*pf5*) for pollen fertility, the major locus (*S5*) for embryo-sac fertility, and one locus on chromosome 8 (*spf8*) for spikelet fertility (Song et al. 2005). The *S5* locus, previously identified as a locus for wide compatibility by spikelet fertility analysis, is a major locus for embryo-sac fertility. Pollen fertility and embryo fertility are controlled by independent loci (Song et al. 2005). These results clearly indicated that embryo-sac fertility is simply controlled by the single *S5* locus, suggesting that embryo-sac fertility, rather than spikelet fertility, is the best criterion for fine mapping of the *S5* locus.

In this study, we made a high-resolution genetic map of the *S5* region and constructed a 540-kb BAC contig spanning the *S5* region. Using recombinant plants from 8,000 F_1 plants derived from a new three-way cross constructed with near isogenic lines (NILs) of the *S5* locus developed in this work, we delimitated the *S5* locus to a 40-kb genomic DNA segment. These results will greatly facilitate the final isolation of the wide compatibility gene in rice.

Materials and methods

Mapping populations

A population of 356 individuals from the three-way cross (02428/Nanjing11//Balilla) used for genome-wide detection of QTLs for wide compatibility (Liu et al. 1997a) was used for the genetic mapping of the *S5* locus. 02428 is a *japonica* WCV and was used as the wide-compatibility parent. Nanjing 11 and Balilla are typical *indica* and *japonica* varieties, respectively, and are widely used as testers for compatibility analysis in Chinese rice breeding programs.

Based on the assumptions that a highly fertile plant carries the $S5^n$ allele (Liu et al. 1997a) while a plant carrying the $S5^n$ allele was not necessarily highly fertile due to modifications by minor loci and environmental factors, 101 plants with spikelet fertility higher than 75% were selected for mapping markers in relation to the *S5* locus according to recombination events in the *S5* region.

Development of NILs of *S5* and a mapping population based on the NILs

To eliminate the influence on fertility by the minor loci from the background, NILs carrying the $S5^n$, $S5^i$, and $S5^j$ alleles were developed using Nanjing 11 as the recurrent parent, and 02428 and Balilla as the donor parents for the $S5^n$ and $S5^i$ alleles, in combination with marker-assisted selection. Briefly, 02428 and Balilla were crossed to Nanjing 11, and true hybrids of F_1 were backcrossed to Nanjing 11. In each generation, all individuals were assayed with two flanking SSR markers, RM253 and RM276, to confirm the transfer of the $S5^n$ and $S5^j$ alleles. To remove alleles of the donor parents at the minor loci and to evaluate the introgression of the Nanjing 11 chromosome fragments for background selection of $S5^j$ and $S5^n$ isogenic lines, respectively, progenies of BC_3F_1 and BC_6F_1 generations were also surveyed with SSR markers which were evenly distributed over the rice genome. One plant with 84% homozygous Nanjing 11 loci and a spikelet fertility of 52% was selected for further selfing for the $S5^i$ isogenic line. In the BC_3F_2 population, homozygous $S5^j$ $S5^j$ plants as identified by RM253 and RM276 were named S5J and used to create a new three-way cross population.

A plant that showed 100% homozygous Nanjing 11 loci at all tested markers was selected for selfing to create the NIL S5N. In the BC_6F_2 generation, heterozygous $S5^nS5^i$ individuals as detected by RM253 and RM276 were selected for further crossing with the NIL S5J to make a new three-way cross, S5N/S5I//S5J. A total of 8,000 F_1 individuals from this cross were planted during the 2003 season in the experimental field of Huazhong Agricultural University in Wuhan.

Spikelet and embryo-sac fertility evaluation

Spikelet fertility was scored as seed setting rate on the upper halves of two to five panicles for each plant. Embryo-sac fertility of F_1 individuals was also investigated as described by Song et al. (2005). For each individual, more than 100 mature spikelets from various parts of different panicles were randomly collected and examined.

DNA extraction and marker assay

Total cellular DNA was extracted from 5 g fresh leaf tissues using the CTAB method (Murray and Thompson 1980). RFLP analysis, including DNA digestion, electrophoresis, blotting and hybridization, followed the method described previously (Liu et al. 1997a). SSR analysis was essentially done as described by Chen et al. (1997). Total DNA of the 8,000 F_1 plants from the S5N/S5I//S5J population was miniprepped using a protocol modified from Saghai Maroof et al. (1996) and used for PCR-based marker genotyping.

Physical mapping of the *S5* locus

To ensure the identification of positive clones, four large-insert BAC libraries, Minghui 63 (MH; Peng et al. 1998), Guanglu Ai (GLA; Tao et al. 1994), Teqing (TQ) and Lemont (LM; both from Texas A&M http://hbz.tamu.edu/bacindex.html), constructed with restriction enzyme *Hind*III or *Bam*HI, were used to increase the coverage of the rice genome in this study. Three strategies were used for screening positive BAC clones. For single-copy RFLP markers, $\alpha-^{32}$P-labeled probes were hybridized with high-density BAC filters using the same protocol as RFLP analysis.

A two-step BAC pool strategy was employed to screen positive clones with SSR marker. First, the MH BAC library was pooled plate by plate. Mixed plasmid DNA was isolated from each plate and used as template for PCR reaction using SSR primers. Then the plates having the same PCR amplicon as that from rice genomic DNA were further pooled column by column and row by row. Mixed plasmid DNA was isolated from these columns and rows and used for PCR amplification. The positive BAC clone was located at the intersection of the columns and rows.

As the third strategy for BAC library screening, a whole BAC clone was digested with *Hind*III, labeled with $\alpha-^{32}$P, and directly used as probe to screen high-density BAC filters. Positive BAC clones were fingerprinted with the original BAC clone to confirm the overlaps by using the original BAC clone as the probe for hybridization.

Isolation of BAC ends and subclones

Bacterial artificial chromosome ends were isolated by TAIL-PCR (Liu and Whittier 1995). To obtain subclones, *Hind*III-digested BAC fragments were ligated to *Hind*III-digested, dephosphorylated pUC18 vector and transformed into DH10B. Clones with different sizes of inserts were used for RFLP analysis. Three overlapping BAC clones, 45M8, 12F9, and 26B7 from the contig, were sequenced using the shotgun sequencing strategy (Birren et al. 1997). The polymorphic BAC ends and subclones were also used for RFLP analysis.

Results

Genetic mapping of the *S5* locus

A previous study located the *S5* locus between two RFLP markers, R2349 and RZ450, with a map distance of 1.0 cM from R2349 and 13.4 cM from RZ450 (Liu et al. 1997a). However, there were two large gaps in the *S5* region on the two available high-density RFLP linkage maps (Causse et al. 1994; Kurata et al. 1994) at the time when we started this work. Fortunately, two SSR markers, RM253 (Chen et al. 1997) and RM276 (Temnykh et al. 2000), were located around the *S5* region on later developed SSR maps. These two markers showed polymorphisms between the parents 02428 and Nanjing 11. From 356 individuals of the population as described by Liu et al. (1997a), 43 informative plants that harbored recombinant events between R2349 and RZ450, the two closest markers spanning the *S5* region, were selected for mapping of newly developed markers. RM276 cosegregated with RG213 and was located outside of the R2349–RZ450 region. RM253 had 35 recombinant events with RZ450 and 8 with R2349. According to recombination events, RM253 was located between R2349 and RZ450, with a distance of 2.2 and 9.8 cM to R2349 and RZ450, respectively (Fig. 1).

From the same population, 101 highly fertile plants that were assumed to have an $S5^n$ allele were selected to determine the order and relationship between markers and the *S5* locus according to recombination events. There was only one recombination event between RM253 and the *S5* locus on one side. A previous study had identified one recombination event between R2349 and the *S5* locus on the other side (Liu et al. 1997a). Therefore, according to recombination events between markers and the $S5^n$ allele, the *S5* locus was bracketed in a 2.2 cM region by RM253 and R2349 (Fig. 1).

Construction of a BAC contig encompassing the *S5* locus

We started chromosome walking from both sides of the *S5* locus with the two closest flanking markers, R2349 and RM253, by screening four BAC libraries. By hybridizing the high-density BAC filters with RFLP marker R2349, a positive BAC clone 23D12 was identified from the TQ BAC library. To determine the right direction of chromosome walking, BAC ends of 23D12

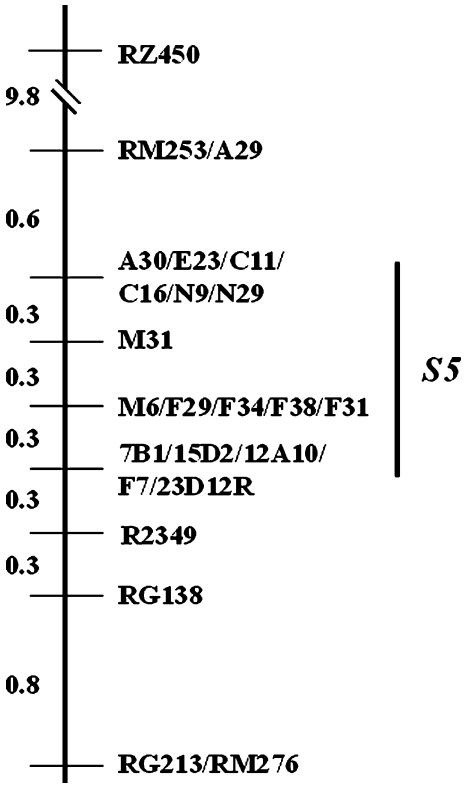

Fig. 1 *S5*-linked markers on the molecular linkage map of chromosome 6 based on 356 individuals derived from the three-way cross 02428/Nanjing11//Balilla. The location of the *S5* locus was determined with highly fertile and sterile plants from this population

were amplified using TAIL-PCR (Liu and Whittier 1995). One BAC end, 23D12F, was a multiple copy sequence, according to its band pattern on a Southern hybridization filter. The other end, 23D12R, was a single-copy sequence and showed polymorphism between 02428 and Nanjing 11. 23D12R was used to survey the 43 recombinant plants and had one recombination event with R2349. Therefore, 23D12R was located between R2349 and RM253 in the direction toward the *S5* locus, the right direction for further chromosome walking (Fig. 2).

The pooled MH BAC library was screened with RM253, the closest marker on the other side of the *S5* locus. A positive clone, 2A20, was identified. To ensure the correct direction of chromosome walking, subclones of 2A20 were isolated and screened for polymorphisms between parents 02428 and Nanjing 11. Two subclones, A29 and A30, were identified to be polymorphic. In the 43 recombinant plants, A29 cosegregated with RM253. A30 had two recombination events with RM253 and was closer to the *S5* locus.

The 101 highly fertile plants were also surveyed with two newly developed markers, 23D12R and A30. On the R2349 side, there was one recombinant event between R2349 and 23D12R. 23D12R cosegregated with the $S5^n$ gene in these highly fertile plants and was closer to the *S5* locus. On the RM253 side, A30 cosegregated with $S5^n$. However, in highly sterile plants, A30 had one recombination event with the *S5* locus on one side, while 23D12R had two recombination events with the *S5* locus on the other side. By using recombination events occurring in both highly fertile and sterile plants, the *S5* locus was bracketed between A30 and 23D12R with a distance of 1.4 cM after the first round chromosome walking and mapping of BAC-ends and subclones.

We started the next round of chromosome walking by screening the BAC libraries with the newly developed and mapped markers 23D12R and A30. 23D12R screened out four positive BAC clones, 15K15, 43L1, 48J3, and 56A20, from the MH BAC library. A30 screened out one positive clone, 3E9, from the MH library. Whole BAC clones digested with *Hind*III or subclones generated from positive BAC clones in the last step were used as probes to screen the BAC libraries for further walking. After six more steps of chromosome walking, a BAC contig covering the *S5* region was constructed. All BAC clones and their relationships are depicted in Fig. 2. Six BAC clones (15K15, 12F19, 26B7, 45M8, 26N9, and 3E9) from the MH BAC library represent a minimal tilling-path covering a genomic region of approximate 540-kb in length (Fig. 2). The ratio of the physical to genetic distance in the *S5* region is about 250 kb/cM, which is consistent with previous results observed in the rice genome (Wu and Tanksley 1993).

In the process of chromosome walking, 12 subclones were isolated from BAC clones in the contig spanning the *S5* region (E23 from 3E9; C11 and C16 from 11C17; M6 and M31 from 45M8; F7, F31, 12A10, 15D2, and 7B1 from 12F9; F29 and F38 from 64F18) and were used to construct a high-density genetic map of the *S5* region using the 43 recombinant plants (Fig. 1). However, all these markers cosegregated with $S5^n$ in the 101 highly fertile plants.

High-resolution mapping of the *S5* locus

To reduce the task of investigating embryo-sac fertility of all 8,000 F_1 plants derived from the newly developed three-way cross S5N/S5I//S5J, it was necessary to identify recombinant plants in the *S5* region by genotyping these F_1 plants with two flanking SSR markers, RM253 and RM276. From this screen, 82 plants carrying recombination events in the RM253–RM276 region were identified. These plants were further analyzed with two closer markers, C11 on the RM253 side and R2349 on the RM276 side, to reduce the number of recombinant plants. Twenty-six recombinant plants were identified in the C11–R2349 region.

Embryo-sac fertility of the 26 plants was carefully examined and ranged from 49.5 to 100% (Table 1). Based on the results obtained by Song et al. (2005), the average embryo-sac fertility of $S5^nS5^j$ plants is 87.4%, while the average embryo-sac fertility of $S5^iS5^j$ plants is 57.8%. To avoid false scoring of the fertility, plants with an embryo-sac fertility higher than 92.5% were scored as

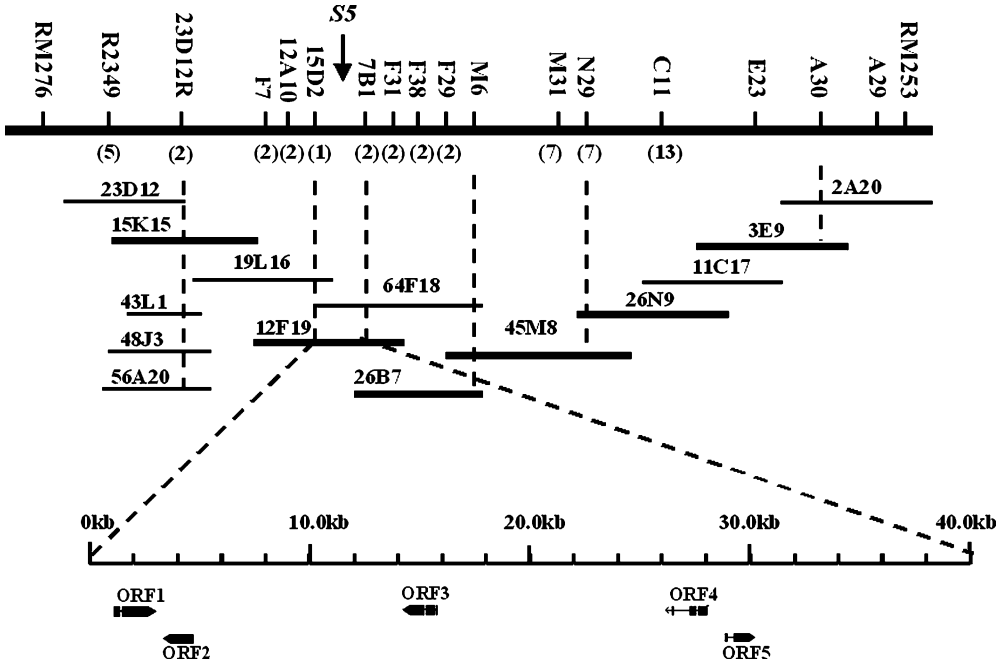

Fig. 2 High-resolution genetic and physical maps of the *S5* locus. The genetic map of *S5* was based on recombination events between markers and the *S5* locus using recombinants from 8,000 F_1 plants derived from the three-way cross S5N/S5I//S5J. The *short horizontal lines* represent BAC clones. The *vertical dashed lines* indicate hybridization relationships between markers and BAC clones. The *thick lines* represent BAC clones forming the minimal tilling-path covering the *S5* region. The *horizontal arrows* represent predicted ORFs using FGENSH

fertile, while ones with an embryo-sac fertility lower than 60% were scored as sterile. Fifteen plants were scored as fertile (with $S5^n$ allele), and three plants were scored as sterile (without $S5^n$ allele; Table 1). Another eight plants with fertility between 60 and 92.5% were not included in further analyses. The fertile and sterile plants were used to map the 12 markers (23D12R, F7, 15D2, 7B1, 12A10, F31, F38, F29, M6, M31, N29, and C11) relative to each other and to $S5^n$. In this analysis, the *S5* locus was mapped between two RFLP markers, 15D2 and 7B1, having two and one recombination events with 15D2 and 7B1, respectively, which corresponded to a genetic region of 0.04 cM (Table 1, Fig. 2). These two markers were subclones from a single BAC clone, 12F9, which was shotgun sequenced and assembled. By comparing the sequences of 15D2 and 7B1 with the sequence of BAC 12F9, a 40-kb DNA fragment in the BAC was identified as containing the wide compatibility gene.

Putative genes in the 40-kb region

Gene prediction analysis of the 40-kb DNA fragment using FGENSH (http://www.softberry.com) identified five putative open reading frames (ORFs; Fig. 2). Both

Table 1 Molecular marker genotypes of eighteen individuals showing recombination events in the *S5* region

Fertility (%)	Genotype	RM253	C11	N29	M31	F29	F38	F31	7B1	15D2	12A10	F7	23D12R	R2349	RM276
58.3	S	1[a]	1	1	1	1	1	1	1	0[b]	0	0	0	0	0
98.1	F	1	1	1	1	1	1	1	1	0	0	0	0	0	0
98.4	F	1	1	1	1	1	1	1	1	0	0	0	0	0	0
98.3	F	1	1	1	1	0	0	0	0	0	0	0	0	0	0
98.9	F	1	1	1	1	0	0	0	0	0	0	0	0	0	0
100	F	1	1	1	1	0	0	0	0	0	0	0	0	0	0
100	F	1	1	1	1	0	0	0	0	0	0	0	0	0	0
92.5	F	1	1	0	0	0	0	0	0	0	0	0	0	0	0
96.9	F	1	1	0	0	0	0	0	0	0	0	0	0	0	0
96.9	F	1	1	0	0	0	0	0	0	0	0	0	0	0	0
97	F	1	1	0	0	0	0	0	0	0	0	0	0	0	0
99	F	1	1	0	0	0	0	0	0	0	0	0	0	0	0
94.8	F	0	0	0	0	0	0	0	0	0	0	0	0	1	1
95.5	F	0	0	0	0	0	0	0	0	0	0	0	0	1	1
96.7	F	0	0	0	0	0	0	0	0	0	0	0	0	1	1
100	F	0	0	0	0	0	0	0	0	0	1	1	1	1	1
58.7	S	0	0	0	0	1	1	1	1	1	1	1	1	1	1
49.5	S	0	0	1	1	1	1	1	1	1	1	1	1	1	1

[a]Genotype 1 of each locus was composed of an allele from Nanjing 11 and an allele from Balilla[b]Genotype 0 of each locus was composed of an allele from 02428 and an allele from Balilla

ORF1 and ORF2 encode xyloglucan fucosyltransferases, enzymes involved in biosynthesis of plant cell walls. ORF3 is similar to dnak-type molecular chaperone BiP. ORF4 encodes a hypothetical protein and did not have homology with any known genes. ORF5 was homologous to a putative eukaryotic aspartyl protease. Except for ORF5, the other ORFs have corresponding full-length cDNAs or ESTs in GenBank. Identification of a candidate gene for the wide compatibility gene by transformation with these ORFs is being investigated.

Discussion

In this study, we constructed a BAC contig covering the *S5* region using a chromosome walking strategy. We delimited the *S5* locus to a DNA fragment of 40-kb in length using recombinants from a population of 8,000 F_1 plants. This result should be very useful for cloning the wide compatibility gene. Furthermore, the close linkage of the *S5* locus with many flanking molecular markers should also be very useful for transferring the $S5^n$ allele to different varieties in rice breeding programs.

In this study, several improvements have been made to increase the accuracy of the wide compatibility gene mapping. To reduce the interference of the genetic background, we developed NILs of the *S5* alleles using recurrent backcrossing in combination with marker-assisted transfer of the target regions and removal of the minor loci, which have significant affect on hybrid fertility (Liu et al. 1997a), and made crosses using these NILs to generate a large segregating population. To avoid false scoring of fertility by the interference of loci controlling pollen fertility, embryo-sac fertility was selected as the criterion for the high-resolution genetic mapping of the *S5* locus based on the results of Song et al. (2005).

Embryo-sac fertility evaluation is a time-consuming and laborious task. It is difficult to avoid false scoring of the fertility in a population of 8,000 plants, as used in this study. To avoid false scoring, we first screened 26 recombinant plants from the 8,000 F_1 plants using flanking markers, then carefully evaluated the embryo-sac fertility of those recombinant plants to ensure the phenotyping accuracy of the fertility trait. Each recombinant plant was scored as fertile or sterile using a very strict standard. Eight plants with fertility between 60 and 92.5% was excluded from the high-resolution mapping analysis of the *S5* locus to avoid misclassification of fertility.

Normal embryo sacs show visible antipodal cells and polar nuclei by the whole-stain clearing method, while abortive embryo sacs show degenerated cells (Song et al. 2005). The degeneration of female gametes starts at or after the first cell division during the process of embryo-sac formation and development (Liu et al. 2004). These observations suggested that mechanism or biochemical pathway exists that could degenerate the normal embryo-sac structure. In the five ORFs in the 40-kb DNA fragment, both ORF1 and ORF2 encode xyloglucan fucosyltransferases, which are involved in plant cell wall biosynthesis. ORF3 is similar to dnak-type molecular chaperone BiP, a master regulator of endoplasmic reticulum (ER) function and responsible for maintaining the permeability barrier of the ER during protein translocation, directing protein folding and assembly, and targeting misfolded proteins for retrograde translocation so they can be degraded by the proteasome (Hammond and Helenius 1995). ORF4 encodes a hypothetical protein. ORF5 encodes a putative eukaryotic aspartyl protease. Aspartic proteinases (APs; EC 3.4.23) constitute one of the four superfamilies of proteolytic enzymes that are believed to participate in various processes during seed development and germination and to play a defensive role against invading pathogens in leaves (Sarkinnen et al. 1992; Ramalho-Santos et al. 1998). None of these five genes could be excluded as candidates of the wide compatibility gene because, based on their functions, they could be involved in the degeneration of embryo-sac structure. We are currently attempting to identify the wide compatibility gene via transformation.

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