

Molecular Mapping of Two Loci Conferring F₁ Pollen Sterility in Inter- and Intraspecific Crosses of Rice

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ABSTRACT

Hybrid sterility, a major form of post-zygotic reproductive barriers, often appears in crosses between relatively distant species as well as between closely related subspecies. To unravel the genetic mechanism and cytological features of F₁ pollen sterility, we identified and characterized the loci causing F₁ pollen sterility using introgression lines of the donor parent *O. nivara* in the genetic background of *O. sativa* ssp. *japonica* cv. 'Taichung 65' and chromosome segment substitution lines carrying *O. sativa* ssp. *indica* cv. 'IR24' segments in the genetic background of ssp. *japonica* cv. 'Asominori'. In this study, we report two F₁ pollen sterility loci, designated as *S36* and *S25*, found in inter- and intraspecific crosses, respectively. Genetic analyses revealed that allelic interaction at the heterozygous locus caused the sterility of male gametes carrying the *japonica* alleles in both cases. Both loci are located on the distal end of the short arm of rice chromosome 12 and comparison of the map positions of *S36* and *S25* indicated that these two loci might be the same locus. Cytological investigation demonstrated that abnormality of sterile pollen grains caused by *S36* occurred mainly at the late bicellular stage after initiation of starch accumulation. The present study provides a better understanding on the genetic nature and the cytological aspect of F₁ pollen sterility and the evolutionary dynamics of post-zygotic reproductive isolation in rice, and consequently, could help to overcome the reproductive barriers in inter- and intraspecific hybridization for the improvement of cultivated rice.

Keywords: cytological abnormality, hybrid sterility, linkage mapping, reproductive barriers

Abbreviations: CSSL, chromosome segment substitution line; FDA, fluorescein diacetate; I₂-KI, iodine-potassium iodide; IL, introgression line; MAS, marker-assisted selection; NIL, near-isogenic line; QTL, quantitative trait locus; RFLP, restriction fragment length polymorphism; T65, Taichung 65

INTRODUCTION

Reproductive isolation, which generally prevents gene flow between any two diverged populations, has been frequently observed in interspecific and intraspecific crosses of rice. F₁ sterility is one of the most common post-zygotic reproductive barriers, in which the hybrids survive but fail to develop male and/or female gametes. F₁ pollen sterility is the most common isolation mechanism among AA genome species (Vaughan and Morishima 2002), and in intraspecific hybrids between *japonica* and *indica*. Therefore, discovering the genetic mechanisms of F₁ pollen sterility is crucial for better understanding of the evolutionary dynamics of post-zygotic reproductive isolation in rice as well as to exploit the valuable genetic resources of wild species. Although 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 (Doi *et al.* 2008; Koide *et al.* 2008; Kubo *et al.* 2008), no F₁ pollen sterility locus has been recognized in hybrids between *O. sativa* and *O. nivara*, an Asian annual wild rice.

The genetic mechanisms of F₁ hybrid sterility have been explained using two models proposed according to the Mendelian pattern of inheritance: the one-locus allelic interaction and the two-locus epistatic interaction (Oka 1988). The one-locus allelic interaction model proposes that the interaction of alleles at a single heterozygous locus causes the abortion of gametes carrying a given allele. The molecular mechanisms of one-locus allelic interaction model have been recently characterized in gametophytic F₁ pollen and embryo sac sterility by gene cloning (Chen *et al.* 2008;

Long *et al.* 2008). In contrast, epistatic interaction between two loci causes F₁ sterility in the two-locus model. The gametophytic F₁ pollen sterility fitting the two-locus epistatic model has recently been analyzed at the molecular level (Yamagata *et al.* 2010).

To understand gene function affecting pollen development, the developmental process in sterile pollen grains caused by allelic interaction at the F₁ 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. The abnormalities of sterile pollen grains differ in these semi-sterile lines, indicating that F₁ pollen sterility which evolved in various loci is controlled by different genetic processes.

Wild species of *Oryza* are a rich source of useful genes for the improvement of cultivated rice (Jena and Khush 1990) and several studies have been conducted to exploit genetic resources of the AA genome wild species. Moreover, the intraspecific hybrids between *indica* and *japonica* showed high yield potential due to their genetic divergence and many studies have been done to transfer the useful genes for agronomically important traits through intraspecific hybridization. However, reproductive barriers often appear in crosses between relatively distant species as well as between closely related subspecies. In this study, we identified two loci causing hybrid pollen sterility in inter- and intraspecific crosses of rice. This information would provide the further understanding on the genetic and cytological mechanisms of F₁ pollen sterility and as a consequence, could facilitate genetic manipulations to overcome reproductive barriers in transferring valuable genes between species or subspecies.

MATERIALS AND METHODS

Plant materials

To generate introgression lines (ILs) of the donor parent *O. nivara* (accession: 'IRGC105444', Sri Lanka), F₁ plants ('T65'/'IRGC105444') were successively backcrossed with the recurrent parent 'Taichung 65' ('T65', *O. sativa* L. ssp. *japonica*) using marker-assisted selection (MAS). The BC₂F₁ population was used for QTL analysis of F₁ pollen sterility. Regarding the detected QTLs, a BC₄F₁ plant, which possess a chromosomal segment of *O. nivara* in the targeted QTL region on chromosome 12, was selected from the BC₄F₁ population using MAS and its derivative BC₄F₃ population was used for molecular mapping of the pollen sterility gene in the targeted QTL region on chromosome 12.

'IR24' chromosome segment substitution lines (CSSLs) with an 'Asominori' (*O. sativa* L. ssp. *japonica*) genetic background were developed by successive backcrossing of selected recombinant inbred lines (RILs; Tsunematsu *et al.* 1996) with 'Asominori' using MAS (Kubo *et al.* 2002). The BC₃F₂ population was genotyped using restriction fragment length polymorphism (RFLP) markers that were evenly distributed over the rice genome. Segregation of pollen sterility was observed in some of the BC₃F₂, BC₃F₃ and BC₄F₁ populations. Among these, some populations showing segregation for pollen 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 map the causal gene, a CSSL of chromosome 12 in BC₃F₃ generation was successively backcrossed with 'Asominori', and the resulting BC₅F₁ population was utilized for linkage mapping of the gene using RFLP markers.

Evaluation of pollen fertility

Panicles at the flowering stage were fixed and stored in 70% (v/v) ethanol (Sigma-Aldrich, St. Louis, MI, USA). Pollen grains collected few days before anthesis were stained with 1% iodine-potassium iodide (I₂-KI; Sigma-Aldrich) solution and more than 200 pollen grains were evaluated for pollen fertility under an Axioplan light microscope (Zeiss, Jena, Germany). Pollen grains that were morphologically the same with those of 'T65' were scored as normal. Empty, unstained, incompletely stained and small pollen grains were scored as sterile.

Observation of postmeiotic pollen development

Panicles in the meiotic to mature stages were continuously collected to observe pollen development from unicellular to mature stages. Panicles were fixed in fixative solution containing 4% (w/v) paraformaldehyde (Sigma-Aldrich), 0.25% (w/v) glutaraldehyde (Sigma-Aldrich), 0.02% (v/v) Triton X-100 (Boehringer Mannheim, Mannheim, W. Germany) and 100 mM sodium phosphate, pH 7.5 (Sigma-Aldrich) for 24 h at 4°C and then rinsed in 100 mM sodium phosphate (Sigma-Aldrich) buffer. The hematoxylin staining procedure used followed that of Chang and Neuffer (1989) with minor modifications.

Evaluation of germination ability and viability of pollen

To evaluate pollen germination on artificial medium, pollen grains were collected just after flowering under natural conditions and shed onto the germination medium containing 15% (w/v) sucrose (Sigma-Aldrich), 0.01% (w/v) boric acid (Sigma-Aldrich), 0.03% (w/v) calcium chloride (Sigma-Aldrich) and 0.6% (w/v) gellan gum (Wako, Osaka, Japan), on a glass microscope slide. After 6 to 8 min of incubation at room temperature, the pollen grains were observed and photographed under a light microscope.

To evaluate the viability of sterile pollen grains, panicles in the heading stage were collected and fresh pollen at a few days before anthesis was stained with 0.05 mM fluorescein diacetate (FDA; Sigma-Aldrich) solution. The stained samples were observed immediately under a fluorescence microscope with a 450-490-nm excitation filter and a 525-nm emission filter according to Heslop-Harrison and Heslop-Harrison (1970).

Molecular mapping of pollen sterility loci

For molecular mapping of the pollen sterility loci in the interspecific cross, 92 individuals of the BC₄F₃ population were used for linkage analysis of the pollen sterility loci and SSR markers *RM3483* (McCouch *et al.* 2002) and *RM453* (Temnykh *et al.* 2001), and the newly developed SSR marker in this study, named *MI-S36*, located in the targeted QTL region. The sequences of primers for *MI-S36* were 5'-CACGGTGAATTTAGAGCCCTC-3' and 5'-GTCGTGAAT CTCCTCCAAGTA-3'.

In the case of the intraspecific cross, 73 BC₅F₁ plants were genotyped using RFLP markers *G24*, *G193* and *G189* (Harushima *et al.* 1998) on chromosome 12 and evaluated for pollen fertility. Recombination values between markers were estimated using the maximum-likelihood equation (Allard 1956) and transformed into genetic map distances, centiMorgans (cM), using Kosambi's mapping function (Kosambi 1944).

RESULTS AND DISCUSSION

Previously, we have conducted genome-wide identification of the F₁ pollen sterility genes in hybrid progeny between *O. sativa* and its AA genome wild relatives as well as between subspecies of *O. sativa* using advanced backcross lines, RILs and CSSLs. We identified a series of hybrid pollen sterility genes *S22*, *S23*, *S27* and *S28* in crosses between *O. sativa* and *O. glumaepatula* (Sobrizal *et al.* 2000a, 2000b, 2001, 2002), *S18*, *S19*, *S20* and *S21* in crosses between *O. sativa* and *O. glaberrima* (Doi *et al.* 1998, 1999; Taguchi *et al.* 1999), and *S24* and *S35* in crosses between *indica* and *japonica* subspecies (Kubo *et al.* 2008). In the present study, during the development of a series of ILs from backcross progeny of *O. nivara* with a genetic background of 'T65', and a series of IR24 CSSLs with 'Asominori' genetic background (Kubo *et al.* 2002), we observed the occurrence of F₁ pollen sterility.

Molecular mapping of a pollen sterility locus, *S36*

The BC₄F₃ population ($n = 92$), which derived from a BC₄F₂ plant (Fig. 1A) exhibited a clear bimodal distribution for pollen fertility, and segregated into 47 pollen semi-sterile and 45 pollen fertile plants (Fig. 1B). Pollen fertility of the fertile plants was more than 95% (Fig. 1B, 1C), while that of semi-sterile plants ranged from 37.5% to 62.3%, with an average of 49.8% (Fig. 1B, 1D). Almost half of the pollen from semi-sterile plants were incompletely stained (Fig. 1D) in I₂-KI staining solution. The newly developed SSR marker, *MI-S36* 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. 1B). Segregation of T65 homozygous plants was not observed in the mapping population (Fig. 1B). The observed segregation ratio of *O. nivara* homozygous and heterozygous plants fits the theoretical 1:1 ratio ($\chi^2 = 0.043$, $P = 0.84$) at *MI-S36*, expected for gametophytic pollen sterility due to the sterility of pollen grains carrying T65 alleles. Therefore, we can conclude that a single gene linked to *MI-S36* on the short arm of chromosome 12, caused pollen sterility in the heterozygous condition due to sterility of pollen grains carrying T65 alleles. Although a series of sterility genes have been reported in AA genome species, no sterility gene has been identified in the hybrid between *O. sativa* and *O. nivara* around this region. Therefore, we designated this gene causing gametophytic pollen sterility in the heterozygous state as *S36*, as a locus for F₁ pollen sterility. To verify the precise location of the causal gene, linkage analysis was conducted in the BC₄F₃ population using SSR markers *RM3483*, *RM453* and *MI-S36*, on the distal end of the short arm of chromosome 12. Linkage analysis showed that the pollen sterility gene was located between *MI-S36* and *RM3483*, with a distance of 0.6 and 3.9 cM, respectively (Fig. 1E).

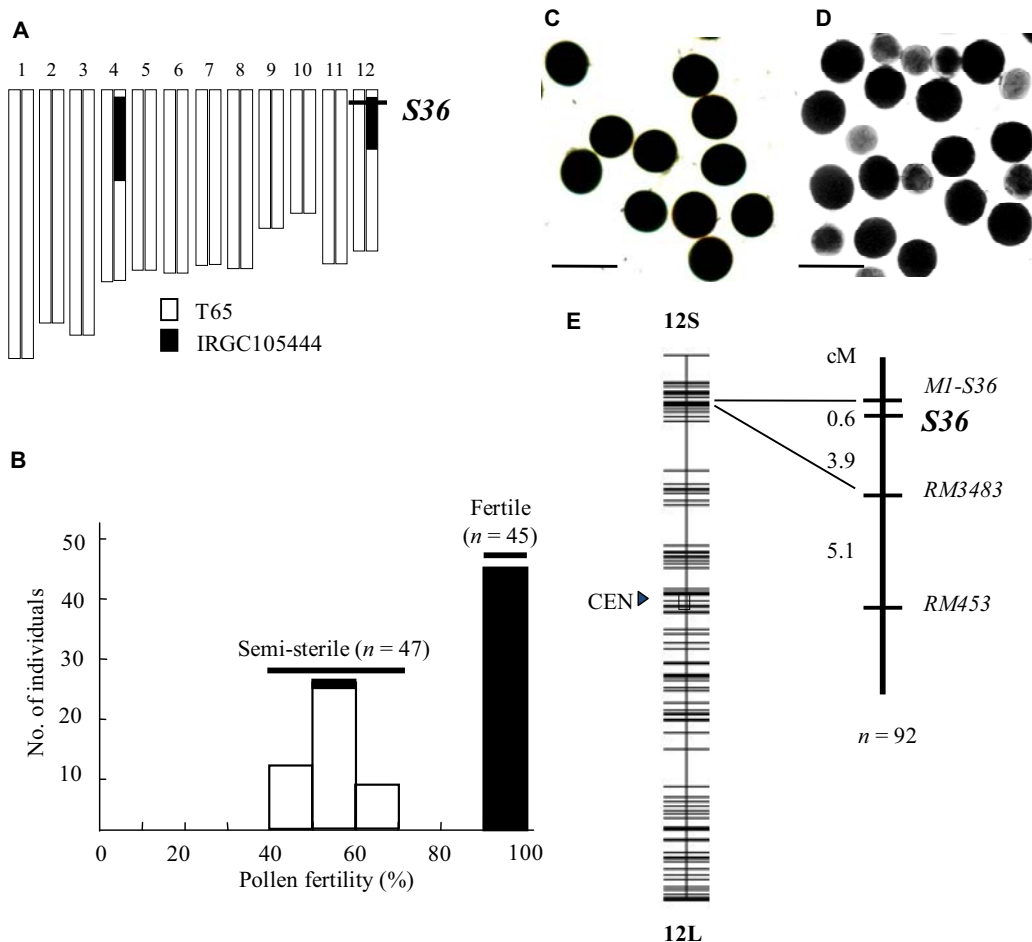


Fig. 1 Identification of *S36* locus causing F₁ pollen sterility. (A) Graphical genotype of the BC₄F₂ plant, a progenitor of the mapping population (BC₄F₃). (B) Frequency distribution of pollen fertility in the BC₄F₃ population, classified by the genotypes of SSR marker *MI-S36*. Black and white bars represent IRGC105444 homozygote and heterozygote, respectively. (C, D) Pollen grains of fertile (C) and semi-sterile (D) plants stained with I₂-KI. Scale bars = 50 μ m. (E) Linkage map showing the location of *S36* for F₁ pollen sterility. Left, RFLP framework map was quoted from the latest high-density rice genetic map including 3267 markers, in the rice genome research program (RGP). Available online: <http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>

Molecular mapping of a pollen sterility locus, *S25*

The donor and recurrent parents, ‘IR24’ and ‘Asominori’, respectively, had above 90% pollen fertility, and the reciprocal F₁ hybrids showed approximately 40% pollen fertility. The BC₅F₁ population consisting of 73 plants, which was derived from a BC₃F₃ plant (Fig. 2A) segregated into 41 pollen-fertile (> 95%) plants and 32 pollen semi-sterile (20–65%) plants, with a clear bimodal distribution for pollen fertility (Fig. 2B–D). This segregation ratio fits the theoretical 1:1 ratio ($\chi^2 = 1.11$, $P = 0.34$) expected for monogenic inheritance of gametophytic pollen sterility. The sterile pollen grains found in semi-sterile plants were incompletely stained by I₂-KI staining (Fig. 2D). All BC₅F₁ plants were genotyped using RFLP markers located on the retained segment of chromosome 12 in the parental BC₃F₃ plants. All fertile plants were ‘Asominori’ homozygous genotypes, whereas all semi-sterile plants were heterozygous genotypes at *G193* (Fig. 2B). This result suggests that the pollen sterility locus was tightly 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 and 5.5 cM, respectively (Fig. 2E).

To examine the genotype of the male gamete that was sterile in the *S25* heterozygous plants, we performed the segregation analysis for pollen sterility in the progeny of reciprocal crosses between the *S25* semi-sterile plants (*S25* SS) and ‘Asominori’. When *S25* SS plants were pollinated

with ‘Asominori’ pollen, 41 fertile and 32 semi-sterile plants were observed, whereas when *S25* SS plants were used as the pollen parents, all progenies were semi-sterile ($n = 160$), indicating that only the male gametes carrying *S25-IR24* alleles are fertile and those carrying *S25-Asominori* alleles were sterile.

Comparison of the 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* alleles 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. When comparing these two loci, it is necessary to recognize the genomic position of both types of markers because *S36* was mapped with SSR markers and *S25* was mapped with RFLP markers. Our basic local alignment search tool (BLAST) search using the international rice genome sequencing project (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. 3). 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*.

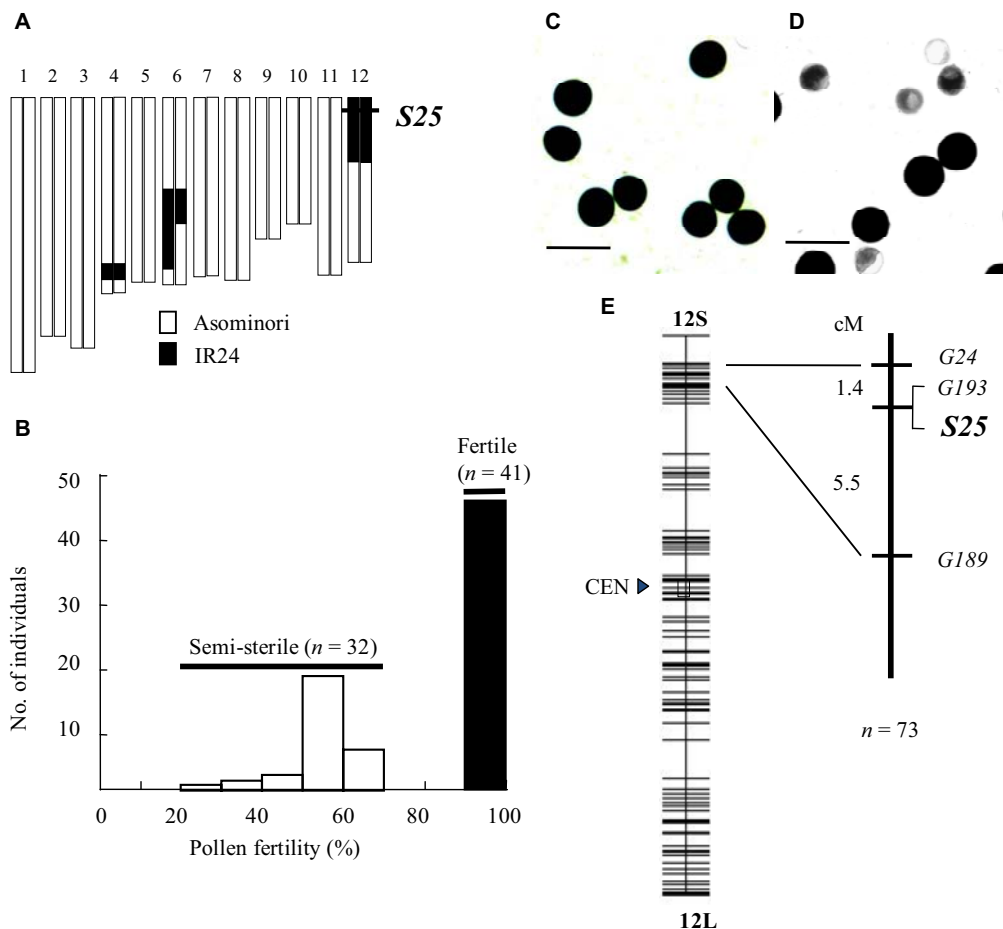


Fig. 2 Identification of *S25* locus causing F_1 pollen sterility. (A) Graphical genotype of the BC_3F_3 plant, a progenitor of the mapping population (BC_5F_1). (B) Frequency distribution of pollen fertility in the BC_5F_1 population, classified by the genotypes of RFLP marker *G193*. Black and white bars represent Asominori homozygote and heterozygote, respectively. (C, D) Pollen grains of semi-sterile (C) and fertile (D) plants stained with I_2 -KI. Scale bars = 50 μ m. (E) Linkage map showing the location of *S25* for F_1 pollen sterility. Left, RFLP framework map was quoted from the latest high-density rice genetic map including 3267 markers, in the rice genome research program (RGP). Available online: <http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>

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 key findings resolving the evolutionary dynamics of post-zygotic reproductive isolation among the Asian cultivated rice and wild relatives.

Characterization of sterile pollen grains caused by *S36*

Since abnormalities of sterile pollen grains differ in various F_1 pollen sterility genes, a diversity of cytological causes of F_1 pollen sterility was suggested to exist in rice (Zhang *et al.* 2005; Jing *et al.* 2007). To elucidate the cytological mechanism of pollen sterility caused by *S36*, pollen development in postmeiotic stages was investigated in the semi-sterile plants using I_2 -KI and hematoxylin staining. No phenotypic abnormality was observed during the unicellular stages. The morphological differences gradually became distinct at the bicellular stage, we identified generative and vegetative cells in all pollen grains at this stage (Fig. 4A, 4B) but half of the pollen grains failed to initiate starch accumulation (Fig. 4C). At the mature stage, almost half of the pollen grains showed typical normal pollen carrying one vegetative cell and two sperm cells (Fig. 4D), but the remainder were mainly at the bicellular stage and could be stained but were not completely stained in I_2 -KI staining (Fig. 4E, 4F). These results suggest that the development of sterile pollen grains caused by *S36* might be arrested at the bicellular

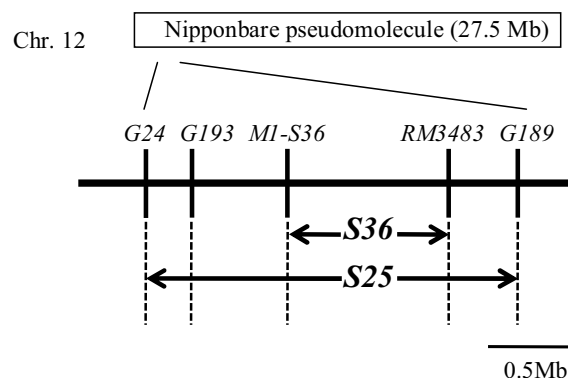


Fig. 3 Comparison between the map positions of *S36* and *S25* based on the genome sequence of chromosome 12. Molecular markers were located on Nipponbare pseudomolecule build 4 of chromosome 12, released from international rice genome sequencing project (IRGSP).

stage after initiation of starch accumulation.

To examine the germination ability, pollen grains from T65 homozygous and *S36* semi-sterile plants collected just after flowering under natural conditions were incubated on artificial germination medium. While about 90% of the pollen from 'IRGC105444' homozygous plants germinated on the artificial medium (Fig. 4G), no germination was observed from all sterile pollen grains in semi-sterile plants (Fig. 4H). These results indicate that the sterile pollen grains lost germination ability.

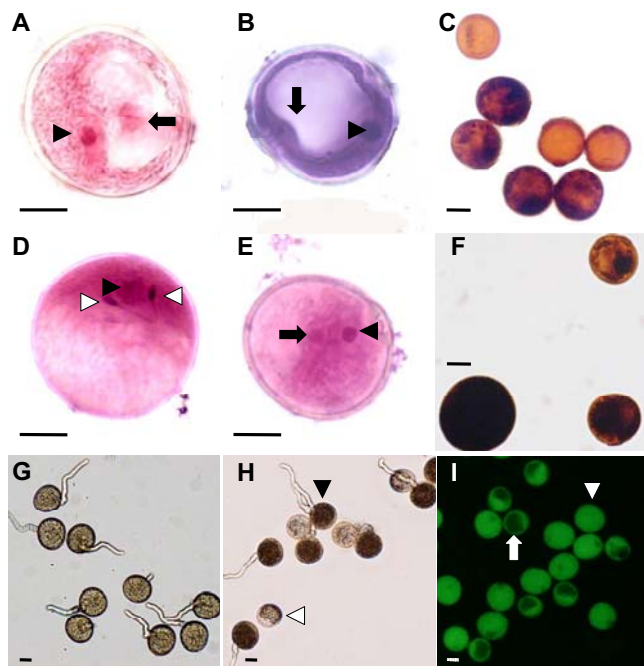


Fig. 4 Morphological features of sterile pollen grains caused by *S36* locus. (A–F) Light-microscopic observation of the postmeiotic pollen development in bicellular (A–C) and mature (D–F) stages in terms of nuclei (A, B, D and E) and starch accumulation (C, F). Normal (A, D) and sterile (B, E) pollen grains. Black and white arrowheads and black arrows indicate nuclei of vegetative cells, sperm cells and generative cells, respectively. (G, H) Germination ability of pollen from IRGC105444 (G) and *S36* semi-sterile plants (H) in an artificial medium. Black and white arrowheads represent fertile and sterile pollen grains, respectively. (I) Fluorescence microscopic observation of the viability of pollen in fluorescein diacetate stain. White arrowhead and arrow indicate fertile and sterile pollen grains, respectively. Scale bars = 10 μ m.

To investigate the pollen viability, pollen grains at a few days before anthesis were stained with FDA. FDA is a non-polar substrate which can pass through cell membrane into the vegetative cell of pollen, and is hydrolyzed by esterase in the cytoplasm to generate the polar product, fluorescein, which is retained by the cell membrane (Heslop-Harrison and Heslop-Harrison 1970). Emission of fluorescein depends on the integrity of the plasmalemma of the pollen grain which closely correlated with viability. Green fluorescence signal was observed in both fertile and sterile pollen grains (Fig. 4I). However, relatively fainter signals which correspond to vacuoles were observed in the center region of sterile pollen grains (Fig. 4I). These results demonstrate that sterile pollen grains caused by *S36* retained viability.

Hybrid sterility, the most common post-zygotic isolating mechanism, plays an important role in speciation and in maintaining species identity (Orr and Presgraves 2000). Understanding the genetic architecture of hybrid sterility at the molecular level still requires further studies, not only to overcome reproductive barriers in transferring valuable genes between species or subspecies but also to clarify divergent evolution of rice species. This study provides some understanding of the genetic mechanisms involved in F₁ pollen semi-sterility and facilitates further clarification of the molecular mechanisms of male gametogenesis and nature of hybrid pollen sterility between cultivated rice and its wild relatives.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation, QTL-5002).

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