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ORIGINAL PAPER

G. W. Wang · Y. Q. He · C. G. Xu · Qifa Zhang

Fine mapping of *f5*-Du, a gene conferring wide-compatibility for pollen fertility in inter-subspecific hybrids of rice (*Oryza sativa* L.)

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Abstract Wide-compatibility varieties (WCVs), comprising a special class of rice germplasm, are able to produce fertile hybrids when crossed with both indica and japonica varieties. Dular, a landrace variety from India, has both a wide spectrum and high level of wide-compatibility when crossed with a range of indica and japonica varieties. In previous studies, an allele at the *f5* locus from Dular (*f5*-Du) was identified as a neutral allele conferring wide-compatibility with a large effect on both pollen and spikelet fertility. Using a population of 1993 hybrid plants derived from a cross between ZS(*f5*-Du/*f5*-ZS) (F_1 of near isogenic line of Zhenshan 97 containing *f5*-Du) and Balilla (a japonica tester), *f5*-Du was genetically mapped to an interval of about 1.6 cM, with 0.8 cM from both SSR markers WFPM3 and WFPR1. Combined with bioinformatic analysis, a contig map was constructed for the *f5* region, consisting of five bacterial artificial chromosome (BAC) or P1 artificial chromosome (PAC) clones and spanning approximately 437 kb in length. By assaying the recombinant events in the region with markers developed using the sequence information, the *f5* locus was further narrowed down to a 70 kb fragment containing nine predicted genes. The result will be very useful for cloning this gene and marker-assisted transferring of the neutral allele in rice breeding programs.

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G. W. Wang · Y. Q. He · C. G. Xu · Q. Zhang (✉)
National Key Laboratory of Crop Genetic Improvement
and National Center of Plant gene Research (Wuhan),
Huazhong Agricultural University, 430070 Wuhan, China
E-mail: qifazh@mail.hzau.edu.cn
Tel.: +86-27-87282429
Fax: +86-27-87287092

Introduction

The Asian cultivated rice (*Oryza sativa* L.) consists of two subspecies, indica and japonica. It has been shown that the fertility of indica–japonica hybrids varies widely from fully fertile to almost completely sterile, with the majority showing significantly reduced fertility (Kato et al. 1928; Oka 1988; Liu et al. 1996; Zhang et al. 1997). The genetic basis of the inter-subspecific hybrid sterility has been extensively investigated in recent decades. Several hypotheses have been proposed to explain the genetic mechanisms of such inter-subspecific hybrid sterility, including allelic interaction at a single locus (Kitamura 1962; Ikehashi and Araki 1986), duplicate gametophytic lethal model (Oka 1974) or epistatic interaction between loci (Wu et al. 1995; Li et al. 1997), and recombination within putative differentiated “supergenes” (Li et al. 1997).

Spikelet fertility of the hybrid is directly a function of male gamete fertility, female gamete fertility, and affinity between the uniting male and female gametes. A large number of loci have been identified as responsible for female gamete abortion (Ikehashi and Araki 1986; Yanagihara et al. 1992; Wan et al. 1993, 1996; Liu et al. 2001b; Song et al. 2005) and pollen sterility (Zhang and Lu 1989, 1993; Zhang et al. 1994; Zhuang et al. 1999, 2002a, b; Li et al. 2002; Song et al. 2005). In addition, segregation distortion was observed at a number of loci in inter-subspecific hybrids (Lin et al. 1992, 1993; Kinoshita 1995; Lu et al. 2000).

Wide-compatibility varieties (WCVs) are a special class of rice germplasm able to produce fertile hybrids when crossed with both indica and japonica varieties (Ikehashi and Araki 1984). The *S5* locus for wide compatibility, as first identified by Ikehashi and Araki (1986) with morphological markers, has been widely confirmed using molecular markers (Liu et al. 1992; Zheng et al. 1992; Yanagihara et al. 1995; Liu et al. 1997). Recently, Song et al. (2005) characterized *S5* as a major locus for embryo-sac fertility, and Qiu et al. (2005), using near isogenic lines (NILs) and a large segregating population, delimited the wide compatibility gene S_5^n to a 40-kb DNA fragment containing five open reading frames.

Dular, a landrace variety from India, conferring both a wide spectrum and high level of wide-compatibility when crossed to a range of indica and japonica varieties (Pan et al. 1990; Liu et al. 1996; Zhang et al. 1997), is considered to be one of the most useful WCV germplasms in rice breeding programs. Using a three-way cross ('Balilla'/'Dular'//'Nanjing 11'), Wang et al. (1998) resolved five QTLs as conferring significant effects on hybrid fertility, with the one on chromosome 5 (*f5*) showing the largest effect, followed by *f6* which was likely to be the same locus as *S5* (Wang et al. 2005). Using NILs, Wang et al. (2005) determined that the *f5* allele from Dular (designated as *f5*-Du) was a neutral allele, compatible with both indica and japonica varieties, and exerted a large effect on spikelet fertility of the hybrid by specifically increasing pollen fertility. It thus proved to be a neutral allele for pollen fertility.

The objectives of this study were: (1) to genetically fine-map the *f5* locus which will also provide tightly linked simple sequence repeat (SSR) markers for transferring the neutral allele in rice breeding programs, and (2) to physically localize the allele to a DNA fragment of known length.

Materials and methods

Plant materials and field planting

According to the study by Wang et al. (2005), Dular, an indica WCV from India, carried a neutral allele (*f5*-Du) conferring wide compatibility for pollen fertility at the *f5* locus, and Zhenshan 97, a typical indica cultivar, contained an indica allele (*f5*-ZS). Balilla, a typical japonica variety introduced in Italy and designated as a tester for screening WCVs in Chinese rice breeding programs, was used as a tester for japonica compatibility.

In this study, a plant with the genotype ZS(*f5*-Du/*f5*-ZS), developed by introgressing the *f5*-Du allele from Dular into Zhenshan 97 with successive backcrossing and marker-assisted selection (Wang et al. 2005), was test-crossed with Balilla. The progeny were planted in the rice growing seasons of 2003 and 2004 at the experimental farm of Huazhong Agricultural University, Wuhan, China. The planting time (May 19 in 2003, and May 16 in 2004) placed the temperature sensitive stage for fertility in late July and early August, during which the average daily temperature was favourable for the fertility of the inter-subspecific hybrids (Li et al. 1996; Lu et al. 2002). The planting density was 16.5 cm between plants in a row, and 26.4 cm between rows, with 12 plants per row. Field management followed essentially the normal agricultural practices. Irrigation of the field was maintained to avoid drought stress.

Pollen fertility examination

One or two panicles per plant were sampled after heading but before flowering, and fixed in 70% (v/v) ethanol. Six florets per panicle were taken from the upper, middle and lower portions of the panicle. One anther per floret was collected, and the six anthers from the same panicle 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) iodine potassium. More than 500 pollen grains from each individual were observed with a microscope for estimating the percentage of fertile stainable pollen.

Molecular markers development and assay

SSR markers around the *f5* locus region were identified from the Gramene database (http://www.gramene.org/). The SSR primers of the RM-series were designed according to Temnykh et al. (2000, 2001) while those of the MRG-series were designed according to the rice genome sequences of Monsanto Company that were made available by McCouch et al. (2002). A number of new SSR markers were also identified based on the publicly available rice genome sequences (http://www.ncbi.nlm.nih.gov/), using the SSR identification tool (SSRIT; http://www.gramene.org/microsat; Temnykh et al. 2001). Primers were designed using the PRIMER 3 program. SSR analysis was carried out essentially according to the procedures described by Wu and Tanksley (1993).

A *Hin*dIII-digested bacterial artificial chromosome (BAC) library of the japonica cultivar Nipponbare from Clemson University (Clemson, SC, USA) was used. Plasmids of BAC clones were extracted as previously described (Liu et al. 2001a). Probes from BAC clones were amplified by PCR following a profile: 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, with a final extension of 72°C for 5 min. The PCR products were then cloned into pGEM-T vector (Promega, USA) according to the manufacturer's specification. The experimental procedures for RFLP assay, including DNA isolation, digestion, electrophoresis, southern blotting and hybridization were essentially as described previously (Liu et al. 1997).

Data processing and statistical analysis

To determine the linkage relationship between the *f5* locus and molecular markers, data were analyzed with Mapmaker/Exp 3.0 program (Lincoln et al. 1992) at a LOD threshold of 3.0 to construct a local genetic map for the *f5* genomic region.

Results

Mapping the *f5* locus to a 1.6-cM interval

Pollen fertility of the 255 hybrid plants from the ZS(*f5*-Du/*f5*-ZS)/Balilla test-cross planted in 2003 showed a bimodal distribution with an apparent valley at 55–65% (Fig. 1). While the individuals with pollen fertility $>65\%$ were classified as fertile, and those with pollen fertility $<55\%$ as partially sterile, the numbers of fertile and partially sterile individuals fit the expected 1:1 ratio ($\chi^2=0.141$, $P>0.50$). In 2004, 1,738 hybrid plants were obtained from this cross, of which 150 individuals were sampled at random for pollen fertility examination. The distribution was also bimodal, with an apparent valley at 50–60% (data not shown).

The 255 plants in the ZS(*f5*-Du/*f5*-ZS)/Balilla test-cross population of 2003 was assayed using a co-segregated marker WFPM5 (see below). It was shown that the genotype having the *f5*-Du allele had 78.64 ± 8.01 (%) darkly stained pollen, and the genotype having the *f5*-ZS allele had 25.46 ± 15.19 (%) darkly stained pollen. The difference was highly significant as determined by *t*-test.

The *f5* locus was previously mapped in the interval between two RFLP markers, R830 and R3166, on the short arm of chromosome 5 (Wang et al. 1998). A number of SSR markers located around this genomic region (Temnykh et al. 2000, 2001; McCouch et al. 2002; http://www.gramene.org/) were selected to screen polymorphism between Dular and Zhenshan 97. Five SSR markers (MRG4361, MRG0200, MRG5110, MRG0259, and RM413) detected polymorphisms between the two parents. Using the 255 individuals obtained in 2003 as the mapping population, the *f5* locus was mapped between markers MRG0200 and MRG5110, with 4.0 cM from MRG0200 on one side and 0.8 cM from MRG5110 on the other side (Fig. 2).

To further map the *f5* locus, BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) was employed to search for sequences matching R830 and MRG5110 in the rice nucleotide database. R830 identified matching sequences in AC093088 (Monsanto BAC clone OJ1001_G01), and MRG5110 was anchored to AC129720 (RGP PAC clone P0683F12). Using the sequences of the BAC/PAC clones, another three overlapping BAC/PAC clones (AC093089, Monsanto BAC clone OJ1729_E02; AC079021, RGP PAC clone P0008AO7; AC134931, CUGI BAC clone OS-JNBb0079L11) covering the *f5* locus region were identified. Sequence alignment analyses of the *Oryza sativa* cv. Nipponbare DNA sequence using the Sequencher 3.0 program (Gene Code) and Pairwise BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) identified a contig spanning approximately 437 kb in length that encompassed the *f5* locus (Fig. 3). Thirty-three SSRs were identified in the contig using the SSR identification tool (SSRIT; http://www.gramene.org/microsat; Temnykh et al. 2001). Five of the 33 primer pairs (WFPL6, W4374, WFPM3, WFPM5, and WFPR1) detected polymorphisms between the two parents (Table 1). These primers were subsequently used to genotype the 255 hybrid plants obtained in 2003, which mapped the *f5* locus to the interval between WFPM3 and WFPR1, at a distance of 0.8 cM from both markers (Fig. 2). In addition, marker WFPM5 co-segregated with the *f5* locus.

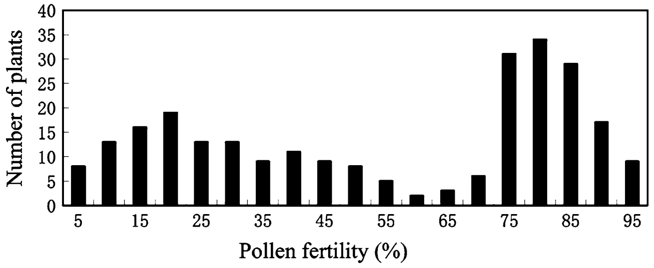

Fig. 1 Distribution of pollen fertility of the 255 hybrid plants obtained from the cross ZS(*f5*-Du/*f5*-ZS)×Balilla in 2003

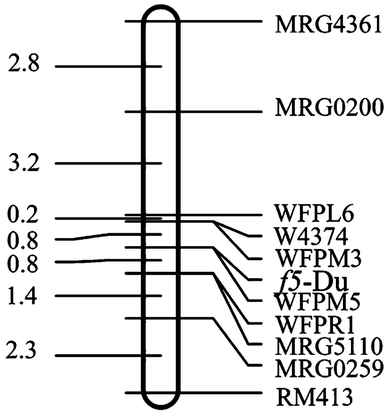

Fig. 2 Location of the *f5* locus on the molecular linkage map of chromosome 5

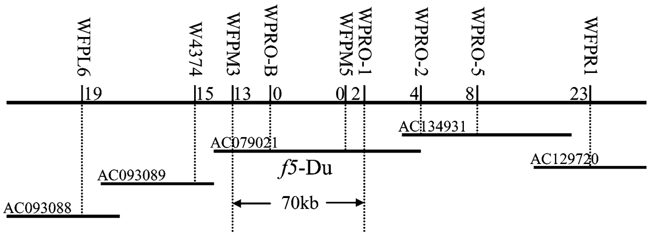

Fig. 3 A contig map covering the *f5*-Du allele region. WFPL6, W4374, WFPM3, WFPM5 and WFPR1 are SSR markers, and WPRO-B, WPRO-1, WPRO-2 and WPRO-5 are RFLP markers. The *numbers* between molecular markers indicate the numbers of recombination events detected between the *f5* locus and the respective markers. The *long horizontal line* indicates the genomic region encompassing the *f5* locus. The *short horizontal lines* represent BAC/PAC clones of cv. Nipponbare with the accession numbers indicated. The *dashed lines* indicate the relative position of the corresponding marker on BAC/PAC clones

Table 1 Primer sequences designed and used for fine mapping of the *f5* locus

Marker	Primer sequence (5′–3′)	Product size in Nipponbare (bp)
WFPL6	F: GAGGAGCACCCCGATGTT R: AGGAGAACCTCACCCTCCTC	152
W4374	F: CCATTGGAGATTTTGATTTGG R: TCAGCAATCATCATAACATGGTC	173
WFPM3	F: TTGTGTCGGTGAGGTGTGTG R: GTGAAACTTGCCTGTCCATC	126
WFPM5	F: ACCTTCTTCTCCAATCCCCAG R: TAAGTGCTCGCGATTCACAC	151
WFPR1	F: GACATGGCATGCTGAAACTG R: CTTCCAAGTCTCCGCAGAAG	142
WPRO-B	F: TCCACTCCATCTTGCAGTTG R: CAACTACCTGTCCGTGGTGA	2,001
WPRO-1	F: GATGGCAATGGTAGGGAATG R: GTTGGCAGGCAGGTTAGGTA	1,044
WPRO-2	F: AGGAGAAGCAGCTGGATGAA R: GCAGCTATTTTGCTCCTTGC	1,147
WPRO-5	F: TTGGGTGTTCCCACCACTAT R: CGACCTCCAATGAGAAGGAA	1,202

Resolving the *f5* locus to a 70-kb fragment

To further reduce the genomic region containing the *f5* locus, a total of 1,993 hybrid plants (1,738 individuals obtained in 2004 and 255 in 2003) were genotyped using SSR markers WFPR1 and WFPL6. Forty-two recombinants with extreme phenotypes (pollen fertility lower than 40%, or higher than 70%) were selected for fine mapping.

To obtain more markers in the WFPM3–WFPR1 interval, a series of primers amplifying unique sequences were designed to obtain fragments between 800–2,000 bp in length using plasmids of the BAC clones (a0085B10, a0031N15, a0067D21, a0070I22, a0012J14, and a0031L19) as the templates, according to information given by http://www.genome.arizona.edu. The PCR products were cloned and used as RFLP probes. Four markers (WPRO-B, WPRO-1, WPRO-2, WPRO-5), showing clear, single copy polymorphic bands between Dular and Zhenshan 97 (Table 1), were integrated in the interval. Thus, together with three SSR markers identified in this region, seven new markers were available for assaying the 42 recombinants. The analysis resolved 13 recombinant events between WFPM3 and *f5*, and two recombinant events between WPRO-1 and *f5*. In addition, WPRO-B and WFPM5 were found to co-segregate with the *f5* locus (Fig. 3). Therefore, the genomic region containing the *f5* locus was narrowed down to the fragment bounded by WFRM3 and WPRO-1 (Fig. 3 and Table 2), approximately 70-kb in length.

Putative genes in the 70-kb region

On the basis of available sequence annotation (http://www.ncbi.nlm.nih.gov/entrez/) that utilizes both the database search and combinations of gene prediction programs, including Fgenesh and GENSCAN, there are nine predicted genes (p0008A07.2–p000A07.10) in the 70-kb region. Of these genes, five had unknown functions, and the functional annotations of the remaining four genes were as follows: (1) p0008A07.2, putative beta3-glucuronyltransferase, a gene of 5,544 bp consisting of 3 exons and having a transcript length of 1,116 bp; (2) p0008A07.3, putative SF16 protein, a gene of 3,126 bp consisting of 5 exons and having a transcript

Table 2 Molecular marker genotypes of some recombinant individuals

Marker	Sterile individuals							Fertile individuals					
	2305	2363	6001	4103	1080	2260	1141	5032	5024	1094	6159	2233	1524
WFPL6	D	D	D	Z	Z	Z	Z	Z	Z	D	D	D	D
W4374	Z	D	D	Z	Z	Z	Z	Z	Z	D	D	D	D
WFPM3	Z	Z	D	Z	Z	Z	Z	Z	Z	D	D	D	D
WPRO-B	Z	Z	Z	Z	Z	Z	Z	D	D	D	D	D	D
WFPM5	Z	Z	Z	Z	Z	Z	Z	D	D	D	D	D	D
WPRO-1	Z	Z	Z	D	Z	Z	Z	D	D	Z	D	D	D
WPRO-2	Z	Z	Z	D	Z	Z	Z	D	D	Z	Z	D	D
WPRO-5	Z	Z	Z	D	D	D	Z	D	D	Z	Z	Z	D
WFPR1	Z	Z	Z	D	D	D	D	D	D	Z	Z	Z	Z

Genotype D of each locus was composed of an allele from Dular and an allele from BalillaGenotype Z of each locus was composed of an allele from Zhenshan 97 and an allele from Balilla length of 1,425 bp; (3) p0008A07.8, putative hydroxyproline-rich glycoprotein, a gene of 3,701 bp consisting of 7 exons and having a transcript length of 3,165 bp; and (4) p0008A07.9, putative polyprotein, a gene of 3,332 bp consisting 2 exons and having a transcript length of 3,192 bp. Six genes (p0008A07.2–p0008A07.5, p0008A07.8, and p0008A07.9) had homology with rice full-length cDNAs or ESTs. Identification of the candidate gene of *f5* by transformation is still in progress.

Discussion

The main accomplishment of this study is the genetic fine mapping and physical delineation of the *f5*-Du allele to a DNA fragment of 70-kb in length. This result should be very useful for cloning the *f5*-Du allele, which is now in progress. The close linkage of the allele with flanking SSR markers should also have utility for transferring the neutral allele in rice breeding programs.

The *f5* locus was previously mapped in the 3.4-cM interval between R830 and R3166 near the distal end on the short arm of chromosome 5 (Wang et al. 1998). Recently, Song et al. (2005) resolved one major QTL *pf5* specifying pollen fertility, which coincided with the *f5* locus. Li et al. (2002) and Zhuang et al. (2002a) also mapped a locus *S-b* for pollen fertility on chromosome 5 located in the same vicinity, indicating *f5* and *S-b* are likely the same locus.

Genetic analysis of the reproductive barriers between indica and japonica may shed light on the process of speciation and differentiation, and provide important information for rice improvement. Harushima et al. (2002) compared diverse reproductive barriers among three different indica–japonica crosses, and found that the number of reproductive barriers in the three crosses were similar, whereas most of the barriers were mapped at different loci. Considering the high genetic similarity within indica and japonica varieties, the large differences in the reproductive barriers among the crosses seemed unexpected. Harushima et al. (2002) further suggested that the reproductive barriers of indica–japonica hybrids evolved more rapidly than other genetic elements. The result of the present study showed that the ratio of genetic to physical distance in the *f5*-Du allele region is quite un-uniform. For example, the physical distance between WFPM3 and WFPRO-B was less than 23 kb, in which 13 recombinants among the 1993 individuals were detected, indicating the existence of hotspots of crossovers in this region. Such recombination hotspots may have contributed to the rapid evolution of the reproductive barriers.

The ORFs identified by the bioinformatics analysis of sequences did not seem to provide useful information about the candidate gene. It is a big challenge to further identify the gene for hybrid sterility and wide-compatibility. Currently, final proof of gene function in map-based cloning approach relies on complementation test by transformation. However, a prerequisite of such a test is a clear dominant–recessive relation between the two alternative alleles, in which the recessive allele is usually a mutant of the dominant allele, and transformation of the dominant allele to an individual carrying the recessive allele would recover the function of the wild type gene. In the case of the inter-subspecific hybrid sterility, however, there is no such dominant–recessive relation between the indica and japonica alleles. Instead, strong evidence clearly established that interactions between the indica and japonica alleles at identified loci cause hybrid sterility, as observed in this and a number of previous studies (Ikehashi and Araki 1986; Liu et al. 1997; Zhuang et al. 1999, 2002a, b; Li et al. 2002). By the current technique, it is almost impossible to obtain a transgenic plant with the transgene to be allelic to the native copy via *Agrobacterium*-mediated transformation, although gene targeting in rice has been reported (Terada et al. 2002). Moreover, the copy number of the transgene in the transgenic plants may pose another constraint to the success of functional test by genetic transformation, since the frequency of transformants with single copy transgene is usually low. Unequal numbers of copies of the indica and japonica alleles in the transgenic plants may mask the interaction effects. Thus, a new approach needs to be developed for functional identification of the gene for indica–japonica hybrid sterility, as ones reported in this and other studies.

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