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Identification of a novel hybrid sterility locus *S67* between temperate *japonica* subgroup and *basmati* subgroup in *Oryza sativa* L

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Asian cultivated rice (*Oryza sativa* L.) is the most important cultivated species in the AA genome species of the genus *Oryza*. *basmati* is a special and famous subgroup in Asian cultivated rice, and temperate *japonica* is one of the most important cultivated subgroup, too. However, hybrid sterility hinders the introgression of favorable traits and the utilization of hybrid vigour between the two subgroups. The genetic basis of intraspecific hybrid sterility between temperate *japonica* and *basmati* remained elusive. In this study, a novel hybrid sterility locus *S67* was identified, which caused hybrid male sterility in hybrids between the temperate *japonica* rice variety Dianjingyou 1 (DJY1) and the *basmati* rice variety Dom-sufid. Initial mapping with BC_1F_1, BC_4F_1, BC_4F_2 populations and DNA markers located *S67* between RM5362 and K1-40.6 on the long arm of chromosome 1. Genetic analysis confirmed that *S67* caused a transmission advantage for the temperate *japonica* rice *S67-te* allele in the hybrid offsprings. This result not only fills the gap in the research on hybrid sterility between *basmati* and temperate *japonica*, but also lays a good foundation for the systematic study of the genetic nature of hybrid sterility between *basmati* and other subgroups, as well as the full exploration and utilization of this subgroup through the creation of wide or specific compatibility lines to overcome hybrid sterility. In addition, this result can also help us broaden our understanding of genetic differentiation within Asian cultivated rice and hybrid sterility between inter-subgroups.

Keywords Hybrid sterility, *Oryza sativa*, *basmati*, Temperate *japonica*, *S67*

Rice is one of the most important food crops in the world, feeding more than half of the global population[1]. The utilization of heterosis played a significant role in improving rice yield per unit area[2]. However, the growth of rice yield entered a bottleneck period, the fundamental reason is that the genetic diversity of hybrid rice parents is narrow[3]. Asian cultivated rice, the most important cultivated species in the AA genome rice species of the genus *Oryza*, mainly comprises two subspecies, *indica* and *japonica*[4,5], and some relatively small ecological subgroups. Currently, most studies classified Asian cultivated rice into five subgroups based on molecular biology, genomics, and other characteristics: temperate *japonica*, tropical *japonica*, *indica*, *aus* and *basmati*[6–12]. Numerous studies and breeding practices shown that the hybridization-introgression of subgroups within Asian cultivated rice played a very important role and had great potential for the utilization of heterosis and the improvement of rice yield, quality, and resistance[10,11,13–15]. Thus, fully mining and introgression of favorable alleles from minor subgroups of Asian cultivated rice is an important way to enrich the genetic diversity of existing breeding populations.

Basmati is a unique subgroup of Asian cultivated rice, mainly distributed in countries in South Asia, Southeast Asia, Central Asia, and West Asia such as Pakistan, India, Bangladesh, Myanmar, Iran, etc.[8,16–19]. It is one of the important agricultural trade commodities in these regions[20,21]. The main feature of *basmati* rice exhibits its excellent longitudinal elongation of rice grains during cooking (about twice as much as before), and the soft and fluffy texture of cooked rice, with a unique nutty aroma, known as the "king of rice". *basmati* rice is rich in trace elements such as zinc and iron, and has a low blood sugar index[19]. In addition, *basmati* rice also contains rich WA-CMS restorer resources and significant potential for nitrogen efficient utilization and storage tolerance[22–25].

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Therefore, *basmati* rice not only has very high economic value and international trade status, but also has a very important position and significance in the classification, genetic research, and breeding application in Asian cultivated rice. Temperate *japonica* is one of the main subgroup of Asian cultivated rice, mainly distributed in a few countries and regions such as East Asia, the Mediterranean, Europe, and North America, including China, Japan, South Korea, Egypt, North Korea, the United States, etc. The annual planting area of temperate *japonica* accounts for about 9% of the world's total rice area, and the total yield accounts for about 14% of the world's total rice production. Given the excellent quality, its market demand continues to increase. However, the breeding and production of temperate *japonica* rice also faces serious problems, such as insufficient genetic diversity of germplasm resources. *basmati* is a very excellent germplasm resource, which can be used for genetic improvement by hybridizing with temperate *japonica* varieties, which helps breed rice varieties with better yield, quality, and adaptability. Unfortunately, the severe hybrid sterility between these two subgroups limits the utilization of heterosis and introgression breeding between them[26]. Identifying and analyzing the hybrid sterility genes between them can help overcome hybrid sterility and better understand the nature of this reproductive barrier, and promote the application of distant parents in hybrid breeding.

Hybrid sterility is the most common form of postzygotic reproductive isolation in plant species. The hybrid sterility between Asian cultivated rice *indica* and *japonica* subgroups is the most classic case of postzygotic reproductive isolation and has always been a focus of genetic research. So far, more than 30 genes/QTL conferring sterility in inter-subspecific hybrids in Asian cultivated rice were reported[12,27–29], of which seven hybrid sterility loci (*S5*, *Sa*, *hsa1*, *S7*, *Sc*, *RHS12/Pf12/Se, DPL1/DPL2*) were cloned[26,30–37]. Twenty-two hybrid sterility loci were described between *indica* and temperate *japonica* cultivars, including major genes such as *S5*, *Sa*, *Sc*, *RHS12/Pf12/Se*[12]. In addition to *indica* and temperate *japonica*, some hybrid sterility loci were also found in other subgroups of Asian cultivated rice. *DPL1*/*DPL2* resulted in male gametes abortion and *qSIG3.1*, *qSIG3.2*, *qSIG6.1* and *qSIG12.1* resulted in female gametes abortion in the crosses between temperate *japonica* and *aus*[33,38],*S7* and *S15* were responsible for female gamete sterility in the hybrid between *indica* and *aus*[37,39], *S8*, *S9*, *S16*, *S17*, *S29*, *S31*, *S32*, *qSS-2*, and *qSS-8b* gave rise to female gametes abortion in the hybrid between tropical *japonica* and temperate *japonica*[40–46], *S7*, *S8*, *S9*, and *S35*(t) led to the female sterility in the cross between tropical *japonica* and *indica*[39,47,48], *S7* and *S9* controlled the hybrid female sterility between tropical *japonica* and *aus*[39,49]. These hybrid sterility genes or QTL identified in the different subgroups will lay the foundation for elucidating the genetic and molecular mechanisms of hybrid sterility in Asian cultivated rice, and for breeding utilization. But, so far, due to the severe hybrid sterility between *basmati* and other subgroups, the hybrid introgression and utilization of favorable agronomic traits are very limited. A few of research was also limited to describing the phenomenon of hybrid sterility between *basmati* and other subgroups or the coincidental utilization of single hybrid sterility gene[50–52]. At present, there are no systematic studies on hybrid sterility between *basmati* and other subgroups, and no hybrid sterility genes or QTL were identified in the cross between them.

In the present study, a major inter-subgroup hybrid male sterility locus *S67* in the hybrids between *basmati* and temperate *japonica* in Asian cultivated rice that conferred selective abortion of male gametes carrying the *basmati* allele, giving a transmission advantage to the temperate *japonica* allele, was identified. *S67* was delimited between RM5362 and K1-40.6 on the long arm of chromosome 1 by linkage analysis. In addition, the degree of segregation distortion and the mode of gamete transmission were analysed by developing reciprocal test crosses between the plants with the NIL-*S67*(H) genotype in BC_4F_2 and recurrent parent DJY1. Judging from the locus location and action mode, this locus was confirmed as a new finding of hybrid sterility between *basmati* and temperate *japonica*.

Results

The confirmation *of hybrid* sterility between *basmati* and temperate *japonica* rice

Severe pollen and spikelet sterility was observed in the F_1 obtained between *basmati* accession Dom-sufid and temperate *japonica* cultivar DJY1 (Fig. 1). The pollen and spikelet fertility distribution of BC_1F_1 to BC_3F_1 was continuous, fertile, semi-sterility, and sterility plants could still be found, and gradually showing a bimodal distribution with the increase of backcross generations (Fig. 2a–c), while BC_4F_1 only had fertile and semi sterility plants (Fig. 2d).

Detection of QTL for hybrid sterility in BC_1F_1

The linkage map comprised of 663 polymorphic SNP markers and spans 2311.93 cM. The mean interval between markers was about 3.49 cM. QTL for pollen and spikelet fertility analyzed by composite interval mapping was presented in Table 1. Two QTL (*qSS3*, *qSS6*) for spikelet fertility were detected on chromosomes 3 and 6, explained 8.48% and 20.35% of the total spikelet fertility variation, respectively, and accounted for 28.83% of the total variation. Three QTL (*qPS1.1* and *qPS1.2*, *qPS2*) for pollen fertility were detected on chromosomes 1, and 2. These QTL individually explained 5.52%–53.80% of the total phenotypic variation, and accounted for 66.14% of the total variation. Among these QTL, *qPS1.2* was the most effective QTL, which explained 53.80% of the total pollen fertility variation, and was selected for in-depth mapping and genetic analysis in this study. Then, based on the molecular marker RM5362 (https://archive.gramene.org/markers/) genotypes (Table 2), tightly linked to *qPS1.2* locus, and the phenotypes in each generation, continuous backcross to obtain BC_4F_1 was made.

Genetic linkage analysis and mapping of the *S67* locus

In BC_4F_1, only two type plants: fertile and semi-sterility could be found, and they were used to detect for genetic background and target fragments introgression using Rice 6 k Chips[53]. The results showed that about 97% of the genetic background of BC_4F_1 plants was the same as that of their recurrent parent DJY1 (Fig. 3), and introgression fragments of the *qPS1.2* locus was also detected in 5 semi-sterility pollen grain plants, 136-1-1, 136-1-5, 136-1-6,

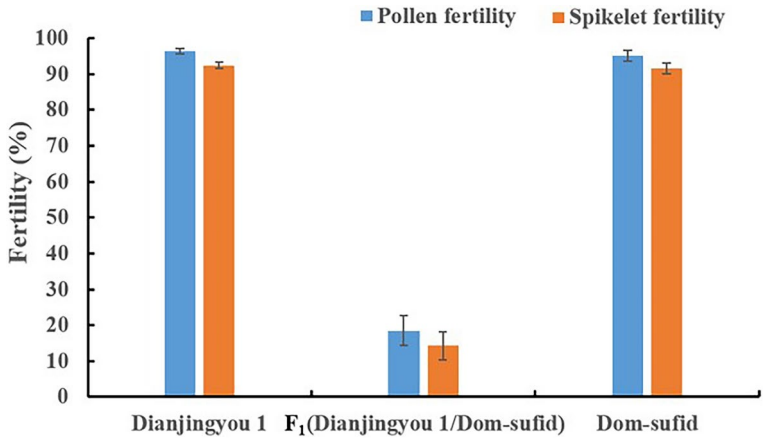

Fig. 1. F_1 (DJY1/Dom-sufid) exhibits pollen and spikelet sterility. Pollen fertility of DJY1, Dom-sufid and F_1 plants was determined based on the average value of five independent florets from DJY1, Dom-sufid and F_1 plants. Data are shown as means ± SD (n = 5). Spikelet fertility of DJY1, Dom-sufid and F_1 plants was determined based on the average value of five independent panicles from DJY1, Dom-sufid and F_1 plants. Data are shown as mean ± SD (n = 5).

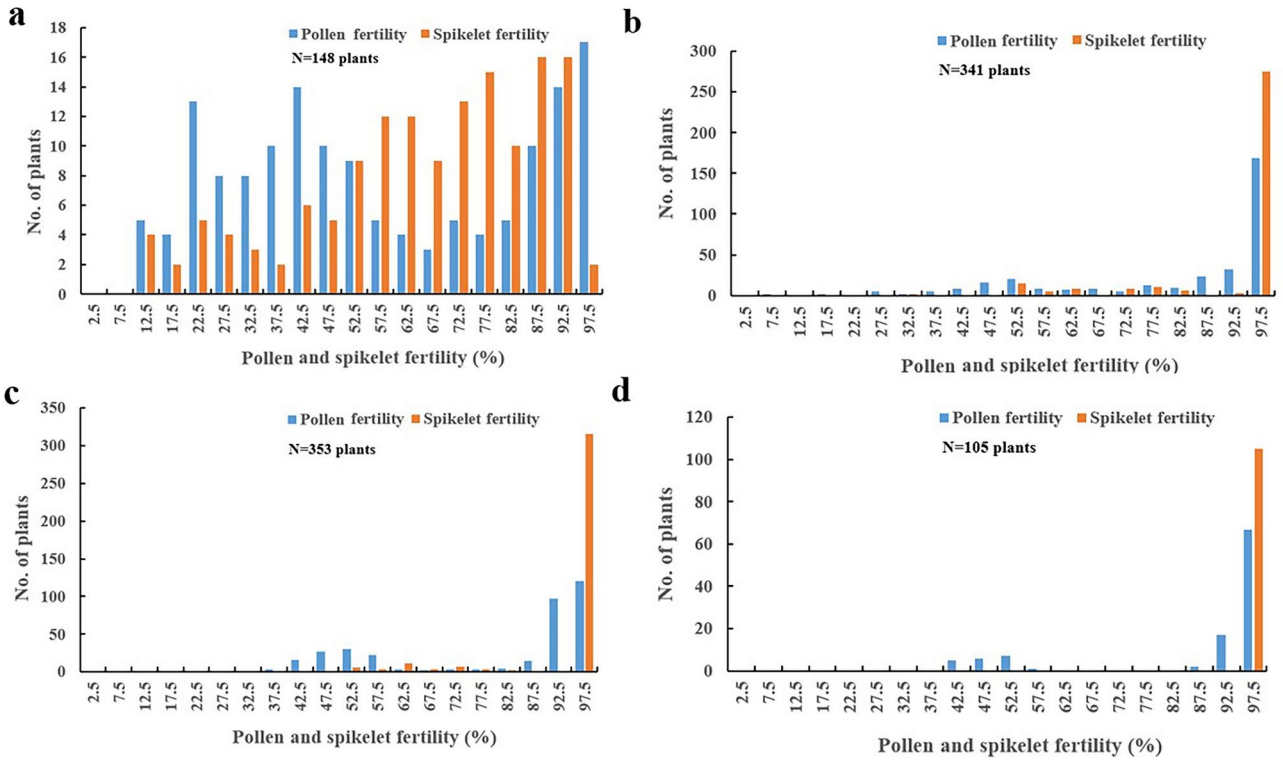

Fig. 2. The frequency distribution of pollen and spikelet fertility in BC_1F_1 (**a**), BC_2F_1 (**b**), BC_3F_1 (**c**), and BC_4F_1 (**d**).

136-1-7, 136-1-10. To map *qPS1.2*, one of the semi-sterility individual 136-1-1, which had a clean background and carried the *qPS1.2* locus in BC_4F_1, was selected to self and form BC_4F_2 mapping population.

Among 390 plants in the BC_4F_2 population, pollen fertility showed bimodal distribution, divided into semi-sterility and fertile and spikelet fertility of all plant was normal (Figs. 4 and 5). By means of linkage analysis using phenotypic data and six polymorphic molecular markers (Table 2), *qPS1.2* was located in a 2.95 cM region flanked by RM5362 and K1-40.6 on the long arm of chromosome 1 (Fig. 6).

Trait	Chromosome	Position (cM)	Left Marker	Right marker	LOD	PVE (%)	QTL
Pollen fertility	1	114.71	OsGRb01924	OsGRg01176	4.81	6.82	*qPS1.1*
Pollen fertility	1	165.70	OsGRb02912	OsGRb02939	26.03	53.80	*qPS1.2*
Pollen fertility	2	89.04	OsGRg03023	OsGRb04514	4.03	5.52	*qPS2*
Spikelet fertility	3	49.01	OsGRg04981	OsGRg04861	6.44	8.48	*qSS3*
Spikelet fertility	6	80.58	OsGRb30999	OsGRg09935	3.71	20.35	*qSS6*

Table 1. QTL detected for pollen and spikelet fertility based on composite interval mapping in the BC_1F_1 population.

Marker name	Primer sequence	Position	Marker type
K1-40.7	GACGAACAATCGGTTGGGTTTCAGAGTTAGCTTCAGAGTAGACAGAACGC[A/G]ACCAAAATCAAATATGAATATGTATTTTTA TACCTGCAAGTTGCAGCAAC	40,448,552	SNP
K1-40.9	TTGAAGTCCTGGCAGACGTGCGCCACCGCTGGCTTCTCCGGTGGGCTGCT[A/C]CGTGCCATCTGCGCGATGATGGCGTTGTT GCTCTCCCAGAATCCCATTTC	41,067,447	SNP
RM5362	F-GCGCTAGGGCTTTGGATC R-TACCTTCCTTACTCTGCCCG	41,087,022	SSR
K1-40.6	TGGTCCGGAGTCGGGTCTCCTCCAGCGCGAAGCTGACGAACTTGTGGTAC[A/G]GCAAGCCGGCGCCCAAGCTCCCGCCGCC GACGCCGCCGCTGCTCATGCTC	41,824,986	SNP
K1-40.11	CTGCCATTTACTAGTAGTAGGGGAGCGTGAAGGTTGCTCGATGCATTTAT[T/C]CCGGACGACGGGATAACTCGATGGATCGCT GCACCGTGTACACAAGCAAA	42,050,647	SNP
RM1067	F- CGATGGAGAGAGAATGTCTAGC R- TAATACGCAAGGCAGAAGGG	42,923,261	SSR

Table 2. Primer sequences of molecular markers for mapping the *S67* locus on rice chromosome 1. The SSR markers can be found in the Gramene Markers Database (https://archive.gramene.org/markers/), and the KASP markers were designed based on the SNP marker information of the Illumina Rice 6K Chips.

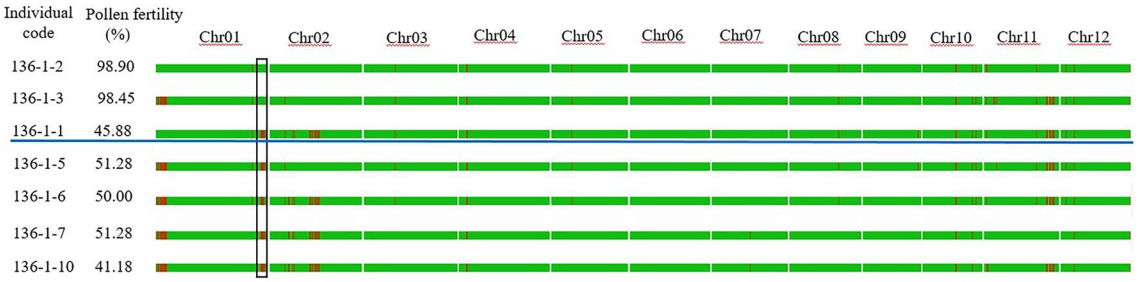

Fig. 3. Rice 6k Chips detection of genetic background and target fragment introgression. The first column is the individual plant code of BC_4F_1 in 2021 Late Crop Season in Xishuangbanna, Yunnan province, P. R. China. Green represents the homozygous genotype of DJY1; Red represents the heterozygous genotype of DJY1 and Dom-sufid; Yellow represents the homozygous genotype of Dom-sufid. The black wireframe represents the area where the target locus is located. The individual with the blue line below had the least background interference and was selected to selfing to form the BC_4F_2 mapping population.

Segregation distortion and gametes transmission of the *S67* locus

To clarify the relationship between the introgression segments and the semi-sterility phenotype, the pollen fertility of 390 BC_4F_2 plants and the segregation of their genotypes of the closely linked molecular marker RM5362 was analysed. One hundred and eighty-six plants exhibited semi-sterility pollen grain and 204 plants exhibited fully fertile pollen grain in BC_4F_2 were found. All plants that exhibited semi-sterility carried heterozygous genotypes and all plants that showed fully fertile pollen grain harbored the homozygous temperate *japonica* DJY1 genotype, which were deteceted with molecular markers RM5362. No any plants with homozygous *basmati* Dom-sufid genotype was found in this population. In this segregating population, the segregation ratio of fertile and semi-sterility was 1:1, which did not conform to classical Mendelian inheritance and was a typical hybrid sterility (Table 3). In fact, no plants with homozygous *basmati* Dom-sufid genotype were detected in another large population of 1344 BC_4F_3 plants (Additional file 1: Table S1). By reviewing previous studies, *qPS1.2* was a novel pollen grain hybrid sterility locus, and was named as *S67*. Plants with heterozygous *S67* (*S67-te*/*S67-b*) genotype in BC_4F_2 were selected as a near isogenic line, NIL-*S67*(H).

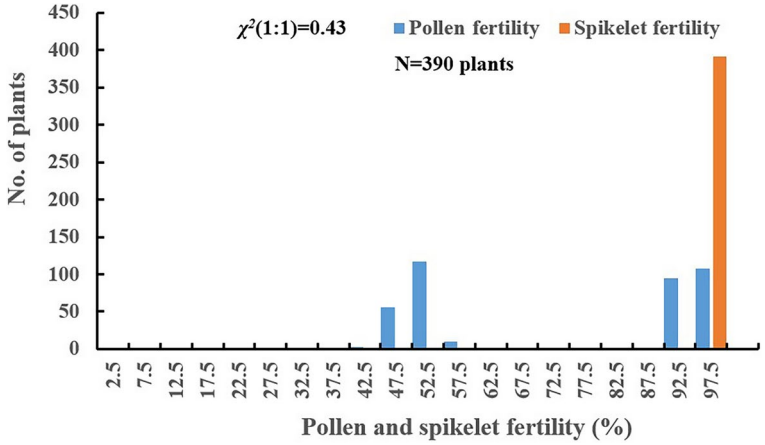

Fig. 4. Distribution of pollen and spikelet fertility in BC_4F_2 mapping population. This population was obtained by selfing of a single plant 136-1-1 in BC_4F_1.

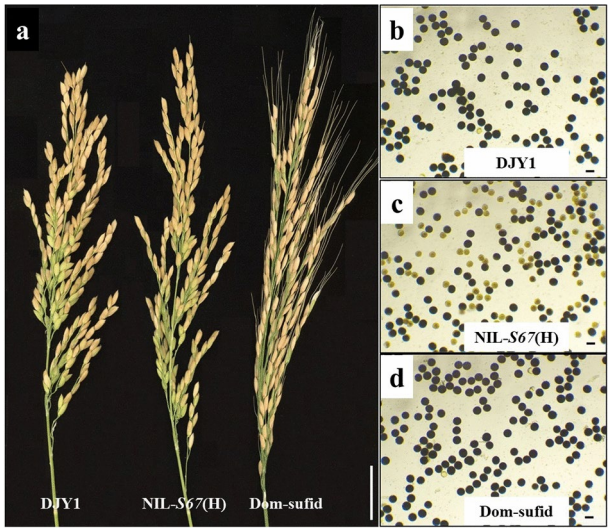

Fig. 5. NIL-*S67*(H) exhibits pollen semi-sterility. (**a**) Panicles of DJY1, Dom-sufid and NIL-*S67*(H) plants. Scale bars, 3 cm. (**b**–**d**) Pollen grains from DJY1, Dom-sufid and NIL-*S67*(H) plants stained with a 1% (w/v) iodine-potassium iodide solution. Scale bars, 100 μm.

For the convenience of description, the *basmati S67* allele was named as *S67-b* and the temperate *japonica* DJY1 *S67* allele as *S67-te*. Given that no homozygous plants having the *S67-b*/*S67-b* genotype using the SSR Marker RM5362 in BC_4F_2 population were found (Table 3), therefore reciprocal test crosses between the NIL-*S67*(H) and DJY1 were made to further analyze the gametes transmission. When the NIL-*S67*(H) was used as the female parent, the segregation ratios of the NIL-*S67*(H) genotype plants and DJY1 genotype plants fitted a 1:1 ratio (Table 3). This indicates that both the *S67-te* and *S67-b* female gametes in the NIL-*S67*(H) were normally fertile, which corresponds to the normal seed setting of the field plants. Nevertheless, when the NIL-*S67*(H) was used as the male parent in a cross with DJY1, only DJY1 genotype F_1 plants were obtained (Table 3 and Fig. 7). This observation showed that the *S67-b* type male gametes in the NIL-*S67*(H) are fully sterile, and *S67-te* has a strong transmission advantage.

Discussion

Hybrid sterility was always considered as a very complex quantitative trait, and the efficiency of using different strategies to map hybrid sterility locus varies greatly. QTL mapping can be performed in low backcross generations, such as BC_1F_1, to prevent the loss of hybrid sterility loci with smaller effects. However, it is important to avoid using the F_2 population as much as possible, as there may be interference from hybrid breakdown. Due to their susceptibility to environmental factors, QTLs that are sensitive to the environment and have minimal effects are often prone to be false positives. Another strategy is to not conduct QTL testing in the low generation, but to select sterile plants in the population through continuous backcrossing based on phenotype, ultimately

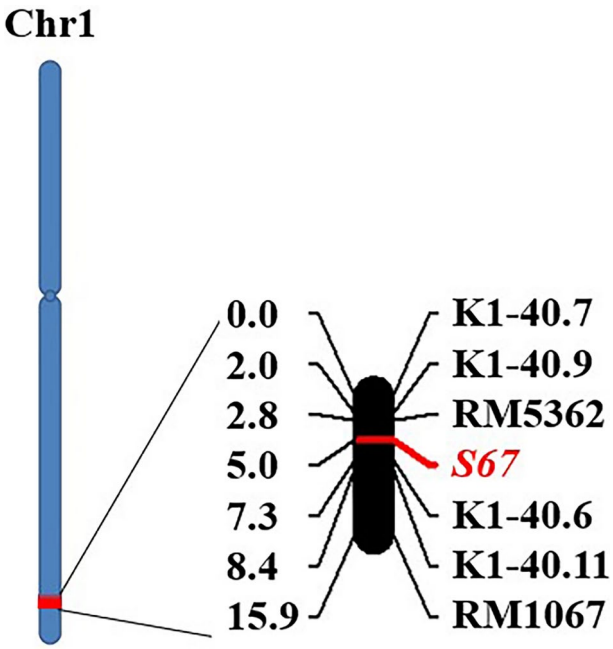

Fig.6. The position of *S67* in BC_4F_2 and segmental linkage maps of locus mapped in BC_4F_2 mapping population for pollen hybrid sterility in rice chromosome 1.

Population	Generation	Number of plants	Pollen fertility	*S67* genotype	χ^2 (1:2:1)	χ^2 (1:1)
S67	BC_4F_2	0 186 204	Fully fertility Semi-sterility Fully fertility	*b/b* *b/te* *te/te*	214.25***	0.83
NIL-*S67*(H)/DJY1	F_1	50 50	Semi-sterility Fully fertility	*b/te* *te/te*	–	0.00
DJY1/ NIL-*S67*(H)	F_1	0 100	Semi-sterility Fully fertility	*b/te* *te/te*	–	100.00***

Table 3. Segregation analysis of *S67* alleles in different populations. The allele *S67-b* and *S67-te* were abbreviated as *b* and *te*, respectively, as determined using the SSR marker RM5362. Fourty to sixty percent of pollen fertility was defined as semi-sterility, ~ 90% pollen fertility was defined as fully fertility. Chi-squared tests (χ^2) were performed for the segregations of the *S67* genotypes in the BC_4F_2 and F_1 and test-cross populations. ***Significant at $P < 0.001$.

transforming quantitative traits into qualitative traits. In this study, we found that the pollen and spikelet fertility distribution of BC_1F_1 to BC_3F_1 was continuous, fertile, semi-sterility, and sterility plants could still be found, and gradually showing a bimodal distribution with the increase of backcross generations (Fig. 2a–c), while BC_4F_1 only had fertile and semi sterility plants (Fig. 2d). The results showed that inter-subgroup hybrid sterility between temperate *japonica* and *basmati* in Asian cultivated rice controlled by multiple genes in the preliminary populations was gradually decomposed into a simple inheritance trait, and a significant amount of genetic noise was removed after four generations phenotypic selection and continuous backcrossing, which facilitated the detection and confirmation of loci in the advanced backcrossing population.

Basmati is a very special and famous subgroups of the Asian cultivated rice germplasm, but it was not fully utilized yet, and the main reason is that the relationship between *basmati* and other groups in Asian cultivated rice is unclear. In terms of grain morphology, some varieties of this group had slender grains, and some had short round grains[17,19]. Some scholars believed that it belonged to the *indica*[54], while others classified them as the *japonica* type[50]. Numerous studies on the genome classified *basmati* as *japonica* or similar to *japonica*[7,10,11,55]. In terms of origin and evolution, some studies suggested that *basmati* was derived from hybridization-introgression between *aus* from South Asia and *japonica*[6,12,56]. Some scholars also believed that *basmati* was formed through domestication and selection of ancient *japonica* rice from China to South Asia after being transmitted[57–59]. There were also views that *basmati* was directly domesticated from local *O. rufipogon* in South Asia[60]. In short, different research led to distinct results. So, as indication of species formation, the relationship between *basmati* and other subgroups from the vision of reproductive isolation is the key to know the difference. In the present study, severe hybrid sterility between *basmati* and temperate *japonica* was found, and a novel hybrid male sterility locus *S67* was identified, which will greatly help understand the hybridization compatibility between *basmati* and other subgroups from a new perspective, and will also help us fully understand the genetic differentiation

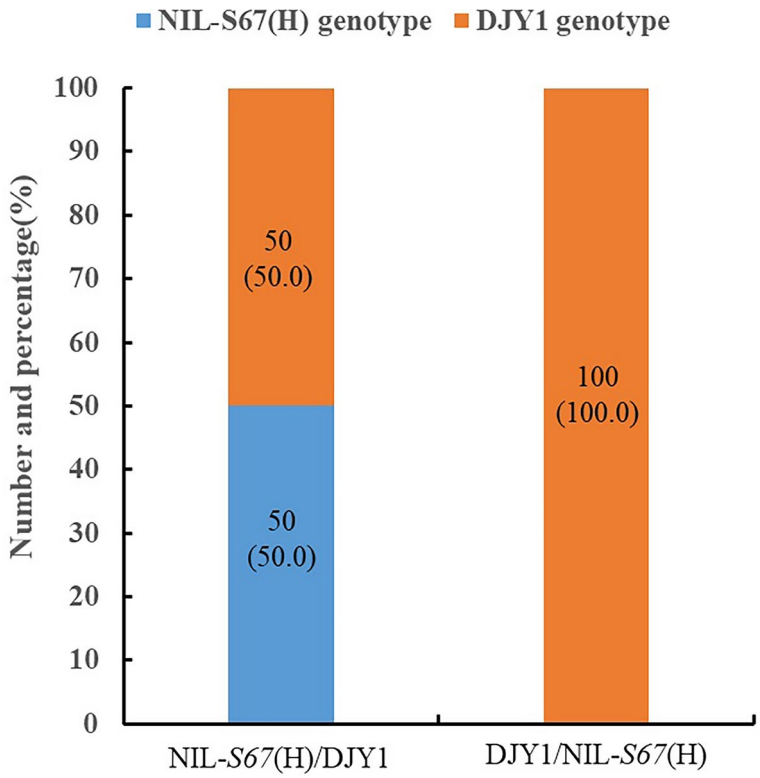

Fig. 7. Number and percentage of the DJY1 genotype and NIL-*S67*(H) genotype plants in the hybrid F_1 generation of NIL-*S67*(H)/DJY1 and DJY1/ NIL-*S67*(H).

of Asian cultivation rice from the perspective of reproductive isolation. However, in order to comprehensively and scientifically explain the hybrid sterility relationship between *basmati* and other subgroups, it is necessary to further systematically map and analyze the corresponding hybrid sterility genes.

To this day, about 50 hybrid sterility loci/QTL were discovered and reported, with more than 30 responsible for the crosses between Asian cultivated rice two subspecies, and these loci mainly contributed to the hybrid sterility between *indica* and temperate *japonica*[12,27,28]. But there are no reports on the hybrid sterility loci of *basmati*. In this study, a new hybrid male sterility locus, *S67*, was discovered in hybrids between the *basmati* variety Dom-sufid and the temperate *japonica* variety DJY1, and mapped between RM5362 and K1-40.6 on the long arm of chromosome 1. In this chromosomal region, previous studies reported one inter-subspecific hybrid sterility locus, *S16*, between *indica* and *japonica*, which was a hybrid sterility locus affecting female gametes[43]. Another locus *S58* controlled the hybrid male sterility in Asian–African cultivated rice hybrids was reported, too[61]. Unlike the gamete elimination of *S1*[62], the finding showed that male gametes carrying the *S58-g* allele (African rice-type *S58* allele) were eliminated, resulting in the male gametes with the *S58-s* (Asian rice-type *S58* allele) allele gaining a transmission advantage. Both *S67* and *S58* are hybrid sterility sites that affect male gametes, and the type of pollen sterility is stained abortion type. The difference is that they lead to hybrid sterility between different subgroups and species, respectively. Interestingly, if we assume that they are different haplotypes on the same locus, the differentiation under different conditions leads to reproductive isolation between *basmati* and temperate *japonica*, *O.sativa* and *O. glaberrima*. In addition, what is the haplotype differentiation of this locus in *indica*, *aus*, tropical *japonica*, and other wild relatives of Asian cultivated rice? It is speculated that in-depth study of the vertical homology relationship of this gene is of great significance and role in understanding the origin and evolution of the AA genome species in the rice genus. This result not only fills the gap in the research on hybrid sterility between *basmati* and temperate *japonica*, but also lays a good foundation for the systematic study of the genetic rules of hybrid sterility between *basmati* and other subgroups.

Basmati is a very excellent germplasm resource with rich genetic variation and great potential for increasing yield, improving quality and adaptability. However, the heterosis generated by hybridization between *basmati* and other subgroups is limited by hybrid sterility, thus overcoming the reproductive barrier by adopting different methods and technical means is the key step to use the diversity of *basmati*. Varieties carrying natural or artificial neutral alleles of hybrid sterility loci are important germplasm resources for overcoming the hybrid sterility[28]. Previous studies have shown that the main hybrid sterility genes between *indica* and *japonica* subspecies are *Sb*, *Sc*, *Sd*, *Se*, *f5*, *pf12*, and *S5*. The *indica*-compatible *japonica* lines developed by pyramiding the *indica* allele (*S-i*) at *Sb*, *Sc*, *Sd* and *Se* loci and the neutral allele (*S–n*) at *S5* locus in *japonica* genetic background through marker-assisted selection are compatible with *indica* rice in pollen fertility and in spikelet fertility[63]. Some researchers constructed and assembled different combinations of naturally compatible alleles of four loci, *S5*, *Sc*, *pf12*, and *f5*, and the improved lines can fully recover pollen and embryo-sac fertility in testcrossed F_1s, thereby completely

fulfilling the demands of inter-subspecific hybrid spikelet fertility in production[36]. Cloning hybrid sterility genes and clarifying their genetic mechanisms are prerequisites for creating widely compatible lines. In this study, only the preliminary localization of *S67* was completed, and the research on cloning and genetic mechanisms is ongoing. But before that, we still have other methods to overcome hybrid sterility between *basmati* and temperate *japonica*. The "bridge parents" developed by fixing the basmati allele at *S67* loci in temperate *japonica* genetic background by repeatedly backcrossing and molecular assisted selection is one way to recover the fertility of hybrid offspring between two subgroups. Nine NILs, including NILs with *O. glaberrima* fragment at six hybrid sterile loci under *O. sativa* genetic background, two lines harboring two hybrid sterile loci, one line harboring three hybrid sterile loci, were used to test cross with *O.glaberrima* accessions. The results showed that compared to single-locus-NILs, the multiple-loci-NILs showed increasing effect on pollen fertility when test crossing with *O. glaberrima* accessions. Further backcrossing can improve the fertility of pollen grain and spikelet of interspecific hybrids, too[64]. Certainly, we need to identify as many major hybrid sterility loci as possible between *basmati* and temperate *japonica*, and analyze their molecular mechanisms. Ultimately, we can overcome the hybrid sterility completely through gene editing plus target chromosome fragment replacement. In addition, extensive test cross can be conducted in temperate *japonica* germplasm to unearth widely compatible varieties carrying natural neutral alleles of *S67* as "bridge parents" for hybridization with *basmati* to overcome hybrid sterility.

Materials and methods

Materials

One *basmati* variety named Dom-sufid introduced from the International Rice Research Institute (IRRI) was as the donor parent, one *O. sativa* ssp. temperate *japonica* variety, Dianjingyou 1 (DJY1), from Yunnan province, P. R. China, as the maternal, were used to obtain F_1. Afterwards, selected the sterility plants in each generation as female parent and continuously backcrossed with DJY1 were made until BC_4F_1. The cultivation of all relevant populations was completed in Xishuangbanna, Yunnan province, P. R. China. In the BC_1F_1, 148 plants were used for QTL detection. Meanwhile, after phenotypic evaluation and marker-assisted selection, sterility individuals (pollen fertility below 90% and the genotype is heterozygous) were randomly selected as the maternal for continuous backcrossing until BC_4F_1. A total of 5 pollen grain semi-sterility plants were selected in the backcross line 136 of BC_4F_1 (Fig. 3). The background and target fragment introgression of plants in BC_4F_1 were screened by using Rice 6 k Chips[53]. BC_4F_2, obtained by selfing of the target individual from the BC_4F_1 population, was used for target locus mapping and near isogenic line raising. To study the gamete elimination and transmission pattern, reciprocal crosses were made between heterozygous individual in BC_4F_2 and recurrent parent to obtain F_1.

Observation of pollen grain and spikelet fertility

Anthers were collected from spikelet of the upper and middle parts of the main panicle at 1 to 2 days before anthesis, fixed and stored in 70% ethanol for determining pollen fertility[65]. Pollen fertility was estimated based on the percentage of pollen grains stained with 1% (w/v) iodine-potassium iodide solution. The pollen grain sterility types were classified as typical, spherical and stained abortion types. Observation requires at least three independent microscopic fields, at least 100 pollen grains in each field were scored for counting the percentage of fertile pollen grains in each plant. Spikelet fertility was calculated as a percentage of fertilized spikelet per panicle for each of the individuals involved.

Detection of QTL and DNA analysis

To detect the QTL, a linkage map of the BC_1F_1 population was constructed using QTL IciMapping Version 4.2[66], with a minimum LOD score of 3.0. Composite interval analysis was conducted to determine QTL related to the tested traits. The experiment-wide LOD (log of the odds ratio) threshold significant level was determined from 1000 permutation tests[67], as implemented by QTL IciMapping. The genotype data of BC_1F_1 was obtained using rice 10 K Liquid Chips by Boradi Biotechnology Co., Ltd, Shijiazhuang, Hebei. BC_4F_2 population was used to target gene mapping and genetic analysis. QTL IciMapping Version 4.2 and MapChart 2.32 were used to construct linkage groups and map hybrid sterility loci.

Ten days after transplanting, fresh leaves from each individual were sampled for extraction of genomic DNA using the CTAB method[68]. SSR and KASP markers, distributed throughout the entire rice genome were used for polymorphism screening and genotyping. Rice 6 k Chips was used to detect target fragments infiltration and background replacement. The protocol of Rice 6 k Chips as Infinium HD Assay Ultra Protocol Guide (https://support.illumina.com.cn/downloads/infinium_hd_ultra_assay_protocol_guide_(11328087_b).html) was used. GGT 2.0 software was used to map and view the infiltration of target trait related fragments and background replacement.

Genetic and gametic transmission assay

To determine the genetic and gametic transmission nature of *S67*, the segregation ratios of the *S67-b* (from *basmati*) and the *S67-te* (from temperate *japonica*) alleles were investigated in three populations: BC_4F_2, NIL-*S67*(H)/DJY1 and DJY1/NIL-*S67*(H). The *S67* genotypes in these populations were checked using the SSR marker RM5362 (Table 3), allowing to analyze the transmission rates to offspring of the *S67-b* allele and the *S67-te* allele.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author (taody12@aliyun.com) on reasonable request.

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Author contributions

D.T and Y.Z conceived and designed the experiments. Y.L participated in phenotyping, genotyping, drafting the manuscript. J.L and Y.Y participated in phenotyping and genotyping. Q.P, J.Z, and X.D participated in phenotyping. D.T and Y.Z corrected manuscript. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethics declarations

The plant collection and use was in accordance with all the relevant guidelines.

Additional information

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