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ORIGINAL PAPER

Two novel loci for pollen sterility in hybrids between the weedy strain Ludao and the *Japonica* variety Akihikari of rice (*Oryza sativa* L.)

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Abstract Partial pollen sterility has been observed in hybrid progeny derived from a *japonica* cultivar, Akihikari and a weedy strain, Ludao, which naturally grows in Jiangsu province of east China. Cytological and histological analyses revealed that pollen abortion occurred largely at the bicellular pollen stage, primarily due to the gradual disaggregation of generative and vegetative cells. A genome-wide analysis was further carried out in a backcross population of Akihikari //Ludao/Akihikari using a total of 118 simple sequence repeat (SSR) markers and an expressed sequence tag (EST) marker distributed on the entire rice linkage map. Two loci controlling hybrid pollen sterility, designated as *S33*(t) and *S34*(t), were located on chromosomes 3 and 11, respectively. Both loci were putatively different from all the previously reported gametophyte genes and hybrid pollen sterility loci. Interaction between the Ludao and Akihikari alleles at each of the two loci resulted in reduction of fertility in the pollens carring the Ludao alleles. To map the precise location of the major locus, *S33*(t), we selected 165 plants of the backcross population with pollen fertility higher than 80.0%, and assayed the recombinant events surrounding the *S33*(t) locus using newly developed SSR markers. The *S33*(t) was delimited to an 86 kb region between SSR markers RM15621 and RM15627. Sequence analysis of this region indicated that there were ten open reading frames. These results will be valuable for cloning this gene and marker-assisted transferring of the corresponding neutral allele in rice breeding programs. Furthermore, the origin of the weedy strain Ludao is discussed.

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Introduction

Crops are often accompanied by their related weedy types, in addition to their wild progenitors. Weedy rice, distributed in rice-growing area, seems to possess a wide variation in characteristics, and differentiate into the *indica* and *japonica* types to some extent (Suh et al. 1997; Tang and Morishima 1988). It is not intentionally cultivated, but grows naturally in and around rice fields. Weedy rice strains reproduce themselves by dispersed seeds, or by being sown together with cultivar seeds, and are highly adapted to disturbed habitats. Studies on the origin of weedy rice and their genetic nature should help in understanding the evolution of rice and rice genetic improvements (Tang and Morishima 1988).

Ludao, a weedy rice strain in lower Yangtze valley, grows naturally in the field of Lianyungang region (34°33′-34°46′N, 119°13′-119°30′E), east China's Jiangsu province (Jiang et al. 1985). It has unique features such as long awn, black hull and red pericarp in the seeds, and high degree of seed shattering and seed dormancy (Tang and Morishima 1988), as well as high salinity and drought tolerance (Jiang et al. 1985). Due

to the specific geographic distribution and the potential impact on the understanding of the origin and differentiation of Chinese rice cultivars, Ludao has attracted much attention since it was discovered in 1960s. However, so far no consensus on its taxonomic status has been reached.

Earlier studies have revealed that the compatibility between Ludao and *japonica* varieties is higher than that between Ludao and *indica* varieties, and low fertility has been observed in hybrids between Ludao and *indica* varieties (Jiang et al. 1985; Chen and Zhu 1990). Two loci, *S10* and *S30*(t) induce the hybrid sterility independently between Ludao and an *indica* cultivar, IR36, via female gamete abortion (Zhu et al. 2005). However, so far little is known about the low pollen fertility in hybrids between Ludao and *japonica* varieties. To our knowledge, we have dissected the cytological mechanism of pollen abortion and identified two novel loci *S33*(t) and *S34*(t) causing the pollen sterility in the hybrids. The precise map location of *S33*(t), the major hybrid pollen sterility gene is further determined. Our results will help in better understanding the origin of Ludao and overcoming the sterility barriers between Ludao and cultivated varieties in order to transfer the genes controlling the desirable traits such as high salinity and drought tolerance in Ludao to cultivated varieties.

Materials and methods

Plant materials

Ludao used in this study is a weedy rice in east China's Jiangsu province. Akihikari is a typical *japonica* variety, and 02428 is a wide compatibility variety developed in Jiangsu Academy of Agriculture Sciences, China (Table 1).

Crossing between Ludao and other varieties was carried out in the winter of 2002-2003 in Hainan province. Then a backcross, Akihikari//Ludao/Akihikari, was performed in the rice-growing summer season of 2003 in Nanjing, Jiangsu Province and off-season in the winter of 2003-2004 in Hainan province. In total 215 BC_1F_1 individual plants from this cross as well as the parents and the F_1 plants were planted in the rice-growing season of 2004 at the experimental station of Nanjing Agricultural University, Jiangsu province of China.

Cytological and histological analysis

Florets of the Ludao/Akihikari hybrid were collected during the period from pistil and stamen differentiation of the young panicle development to 1 or 2 days before flowering and immediately fixed in FAA solution (containing an 18:1:1 mixture of 70% ethanol, formalin and acetic acid). Anthers were collected from fixed floret tissues, and stained with acetocarmine. The remaining tissues containing anther and ovary were dehydrated with ethanol, infiltrated with xylene and embedded in paraffin. Serial 8-μm-thick sections were cut with Leica RM2235 rotary microtome and mounted on glass slides. Histochemical staining of protein and total carbohydrates were performed according to Yeung (1984) with minor modifications. The sections were stained with the periodic acid-Schiff reaction (PAS reaction) for total carbohydrates, and counterstained with amino black 10B for proteins. The preparations were examined and photographed with a photomicroscope (Olympus BH-2).

Pollen and spikelet fertility

Spikelets were collected from parents and F_1, BC_1F_1 individuals 1 or 2 days before anthesis, and then fixed in Carnoy's fluid. Three anthers were sampled randomly from collected spikelets. Pollen grains were stained with 1% iodine potassium iodide (I_2-KI) solution, and observed under microscope. A total of 200-300 pollen grains were randomly scanned on each slide and classified as sterile or fertile according to their staining behavior (Chaudhary et al. 1981). All round and dark brown stained pollen grains were scored as

Table 1 Pollen and spikelet fertility of parents and F_1

Parental varieties and F_1	Pollen fertility (%) with I_2-KI staining	In vitro pollen germination rate (%)	Spikelet fertility (%)
Ludao	97.6 ± 0.2	96.2 ± 1.2	91.5 ± 0.3
Akihikari	94.2 ± 1.8	91.2 ± 0.8	95.2 ± 2.4
02428	94.2 ± 3.0	94.7 ± 1.5	94.2 ± 1.0
Ludao/Akihikari	49.4 ± 3.5	44.6 ± 3.2	82.5 ± 5.7
Akihikari/Ludao	51.5 ± 2.7	46.2 ± 3.8	88.1 ± 5.0
02428/Ludao	91.6 ± 2.2	88.0 ± 1.1	87.9 ± 5.0
02428/Akihikari	98.8 ± 0.7	97.9 ± 1.5	98.7 ± 0.5

fertile and irregular-shaped, yellowish or unstained pollen grains were scored as sterile. Pollen fertility was estimated as the percentage of fertile pollen grains to all the pollen grains examined.

For in vitro pollen germination tests, Brewbaker and Kwach (BK) medium (10% sucrose, 100 mg l^{-1} boric acid, 300 mg l^{-1} calcium nitrate, 200 mg l^{-1} magnesium sulphate, and 100 mg l^{-1} potassium nitrate) was used. Pollen grains were placed on a clean 24 × 50 mm cover glass and 20 µl of the liquid medium were added. The cover glass was placed on a piece of moist filter paper in a plastic dish. Then the dish was sealed tightly and incubated for 60 min at 25°C in the dark. The pollen grains were then observed under a microscope. More than 200 pollen grains were observed to determine the percentage of germination. Pollen grains in which the pollen tube had elongated longer than the diameter of the pollen grain were scored as germinated.

Spikelet fertility of parents and F_1 hybrids were estimated as the mean of seed setting rate of five plants, by counting fertile and sterile spikelets on the upper half of three main panicles for each plant.

DNA preparation and PCR analyses

The genomic DNA was extracted from fresh leaves of each plant as described by Dellapporta et al. (1983) with minor modifications. The extracted DNA samples were dissolved in TE buffer (10 mM Tris–HCl pH8.0, 0.1 mM EDTA) and tested for quality and quantity using a MBA 2000 UV/VIS Spectrometer (Perkin Elmer Co.). The eligible samples were diluted into 20 ng/µl, and stored at 4°C for further analysis.

The original sources and motifs for the 858 SSR primers and 10 EST primers are obtained from the Gramene database (http://www.gramene.org/microsat) (Temnykh et al. 2000; McCouch et al. 2002). SSR and EST analysis was performed following the procedure of Chen et al. (1997) with minor modifications. Amplification reactions were performed in 10 µl mix containing 10 mM Tris–HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 50 µM each of dNTPs, 0.2 µM each primers, 0.5 U Taq polymerase (TaKaRa, Dalian) and 20 ng of DNA template, using a PTC-200 thermal cycler (MJ Research Inc.) programmed as 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C with a final extension of 7 min at 72°C. Amplified DNA were electrophoresed on 8% polyacrylamide non-denaturing gels in 0.5 × TBE buffer, and observed by silver staining method based on Sanguinetti et al. (1994). Amplified DNA fragments showing clear polymorphism were used for analysis of the backcross population and linkage mapping.

The development of SSR markers

To develop simple sequence repeats (SSR) markers, the sequences of the delimited region on chromosome 3 of rice were downloaded from International Rice Genome Sequencing Project (IRGSP) (http://www.rgp.dna.affrc.go.jp/IRGSP/index.html). Then suitable microsatellite sequences were screened using online SSR searching software SSRIT (http://www.Gramene.org/db/searches/ssrtool) and subjected to SSR primers design using software Primer Premier 5.0.

Linkage map construction and QTL analyses

Linkage groups and the order of markers were determined using MAPMAKER/EXP 3.0 (Lander et al. 1987). The Kosambi mapping function was used to transform the recombination frequency to genetic distances (cM). QTL mapping for hybrid pollen sterility ($\arcsin\sqrt{x}$ transformed) was performed by composite interval mapping using the software package QTL CARTOGRAPHER with forward-backward regression (Basten et al. 1998). The experiment-wise LOD (log of the odds ratio) threshold significance level was determined by computing 1,000 permutations (Churchill and Doerge 1994), as implemented by the QTL CARTOGRAPHER. These permutations can account for non-normality in marker distribution and traits values. The level of significance for QTLs was determined to be $P \leq 0.05$: LOD 2.52. Analysis of variance using marker genotypes as the groups was conducted using the general linear model (GLM) procedure of SAS (SAS Institute 1989).

Results

Pollen sterility in F_1 hybrids between Ludao and Akihikari

The reciprocal F_1 hybrids between Ludao and Akihikari showed normal spikelet fertility, as reflected in minor differences between the parents and hybrids (Table 1). Meanwhile, the studies of I_2-KI staining and in vitro germination of pollen grains revealed that pollens of the two parents were fully fertile, whereas their F_1 progenies showed partial pollen sterility, and spherical abortion was observed in most of aborted pollens (Table 1; Fig. 1). The reciprocal F_1 hybrids exhibited similar pollen fertility (Table 1; Fig. 1), indicating that gene(s) in the cytoplasmic genome had no significant impact on the pollen fertility in the F_1 hybrids.

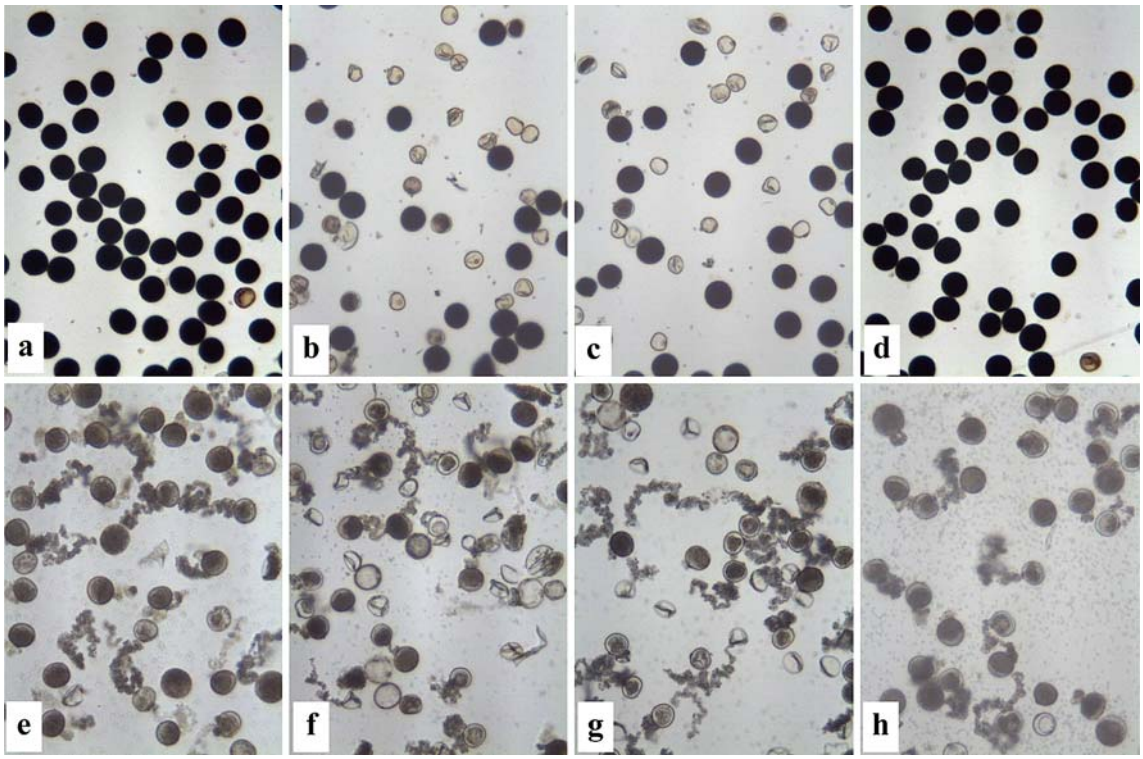


Fig. 1 Evaluation of pollen fertility by staining with I_2-KI (**a**–**d**) or observing pollen tube elongation (**e**–**h**) in an artificial medium. **a** and **e** show the pollen grains of Ludao. **b** and **f** show the pollen grains of the Ludao/Akihikari hybrid. **c** and **g** show the pollen grains of Akihikari/Ludao. **d** and **h** show the pollen grains of Akihikari

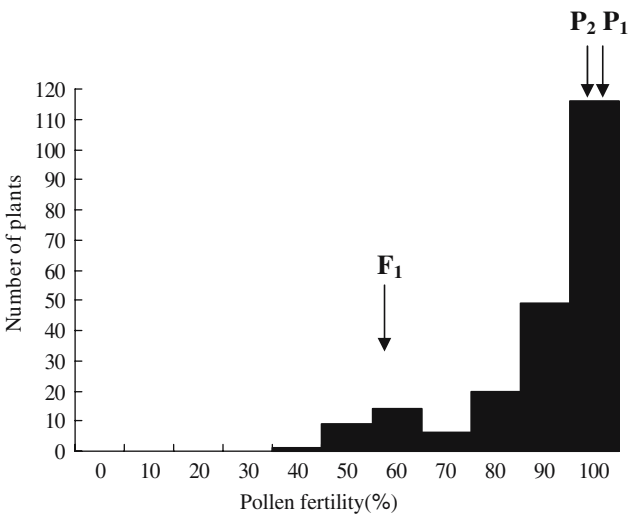


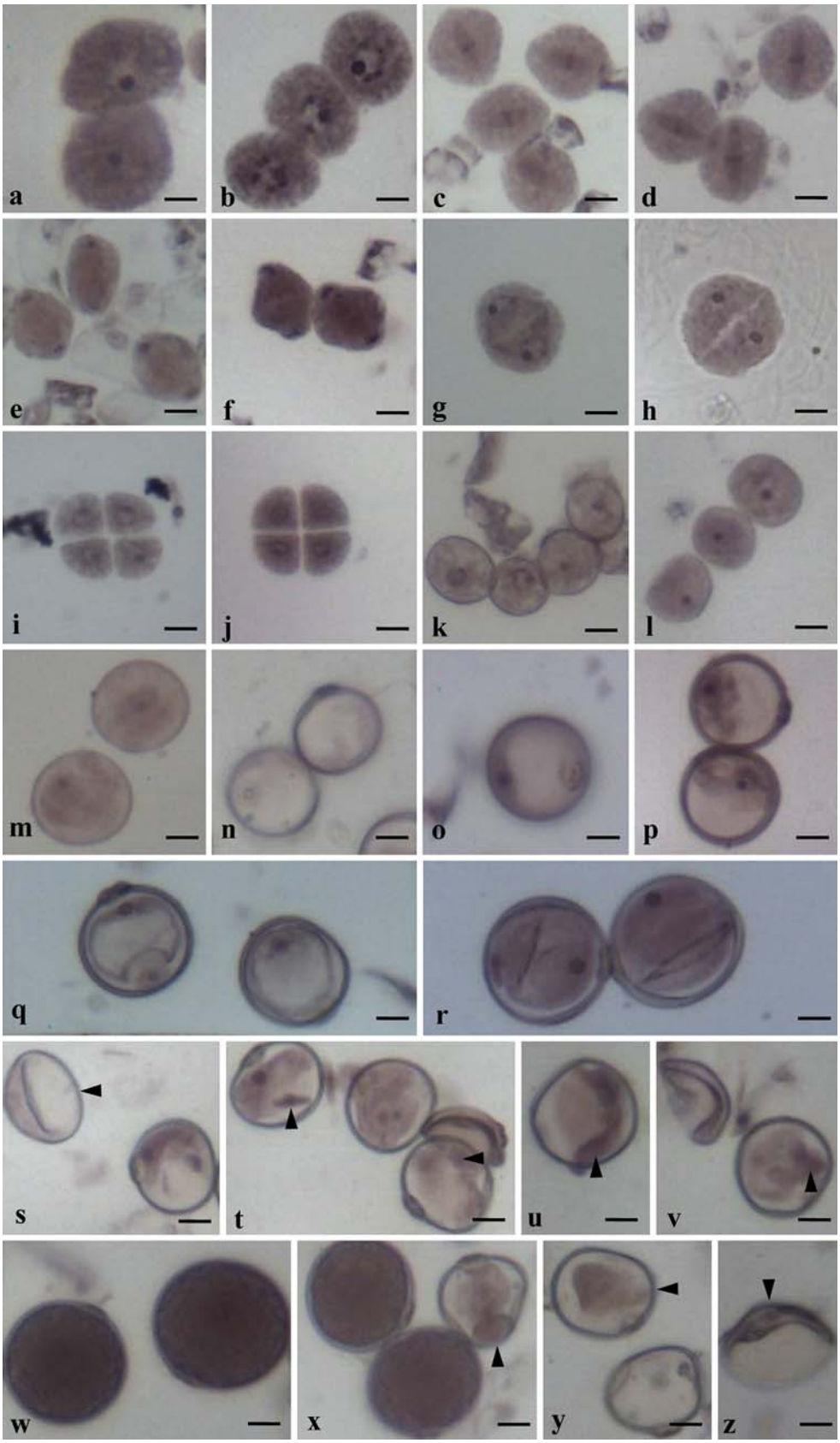
Fig. 2 Frequency distribution of pollen fertility in Akihikari// Ludao/Akihikari population. P_1, P_2 and F_1 represent Ludao, Akihikari and Ludao/ Akihikari, respectively

Pollen fertility in the Akihikari//Ludao/Akihikari population exhibited a skewed distribution, with more individuals in the high-fertility class than in the low-fertility class (Fig. 2). These results suggested that the majority of the aborted pollens in the F_1 hybrids carried the Ludao alleles.

Cytological mechanism of pollen abortion in the Ludao/Akihikari hybrid

During the process of microsporogenesis and uninucleate pollen formation, the pollen development of Ludao/Akihikari hybrid is indistinguishable from Akihikari (Figs. 3a–p, 4a–q, u). Visible differences of

Fig. 3 The process of microsporogenesis (**a**–**l**) and male gametophytes development (**m**–**z**) in Akihikari and the Ludao/Akihikari hybrid stained with acetocamine. **a** Pollen mother cells of Akihikari. **b** Pollen mother cells of the Ludao/Akihikari hybrid. **c**–**j** the process of meiosis. **c** Meiotic metaphase 1 of Akihikari. **d** Meiotic metaphase 1 of the Ludao/Akihikari hybrid. **e** Meiotic telophase 1 of Akihikari. **f** Meiotic telophase 1 of the Ludao/Akihikari hybrid. **g** Dyads of Akihikari. **h** Dyads of the Ludao/Akihikari hybrid. **i** Tetrads of Akihikari. **j** Tetrads of the Ludao/Akihikari hybrid. **k** Early microspore of Akihikari. **l** Early microspore of the Ludao/Akihikari hybrid. **m** Middle microspore of Akihikari. **n** Middle microspore of the Ludao/Akihikari hybrid. **o** Late microspore of Akihikari. **p** Late microspore of the Ludao/Akihikari hybrid. **q** Early bicellular pollen of Akihikari. **r** Late bicellular pollen of Akihikari. **s** Early bicellular pollen of the Ludao/Akihikari hybrid (*arrowhead* indicates microspores become irregular in shape). **t**–**v** Late bicellular pollen of the Ludao/Akihikari hybrid (*arrowheads* in **t** indicate chromatin conglomeration in disaggregated generative cells; *arrowheads* in **u** and **v** indicate a bigger chromatin conglomeration across pollen center resulting from disaggregation of both generative and vegetative cells). **w** Mature pollen of Akihikari. **x**–**z** Mature pollen of the Ludao/Akihikari hybrid (*arrowheads* indicate abortive pollens). Bar = 10 μm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pollens between Ludao/Akihikari hybrid and Akihikari began at the transition from late microspores to mature pollens. Two types of abnormalities were observed in hybrid pollens. In the first type, at the stage of a single nucleus to two nuclei of microspore development, some microspores became abortive as indicated by their irregular shape (Figs. 3s, 4v). In the second type, during the late bicellular pollen stage, a large proportion of pollen generative and vegetative cells began to disaggregate and form chromatin conglomeration, which further integrated to shape a bigger chromatin conglomeration across pollen center, eventually leading to abortion of the pollens (Figs. 3t–v, 4w). Further abortion of microspores also took place during pollen maturation (Figs. 3x–z, 4x). No visible abnormality in the development and disintegration of tapetum and other anther walls during microspore formation and development in the Ludao/Akihikari hybrid (Fig. 4a–x). Taken together, these results suggested that the abnormalities of late microspores and bicellular pollens, rather than the anther walls development, caused the pollen abortion in the Ludao/Akihikari hybrid.

Construction of molecular linkage map

A total of 858 SSR primers covering the entire rice linkage map and 10 EST primers on chromosome 3 were used for the parental polymorphism screening between Ludao and Akihikari. Among these, 191 primers (22.0%) showed polymorphism. The frequency of polymorphism varied among the chromosomes, ranging from 18.5 to 30.6% (data not shown).

The molecular linkage map was constructed using 215 truly hybrid plants of Akihikari//Ludao/Akihikari, as confirmed by both morphological and molecular markers. The map, consisting of 118 SSR loci and 1 EST locus, spanned a total of 1597.0 cM on all 12 chromosomes with an average interval of 13.4 cM between adjacent markers (data not shown). The linear order of the markers on the chromosomes in the map was in good agreement with earlier reports by Temnykh et al. (2000) and McCouch et al. (2002).

Identification of the hybrid pollen sterility genes

Based on the constructed molecular linkage map, two putative QTLs controlling hybrid pollen sterility were detected on chromosomes 3 and 11, and designated as *qPS-3* and *qPS-11*, respectively (Table 2; Fig. 5). *qPS-3* was linked with the EST marker C0729 with LOD score of 52.6 and PVE (phenotypic variance explained) of 57.9%. Another QTL, *qPS-11* with LOD score of 32.1 was linked with the SSR marker RM552, and accounted for 32.5% of the phenotypic variance of hybrid pollen sterility (Table 2). Both QTLs affected the hybrid pollen fertility in the same direction (Tables 2, 3). For each locus, the homozygote exhibited significantly higher pollen fertility than the heterozygote (Table 3).

Genetic modes of the gene on pollen fertility

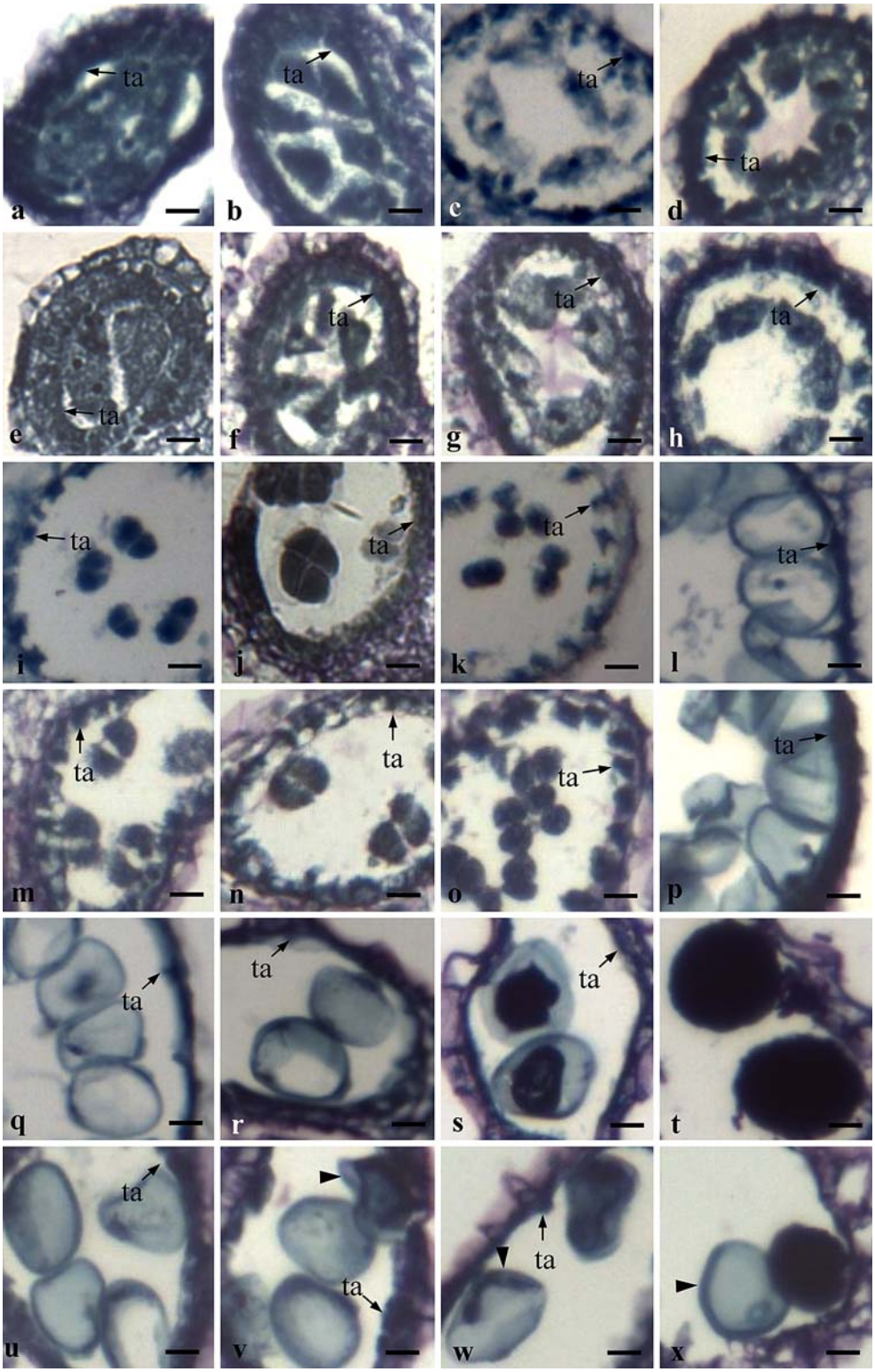
To assess possible effects of interactions between the two loci, a two-way analysis of variance was performed using the genotypes of the most closely linked markers. The results showed that the two loci did not have significant interaction effect and acted independently of each other (Table 4). Thus, the F_1 fertility reduction was a result of allelic interaction within each of the two loci.

The two-locus combination with Akihikari alleles at both loci displayed the highest pollen fertility, and had completely normal fertility of pollens (Table 5). The plants homozygous only at one of the two loci, especially the heterozygote at *qPS-3* showed significant reduction of pollen fertility (Table 5). The lowest pollen fertility was observed in the plants heterozygous at both loci (Table 5). The results indicated that the genetic effects of two loci were additive, and *qPS-3* conferred more effect on pollen sterility than *qPS-11*.

Determination of neutral allele at loci *qPS-3* and *qPS-11*

Allelic difference at *qPS-3 and qPS-11* was determined on the basis of pollen fertility in related hybrid crosses.

Fig. 4 The transverse sections of anthers during the process of microsporogenesis and male gametophytes development in Akihikari and the Ludao/Akihikari hybrid. **a** Pollen-mother cell stage of Akihikari. **b** Meiotic prophase 1 of Akihikari. **c** Meiotic metaphase 1 of Akihikari. **d** Meiotic anaphase 1 of Akihikari. **e** Pollen-mother cell stage of the Ludao/Akihikari hybrid. **f** Meiotic prophase 1 of the Ludao/Akihikari hybrid. **g** Meiotic metaphase 1 of the Ludao/Akihikari hybrid. **h** Meiotic anaphase 1 of the Ludao/Akihikari hybrid. **i** Dyad stage of Akihikari. **j** Tetrad stage of Akihikari. **k** Early microspore stage of Akihikari. **l** Middle microspore stage of Akihikari. **m** Dyad stage of the Ludao/Akihikari hybrid. **n** Tetrad stage of the Ludao/Akihikari hybrid. **o** Early microspore stage of the Ludao/Akihikari hybrid. **p** Middle microspore stage of the Ludao/Akihikari hybrid. **q** Late microspore stage of Akihikari. **r** Early bicellular pollen stage of Akihikari. **s** Late bicellular pollen stage of Akihikari. **t** Mature pollen stage of Akihikari. **u** Late microspore stage the Ludao/Akihikari hybrid. **v** Early bicellular pollen stage the Ludao/Akihikari hybrid (*arrowhead* indicates microspores become irregular in shape). **w** Late bicellular pollen stage the Ludao/Akihikari hybrid (*arrowhead* indicates generative and vegetative cell gradually disaggregated). **x** Mature pollen stage the Ludao/Akihikari hybrid (*arrowhead* indicates abortive pollen). *ta* tapetum. Bar = 10 μm



Generally, for three given varieties, A, B and N, if the A/B hybrids show gamete abortion at a locus *SX* by allelic interaction between SX^a and SX^b while both the N/A and N/B hybrids do not, the variety N may posses a neutral allele SX^n (Ikehashi and Araki 1986). The F_1 hybrids between Ludao and Akihikari showed pollen sterility due to the allelic interaction at loci *qPS-3* and *qPS-11* while those of 02428/Ludao and 02428/Akihikari

were normal (Table 1). Therefore, 02428 was considered to possess the neutral allele at loci *qPS-3* and *qPS-11* respectively.

The precise map location of *qPS-3*

To determine the precise map location of *qPS-3*, a major locus, we selected 165 highly fertile plants with pollen fertility higher than 80.0% from the backcross population and assayed these individuals using SSR and EST markers surrounding the *qPS-3* locus. According to the recombination frequencies, as well as the linkage map constructed in this study, the *qPS-3* locus was located to the interval between C0729 and RM3350, at a distance of 4.7 cM from both markers (Fig. 5), which was in accordance with the results of QTL mapping (Table 2; Fig. 5).

To further map the *qPS-3* locus, BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) was employed to search for sequences matching C0729 and RM3350 in the rice nucleotide database. As a result, matching sequences of C0729 and RM3350 were identified in BAC clone OSJNBb0023J24, and BAC clone OSJNBb0009F04, respectively. Further, other twelve overlapping BAC/PAC clones (OSJNBa0075A22, OSJNBb0047K21, OSJNBa0069E15, OSJNBb0065L20, OSJNBa0034D21, OSJNBb0031A14, OSJNBa0056E06, OSJNBb0036M02, OSJNBa0039O18, B1130H05, OSJNBb0113I20, and OSJNBa0035I24) covering the *qPS-3* locus region were identified. Eighty new SSR markers were identified using the SSR identification tool (SSRIT; http://www.gramene.org/microsat; Temnykh et al. 2001) based on the nucleotide sequence of these fourteen BAC clones. These markers together with 8 SSR markers reported by International Rice Genome Sequencing Project (2005) were used for the polymorphism screening between Ludao and Akihikari. Of them, five (JW56, RM15621, JW78, RM15627 and JW51) showed polymorphisms between the two parents. These five primer pairs were subsequently used to assay the recombination events of the 165 highly fertile BC_1F_1 plants. *qPS-3* was then mapped between RM15621 and RM15627, having one recombination event with each marker (Fig. 6). In addition, marker JW78 cosegregated with the *qPS-3* locus (Fig. 6). By comparing the genomic region of RM15621 and RM15627 with the map-based sequence of rice genome (International Rice Genome Sequencing Project, 2005), the *qPS-3* locus was located in an 86-kb DNA fragment on chromosome 3.

Putative genes in the 86-kb region

Based upon 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 ten predicted genes in the 86-kb region. Of these genes, four were hypothetical proteins, and three were expressed proteins (EST cDNA AK100254, AK069601 and AK072616; http://www.cdna01.dna.affrc.go.jp/cDNA). The functional annotations of the remaining three genes were as follows: (1) tubulin beta subunit, a gene of 495 bp, having no intron; (2) tubulin beta subunit (EST cDNA AB104733; http://www.cdna01.dna.affrc.go.jp/cDNA), a gene of 3,753 bp consisting of 3 exons and having a transcript length of 1,341 bp; and (3) putative pathogenesis-related thaumatin-like protein (EST cDNA AK058637, http://www.cdna01.dna.affrc.go.jp/cDNA), a gene of 1,248 bp, having no intron.

Discussion

Ludao is a weedy rice growing naturally in field of Lianyungang, Jiangsu province, China. The morphological characteristics of Ludao are similar to those of *japonica* varieties (Jiang et al. 1985; Chen and Zhu 1990), which may be an intermediate *japonica* type between *O. sativa* L. and common wild rice (*O. rufipogon* Griff) (Chen and Zhu 1990). In this study, we found that the hybrids between Ludao and a typical *japonica* variety, Akihikari, showed partial pollen sterility. Cytological and histological analysis of the anther in the Ludao/Akihikari hybrid showed that pollen abortion occurred mainly during the late stages of pollen development, which is consistent with the previous reports on other inter-subspecific rice hybrids (Wang et al. 1991; Liu et al. 1997, 2004; Zhu et al. 1996; Zhang et al. 2006). In general, persistence or

Table 2 QTLs for hybrid pollen sterility in Akihikari//Ludao/Akihikari detected by composite interval mapping

QTLs	Chromosome	NML[a]	LOD score	PVE (%)[b]	Additive effect
qPS-3	3	C0729	52.6	57.9	51.7
qPS-11	11	RM552	32.1	32.5	25.4

[a] Nearest marker locus of putative QTLs

[b] Phenotypic variance explained (%)

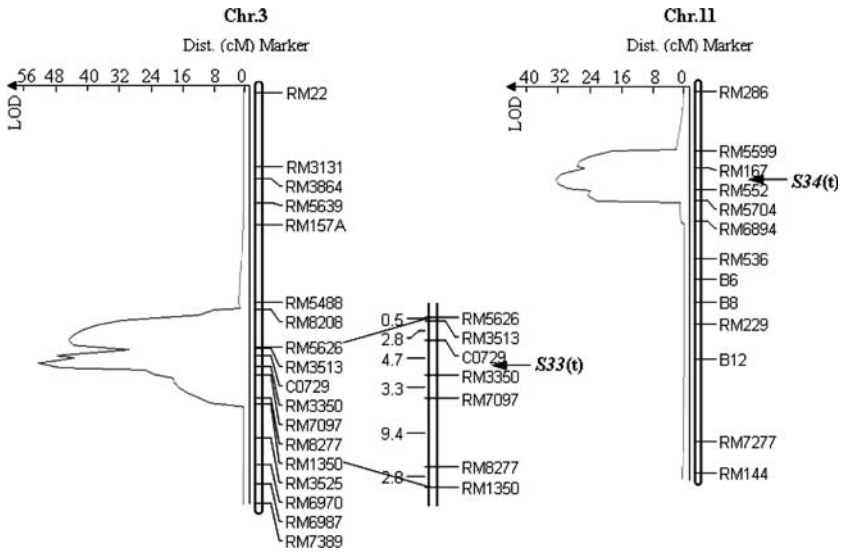


Fig. 5 Chromosome locations of *S33*(t) and *S34*(t)controlling pollen fertility in the genome based on the backcross population Akihikari//Ludao/Akihikari. The *arrow* on the chromosome 3 indicates the precise map location of *S33*(t) based on the 165 highly fertile plants with pollen fertility higher than 80.0% from the backcross population. The *arrow* on the chromosome 11 indicates the position of the LOD score peak for *S34*(t)

Table 3 Distribution of pollen fertility classified by marker genotype in Akihikari//Ludao/Akihikari population

Markers	Genotype	Number of plants in pollen fertility (%)							Total	Mean pollen fertility (%)
		40	50	60	70	80	90	100		
C0729	Akihikari	0	2	3	5	19	49	115	193	89.8**
	Hetero	1	7	11	1	1	0	1	22	53.6
RM552	Akihikari	0	3	8	2	1	2	86	102	91.8**
	Hetero	1	6	6	4	19	47	30	113	81.0

**Shows significant difference between two genotypes at 1%. Akihikari and Hetero denote homozygote and heterozygote, respectively

Table 4 A two-way ANOVA using one marker locus from each of the genomic regions showing effects on pollen fertility

Effect	*df*	MS	*F*	*P*
1 (C0729, Chr.3)	1	2.5906	284.44	0.0001
2 (RM552, Chr.11)	1	0.6613	72.62	0.0001
1 × 2	1	0.0006	0.07	0.7906
Error	211	0.0091		

Table 5 Pollen fertility averaged for each of the two-locus genotypes

C0729 (Chr.3)	RM552 (Chr.11)	Number	Pollen fertility (%)
Akihikari	Akihikari	91	95.6 a*
Akihikari	Hetero	102	84.6 b
Hetero	Akihikari	11	59.7 c
Hetero	Hetero	11	47.5 d

Note: Chromosomal locations of the markers are in parentheses;
* Significance level: $P \leq 0.05$ (Duncan's test)

premature breakdown of the tapetum was believed to be the main reason of male sterility, and the similar cases were also reported in the inter-subspecific hybrids of rice (Wang et al. 1991; Liu et al. 2004; Zhang et al. 2006). However, in this study, the tapetal cells developed and degraded normally during the process of pollen formation and development in the Ludao/Akihikari hybrid. In many cases, it is likely that tapetal abnormality was one of pleiotropic consequences rather than the primary cause of pollen abortion (Grant and Beversdorf 1986).

In the present study, we document that the pollen sterility of the hybrids between Ludao and Akihikari is independently controlled by two loci on chromosome 3 and chromosome 11 with LOD scores of 52.6 and 32.1 and PVE of 57.9 and 32.5%, respectively. So far, three gametophyte genes for segregation distortion and two F_1 pollen sterility genes have been located on chromosome 3. Nakagahra et al. (1972) and Nakagahra (1972) discovered two gametophyte genes *ga2* and *ga3* near the loci of marker genes *bc-1* and *dl* on chromosome 3, of which, the *ga2* led to the increase of *indica* allele in the F_1 hybrids, while the *ga3* had the opposite effect. Lu et al. (2000a, b) reported that the locus, *ga14* was located between *Pgi-1* and *bc-1* on chromosome 3 and caused the abortion of male gamete carrying *ga14-j*. *S19*, a gene for F_1 pollen semi-sterility was mapped to the end of the short arm of chromosome 3 (Taguchi et al. 1999). Zhang and Lu (1989) and Zhuang et al. (2002) identified and fine mapped another F_1 pollen sterility gene, *Sc*, which was also located on the short arm of chromosome 3. In contrast, the hybrid pollen

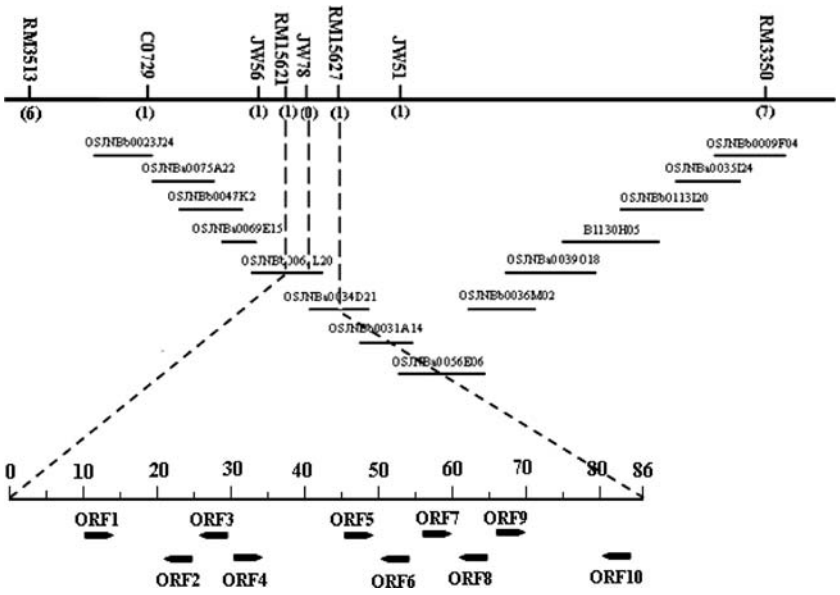


Fig. 6 Genetic and physical maps of the *S33*(t) locus. RM3513, RM15621, RM15627, RM3350, JW51, JW56 and JW78 are SSR markers, and C0729S are EST marker. The numbers between molecular markers indicate the numbers of recombination events detected between the *qPS-3* locus and the respective markers. The *long horizontal line* indicates the genomic region encompassing the *qPS-3* locus. The *short horizontal lines* represent BAC/PAC clones of cv. Nipponbare. The *dashed lines* indicate the relative position of the corresponding marker on BAC/PAC clones. The *horizontal arrows* represent predicted 10 ORFs based on the available sequence annotation (http://www.ncbi.nlm.nih.gov/entrez/)

sterility locus, *qPS3*, detected in this study was located on the long arm of chromosome 3, and led to the increase of *japonica* allele in the F_1 hybrids. Thus this locus appears to be different from all the genes abovementioned. According to the hybrid sterility nomenclature, the new locus is tentatively designated as *S33*(t). To date, no locus for hybrid pollen sterility has been reported on chromosome 11. Therefore, the locus, *qPS-11*, on chromosome 11, could be another novel gene controlling pollen sterility, and is tentatively designated as *S34*(t).

By assaying the recombinant events with SSR markers developed surrounding the *S33*(t) locus, we have fine mapped it within an 86 kb region on chromosome 3, which contains ten predicted genes. Among them, three expressed proteins and two tubulin beta subunits are expressed in flower of rice (http://www.ncbi.nlm.nih.gov/entrez/, http://www.cdna01.dna.affrc.go.jp/cDNA). These genes could be strong candidates of the pollen sterility gene. Further studies are necessary to determine the identity of *S33*(t).

Our data support that the pollen sterility of hybrids between Ludao and Akihikari was independently caused by two novel loci, *S33*(t) and *S34*(t), and Akihikari possess the alleles *S33*(t)-*j* and *S34*(t)-*j* respectively at the two loci. Previous studies have indicated that typical *indica* varieties such as IR36 and typical *japonica* varieties such as Akihikari carry the alleles *ga11-i* and *ga11-j*, and *ga14-i* and *ga14-j* respectively, at two gametophyte genes, *ga11* and *ga14* loci on chromosome 7 and 3 (Lu et al. 2000). Zhu et al. (2005) have found that the low fertility of hybrids between Ludao and a typical *indica* variety, IR36, was caused by female gamete abortion and independently controlled by two loci, *S10* and *S30*(t), while the hybrid female sterility between *indica* and *japonica* types in the current Chinese cultivars was mostly due to allelic interaction at the *S5* locus (Wan and Ikehashi 1997) and the native varieties in the Tai-hu Lake region carried the sterility-associated allele at the *S16* locus (Wan and Ikehashi 1995). It has been suggested that at the *S10* and *S30*(t) loci, the alleles causing gamete abortion in Ludao were from ancient rice strain which had undergone differentiation, then transmitted to Ludao by crossing-out between ancient rice strains and a *japonica*-like wild rice, and maintained in Ludao (Zhu et al. 2005). The *S33*(t) and *S34*(t) loci identified in this study might have undergone the similar differentiation process to the *S10* and *S30*(t) locus. Alternatively, Ludao might have been originated from natural hybridization between *japonica* cultivars and *japonica*-like wild rice (Tang and Morishima 1997). Since the hybrids between Ludao and a typical *japonica* variety, Akihikari, show partial pollen sterility, the origin of Ludao might be earlier than that of the current cultivars in China (Zhu et al. 2005).

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