Identification of two loci causing F₁ pollen sterility in inter- and intraspecific crosses of rice

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F₁ sterility, one of the most common post-zygotic reproductive barriers, is frequently observed in both inter-specific and intraspecific crosses of rice. Elucidating the genetic and cytological mechanisms of F₁ pollen sterility is important to exploit genetic resources and to understand the evolutionary dynamics of post-zygotic reproductive isolation in rice. Here, we report two F₁ pollen sterility loci, designated *S36* and *S25*, found in an interspecific cross between *O. sativa* ssp. *japonica* (Taichung 65) and *O. nivara* (IRGC105444), and an intraspecific cross between *O. sativa* ssp. *japonica* (Asominori) and ssp. *indica* (IR24). Genetic analyses revealed that both loci are located on distal end of the short arm of chromosome 12 and that allelic interaction at the heterozygous locus caused the sterility of male gametes carrying the *japonica* alleles in both cases. Comparison of map positions of *S36* and *S25* suggested that these two loci might be the same locus. Cytological investigation revealed that abnormality of sterile pollen grains caused by *S36* occurred mainly at the late bicellular stage after initiation of starch accumulation. This study would provide the better understanding on the genetic nature and the cytological mechanism of F₁ pollen sterility in rice.

Key Words: F₁ pollen sterility, interspecific cross, intraspecific cross, linkage mapping, cytological abnormality, rice.

Introduction

Rice is one of the most important food crops in the world, being the staple food for almost half of the world population. The genus *Oryza* consists of two cultivated and 21 wild species (Khush 1997) that can be classified into several complexes according to genome groups. Asian cultivated rice (*Oryza sativa* L.) and its closely related Asian wild species, *O. rufipogon* and *O. nivara*, were categorized into AA genome species and gene transfer among them can be accomplished through conventional hybridization breeding. Several studies have been conducted to develop and exploit genetic resources of the AA genome wild species and some useful genes have been introgressed to cultivated rice through interspecific hybridization (Khush 1997). In addition, *indica* and *japonica* subspecies possess wide genetic resources and many studies have been carried out to transfer the useful genes between them through intraspecific hybridization.

Reproductive isolation, which interferes with gene exchange between the parents, has been frequently observed in interspecific and intraspecific crosses of rice. Reproductive

isolation has been classified into two general categories (Smith 1989): pre-zygotic isolation mechanisms, which prevent the formation of hybrids, and post-zygotic isolation mechanisms, which act after the formation of a hybrid and can be classified into several subtypes (Stebbins 1950). F₁ sterility is one of the most common post-zygotic reproductive barriers, in which the hybrids survive but fail in the development of male and/or female gametes. F₁ pollen sterility is the most common isolation mechanism among AA genome species (Vaughan and Morishima 2002), as well as it was frequently observed in intraspecific hybrids between *japonica* and *indica*. Therefore, clarifying genetic mechanisms of F₁ pollen sterility is crucial for better understanding the evolutionary dynamics of post-zygotic reproductive isolation in rice as well as to exploit the valuable genetic resources of wild species. To date, several F₁ pollen sterility loci have been reported in F₁ hybrids between cultivated rice and its wild relatives carrying the AA genome, and also in intraspecific *indica*–*japonica* crosses (for review, Doi *et al.* 2008 and Koide *et al.* 2008, Kubo *et al.* 2008). However, no F₁ pollen sterility locus has been recognized in hybrids between *O. sativa* and *O. nivara*, an annual Asian common wild rice.

The genetic mechanisms of F₁ sterility have been explained using one-locus or two-locus models that affect the development of male and/or female gametophytes (for review, Oka 1988). One-locus allelic interaction model proposes

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that the interaction of alleles at a single heterozygous locus causes the abortion of gametes carrying a given allele. In contrast, epistatic interaction between two loci causes F_1 sterility in the two-locus model. Molecular mechanism of the one-locus allelic interaction model has been recently characterized by gene cloning (Chen *et al.* 2008, Long *et al.* 2008).

To understand gene function affecting pollen development, altered developmental processes in sterile pollen grains have been extensively characterized using mutants in *Arabidopsis* (McCormick 2004, Wilson and Yang 2004). Likewise, the developmental process in sterile pollen grains caused by allelic interaction at the F_1 pollen sterility loci, *S-a*, *S-b* and *S-c* (Zhang *et al.* 2005), and *S33* and *S34* (Jing *et al.* 2007) have been investigated in rice. Since abnormalities of sterile pollen grains in these semi-sterile lines differ, a diversity of cytological causes of F_1 pollen sterility is suggested to exist in rice.

Isogenic lines (Oka 1974) and three-way crosses (Ikehashi and Araki 1986) have been used in the genetic analysis of F_1 sterility. Recent advances of molecular marker technology allow us to recognize the gene for F_1 pollen sterility more effectively using advanced backcrossed lines through marker-assisted selection (MAS). In the process of developing a series of introgression lines (ILs) from backcrossed progeny of *O. nivara* with a genetic background of a *japonica* cultivar, and a series of chromosome segment substitution lines (CSSLs) between a *japonica* variety and an *indica* variety of rice (Kubo *et al.* 2002), we discovered the occurrence of F_1 pollen sterility. In this study, we identified two loci that cause pollen sterility in hybrids of inter- and intraspecific crosses of rice. In addition, cytological mechanisms of pollen abnormality in sterile pollen grains were characterized by observing starch accumulation and morphological changes in nuclei and cytoplasm, to clarify gene function affecting pollen development.

Materials and Methods

Plant materials

To generate ILs of the donor parent *O. nivara* (Accession Number IRGC105444), F_1 plants (T65/IRGC105444) were continuously backcrossed with the recurrent parent Taichung 65 (T65, *O. sativa* L. ssp. *japonica*) using MAS. In this process, the BC_2F_1 population was employed for QTL analysis of F_1 pollen sterility. With regard to the detected QTLs, a BC_4F_1 plant, which possessed a chromosomal segment of *O. nivara* in the targeted QTL region on chromosome 12, was selected from the BC_4F_1 population using MAS. The BC_4F_3 population derived from self-pollination of the BC_4F_1 plant was used for molecular mapping and characterizing the pollen sterility gene in the targeted QTL region on the chromosome 12 and cytological investigation of abnormalities in sterile pollen grains.

IR24 CSSLs with an Asominori genetic background were developed by successive backcrossing of recombinant inbred lines (RILs) (Tsunematsu *et al.* 1996) with Asominori

using MAS (Kubo *et al.* 2002). Segregation of pollen sterility was observed in some of the BC_3F_2 , BC_3F_3 and BC_4F_1 populations. The BC_3F_2 population was genotyped using restriction fragment length polymorphism (RFLP) markers that were evenly distributed over the rice genome. To identify and map the causal gene, a CSSL of chromosome 12 in BC_3F_3 generation was successively backcrossed with pollen of Asominori, and the resulting BC_5F_1 population was utilized for linkage mapping of the gene using RFLP markers.

Investigation of pollen fertility

Pre-flowering panicles from each individual in the two mapping populations were fixed and stored in 70% ethanol. Pollen grains of six anthers from one spikelet a few days before anthesis were stained with 1% iodine–potassium iodide (I_2 –KI) solution and more than 200 pollen grains were evaluated for pollen fertility of each individual under an Axioplan light microscope (Zeiss, Jena, Germany). Dark brown-stained pollen grains of normal size were scored as fertile; empty or unstained, incompletely stained and stained small pollen grains were evaluated as sterile. Pollen from each individual under the light microscope were photographed with a digital camera and pollen fertility was investigated.

Observation of postmeiotic development of sterile pollen grains

Panicles in meiotic to mature stages for *O. nivara* homozygous and heterozygous segregants at *S36* were continuously collected to cover unicellular to mature stages of pollen grains. Panicles were fixed for 24 h in fixative solution containing 4% (w/v) paraformaldehyde, 0.25% (w/v) glutaraldehyde, 0.02% (w/v) Triton X-100 and 100 mM sodium phosphate. After rinsing in 100 mM sodium phosphate buffer, the fixed panicles were stored in 100 mM sodium phosphate buffer containing 0.1% (w/v) sodium azide (NaN_3).

The hematoxylin staining procedure was used (Chang and Neuffer 1989) with minor modifications. The staining solution was prepared by mixing 2 ml of hematoxylin solution [1% (w/v) hematoxylin, 50% (w/v) propionic acid, 0.25% (w/v) $FeNH_4(SO_4)_2$] and 8 ml of acetocarmine solution [1% (w/v) carmine, 40% (w/v) acetic acid] with 0.2 mg of chloral hydrate. Six anthers from one spikelet at each developmental stage were squashed in the staining solution on microscope slides to release pollen grains. After debris was removed, a coverslip was applied. The preparation was gently heated with an alcohol lamp to just before boiling to activate the staining process and then cooled. The procedure of alternate heating and cooling was repeated three to four times. After keeping at room temperature for about 12 h to enhance stain contrast, samples were observed and photographed as indicated above.

DNA extraction and genotyping

Genomic DNA for simple sequence repeat (SSR) analysis was extracted from freeze-dried leaf samples according to Dellaporta *et al.* (1983) with minor modifications. PCR

reactions were performed in a volume of 15 µl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 200 µM of each dNTP, 0.2 µM of each primer, 0.75 units *Taq* polymerase (Takara, Otsu, Japan) and approximately 25 ng template DNA in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The PCR program used was 95°C for 5 min for initial denaturation, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. PCR products were run in 4% agarose gels (Agarose HT; Amresco Inc., Solon, OH, USA) in 0.5× TBE buffer.

Genomic DNA for RFLP analysis was extracted from the frozen leaf samples using the CTAB method (Murray and Thompson 1980). The extracted DNA (2.0 µg) was digested with the restriction enzymes *Bgl*II, *Dra*I, *Eco*RV, *Hind*III and *Kpn*I, separated by 0.8% agarose gel electrophoresis and blotted onto Hybond-N⁺ membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by capillary transfer mediated by 0.4 M NaOH. Blotted membranes were rinsed in 2× SSC, dried and baked at 120°C for 20 min. DNA clones previously mapped by Tsunematsu *et al.* (1996) and Harushima *et al.* (1998) were used. DNA labeling, hybridization and signal detection were conducted with the ECL detection system (GE Healthcare).

QTL analysis

In the interspecific cross, 56 BC₂F₁ plants were genotyped using a total of 87 SSR markers that were evenly distributed throughout the 12 rice chromosomes. The map positions of the SSR markers used in this study were based on those of the nearest RFLP markers on the latest high-density rice genetic map provided in Rice genome research program (<http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>). QTL analysis for pollen sterility was performed by one-way analysis of variance (ANOVA) using single-marker analysis method of the QGene 3.07a program (Nelson 1997). The proportion of phenotypic variance explained by a QTL was estimated as the coefficient of determination (R^2) of the closest marker. A QTL was declared significant if the probability of *F* statistic was equivalent to or less than 1% significant level ($P \leq 0.01$). QTLs were named according to the suggestions of the latest rice nomenclature (McCouch and CGSNL 2008).

Molecular mapping of pollen sterility genes

For molecular mapping of the pollen sterility gene in interspecific cross, 92 individuals of the BC₄F₃ population (T65/IRGC105444/4*T65) were used for linkage analysis of the pollen sterility gene and SSR markers located in the targeted QTL region. The sequences of primers for the newly developed SSR marker, named *M1-S36*, were 5'-CAC GGT GAA TTT AGA GCC CTC-3' and 5'-GTC GTG AAT CTC CTC CAA GTA-3'.

In the case of the intraspecific cross, 73 BC₃F₁ (IR24/6*Asominori) plants were genotyped using RFLP markers on the chromosome 12 and evaluated for pollen sterility. Re-

combination values between markers were estimated using the maximum-likelihood equation (Allard 1956) and transformed into genetic map distances, centiMorgans (cM), using Kosambi's (1944) mapping function.

Results

Detection of QTLs for F₁ pollen sterility in interspecific cross

Both parents, T65 and IRGC105444, showed more than 90% pollen fertility. The 56 individuals of BC₂F₁ population exhibited a continuous frequency distribution in pollen fertility, ranging from 35.7% to 99.0%, except for one plant that had 13.1% fertility (Fig. 1). To clarify genetic factor underlying the pollen sterility in the BC₂F₁ population, we performed QTL analysis using 87 SSR markers evenly distributed throughout the 12 rice chromosomes. Two putative QTLs for pollen sterility were detected on chromosomes 1 and 12, designated as *qPS1* and *qPS12*, respectively (Table 1). Both QTLs independently reduced pollen fertility under the heterozygous condition.

Molecular mapping of a pollen sterility gene, *S36*

To identify the pollen sterility gene from the targeted QTL region on chromosome 12 (the more promising locus between two detected QTLs), we performed MAS to develop the BC₄F₃ population from the BC₂F₁ plant possessing a chromosomal segment of *O. nivara* on chromosome 12. The parental BC₄F₂ plant of the BC₄F₃ population retained the *O. nivara* chromosomal segment under the heterozygous condition on chromosomes 4 and 12 (Fig. 2A). The BC₄F₃ population ($n=92$) exhibited a clear bimodal distribution for pollen fertility, and segregated into 47 pollen semi-sterile and 45 pollen-fertile plants (Fig. 2B). Pollen fertility of the fertile plants was more than 95% (Fig. 2B and 2D), while that of semi-sterile plants ranged from 37.5% to 62.3%, with an average of 49.8% (Fig. 2C and 2D). In I₂-KI staining, almost half of pollen of the semi-sterile plants were incompletely

Fig. 1. Frequency distribution of pollen fertility in the BC₂F₁ population (T65/IRGC105444/T65).

Table 1. Quantitative trait loci affecting pollen sterility in the BC₂F₁ population (T65/IRGC105444/T65)

QTL	Chromosome	Marker	Map position (cM)	Peak LOD score ^a	PVE ^b (%)	Additive effect ^c	Probability
qPS1	1	RM6827	157.1	2.2	16.6	19.3	<0.01
qPS12	12	RM3483	10.8	2.7	20.2	21.6	<0.001

^a LOD score from the most significant location by single-marker analysis

^b Percentage of phenotypic variation explained by the QTL

^c Plus values indicate that pollen fertility decreased under the heterozygous condition.

stained (Fig. 2B and 2C). The observed segregation ratio fit the theoretical 1 : 1 ratio ($\chi^2=0.043$, $P=0.84$) expected for monogenic inheritance of gametophytic pollen sterility.

To verify the precise location of the gene causing pollen sterility, linkage analysis was conducted in the BC₄F₃ population using SSR markers *RM3483* (McCouch *et al.* 2002) and *RM453* (Temnykh *et al.* 2001), and the newly developed SSR marker, *M1-S36*, on the distal end of the short arm of chromosome 12. The *M1-S36* marker revealed that all fertile plants were *O. nivara* homozygous genotypes, while all semi-sterile plants were heterozygous genotypes, except for one plant that had the *O. nivara* homozygous genotype (Fig. 2B). This result suggests that the single gene for pollen semi-sterility was tightly linked to *M1-S36* on the short arm of chromosome 12, and induced pollen sterility under the heterozygous condition. Although a series of sterility genes have been reported in AA genome species, no sterility gene has been identified in hybrid between *O. sativa* and *O. nivara* around this region. Therefore, we tentatively designated this gene as *S36*. Linkage analysis showed that the pollen sterility gene was located between *M1-S36* and *RM3483*, with a distance of 0.6 cM and 3.9 cM, respectively (Fig. 2E).

In the BC₄F₃ population, segregation of *S36-T65* homozygous plants was not observed. This segregation distortion was considered to be caused by sterility of pollen grains carrying *S36-T65* alleles. To confirm the transmission pattern of the alleles in pollen semi-sterile plants, we performed the reciprocal crossing between *S36* pollen semi-sterile (*S36* SS) plants and T65, and evaluated pollen fertility in the F₁ population (Table 2). When *S36* SS plants were used as female parents, 23 fertile and 22 semi-sterile plants segregated ($\chi^2_{1:1}=0.022$, $P=0.88$). On the other hand, when *S36* SS plants were used as male parents, all F₁ plants showed semi-sterility. These results demonstrated that *S36-T65* allele was eliminated in male gametophytes due to sterility of pollen grains carrying *S36-T65* alleles.

Molecular mapping of a pollen sterility gene, *S25*

The donor and recurrent parents, IR24 and Asominori, respectively, had above 90% pollen and seed fertility, and the reciprocal F₁ hybrids showed approximately 40% pollen fertility and 60% seed fertility. Segregation of pollen sterility was observed in some of the BC₃F₂, BC₃F₃ and BC₄F₁ populations, which were developed in the process of generating a series of IR24 (CSSLs) with Asominori genetic background by successive backcrossing and MAS (Kubo *et al.* 2002). Among these, some populations showing segregation of pol-

Fig. 2. Identification of the *S36* locus causing F₁ pollen sterility. (A) Graphical genotype of the BC₄F₂ plant, a progenitor of the mapping population (BC₄F₃). (B) Frequency distribution of pollen fertility in the BC₄F₃ population, classified by the genotypes of SSR marker *M1-S36*. (C, D) Pollen grains of semi-sterile (C) and fertile (D) plants stained with I₂-KI. Scale bars = 50 μm. Asterisks indicate sterile pollen. (E) Linkage map showing the location of *S36* for F₁ pollen sterility. Left, RFLP framework map from Harushima *et al.* (1998).

Table 2. Segregation of pollen sterility in the backcross populations derived from the reciprocal crosses of *S36* pollen semi-sterile (*S36* SS) plants and T65

Cross combination	No. of plants		
	Fertile	Semi-sterile	Total
<i>S36</i> SS plant × T65	23	22	45
T65 × <i>S36</i> SS plant	0	104	104

len sterility carried the IR24 segment on the short arm of chromosome 12 (data not shown), indicating that the F₁ pollen sterility gene is possibly located in this chromosomal region.

To identify and characterize the causal gene for F₁ pollen sterility, the BC₅F₁ population was developed from a BC₃F₃ plant (Fig. 3A) carrying the substituted segment of IR24 on the short arm of chromosome 12 by successive backcrossing with Asominori. The BC₅F₁ population consisting of 73 plants segregated into 32 pollen semi-sterile (20–65%) plants and 41 pollen-fertile (>95%) plants, with a clear bimodal distribution for pollen fertility (Fig. 3B, 3C and 3D). This segregation ratio fit the theoretical 1 : 1 ratio ($\chi^2=1.11$,

$P=0.34$) expected for gametophytic pollen sterility under control of a single nuclear gene. The sterile pollen grains found in semi-sterile plants were incompletely stained by I₂–KI (Fig. 3C). All BC₅F₁ plants were genotyped using RFLP markers located on the retained segment of chromosome 12 in the parental BC₃F₂ plant. The genotyping of *G193* marker revealed that all fertile plants were Asominori homozygous genotypes, whereas all semi-sterile plants were heterozygous genotypes (Fig. 3B). This result suggests that the pollen sterility gene was completely linked to *G193* on the distal end of the short arm of chromosome 12, and caused pollen sterility in the heterozygous state. Linkage analysis showed that the pollen sterility gene, named *S25* (Kubo *et al.* 2001), completely segregated with the marker *G193* and was located between *G24* and *G189*, with map distances of 1.4 cM and 5.5 cM, respectively (Fig. 3E).

Based on segregation analysis for pollen sterility in the progeny of reciprocal crosses between the *S25* pollen semi-sterile (*S25* SS) plants and Asominori, the genotype of male gamete which was abortive in the *S25* heterozygous plants was examined. In Table 3, when the *S25* SS plants were pollinated with Asominori pollen, 41 fertile and 32 semi-sterile plants that fitted to the expected 1 : 1 ratio were observed ($\chi^2 = 1.11$, $P=0.34$). However, when *S25* SS plants were used as the pollen parents, all progeny were semi-sterile. These reciprocal crosses indicated that only the male gametes carrying *S25-IR24* alleles were fertile and those carrying *S25-Asominori* alleles were sterile.

Comparison of map positions of *S36* and *S25*

Both loci, *S36* and *S25*, were located at the distal end of the short arm of chromosome 12, and the pollen grains carrying the *japonica* allele were sterile in the heterozygous state in both cases. In addition, the sterile pollen grains of the *S36* heterozygotes and the *S25* heterozygotes showed similar phenotypes in I₂–KI staining (Fig. 2C and 3C, asterisks). Since *S36* was mapped with SSR markers and *S25* was mapped with RFLP markers, it is necessary to recognize the genomic position of both types of marker for comparison of these two loci. Our BLAST search using the IRGSP genome sequence build 4 (<http://rapdb.dna.affrc.go.jp/rapdownload/>) demonstrated that SSR markers *MI-S36* and *RM3483* are located between RFLP markers *G24* and *G189* on the rice reference sequence of Nipponbare (Fig. 4). In addition, the *japonica* alleles, *S36-T65* and *S25-Asominori*, were not transmitted to the progeny via male gametes. These results suggest that these two loci might be the same locus. However, we tentatively named the F₁ pollen sterility locus detected in the hybrid between *japonica* and *O. nivara* as *S36* to discriminate it from the *S25* locus found in the hybrid between *japonica* and *indica*.

Postmeiotic development of aborted pollen grains caused by *S36*

To elucidate the cytological mechanism of pollen sterility induced by *S36*, pollen development in postmeiotic stages

Fig. 3. Identification of *S25* causing F₁ pollen sterility. (A) Graphical genotype of the BC₃F₃ plant, a progenitor of the mapping population (BC₅F₁). (B) Frequency distribution of pollen fertility in the BC₅F₁ population, classified by the genotypes of RFLP marker *G193*. (C, D) Pollen grains of semi-sterile (C) and fertile (D) plants stained with I₂–KI. Scale bars = 50 μ m. Asterisks indicate sterile pollen. (E) Linkage map showing the location of *S25* for F₁ pollen sterility. Left, RFLP framework map from Harushima *et al.* (1998).

Table 3. Segregation of pollen sterility in the backcross populations derived from the reciprocal crosses of *S25* pollen semi-sterile (*S25* SS) plants and Asominori

Cross combination	No. of plants		
	Fertile	Semi-sterile	Total
<i>S25</i> SS plant $\times$ Asominori	41	32	73
Asominori $\times$ <i>S25</i> SS plant	0	160	160

was investigated in the semi-sterile plants using I₂–KI and hematoxylin staining. During the unicellular stages, from tetrad to uninucleate microspore, all microspores of both *S36-niv* homozygotes (Fig. 5A and 5C) and heterozygotes (Fig. 5B, 5D and 5E) had a normal phenotype. At the early bicellular pollen stage, all pollen grains of the *S36-niv* homozygotes exhibited normal phenotype; generative cell (Fig. 5F, arrow) attached to the pollen wall at the opposite side of the pollen pore, where pollen mitosis I generally occurs, and vegetative nucleus (Fig. 5F, arrowhead) migrated toward the pollen pore along the pollen wall. All pollen grains of the *S36-niv* heterozygotes showed similar phenotype to that of the *S36* homozygotes (Fig. 5G). In addition, all pollen grains of *S36-niv* heterozygotes were of similar size in I₂–KI staining at this stage (Fig. 5H).

The morphological difference gradually became distinct at the late bicellular stage (Fig. 5I–5K). In the normal development as observed in the *S36-niv* homozygotes, the generative cell detached from the pollen wall at a position opposite to the pollen pore and moved toward the vegetative

Fig. 4. Comparison between the map positions of *S36* and *S25* based on the genome sequence of chromosome 12. Molecular markers were located on Nipponbare pseudomolecule build 4 of chromosome 12, released from international rice genome sequence project (IRGSP).

cell (Fig. 5I, arrow). Meanwhile, almost half of the pollen grains in the *S36-niv* heterozygotes exhibited the normal development and the generative cells of the other half still attached to the pollen wall, it means that nuclear migration of generative cells began to slowdown at this stage (Fig. 5J, white arrow) and these pollen grains were smaller than normal pollen grains. The initiation of the starch accumulation at the late bicellular stage was observed in normal plants, including T65 and *S36-niv* homozygotes (data not shown). In contrast, half of the pollen in *S36* heterozygotes remained unstained, whereas the other half of the pollen grains gradually stained brown with I_2 -KI (Fig. 5K). This indicates that starch accumulation is not yet initiated in abnormal pollen grains.

At the tricellular stage after hematoxylin staining, half of the pollen grains in the *S36-niv* heterozygotes were densely stained, with a round vegetative nucleus and two rounded sperm cells, as observed in *S36-niv* homozygotes (Fig. 5L). The other half of the pollen grains remained at the bicellular stage, and the generative nucleus migrated to a position below the pollen pore where pollen mitosis II occurs (Fig. 5M, arrow). At this stage, the lately developed pollen could be stained with I_2 -KI, indicating that it accumulates some starch granules (Fig. 5N). These results suggest that development of abnormal pollen might be arrested in the late bicellular stage after migration of the generative cell from the periphery into the vegetative cytoplasm below the pollen pore.

At the mature stage, pollen of *S36-niv* homozygotes had typical normal pollen grains carrying one vegetative nucleus and two sperm cells (Fig. 5O). The normal vegetative nucleus was only slightly stained and obscure and normal sperm cells were compact and ellipsoid in shape. In contrast, in semi-sterile plants, almost half of the pollen was normal, similar to pollen of *S36-niv* homozygotes, and the remaining half of the sterile pollen was mainly at the bicellular stage with the generative cells located in the vegetative cytoplasm. The bicellular sterile pollen grain had a densely stained and round vegetative nucleus and a round generative cell

(Fig. 5P). Some tricellular sterile pollen was also observed, with densely stained and round vegetative nuclei and round sperm cells, which were not compact and ellipsoidal, unlike normal pollen grains (Fig. 5Q). Approximately 90% of sterile pollen grains were retaining in the bicellular stage. After I_2 -KI staining, *S36-niv* homozygotes had a normal phenotype with rounded black pollen grains. In semi-sterile plants, almost half of the pollen grains could be stained but were not completely stained with I_2 -KI even though the remaining pollen grains were normal as in *S36-niv* homozygotes (Fig. 5R). These results also revealed that the abnormality in sterile pollen grains occurs largely at the bicellular pollen stage after initiation of starch accumulation.

Discussion

In this study, we conducted genetic analysis of F_1 pollen sterility using inter- and intraspecific crosses of rice. Two putative QTLs, *qPS1* on chromosome 1 and *qPS12* on chromosome 12, were found in the interspecific cross between *O. sativa* L. and *O. nivara*. The more significant QTL on chromosome 12 was successfully mapped on distal end of the short arm of the chromosome 12, and designated as *S36*. Moreover, we identified an F_1 pollen sterility locus, *S25*, on the short arm of chromosome 12, in intraspecific *indica-japonica* cross. Both loci located on the distal end of the short arm of chromosome 12, and the *japonica* allele was aborted in the heterozygous state in both cases. This result suggests the possibility that *S36* and *S25* might be the same locus. The genetic analyses demonstrated that F_1 pollen sterility caused by both loci fit a one-locus allelic interaction model, since pollen semi-sterility was observed in the heterozygous state. The common occurrence of the F_1 pollen sterility gene against *japonica*, *S25* (*indica*) and *S36* (*O. nivara*), might be one of the keys resolving the evolutionary dynamics of post-zygotic reproductive isolation among the complex of the Asian cultivated and wild species. On the other hand, we did not perform confirmation of the *qPS1* as a single Mendelian factor because the advanced backcross line carrying only *O. nivara* chromosomal segment around *qPS1* region was not available. Since the *qPS1* exhibited a major effect as well as *qPS12* (*S36*), analysis of *qPS1* using the advanced backcross population is required.

According to the cytological investigation, stainable sterile pollen grains were observed in *S36* heterozygous plants (Fig. 5R) and the typical sterile pollen grain was bicellular with the generative cell located in the vegetative cytoplasm (Fig. 5P). Thus, development of most of sterile pollen grains appeared to be arrested in the late bicellular stage after migration of generative cell from the periphery into the vegetative cytoplasm. Zhang *et al.* (2005) reported cytological observation of F_1 pollen sterility caused by allelic interaction of the respective genes, *S-a*, *S-b* and *S-c*, using nearly isogenic lines (NILs). Since NILs facilitate gene characterization precisely, the comparison of sterile pollen grains caused by various F_1 pollen sterility genes is possible. The sterile pollen

Fig. 5. The process of postmeiotic pollen development in *S36-niv* homozygous and heterozygous plants. (A, C, F, I, L, O) Pollen of *S36-niv* homozygous plants. (B, D, E, G, H, J, K, M, N, P–R) Pollen of *S36-niv* heterozygous plants. (A, B) Tetrad stage. (C–E) Unicellular stage. (F–H) Early bicellular stage. (I–K) Late bicellular stage. (L–N) Tricellular stage. (O–R) Mature stage. (A–C, E–G, I, J, L, M, O–Q) Hematoxylin staining. (D, H, K, N, R) I₂–KI staining. Scale bars=20 μm. Black and white arrows indicate generative cells of fertile and sterile pollen, and black and white arrowheads indicate vegetative cells and sperm cells, respectively. Asterisks indicate pollen pores.

grains caused by the *S-a* locus degenerate at the early bicellular pollen stage with empty abortive pollen. In the case of *S-b* locus, the sterile pollen grains abort at the mature pollen stage with two sperm cells and a vegetative nucleus, and accumulate many starch grains. Therefore, the abnormalities of sterile pollen caused by *S-a* and *S-b* were apparently

different from that of *S36*. The sterile pollen grains caused by *S-c* locus abort at the late bicellular stage due to the failure of migration of the generative cells into the cytoplasm of the vegetative cell. In the case of *S36*, the sterile pollen aborted also at the late bicellular stage, but the migration of the generative cell from the periphery into the vegetative

cytoplasm occurred in this stage. Therefore, the cytological abnormality of sterile pollen grains caused by *S-c* was also different from that of *S36* locus. These diversities of cytological causes of F_1 pollen sterility reveal that postmeiotic pollen development is a genetically complex process, and that F_1 pollen sterility which evolved in various loci is controlled by different genetic processes.

Hybrid sterility, the most common post-zygotic isolating mechanism, plays an important role in speciation and provides an initial force driving genetic differentiation (Orr and Presgraves 2000). Understanding the genetic architecture of hybrid sterility at the molecular level still requires further study, not only to overcome reproductive barriers in transferring valuable genes between species or subspecies but also to clarify divergent evolution of rice species. Consequently, this study could provide the understanding of genetic and cytological mechanisms of pollen semi-sterility and facilitate further clarification of molecular mechanism of male gametogenesis and nature of hybrid pollen sterility between cultivated rice and its wild relatives.

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