# Non-redundancy of Rice Mutant Library for Male Gametogenesis Confirmed by Bulked Segregants Analysis Using Illumina BeadsArray

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# Non–redundancy of Rice Mutant Library for Male Gametogenesis Confirmed by Bulked Segregants Analysis Using Illumina BeadsArray

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Mutant resources play a crucial role in unraveling the genetic network controlling traits of interest. In this study, we focused on pollen–sterile mutants as important genetic resources for understanding the genetic regulation of male gametogenesis. We employed high–throughput genotyping technologies using Illumina BeadArray to map the responsible genes for male gametogenesis in rice; four *SPOROPHYTIC POLLEN STERILITY* (*SPS2, SPS3, SPS4, SPS7*) and four *GAMETOPHYTIC POLLEN STERILITY* (*GPS1, GPS2, GPS9, GPS10*). Using 287 single nucleotide polymorphism (SNP) markers, we detected polymorphisms between the *japonica* cultivar Taichung 65 (T65) and Hinohikari. Bulked segregant analysis (BSA) based on DNA marker analysis was performed to identify candidate markers tightly linked to the causal genes. The analysis revealed candidate markers for each mutant line, such as Mk240 for *SPS2* and Mk94 and Mk126 for *GPS1*. Linkage mapping using the PCR–based markers confirmed the map positions of these candidate markers. Our study demonstrates the utility of high–throughput genotyping technologies combined with BSA for the genetic characterization of mutant stocks. The identified candidate markers provide valuable resources for future studies aiming to understand the molecular mechanisms underlying pollen development and male gametogenesis in rice. The systematic gathering and reduction of redundancy in pollen sterile mutants are essential for a comprehensive understanding of the molecular networks involved in post–meiotic male gametogenesis.

**Key words**: Rice, Mutant, Pollen sterility, Bulked segregant analysis, Illumina Beadsarray

## INTRODUCTION

Mutants are important genetic resources to reveal genetic networks controlling traits of interest by cloning causative genes and their physiological and biological characterizations. To understand the functions of the genes that regulate biological processes, molecular biological and biochemical analyses using mutant resources are extensively used in plant model species. At the beginning of a post–genome era, the forward genetics approach by positional cloning and reverse genetics approaches by T–DNA or transposon tagging and targeting induced local lesions in genomes (TILLING) have dramatically promoted cloning and characterization of genes functionally unknown (Osborne *et al*., 1991; Hirochika *et al*., 2001; Tzfira *et al*., 2004; Comai *et al*., 2006). Moreover, high–throughput sequencing technologies by next–generation sequencers (NGS) would allow rapid identification of causal mutation (for review, Schneeberger, 2014). Therefore, developing a series of mutant libraries associated with specific biological phenomena of interest becomes more important to clarify molecular players on a pathway.

Pollen–sterile mutants have been considered critical genetic resources for understanding male gametogenesis's genetic regulation (Twell, 2011; McCormick, 2004). On male gametogenesis, one male meiocyte produces four haploid cells (microspores) through the meiosis, and a microspore generates a tricellular pollen grain containing one vegetative cell and two sperm cells by two rounds of asymmetric mitosis. During the development, zygotic nursery tissues, tapetum, supply nutrition indispensable for developing male gametophytes and eventually disappears at bicellular to mature stages of male gametogenesis.

Genetic defects for gametogenesis can be classified into zygotic (sporophytic) and gametic (gametophytic) types based on their genetic basis. Typical mutants for sporophytic pollen sterility expressed aberrant zygotic phenotypes in a recessive manner in anther wall layer, tapetum formation and degradation, and lipid metabolism and transportation (Ariizumi and Toriyama, 2011; Jiang *et al*., 2013). In contrast, the sterility of pollen grains is controlled by the genotype of gametophytes in gametophytic pollen sterility mutants. *MOR1/GEM* encodes a member of the MAP215 family of microtubule-associated proteins for the establishment of interphase arrays of cortical microtubules in plant cells, and the *MOR1/GEM* deficient mutant in *Arabidopsis*, *gemini pollen1* (*gem1*), showed pollen semi–sterility due to defective cytokinesis of pollen containing a T–DNA insertion (Twell *et al*., 2002). The male gametophytic rice mutant, *rice immature pollen1* (*rip1*), carry defect protein with conserved five WD40 repeats sequences and showed semi–sterility in pollen in heterozygous condition (Han *et al*., 2006). An arabinokinase–like protein defective mutant, *collapsed abnormal pollen1* (*cap1*), showed pollen sterility in a gametophytic manner, possibly due to the lack of UDP–L–arabinose (Ueda *et al*., 2013). The *RICE GLYCOSYLTRANSFERASE1* (*OsGT1*) gene is

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suggested to localize in a Golgi apparatus of pollen essential for intine wall formation and pollen maturation (Moon *et al*., 2013). Although gene cloning and characterization studies for pollen formation have been extensively conducted in the mutant approach, the isolated genes involved in diverged biological processes to each other and their spatially and temporally different expression and function let our knowledge be partially limited. Therefore, systematic gathering and reduction of redundancy in the pollen sterile mutants are necessary for a more efficient comprehensive understanding of complicated molecular networks during post–meiotic male gametogenesis.

For the genetic characterization of mutant stocks, an allelic test is a simple and powerful approach to classify mutant stocks into allelic groups. However, an allelic test among pollen–sterile mutants is generally tricky because mutant alleles do not transmit to progeny via male gamete due to sterility, especially, for the gametophytic pollen sterility. In this case, gene tagging using a DNA marker is available to investigate the allelism of mutants. Bulked segregant analysis (BSA) has been frequently used for rapid gene identification (Michelmore *et al*., 1991). The BSA uses two types of pooled (bulked) DNA samples from normal and mutant segregants for DNA marker analysis, and the DNA markers with a high frequency of alleles derived from the mutant stock have a high possibility to tightly link to the causal gene in the mutant bulked DNA. Today, single nucleotide polymorphisms (SNP) markers are rapidly replacing simple sequence repeats (SSR) markers because they are more abundant, stable, conveniently automated, efficient, and increasingly cost–effective (McCouch *et al*., 2010; Thomson *et al*., 2012). Furthermore, modern SNP genotyping techniques provide a high–throughput and cheaper allele calling process. It also produces accurate and stable data that can be flexible, merged across groups, and stored in databases no matter which genotyping platform is used. (Thomson *et al*., 2012).

So far, we have identified twelve mutants of *SPOROPHYTIC POLLEN STERILITY* (*SPS1–SPS12*) and twelve *GAMETOPHYTIC POLLEN STERILITY* (*GPS1–GPS12*) by gamma–ray mutagenesis to *japonica* cultivar Taichung 65 (T65) (Yamagata *et al*., 2007). To understand the genetic basis of pollen development, linkage mapping of these mutants has been conducted. Although *SPS6, SPS9, SPS12, GPS4, GPS5, GSP6,* and *GPS12* have been mapped on the rice linkage map. However, the map position of the causal gene for the other mutant lines has not been revealed. Here, we conducted bulked segregants analysis assisted by Illumina Beadarray–based genotyping system in the $F_2$ population for four *SPS* mutant lines and four *GPS* mutant lines. Using 287 SNP markers designed for Japanese rice accessions, we detected signal peaks on candidate SNP markers involved in phenotypes. Linkage analysis was conducted by PCR–based markers which are linked to the candidate SNP markers for identification of the genes for sporophytic or gametophytic pollen sterility.

## MATERIALS AND METHODS

### Plant materials

Eight gamma–ray–induced mutants derived from T65 were used in this study (Table 1). For bulked segregant analysis and linkage analysis of the mutants, the $F_2$ population derived from a cross between the mutants and the Japonica rice cultivar Hinohikari was grown. For the recessive *SPS2, SPS3, SPS4,* and *SPS5* mutants showing complete male and female sterility, pollen–fertile plant segregated in mutant lines were crossed by Hinohikari pollen, and the $F_2$ populations in which segregation of pollen sterile plants was observed were used for the gene mapping. For the *GPS1, GPS3, GPS9,* and *GPS10* mutants for gametophytic pollen sterility, pollen semi–sterile plants were pollinated by Hinohikari pollen and the $F_1$ plants showing pollen semi–sterility were screened to the generation of the $F_2$ population used for the mapping.

**Table 1.** Mutant lines used in this study

| Mutant line | Gene | Type of pollen sterility |
|---|---|---|
| PSML2 | *SPS2* | Sporophytic |
| PSML3 | *SPS3* | Sporophytic |
| PSML4 | *SPS4* | Sporophytic |
| PSML7 | *SPS7* | Sporophytic |
| PSML101 | *GPS1* | Gametophytic |
| PSML103 | *GPS3* | Gametophytic |
| PSML109 | *GPS9* | Gametophytic |
| PSML110 | *GPS10* | Gametophytic |

### Observation of pollen fertility

Panicles at the heading stage were fixed and stored in 70% ethanol. Six anthers from the spikelet just before flowering were squashed on a slide glass in 1% $I_2$–KI solution. After removing the anthers and debris, pollen grains were observed under a microscope (Zeiss, Jena, Germany). Pollen grains without difference from those of Taichung 65 were described as fertile. Aborted, unstained, incompletely stained, or small pollen grains were scored as sterile.

### Bulked segregants analysis

Genomic DNA was extracted from freeze–dried leaf samples of the individual plants in the $F_2$ population according to the minipreparation method (Dellaporta *et al*., 1983) with minor modifications. The DNA concentration of the individual plants was measured by a spectrophotometer. For the BSA of the sporophytic mutants, we prepared bulked DNA samples equally from eight pollen sterile plants ($S_{spo}$ bulked DNA) and eight normal plants ($N_{spo}$ bulked DNA). Similarly, for the BSA of the gametophytic mutants, we bulked DNA samples equally for eight pollen semi-sterile plants ($S_{ga}$ bulked DNA) and eight normal plants ($N_{ga}$ bulked DNA). For the detection of the genomic region for segregation distortion

observed in the $F_2$ population between T65 and Hinohikari, we prepared a control DNA bulked sample equally from eight fertile plants randomly selected from the $F_2$ population (C bulked DNA). The concentration of the bulked DNA samples was adjusted to 20 ng/$\mu$l after purification by spin column kit (GeneAll Expin PCR SV, Singapore). A core set of 768 SNP markers designed for diversity analysis in Japanese rice accessions (Nagasaki *et al*., 2010) were used for the bulked segregant analysis by Infinium assay using iScan Beadarray Reader (Illumina, San Diego, USA). The obtained data were analyzed by the Illumina Genome Studio Genotyping module.

**Linkage mapping using PCR–based marker**

Linkage maps of the gene for pollen sterility were constructed by PCR–based markers, cleaved amplified polymorphic sequences (CAPS) markers, derived CAPS (dCAPS) markers, sequence–tagged sites (STS) markers, and simple sequence repeats (SSR) markers (Table 2). The CAPS or dCAPS markers were converted from the Infinium SNP markers to detect the corresponding SNPs by the dCAPS Finder 2.0 program (Neff *et al*., 1998). The STS markers used in this study were designed from polymorphic insertion–deletion sites found in T65 resequencing data using the Roche–454 Titanium FLX system. The SSR markers were referred from McCouch *et al*. (2002). PCR reactions were performed in 15 $\mu$L of reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer, 0.75 units *Taq* polymerase (Takara, Otsu, Japan), and approximately two ng of template DNA in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). PCR was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. For genotyping, the PCR amplified products of the CAPS and dCAPS markers were digested by restriction enzyme after conventional isopropanol precipitation of DNA. The obtained DNA fragments were run in 4% agarose gels in 0.5×TBE buffer. The linkage map was constructed by MAPMAKER/EXP 3.0 program (Lander *et al*., 1987) using Kosambi's mapping function.

## RESULTS

**Polymorphism detection between T65 and Hinohikari**

Using the core set of 768 SNP markers designed for diversity analysis in Japanese rice accessions at the National Institute of Agrobiological Sciences (NIAS) (Nagasaki *et al*., 2010), we surveyed bulked DNA from normal and sterile plants by the Illumina BeadArray. The scatter plot of the Cye3 and Cye5 signal intensities on each SNP marker were obtained and converted to standardized polar coordinates using $R$ and $\theta$ by Illumina Genome Studio Genotyping module (Fig. 1A, B). For example, adenine (A) and guanine (G) nucleotides at the SNP marker locus NIAS_Os_ ab05000327 (Mk139) were labeled with Cye3 and Cye5 dyes, respectively (Fig. 1C, D). Hinohikari (Fig. 1C, D; white inverted triangle) and T65 (Fig. 1C, D; white box) have been found to possess homozygous genotypes for A and G nucleotides (AA and GG), respectively. The five cultivars carrying AA genotypes and Hinohikari and the other five carrying GG genotypes and T65 formed each of two separable linear plots on the Cye3–Cye5 scatter plot and the standardized polar coordinates plot (Fig. 1C, D). Whereas plots of the bulked DNA were distributed between the two scatter lines of cultivars (plus symbol and white circles,

**Table 2.** PCR–based markers used in this study

| Gene | Marker | Marker type | Forward primer[1] | Reverse primer[1] | Restriction enzyme | Chr. | Start[2] | End[2] | Product size |
|---|---|---|---|---|---|---|---|---|---|
| *SPS2* | *QSTS237* | STS | GACCAGAGACTGCCCCATC | TCACTTCAAGTCTGTGGTTTCAG | | 10 | 2,522,458 | 2,522,561 | 104 |
| *SPS2* | *RM5304* | SSR | CATCTTGAATCCTCCTTCGACTCC | GGCAGCGATAGCAGGAAGAGG | | 10 | 16,874,853 | 16,875,188 | 336 |
| *SPS3* | *Mk137dCAPS* | dCAPS | CCCTTATAATGAGAAATTGGAAGAG | GCAGCACCTCATTTTGTAGATT | *Sac*I | 5 | 26,896,716 | 26,897,014 | 299 |
| *SPS3* | *Mk138CAPS* | CAPS | CCATTTCTGCAAACTGGAAG | GAGCCAAAATTTGCAAAGCA | *Mbo*I | 5 | 28,986,332 | 28,986,590 | 259 |
| *SPS3* | *QSTS154* | STS | GAAGCCAGGTCCCAAGATG | GTAGCTAGGGCCCACTCTGG | | 5 | 29,659,648 | 29,659,789 | 142 |
| *SPS4* | *Mk97dCAPS* | dCAPS | GTTATTAACTCGTCCGTTTCATATT | TGCAAATAGCTTAAAATAAGTGGTC | | 3 | 26,482,714 | 26,483,013 | 300 |
| *SPS4* | *RM1350* | SSR | AGGAACACCCAAGAGAGTCATGC | GCAAGAAAGCTCTGCTCCATGC | | 3 | 29,443,733 | 29,443,940 | 208 |
| *SPS7* | *Mk97dCAPS* | dCAPS | GTTATTAACTCGTCCGTTTCATATT | TGCAAATAGCTTAAAATAAGTGGTC | *Taq*I | 3 | 26,482,714 | 26,483,013 | 300 |
| *SPS7* | *RM1350* | SSR | AGGAACACCCAAGAGAGTCATGC | GCAAGAAAGCTCTGCTCCATGC | | 3 | 29,443,733 | 29,443,940 | 208 |
| *GPS1* | *Mk97dCAPS* | dCAPS | GTTATTAACTCGTCCGTTTCATATT | TGCAAATAGCTTAAAATAAGTGGTC | | 3 | 26,482,714 | 26,483,013 | 300 |
| *GPS1* | *RM1350* | SSR | AGGAACACCCAAGAGAGTCATGC | GCAAGAAAGCTCTGCTCCATGC | | 3 | 29,443,733 | 29,443,940 | 208 |
| *GPS3* | *Mk82dCAPS* | dCAPS | TGCATTGATCGATAGAGTCGATCCG | GAATGGTATTTCGGCATGTTCT | *Bsr*BI | 3 | 6,769,688 | 6,769,916 | 229 |
| *GPS3* | *Mk83dCAPS* | dCAPS | AAGAGAATCTTTTCACTGTAGCATA | ATTCCGTATGGGTATTAAGGTG | *Nde*I | 3 | 7,846,383 | 7,846,674 | 292 |
| *GPS9* | *QSTS134* | STS | CTTGTATTGCGTGCAGGATG | TTGGCTTCAATGCATACACC | | 4 | 13,161,035 | 13,161,154 | 120 |
| *GPS10* | *RM5847* | SSR | CTTTAGGTAGCGTCATCTTCC | TGGAAATACAGAAGGAGTCG | | 7 | 24,309,502 | 24,309,991 | 490 |
| *GPS10* | *RM1364* | SSR | AAGAAATTCAAAACACATGA | AAAACATCTACTTTGATCCA | | 7 | 27,435,157 | 27,435,314 | 158 |

[1]Modified nucleotide sequences for derived–cleaved amplified sequence (dCAPS) marker are represented in underline.
[2]Genomic positions in the reference genome Nipponbare IRGSP–1.0.

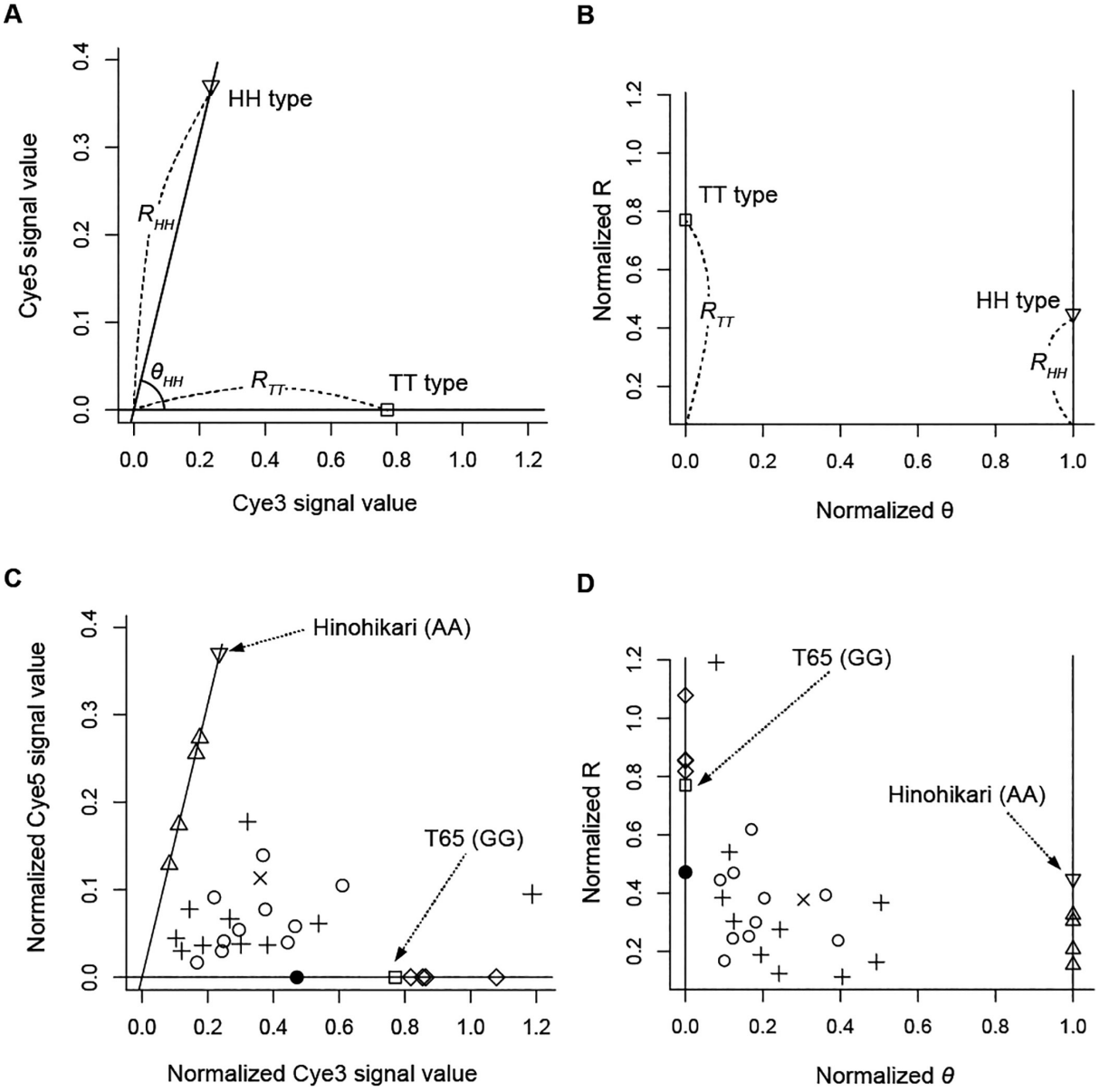


**Fig. 1.** Definition of normalized $\theta$ and $R$ values converted from the Cye3 and Cye5 signals by Illumina Genome Studio software. (**A**) White inverted triangles and rectangles represent Hinohikari and T65, respectively. The Cye3 and Cye5 signals of the parents, T65 and Hinohikari, are plot on the polar coordinate plane of $\theta$ and $R$. (**B**) The angle Hinohikari from the one of T65 ($\theta_{HH}$) were normalized to one. (**C**–**D**) The bulked DNA samples of fertile and sterile segregants in $F_2$ populations were shown in white circles and daggers, respectively. The black circle and cross represent the signals from the sterile and fertile bulks for *SPS3* mapping in the nearest SNP marker *Mk139* (see result). These samples were plotted on the original Cye3 and Cye5 coordinate plane (**C**) and normalized $\theta$ and $R$ coordinate plane (**D**).

Fig. B, D). This indicates that the Mk139 marker is bimorphic between T65 and Hinohikari, whereas bulked DNA samples from normal or pollen sterile plants in the $F_2$ population would have variation in T65 DNA and Hinohikari DNA ratio. Consequently, we found 287 bimorphic markers between T65 and Hinohikari distributed on a whole genomic region on the rice chromosome (Fig. 2; Table 3). We found a large gap region where a bimorphic marker was not discovered in more than 9 Mb on chromosomes 4, 10, and 11 probably due to the identity–by–descent in the ancestral pedigree.

**Bulked segregants analysis by BeadArray**

The bulked DNA of the normal plants ($N_{spo}$ bulk) and sterile plants ($S_{spo}$ bulk) in the $F_2$ population for the sporophytic pollen sterile mutants and the bulked DNA of the normal ($N_{ga}$ bulk) and semi–sterile ($S_{ga}$ bulk) plants in the $F_2$ population for the gametophytic pollen sterile mutants were examined by the Illumina BeadArray. Theoretically, allele frequencies of T65 and Hinohikari in bulks are estimated by a function of recombination fraction $r$ between causal genes and given markers (Table 4). To estimate $r$ between marker and gene, deviation of T65 allele frequency from 1 in the $N_{spo}$ and $S_{spo}$ bulks ($\Delta f_{Nspo}$ and $\Delta f_{Sspo}$) (Fig. 3A, B) and Hinohikari allele frequency from 1 in the $N_{ga}$ and $S_{ga}$ bulks ($\Delta f_{Nga}$ and $\Delta f_{Sga}$) were calculated (Fig. 3C, D). At the SNP marker completely linking to the gene ($r$=0) in the bulked seg-

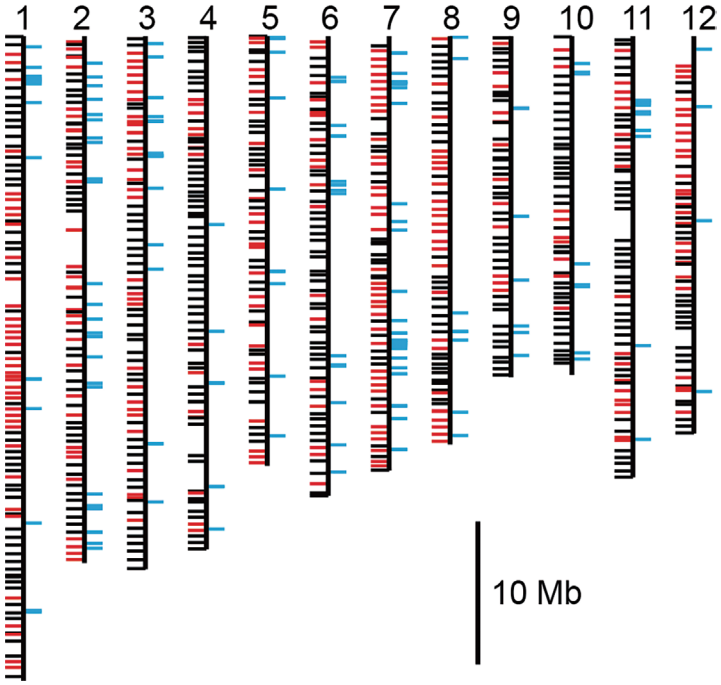


**Fig. 2.** Whole genome coverage of the polymorphic markers used in this study. Twelve vertical lines represent the rice chromosomes (n=12). Horizontal bars on the left side of the vertical lines show the core set of 768 SNP markers designed for diversity analysis in Japanese rice accessions (Nagasaki *et al*. 2010). The SNP markers harboring polymorphism between T65 and Hinohikari are shown in red. Horizontal bars on the right side of the vertical lines show the polymorphic PCR–based markers.

**Table 3.** SNP markers showing clear polymorphism between Taichung 65 and Hinohikari

| Marker | Original marker ID | Chr | Position[1] | Marker | Original marker ID | Chr | Position[1] |
|---|---|---|---|---|---|---|---|
| Mk1 | NIAS_Os_aa01000201 | 1 | 1,268,484 | Mk73 | NIAS_Os_aa03000455 | 3 | 2,174,753 |
| Mk2 | NIAS_Os_aa01000889 | 1 | 1,841,582 | Mk74 | NIAS_Os_aa03000464 | 3 | 2,722,161 |
| Mk3 | NIAS_Os_aa01003605 | 1 | 3,027,485 | Mk75 | NIAS_Os_aa03000481 | 3 | 3,223,584 |
| Mk4 | NIAS_Os_ac01000670 | 1 | 8,035,380 | Mk76 | NIAS_Os_aa03000493 | 3 | 3,884,127 |
| Mk5 | NIAS_Os_aa01005640 | 1 | 11,019,483 | Mk77 | NIAS_Os_aa03000513 | 3 | 4,446,439 |
| Mk6 | NIAS_Os_aa01005935 | 1 | 11,424,453 | Mk78 | NIAS_Os_ab03000076 | 3 | 5,625,905 |
| Mk7 | NIAS_Os_aa01006068 | 1 | 12,018,297 | Mk79 | NIAS_Os_ab03000079 | 3 | 5,966,048 |
| Mk8 | NIAS_Os_aa01006075 | 1 | 12,480,565 | Mk80 | NIAS_Os_ac03000074 | 3 | 6,176,568 |
| Mk9 | NIAS_Os_aa01006089 | 1 | 13,142,274 | Mk81 | NIAS_Os_ac03000079 | 3 | 6,738,586 |
| Mk10 | NIAS_Os_aa01006150 | 1 | 15,463,045 | Mk82 | NIAS_Os_ab03000088 | 3 | 6,769,713 |
| Mk11 | NIAS_Os_aa01006249 | 1 | 16,958,784 | Mk83 | NIAS_Os_ac03000086 | 3 | 7,846,649 |
| Mk12 | NIAS_Os_aa01006278 | 1 | 18,853,746 | Mk84 | NIAS_Os_ab03000111 | 3 | 8,251,245 |
| Mk13 | NIAS_Os_aa01006340 | 1 | 19,767,057 | Mk85 | NIAS_Os_ac03000229 | 3 | 9,345,120 |
| Mk14 | NIAS_Os_aa01006478 | 1 | 20,246,725 | Mk86 | NIAS_Os_aa03000528 | 3 | 10,014,890 |
| Mk15 | NIAS_Os_aa01006756 | 1 | 20,670,591 | Mk87 | NIAS_Os_aa03000595 | 3 | 10,734,126 |
| Mk16 | NIAS_Os_aa01006765 | 1 | 21,077,064 | Mk88 | NIAS_Os_aa03000667 | 3 | 11,264,731 |
| Mk17 | NIAS_Os_aa01006770 | 1 | 21,547,487 | Mk89 | NIAS_Os_aa03001638 | 3 | 17,043,482 |
| Mk18 | NIAS_Os_aa01006817 | 1 | 22,533,243 | Mk90 | NIAS_Os_ac03000493 | 3 | 17,995,961 |
| Mk19 | NIAS_Os_aa01006855 | 1 | 23,033,795 | Mk91 | NIAS_Os_aa03002083 | 3 | 18,373,459 |
| Mk20 | NIAS_Os_aa01006881 | 1 | 23,530,660 | Mk92 | NIAS_Os_aa03002095 | 3 | 18,720,599 |
| Mk21 | NIAS_Os_aa01006903 | 1 | 23,769,255 | Mk93 | NIAS_Os_aa03002290 | 3 | 23,208,308 |
| Mk22 | NIAS_Os_aa01006939 | 1 | 24,017,033 | Mk94 | NIAS_Os_ac03000587 | 3 | 24,512,928 |
| Mk23 | NIAS_Os_aa01006944 | 1 | 24,535,890 | Mk95 | NIAS_Os_ab03000476 | 3 | 25,569,893 |
| Mk24 | NIAS_Os_aa01006950 | 1 | 24,940,615 | Mk96 | NIAS_Os_ac03000619 | 3 | 26,104,995 |
| Mk25 | NIAS_Os_aa01008076 | 1 | 25,274,929 | Mk97 | NIAS_Os_ac03000633 | 3 | 26,482,739 |
| Mk26 | NIAS_Os_aa01008849 | 1 | 26,063,580 | Mk98 | NIAS_Os_aa03002367 | 3 | 27,619,018 |
| Mk27 | NIAS_Os_aa01008907 | 1 | 26,509,357 | Mk99 | NIAS_Os_aa03002478 | 3 | 30,362,290 |
| Mk28 | NIAS_Os_aa01009141 | 1 | 26,909,316 | Mk100 | NIAS_Os_aa03002548 | 3 | 32,048,002 |

**Table 3.** (continued)

| Marker | Original marker ID | Chr | Position[1] | Marker | Original marker ID | Chr | Position[1] |
|---|---|---|---|---|---|---|---|
| Mk29 | NIAS_Os_aa01009804 | 1 | 27,303,987 | Mk101 | NIAS_Os_aa03002554 | 3 | 32,280,379 |
| Mk30 | NIAS_Os_aa01009851 | 1 | 28,636,306 | Mk102 | NIAS_Os_ab03000579 | 3 | 33,828,281 |
| Mk31 | NIAS_Os_aa01010071 | 1 | 30,801,732 | Mk103 | NIAS_Os_aa04002698 | 4 | 4,433,214 |
| Mk32 | NIAS_Os_aa01010140 | 1 | 33,078,597 | Mk104 | NIAS_Os_aa04002724 | 4 | 4,765,053 |
| Mk33 | NIAS_Os_aa01010145 | 1 | 33,552,000 | Mk105 | NIAS_Os_aa04002941 | 4 | 5,369,672 |
| Mk34 | NIAS_Os_aa01010816 | 1 | 39,263,468 | Mk106 | NIAS_Os_aa04003046 | 4 | 6,475,663 |
| Mk35 | NIAS_Os_aa01010935 | 1 | 41,221,259 | Mk107 | NIAS_Os_aa04003107 | 4 | 6,892,422 |
| Mk36 | NIAS_Os_aa01010937 | 1 | 41,804,180 | Mk108 | NIAS_Os_aa04003679 | 4 | 7,786,585 |
| Mk37 | NIAS_Os_aa01010967 | 1 | 43,717,183 | Mk109 | NIAS_Os_aa04003724 | 4 | 8,263,746 |
| Mk38 | NIAS_Os_ab01001286 | 1 | 44,114,080 | Mk110 | NIAS_Os_ac04001045 | 4 | 21,548,924 |
| Mk39 | NIAS_Os_aa02000006 | 2 | 390,336 | Mk111 | NIAS_Os_ab04001157 | 4 | 23,524,912 |
| Mk40 | NIAS_Os_aa02000031 | 2 | 891,366 | Mk112 | NIAS_Os_ac04001067 | 4 | 26,235,224 |
| Mk41 | NIAS_Os_aa02000032 | 2 | 1,464,369 | Mk113 | NIAS_Os_ac04001069 | 4 | 26,626,506 |
| Mk42 | NIAS_Os_aa02000034 | 2 | 2,153,815 | Mk114 | NIAS_Os_ab04001335 | 4 | 31,918,946 |
| Mk43 | NIAS_Os_aa02000675 | 2 | 5,100,626 | Mk115 | NIAS_Os_ac04001259 | 4 | 34,104,276 |
| Mk44 | NIAS_Os_aa02000707 | 2 | 5,602,214 | Mk116 | NIAS_Os_aa04009710 | 4 | 34,544,780 |
| Mk45 | NIAS_Os_ab02000190 | 2 | 6,550,893 | Mk117 | NIAS_Os_aa05000026 | 5 | 142,341 |
| Mk46 | NIAS_Os_ac02000121 | 2 | 6,614,944 | Mk118 | NIAS_Os_ac05000011 | 5 | 441,754 |
| Mk47 | NIAS_Os_aa02000729 | 2 | 8,785,514 | Mk119 | NIAS_Os_aa05000045 | 5 | 2,338,894 |
| Mk48 | NIAS_Os_aa02000735 | 2 | 9,135,316 | Mk120 | NIAS_Os_ac05000021 | 5 | 2,816,864 |
| Mk49 | NIAS_Os_aa02000798 | 2 | 10,149,289 | Mk121 | NIAS_Os_aa05000263 | 5 | 3,868,405 |
| Mk50 | NIAS_Os_ac02000212 | 2 | 13,550,474 | Mk122 | NIAS_Os_aa05000340 | 5 | 5,320,109 |
| Mk51 | NIAS_Os_ab02000310 | 2 | 13,558,615 | Mk123 | NIAS_Os_ac05000101 | 5 | 7,492,024 |
| Mk52 | NIAS_Os_ac02000216 | 2 | 16,109,468 | Mk124 | NIAS_Os_ab05000119 | 5 | 9,327,890 |
| Mk53 | NIAS_Os_ab02000319 | 2 | 16,818,748 | Mk125 | NIAS_Os_ab05000124 | 5 | 11,349,470 |
| Mk54 | NIAS_Os_ab02000327 | 2 | 17,534,032 | Mk126 | NIAS_Os_ab05000128 | 5 | 12,410,382 |
| Mk55 | NIAS_Os_ab02000329 | 2 | 17,603,774 | Mk127 | NIAS_Os_ab05000132 | 5 | 12,998,736 |
| Mk56 | NIAS_Os_ac02000233 | 2 | 19,126,740 | Mk128 | NIAS_Os_ac05000151 | 5 | 14,518,855 |
| Mk57 | NIAS_Os_ab02000375 | 2 | 19,545,260 | Mk129 | NIAS_Os_ac05000153 | 5 | 14,752,316 |
| Mk58 | NIAS_Os_ab02000380 | 2 | 20,038,083 | Mk130 | NIAS_Os_ab05000169 | 5 | 16,556,457 |
| Mk59 | NIAS_Os_aa02001232 | 2 | 21,193,559 | Mk131 | NIAS_Os_ac05000227 | 5 | 18,203,829 |
| Mk60 | NIAS_Os_aa02001425 | 2 | 23,000,346 | Mk132 | NIAS_Os_ac05000233 | 5 | 18,857,649 |
| Mk61 | NIAS_Os_aa02001836 | 2 | 25,147,471 | Mk133 | NIAS_Os_aa05000835 | 5 | 20,189,252 |
| Mk62 | NIAS_Os_aa02002928 | 2 | 26,478,941 | Mk134 | NIAS_Os_aa05000842 | 5 | 21,650,823 |
| Mk63 | NIAS_Os_aa02003208 | 2 | 28,746,716 | Mk135 | NIAS_Os_ab05000280 | 5 | 22,851,067 |
| Mk64 | NIAS_Os_aa02003253 | 2 | 29,068,140 | Mk136 | NIAS_Os_ac05000298 | 5 | 23,262,105 |
| Mk65 | NIAS_Os_aa02003269 | 2 | 29,406,298 | Mk137 | NIAS_Os_aa05000868 | 5 | 26,896,741 |
| Mk66 | NIAS_Os_aa02003371 | 2 | 30,963,801 | Mk138 | NIAS_Os_aa05001022 | 5 | 28,986,433 |
| Mk67 | NIAS_Os_aa02003947 | 2 | 35,131,698 | Mk139 | NIAS_Os_ab05000327 | 5 | 29,388,932 |
| Mk68 | NIAS_Os_aa02003989 | 2 | 35,683,641 | Mk140 | NIAS_Os_ac05000360 | 5 | 29,809,566 |
| Mk69 | NIAS_Os_aa02004105 | 2 | 36,143,511 | Mk141 | NIAS_Os_aa06000004 | 6 | 396,429 |
| Mk70 | NIAS_Os_aa02004112 | 2 | 36,572,369 | Mk142 | NIAS_Os_aa06000024 | 6 | 775,093 |
| Mk71 | NIAS_Os_aa03000026 | 3 | 1,192,473 | Mk143 | NIAS_Os_ac06000061 | 6 | 3,252,868 |
| Mk72 | NIAS_Os_aa03000452 | 3 | 1,741,544 | Mk144 | NIAS_Os_ac06000078 | 6 | 4,439,073 |
| Mk145 | NIAS_Os_ab06000075 | 6 | 5,343,225 | Mk217 | NIAS_Os_ab08000952 | 8 | 26,181,049 |
| Mk146 | NIAS_Os_aa06000302 | 6 | 5,532,677 | Mk218 | NIAS_Os_ac08000961 | 8 | 26,216,441 |
| Mk147 | NIAS_Os_ac06000121 | 6 | 7,237,880 | Mk219 | NIAS_Os_ac08000965 | 8 | 26,823,571 |
| Mk148 | NIAS_Os_ac06000385 | 6 | 8,658,335 | Mk220 | NIAS_Os_aa08006353 | 8 | 27,309,655 |
| Mk149 | NIAS_Os_ac06000397 | 6 | 9,137,292 | Mk221 | NIAS_Os_aa08006402 | 8 | 27,816,975 |
| Mk150 | NIAS_Os_ab06000799 | 6 | 10,381,181 | Mk222 | NIAS_Os_ac08000993 | 8 | 28,304,764 |
| Mk151 | NIAS_Os_ac06000592 | 6 | 11,912,372 | Mk223 | NIAS_Os_ac09000003 | 9 | 495,078 |
| Mk152 | NIAS_Os_ab06001118 | 6 | 18,117,938 | Mk224 | NIAS_Os_ac09000009 | 9 | 2,558,972 |
| Mk153 | NIAS_Os_ac06000665 | 6 | 19,763,293 | Mk225 | NIAS_Os_ac09000010 | 9 | 3,510,493 |
| Mk154 | NIAS_Os_ac06000790 | 6 | 24,118,399 | Mk226 | NIAS_Os_ab09000234 | 9 | 5,359,013 |
| Mk155 | NIAS_Os_aa06000879 | 6 | 24,704,577 | Mk227 | NIAS_Os_ac09000150 | 9 | 5,978,368 |
| Mk156 | NIAS_Os_aa06000895 | 6 | 25,874,054 | Mk228 | NIAS_Os_ac09000154 | 9 | 7,581,539 |
| Mk157 | NIAS_Os_aa06001139 | 6 | 28,752,213 | Mk229 | NIAS_Os_aa09000044 | 9 | 10,008,700 |
| Mk158 | NIAS_Os_aa06001150 | 6 | 29,230,199 | Mk230 | NIAS_Os_ac09000231 | 9 | 11,696,860 |
| Mk159 | NIAS_Os_aa06001164 | 6 | 31,279,600 | Mk231 | NIAS_Os_ac09000238 | 9 | 13,333,020 |
| Mk160 | NIAS_Os_aa07000004 | 7 | 1,016,334 | Mk232 | NIAS_Os_ac09000278 | 9 | 16,768,906 |

**Table 3.** (continued)

| Marker | Original marker ID | Chr | Position[1] | Marker | Original marker ID | Chr | Position[1] |
|---|---|---|---|---|---|---|---|
| Mk161 | NIAS_Os_aa07000201 | 7 | 1,597,698 | Mk233 | NIAS_Os_ab09001035 | 9 | 17,235,555 |
| Mk162 | NIAS_Os_aa07000566 | 7 | 2,104,295 | Mk234 | NIAS_Os_ac09000281 | 9 | 17,755,974 |
| Mk163 | NIAS_Os_aa07000615 | 7 | 2,683,214 | Mk235 | NIAS_Os_aa10000007 | 10 | 952,586 |
| Mk164 | NIAS_Os_aa07000697 | 7 | 3,168,158 | Mk236 | NIAS_Os_aa10000749 | 10 | 2,129,081 |
| Mk165 | NIAS_Os_aa07001067 | 7 | 3,686,778 | Mk237 | NIAS_Os_aa10002652 | 10 | 12,225,180 |
| Mk166 | NIAS_Os_aa07001130 | 7 | 4,276,229 | Mk238 | NIAS_Os_aa10002752 | 10 | 12,804,781 |
| Mk167 | NIAS_Os_aa07001807 | 7 | 4,800,059 | Mk239 | NIAS_Os_aa10002866 | 10 | 14,081,991 |
| Mk168 | NIAS_Os_aa07001816 | 7 | 5,238,134 | Mk240 | NIAS_Os_aa10002874 | 10 | 14,370,080 |
| Mk169 | NIAS_Os_aa07001842 | 7 | 7,213,462 | Mk241 | NIAS_Os_ac10000399 | 10 | 15,585,702 |
| Mk170 | NIAS_Os_ab07000442 | 7 | 8,456,603 | Mk242 | NIAS_Os_aa10002908 | 10 | 16,771,274 |
| Mk171 | NIAS_Os_ab07000446 | 7 | 8,915,167 | Mk243 | NIAS_Os_ac10000429 | 10 | 19,009,956 |
| Mk172 | NIAS_Os_ab07000462 | 7 | 10,583,608 | Mk244 | NIAS_Os_aa11000032 | 11 | 994,653 |
| Mk173 | NIAS_Os_ab07000482 | 7 | 11,092,773 | Mk245 | NIAS_Os_aa11000115 | 11 | 3,266,163 |
| Mk174 | NIAS_Os_ab07000535 | 7 | 11,987,250 | Mk246 | NIAS_Os_aa11000146 | 11 | 3,902,699 |
| Mk175 | NIAS_Os_ab07000574 | 7 | 12,456,450 | Mk247 | NIAS_Os_aa11000283 | 11 | 4,387,906 |
| Mk176 | NIAS_Os_ab07000628 | 7 | 13,520,623 | Mk248 | NIAS_Os_ab11000174 | 11 | 5,001,838 |
| Mk177 | NIAS_Os_ac07000428 | 7 | 16,305,134 | Mk249 | NIAS_Os_aa11000573 | 11 | 6,868,556 |
| Mk178 | NIAS_Os_ac07000431 | 7 | 17,282,400 | Mk250 | NIAS_Os_aa11002695 | 11 | 7,733,203 |
| Mk179 | NIAS_Os_ac07000440 | 7 | 17,631,034 | Mk251 | NIAS_Os_aa11002722 | 11 | 9,097,827 |
| Mk180 | NIAS_Os_aa07001862 | 7 | 18,079,458 | Mk252 | NIAS_Os_ac11000385 | 11 | 18,200,567 |
| Mk181 | NIAS_Os_ac07000529 | 7 | 18,544,719 | Mk253 | NIAS_Os_aa11004155 | 11 | 22,189,282 |
| Mk182 | NIAS_Os_aa07001872 | 7 | 18,897,313 | Mk254 | NIAS_Os_aa11004494 | 11 | 22,902,061 |
| Mk183 | NIAS_Os_aa07001881 | 7 | 19,448,188 | Mk255 | NIAS_Os_aa11004535 | 11 | 24,050,290 |
| Mk184 | NIAS_Os_aa07002123 | 7 | 20,485,611 | Mk256 | NIAS_Os_aa11004652 | 11 | 24,778,955 |
| Mk185 | NIAS_Os_aa07002833 | 7 | 21,476,529 | Mk257 | NIAS_Os_aa11005083 | 11 | 25,456,528 |
| Mk186 | NIAS_Os_aa07004070 | 7 | 23,408,558 | Mk258 | NIAS_Os_aa11005455 | 11 | 25,754,577 |
| Mk187 | NIAS_Os_aa07005154 | 7 | 23,897,779 | Mk259 | NIAS_Os_aa11006712 | 11 | 26,298,510 |
| Mk188 | NIAS_Os_aa07005205 | 7 | 24,372,658 | Mk260 | NIAS_Os_aa11007380 | 11 | 28,042,026 |
| Mk189 | NIAS_Os_aa07005234 | 7 | 24,806,873 | Mk261 | NIAS_Os_aa11007953 | 11 | 28,251,660 |
| Mk190 | NIAS_Os_aa07006696 | 7 | 25,842,928 | Mk262 | NIAS_Os_aa12000015 | 12 | 2,091,263 |
| Mk191 | NIAS_Os_aa07007162 | 7 | 27,301,089 | Mk263 | NIAS_Os_aa12000060 | 12 | 2,442,856 |
| Mk192 | NIAS_Os_aa07007461 | 7 | 27,710,942 | Mk264 | NIAS_Os_aa12000100 | 12 | 2,837,321 |
| Mk193 | NIAS_Os_aa07007493 | 7 | 28,145,394 | Mk265 | NIAS_Os_aa12001361 | 12 | 4,432,378 |
| Mk194 | NIAS_Os_aa07007512 | 7 | 28,953,150 | Mk266 | NIAS_Os_aa12001740 | 12 | 5,123,887 |
| Mk195 | NIAS_Os_aa07007522 | 7 | 29,724,834 | Mk267 | NIAS_Os_aa12001794 | 12 | 5,528,490 |
| Mk196 | NIAS_Os_aa07007554 | 7 | 30,021,987 | Mk268 | NIAS_Os_aa12002375 | 12 | 6,212,403 |
| Mk197 | NIAS_Os_aa08000002 | 8 | 172,474 | Mk269 | NIAS_Os_aa12002973 | 12 | 6,759,198 |
| Mk198 | NIAS_Os_ac08000095 | 8 | 3,334,435 | Mk270 | NIAS_Os_aa12004168 | 12 | 7,306,761 |
| Mk199 | NIAS_Os_aa08001066 | 8 | 5,648,692 | Mk271 | NIAS_Os_aa12004213 | 12 | 7,986,190 |
| Mk200 | NIAS_Os_aa08001334 | 8 | 8,023,300 | Mk272 | NIAS_Os_aa12004255 | 12 | 8,402,485 |
| Mk201 | NIAS_Os_aa08001338 | 8 | 8,442,466 | Mk273 | NIAS_Os_aa12004405 | 12 | 9,771,738 |
| Mk202 | NIAS_Os_aa08001560 | 8 | 8,838,173 | Mk274 | NIAS_Os_aa12004429 | 12 | 10,798,350 |
| Mk203 | NIAS_Os_aa08001638 | 8 | 9,237,171 | Mk275 | NIAS_Os_aa12004430 | 12 | 11,037,889 |
| Mk204 | NIAS_Os_aa08002627 | 8 | 10,335,140 | Mk276 | NIAS_Os_aa12004439 | 12 | 11,771,031 |
| Mk205 | NIAS_Os_aa08002710 | 8 | 11,543,528 | Mk277 | NIAS_Os_aa12004460 | 12 | 12,333,911 |
| Mk206 | NIAS_Os_aa08002774 | 8 | 12,108,262 | Mk278 | NIAS_Os_aa12004471 | 12 | 13,085,252 |
| Mk207 | NIAS_Os_aa08002815 | 8 | 12,536,246 | Mk279 | NIAS_Os_aa12004494 | 12 | 14,476,268 |
| Mk208 | NIAS_Os_aa08002874 | 8 | 13,145,021 | Mk280 | NIAS_Os_aa12004528 | 12 | 15,029,125 |
| Mk209 | NIAS_Os_aa08002899 | 8 | 13,648,707 | Mk281 | NIAS_Os_aa12004614 | 12 | 15,815,263 |
| Mk210 | NIAS_Os_aa08002979 | 8 | 14,354,648 | Mk282 | NIAS_Os_aa12004637 | 12 | 16,842,118 |
| Mk211 | NIAS_Os_aa08004016 | 8 | 14,874,393 | Mk283 | NIAS_Os_aa12004649 | 12 | 17,627,663 |
| Mk212 | NIAS_Os_ac08000582 | 8 | 16,038,090 | Mk284 | NIAS_Os_aa12005155 | 12 | 23,864,601 |
| Mk213 | NIAS_Os_aa08005445 | 8 | 17,903,867 | Mk285 | NIAS_Os_aa12005164 | 12 | 24,591,137 |
| Mk214 | NIAS_Os_aa08006226 | 8 | 21,226,060 | Mk286 | NIAS_Os_aa12005168 | 12 | 24,759,985 |
| Mk215 | NIAS_Os_aa08006250 | 8 | 21,821,034 | Mk287 | NIAS_Os_aa12005291 | 12 | 26,294,072 |
| Mk216 | NIAS_Os_ab08000934 | 8 | 24,927,823 | | | | |

[1] Position in rice pseudomolecule build4.

**Table 4.** Theoretical allele frequencies of T65 and Hinohikari in bulked DNA

| Mutant type | Bulk | T65 allele frequency | | | Hinohikari allele frequency | | | Total |
|---|---|---|---|---|---|---|---|---|
| | | Completely linked ($r$=0) | Linked | Independent ($r$=0.5) | Completely linked ($r$=0) | Linked | Independent ($r$=0.5) | |
| Sporophytic | $N_{spo}$ | 2/3 | 2/3–$r$/3 | 0.5 | 1/3 | $r$/3+1/3 | 0.5 | 1 |
| | $S_{spo}$ | 0 | 1–$r$ | 0.5 | 1 | $r$ | 0.5 | 1 |
| Gametophytic | $N_{ga}$ | 0 | $r$ | 0.5 | 1 | 1–$r$ | 0.5 | 1 |
| | $S_{ga}$ | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1 |

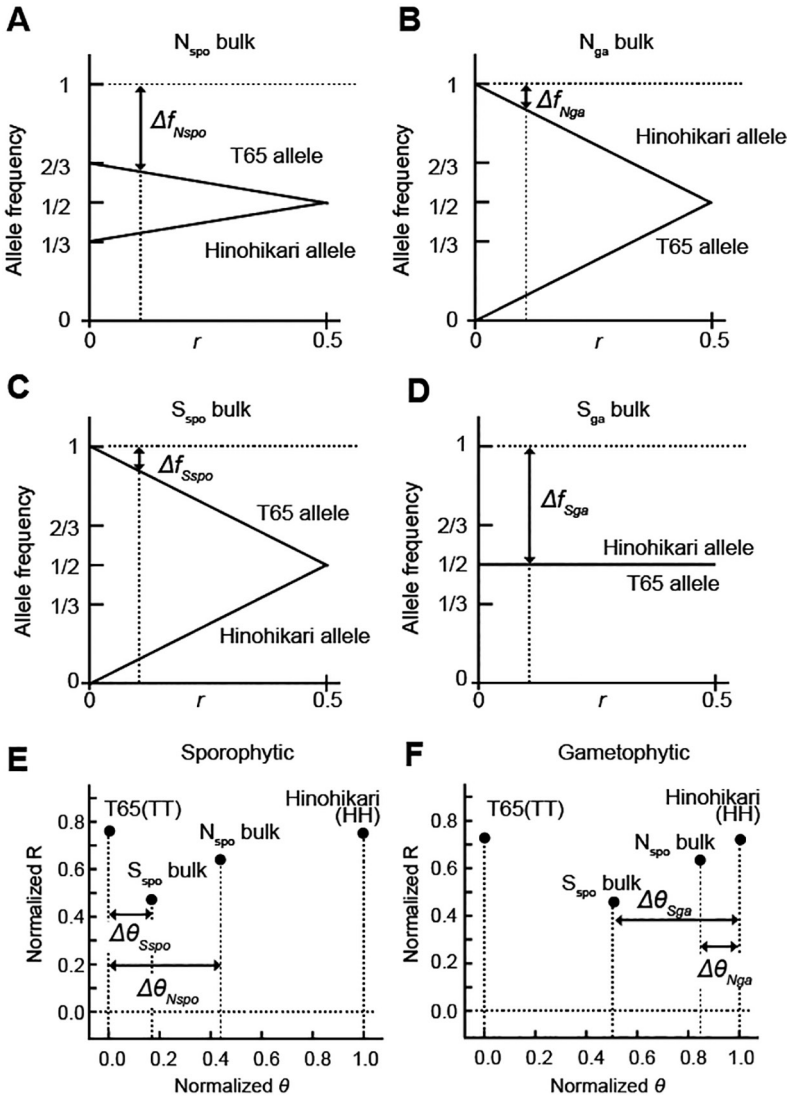


**Fig. 3.** Definition of genetic parameters in this study. (**A**–**D**) Theoretical allele frequency on recombination fraction ($r$) between the causal gene and marker loci. The bulked DNA from normal (fertile) segregants in $F_2$ populations in *SPS* and *GPS* mutants were designated $N_{spo}$ and $N_{ga}$ whereas the bulked DNA from sterile segregants in $F_2$ populations in *SPS* and *GPS* mutants were designated $S_{spo}$ and $S_{ga}$, respectively. The deviation of T65 allele frequency from 1 in the $N_{spo}$ and $S_{spo}$ bulks [$\Delta f_{Nspo}$ (**A**) and $\Delta f_{Sspo}$ (**B**)] and Hinohikari allele frequency from 1 in the $N_{ga}$ and $S_{ga}$ bulks [$\Delta f_{Nga}$ (**C**) and $\Delta f_{Sga}$ (**D**)] were defined. Note that $\Delta f_{Nga}$ and $\Delta f_{Sspo}$ is 0 when the SNP marker of interest is completely linked to the causal locus $r$=0. (**E**–**F**) Differences of $\theta$ values of the $N_{spo}$ bulk and the $S_{spo}$ bulk from T65 as $\Delta\theta_{Nspo}$ and $\Delta\theta_{Sspo}$ (**E**), and difference of $\theta$ values of the $N_{ga}$ bulk and the $S_{ga}$ bulk from Hinohikari as $\Delta\theta_{Nga}$ and $\Delta\theta_{Sga}$ (**F**).

regants analysis of sporophytic pollen sterility, $\Delta f_{Nspo}$ is 1/3 in the $N_{spo}$ bulk (Fig. 3A), and $\Delta f_{Sspo}$ is zero in the $S_{spo}$ bulk (Fig. 3B). Whereas, $\Delta f_{Nspo}$ and $\Delta f_{Sspo}$ at SNP marker independently assorted to the gene ($r$=0.5) are 0.5 (Fig. 3A, B). Similarly, $\Delta f_{Nga}$ in the $N_{ga}$ bulk is zero at $r$=0 (Fig. 3C). In contrast, $\Delta f_{Sga}$ in the $S_{ga}$ bulk is 0.5 at $r$=0.5 (Fig. 3D). In the standardized polar coordinates plot, we defined differences of $\theta$ values of the $N_{spo}$ bulk and the $S_{spo}$ bulk from T65 as $\Delta\theta_{Nspo}$ and $\Delta\theta_{Sspo}$ (Fig. 3E), and difference of $\theta$ values of the $N_{ga}$ bulk and the $S_{ga}$ bulk from Hinohikari as $\Delta\theta_{Nga}$ and $\Delta\theta_{Sga}$ (Fig. 3F). Since $\Delta\theta_{Nspo}$, $\Delta\theta_{Sspo}$, $\Delta\theta_{Nga}$, and $\Delta\theta_{Sga}$ were proportional to $\Delta f_{Nspo}$, $\Delta f_{Sspo}$, $\Delta f_{Nga}$, and $\Delta f_{Sga}$, we assumed that $\Delta\theta_{Sspo}$ and $\Delta\theta_{Nga}$ are closed to zero at $r$=0.

For simple detection of candidate markers linked to the gene, we defined a common logarithm of $\Delta\theta$ for the normal bulk to the sterile bulk (*CLT*) for sporophytic pollen sterility and gametophytic pollen sterility as follows.

$$CLT_{spo} = \log_{10}(\Delta\theta_{Nspo}/\Delta\theta_{Sspo}) \quad (1)$$

$$CLT_{ga} = \log_{10}(\Delta\theta_{Sga}/\Delta\theta_{Nga}) \quad (2)$$

When $r$ is 0.5, $CLT_{spo}$ and $CLT_{ga}$ values should show zero because $\Delta f_{Nspo} = \Delta f_{Sspo} = \Delta f_{Nga} = \Delta f_{Sga} = 0.5$ and $\Delta\theta_{Nspo}/\Delta\theta_{Sspo}$ and $\Delta\theta_{Sga}/\Delta\theta_{Nga}$ is expected to be 1. Meanwhile, when $r$ gets close to 0, $CLT_{spo}$ and $CLT_{ga}$ value approach positive infinity. For practical implementation, the maximum values of $CLT_{spo}$ and $CLT_{ga}$ were limited to 15. Using the 287 bimorphic markers between T65 and Hinohikari, we plot $CLT_{spo}$ and $CLT_{ga}$ on chromosomal order (Fig. 4). In *sps2*, $CLT_{spo}$ showed the highest peak score at the SNP marker *Mk240* (Fig. 4A). In the control bulked sample (C bulk), peak at *Mk240* was not observed (Fig. 4E). Similarly, we selected candidate markers, *Mk139* and *Mk140* for *SPS3*, *Mk99*, *Mk193*, and *Mk251* for *SPS4*, and *Mk83* and *Mk97* for *SPS7*. The bulked segregants analysis of *GPS1* suggested that *Mk94* and *Mk126* were possibly involved in pollen semi–sterility (Fig. 4). We detected candidate markers *Mk81* for *GPS3*, *Mk116*, *Mk153*, *Mk212*, and *Mk271* for *GPS9*, and *Mk192* for *GPS10*, respectively.

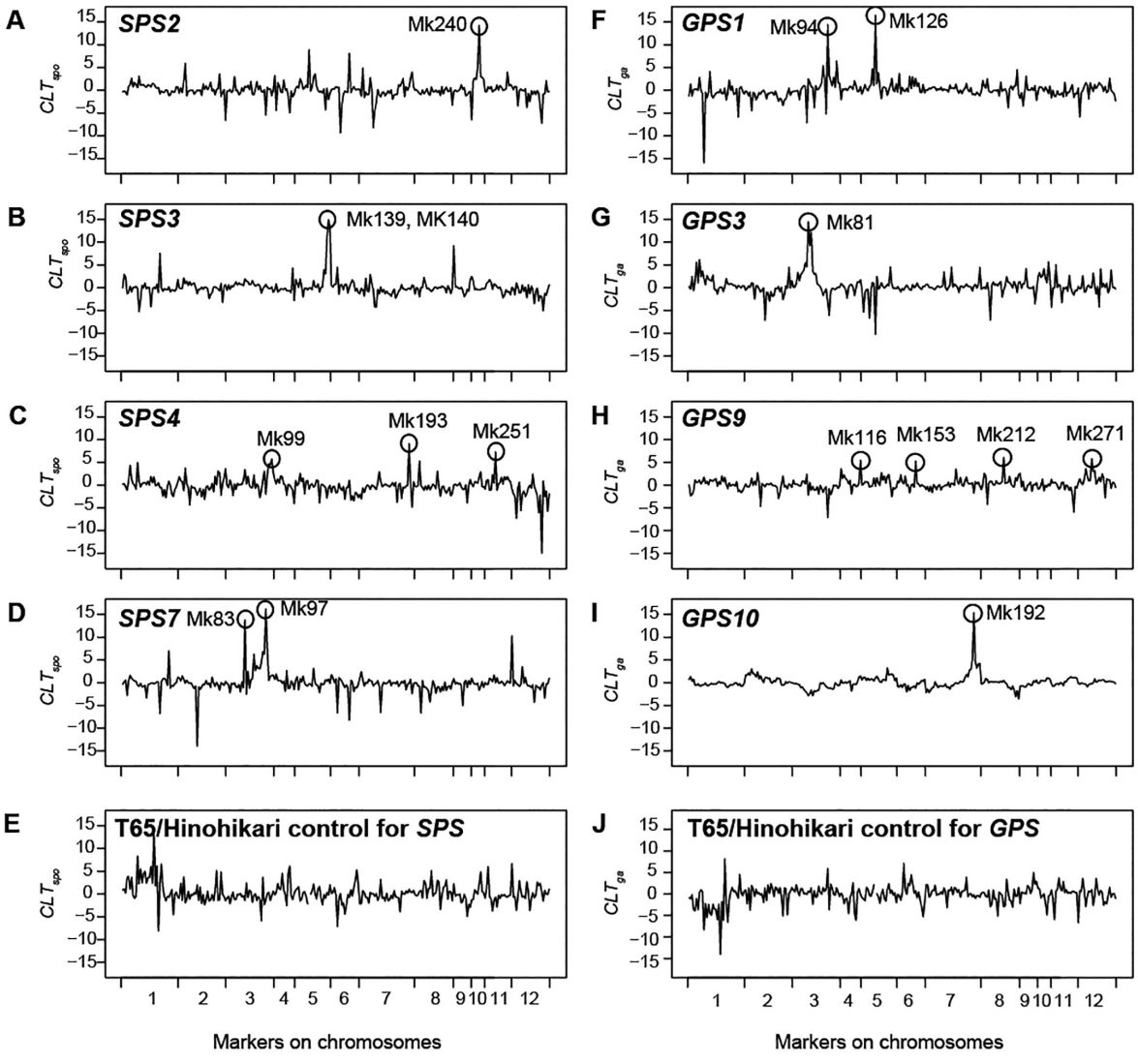


**Fig. 4.** $CLT_{spo}$ for the *SPS* mutants and $CLT_{ga}$ plots for the *GPS* mutants on the twelve chromosome in rice. The candidate peaks are marked in circles.

**Confirmation of candidate peaks by linkage mapping**

To confirm genomic region around the candidate markers was involved in pollen sterility, linkage analysis was performed using PCR–based markers, SSR, STS, CAPS, and dCAPS markers. dCAPS and CAPS markers used in the linkage mapping were newly developed by converting SNP markers of the Beadsarray. In the genetic analysis for *SPS2* using $F_2$ population ($n$=95), *SPS2* was mapped between *QSTS237* and *RM5304* on chromosome 10 with map distances of 32.1 cM and 13.2 cM, respectively (Fig. 5A). *SPS3* was determined to be co–segregated with *Mk138CAPS* and located between *Mk137dCAPS* and *QSTS154* on chromosome 5 at a map distance of 11.0 cM and 2.3 cM (Fig. 5B). *SPS4* was co–segregated with marker *RM1350* and linked to *Mk97dCAPS* on chromosome 3 at a map distance of 16.3 cM (Fig. 5C). And *SPS7* was located between *Mk97dCAPS* and *RM1350* on chromosome 3 at a map distance of 2.9 cM and 21.3 cM, respectively (Fig. 5D). For gametophytic pollen sterility, *GPS1* was linked to marker *Mk97dCAPS* with a map distance of 23.8 cM (Fig. 5E). *GPS3* was also located between markers *Mk82dCAPS* and *Mk83dCAPS* on chromosome 3 at a map distance of 0.5 cM and 5.0 cM, respectively (Fig. 5F). Linkage analysis showed that *GPS9* linked to the marker *QSTS134* on chromosome 4 at map distance of 17.5 cM (Fig. 5G). *GPS10* was located between markers *RM5847* and *RM1364* at a map distance of 11.3 cM and 6.1 cM (Fig. 5H).

## DISCUSSION

Biological studies using mutants has been a widely accepted approach to clarify genomic architectures and physiological and biochemical pathway in the functional genomics era (Sikora *et al*., 2011; Adamski *et al*., 2020). To understand gene function associated with male gametogenesis, mutant resources play an important role in rice as a model crop species for monocotyledon. For gene cloning of gamma–ray or heavy–ion beam irradiated mutants in rice, developing of segregating population derived from an inter–subspecific hybridization between so-called *japonica* and *indica* cultivars is widely used in a variety of analyses such as coarse linkage mapping and high–resolution mapping (Hanzawa *et al*., 2013). However, genetic analysis for pollen sterile mutants should include some considerations. First, pollen sterility controlled by hybrid pollen sterility genes caused by *japonica* and *indica* cross confuses phenotyping of pollen sterility governed by mutations. To avoid this matter, the selection of a more appropriate crossing parent, which shows a *more* polymorphic rate but *less* hybrid pollen sterility, is important for mapping and cloning mutant genes conferring sporophytic and gametophytic pollen sterility. So far, many hybrid pollen sterility genes have been found in *japonica* and *indica*, whereas more genetically related cross combinations between intra–subspecific combinations appear less frequently in hybrid sterility in pollen and embryo sac.

Recent SNP–based high–density and high–through-

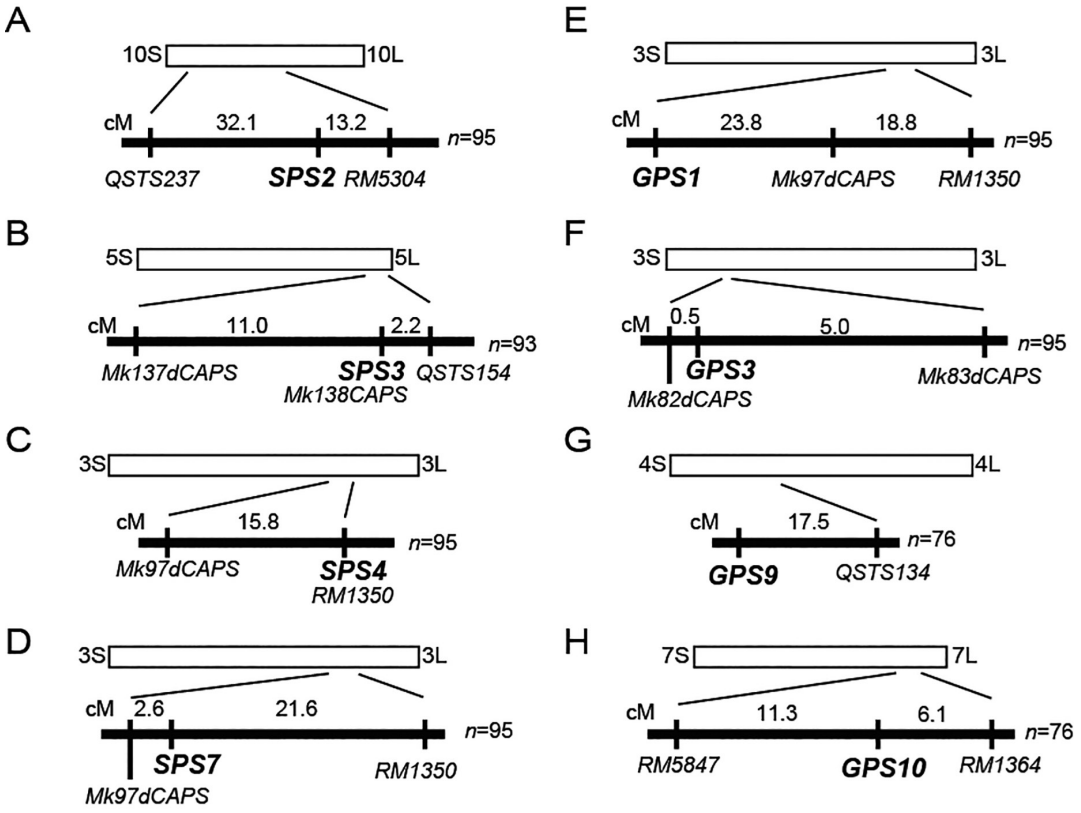


**Fig. 5.** Linkage mapping of the sporophytic pollen sterile mutants (*SPS*) and the gametophytic pollen sterile mutant (*GPS*). (**A**) *SPS2*. (**B**) *SPS3*. (**C**) *SPS4*. (**D**) *SPS7*. (**E**) *GPS1*. (**F**) *GPS3*. (**G**) *GPS9*. (**H**) *GPS10*.

put genotyping systems increased the availabilities of relatively rare SNPs scattered on the rice genome (McCouch *et al*., 2010; Thomson *et al*. 2012). A genotyping–by–sequencing (GBS) and genotype imputation with a hidden Markov model (HMM) approach are powerful in investigating genome–wide genotypes in biparental crosses (Fragoso *et al*., 2016; Furuta *et al*., 2017). However, GBS does not enable to capture the sufficient numbers of polymorphisms, especially on cross combination of the parents with less frequent polymorphisms due to shallow short–read coverage by stochastic sequencing at vast numbers of restriction sites. On the other hand, the SNP genotypes at the genomic region of interest can be rationally designed in Illumina infinium assay and would be suitable for the cross combination with less polymorphism rate, such as Japanese rice (Nagasaki *et al*., 2010).

In rice breeding, pollen sterility was applied widely to exploit rice genetic resources. Thermal–sensitive genic male sterility (TGMS), one kind of nuclear male sterility, was used to develop two–line hybrid rice that utilizes the heterosis of $F_1$ progenies between inbreed varieties. Elucidating the genetic and cytological mechanisms of $F_1$ pollen sterility is essential to overcome the reproductive barrier, an obstacle in exploiting new genetic resources from other subspecies or wild rice.

## AUTHOR CONTRIBUTIONS

A. Yoshimura and Y. Yamagata conceptualized and administrated this study. A. T. Nguyen and Y Yamagata performed the BeadsArray experiment. A. T. Nguyen conducted linkage mapping. The manuscript was originally written by A. T. Nguyen and reviewed and edited by Y. Yamagata. All authors assisted in editing the manuscript and approved the final version.

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