Identification of a single nucleotide deletion causing frameshift mutation in OsDFR2A in a genic male sterile mutant of rice and its possible application to F1 hybrid breeding

doi: 10.?1007/?s11032-013-9835-y

Published in Mol Breeding (2013) 31: 805-814
Postprint; The final publication is available at Springer via
http://dx.doi.org/10.1007/s11032-013-9835-y

Identification of a single nucleotide deletion causing frameshift mutation in *OsDFR2A* in a genic male sterile mutant of rice and its possible application to F_1 hybrid breeding

Sachiko Shirasawa[1], Yasuko Kifuji[1], Reina Komiya[1], Hiroyasu Kitashiba[1], Minoru Nishimura[2], Takeshi Nishio[1]*

1. Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-Amamiyamachi 1-1, Aoba-ku, Sendai, Miyagi 981-8555 Japan
2. Institute of Radiation Breeding, National Institute of Agrobiological Science, Hitachi-Omiya, Ibaraki 319-2293, Japan
* Corresponding author
Present address: Y. Kifuji, Kaneko Seeds Co. Ltd, Isesaki, Gunma 379-2211, Japan
R. Komiya, National Institute of Genetics, Mishima 411-8540, Japan

Abstract

Stable genic male sterility (GMS), which is not influenced by environmental factors, has not been used for F_1 hybrid seed production because male-sterile inbred lines cannot be developed and male-sterile plants must be selected from segregating populations every time. However, the stability of male sterility may provide a reliable system for F_1 seed production without contamination of selfed seeds. A genic male-sterile mutant in rice, 'C204', which was selected from progeny of the cultivar 'Koshihikari' irradiated by gamma-rays, has shorter and whiter anthers than those of 'Koshihikari' and has no pollen grains. Segregation analysis of 'C204' suggested the male sterility of this mutant to be controlled by a recessive allele of a single gene. Linkage analysis of a mutated gene responsible for the male sterility revealed the gene to be in a region of ca. 75 kb on the long arm of chromosome 9. The nine genes predicted in the 75-kb region were sequenced, and compared with the published 'Nipponbare' genome sequences. A single-base deletion was found in the first exon of a 'C204' allele of *Os09g0493500*, which encodes an NAD-dependent epimerase/dehydratase family protein, resulting in frameshift causing a premature stop codon. A dot-blot SNP marker for detection of the single-base deletion in *Os09g0493500* was developed. We herein propose an F_1 hybrid seed production system using stable GMS with a simple selection method of GMS plants.

Keywords: stable GMS, fine mapping, NAD-dependent epimerase/dehydratase, dot-blot-SNP marker, DNA marker selection

Introduction

Male sterility, which is a trait of producing no functional pollen grain with fertile pistils, is useful for F_1 hybrid breeding. Use of a male-sterile line as a maternal parent enables efficient F_1 hybrid seed production without contamination of selfed seeds. Male sterility is classified into cytoplasmic male sterility (CMS) and genic male sterility (GMS). In GMS, there are temperature-sensitive genic male sterility (TGMS), photoperiod-sensitive genic male sterility (PGMS), and stable GMS not influenced by environmental factors (Kurata et al. 2005).

CMS is caused by abnormal transcripts in mitochondria, which disturb the process of pollen development (Pruitt and Hanson 1991; Singh and Brown 1991; Akagi et al. 1994), and pollen fertility is restored by a nuclear restorer gene, i.e., *Rf* (Cui et al. 1996; Bentolila et al. 2002; Brown et al. 2003). CMS lines can be developed by repeated backcrossings of nuclear-donor lines to cytoplasm-donor lines. In F_1 hybrid breeding using CMS lines, a male sterile line is maintained by pollination of a maintainer line that has a normal cytoplasm without an *Rf* gene, and the CMS line is crossed with a restorer line having the *Rf* gene for F_1 hybrid seed production. In this F_1 hybrid breeding system, all the plants in the male sterile line are sterile. However, to maintain three lines, this system requires a significant amount of labor. Furthermore, pollen fertility restored by *Rf* genes functioning gametophytically, e.g., *Rf1* in rice (Kazama and Toriyama 2003; Komori et al. 2004; Akagi et al. 2004; Wang et al. 2006), is 50% fertile, resulting in low seed fertility of F_1 hybrid cultivars.

TGMS and PGMS, which are male sterile under some temperature or photoperiod conditions, e.g., higher than 28°C or longer than 14 h day length, have been reported to be controlled epigenetically (Ding et al. 2012; Zhou et al. 2012). In F_1 hybrid breeding using TGMS or PGMS lines, control of temperature or photoperiod enables development of inbred lines having TGMS or PGMS, respectively, and production of F_1 hybrid seeds (Virmani et al. 2003). This F_1 hybrid breeding system does not require a maintainer line and is thus simple. However, it is not easy to control temperature in the field for large-scale seed production using TGMS, and therefore there is a risk of contamination of selfed seeds in F_1 hybrid seeds.

Although stable GMS not influenced by environmental factors may be considered to be useful for production of F_1 hybrid seeds with high purity, it is impossible to maintain male-sterile inbred lines. Selection of male-sterile plants from a segregating population for a GMS gene is necessary for F_1 hybrid seed production. In the crops whose F_1 hybrid seeds are produced by hand pollination, e.g., tomato, sweet pepper, and eggplant, male-sterile plants can be selected for commercial seed production. On the other hand, in crops which have a short flowering period and are grown in a large field for seed production, e.g., rice, DNA markers closely linked with a GMS gene, which enable selection of plantlets homozygous for the GMS gene, are indispensable for practical F_1 hybrid seed production. Few reports on DNA marker production for GMS have been reported so far (Woo et al. 2008).

We have developed several methods of DNA polymorphism analysis (Sato and Nishio. 2002; Sato et al. 2006; Shirasawa et al. 2006) for selection of plants in a large breeding field. Among them, dot-blot-SNP method is expected to be useful, because it enables single nucleotide polymorphism (SNP) and insertion/deletion genotyping of a large number of plants at low cost without using electrophoresis. In this method, polymerase chain reaction (PCR) products containing SNP sites are dot-blotted on a nylon membrane after denaturation with alkaline solution and are hybridized with digoxigenin-labeled oligonucleotide probes together with unlabeled oligonucleotides having the sequence of the other allele (Shirasawa et al. 2006; Shiokai et al. 2010a; Shiokai et al. 2010b). Furthermore, we have developed a simple method for preparation of PCR templates, named the "leaf-punch method", in which leaf disks excised from leaves using a mini cork borer 1 mm in diameter are directly used as PCR templates (Shiokai et al. 2009).

In the present study, a stable GMS mutant, C204, was selected from progeny of the rice cultivar 'Koshihikari' irradiated by gamma-rays, and a candidate gene responsible for this mutation was identified by genetic analysis. A dot-blot-SNP marker for this mutated gene was developed for establishing an F_1 hybrid breeding system using the stable GMS mutant with the leaf punch method.

Materials and methods

Plant materials

Plants of the Japonica rice cultivar 'Koshihikari' were irradiated with 20 Gy/day of gamma rays for 20 days in the Gamma Field of the Institute of Radiation Breeding, National Institute of Agrobiological Sciences, Japan. A line 'C204', from which male-sterile and fertile plants were segregated with a 1:3 ratio, was selected from M_4 plants. For linkage analysis, 95 F_2 plants derived from a cross between 'C204' and 'Akihikari', which is a Japonica cultivar, were used. For delimitation of a candidate region, 256 F_2 and 1536 F_3 plants derived from a cross between 'C204' and 'CSSL (Chromosome Segment Substitution Line) 228', which is a 'Koshihikari' line carrying a chromosome segment from 'Kasalath' on the long arm of chromosome 9 (Ebitani et al. 2005), were used.

Examination of fertilities of pollen and seeds

For examining pollen fertility, pollen grains were collected just before anthesis and stained with 1% I_2/KI solution. Stained pollen grains were counted under a light microscope. The percentage of fertile seeds per panicle was investigated using five mature panicles per plant harvested in a greenhouse.

Isolation of DNA

Genomic DNAs were prepared from leaves of 'C204' and 'Koshihikari' by the CTAB method (Murray and Thompson 1980). DNAs from individuals of the F_2 mapping populations were extracted by the method of Edwards et al. (1995). For fine mapping using the F_3 population, leaf discs were prepared with a mini cork borer 1 mm in diameter and used directly as templates of PCR (Shiokai et al. 2009).

Linkage analysis and delimitation of a candidate region

Ninety-five F_2 individuals derived from a cross between 'C204' and 'Akihikari' were genotyped with 2 simple sequence repeat (SSR) (McCouch et al. 2002), 2 sequence characterized amplified region (SCAR) (Shirasawa. et al. 2004), 5 cleaved amplified polymorphic sequence (CAPS) (Shirasawa et al. 2004), and 29 dot-blot-SNP markers (Shirasawa et al. 2006) (Supplementary Table S1). These genotype data were used for linkage analysis of GMS by Mapmaker/EXP 3.0 software (http://www.broad.mit.edu/ftp/distribution/software/mapmaker3/) with lod score 3.0. As other mapping populations, 256 F_2 and 1536 F_3 plants derived from a cross between 'C204' and 'CSSL228' were used for the genotyping of seven markers, including two SSRs, one SCAR, one CAPS, and two dot-blot-SNP markers (Supplementary Table S2). In addition, 25 PCR-restriction fragment-single strand conformation polymorphism (PCR-RF-SSCP) markers (Shirasawa et al. 2004) were designed to narrow the region containing the GMS gene (Supplementary Table S3). PCR and signal detections were performed as previously described by McCouch et al. (2002), Shirasawa et al. (2004), and Shirasawa et al. (2006).

Sequencing of a candidate of the GMS gene

DNA fragments were amplified from genomic DNA of 'C204' by PCR using primers covering the entire coding regions and 1-kb upstream regions of candidate genes (Supplementary Table S4). A reaction mixture of 20 µl contained 20 ng plant genomic DNA, 20 pmol of each primer, 1 x PCR buffer, 2 nmol dNTPs, and 0.5 units of ExTaq DNA polymerase (TaKaRa Biomedicals, Japan). The PCR condition was 1-min denaturation at 94ºC, followed by 40 cycles of 30-sec denaturation at 94ºC, 30-sec annealing at 62ºC, and 90-sec extension at 72ºC, and a 3-min final extension at 72ºC. PCR products separated by electrophoresis on 1% agarose gel (AgaroseS, Nippongene, Japan) were purified using Ultraclean 15 DNA Purification Kit (MO BIO, USA). Nucleotide sequences of the PCR products were determined by the direct sequencing method using a CEQ2000 DNA sequencer (Beckman Coulter, USA).

Development of dot-blot-SNP markers for the mutated gene and screening of GMS plants homozygous for the mutant allele

Leaf discs were prepared from young leaves of F_2 plants derived from a cross between 'C204' and 'Akihikari' with a mini cork borer 1 mm in diameter. PCR was performed with these discs as templates to amplify DNAs containing the mutation. A reaction mixture of 20 µl contained the leaf disc, 10 pmol of each primer (5'-GGCAAAGTGTGTGTAACTGGTG-3' and 5'-TTGGGTCTCTCACAGTTCCTATC-3'), 1 x PCR buffer, 2 nmol dNTPs, and 0.5 units of ExTaq DNA polymerase (TaKaRa Biomedicals, Japan). The PCR condition was as follows: 1-min denaturation at 94ºC; 40 cycles of 30-sec denaturation at 94ºC, 30-sec annealing at 58ºC, and 30-sec extension at 72ºC, and a 1-min final extension at 72ºC. Dot-blot SNP analysis was performed as described by Shiokai et al. (2010b). The PCR products were mixed with an equal volume of the denaturing solution containing 0.4 N NaOH and 10 mM EDTA, and then dot-blotted onto two nylon membranes (Hybond N, GE healthcare, USA) with a Multi-pin Blotter (ATTO, Japan). Oligonucleotides were prepared as probes for detecting 'Koshihikari'-allele (5'-TCGCCTCTTGGCTCATC-3') and 'C204'-allele (5'-GTCGCCTCTGGCTCATC-3'). Oligonucleotide probes of 100 pmol labeled with biotin at the 5' end were mixed with five volumes of a 100-pmol unlabeled counterpart oligonucleotide DNA as a competitor. Each membrane was hybridized with each probe solution at 40ºC and washed with 1x SSC/0.1% SDS twice. Signals were detected with streptavidin-alkaline phosphatase conjugate (Promega, USA).

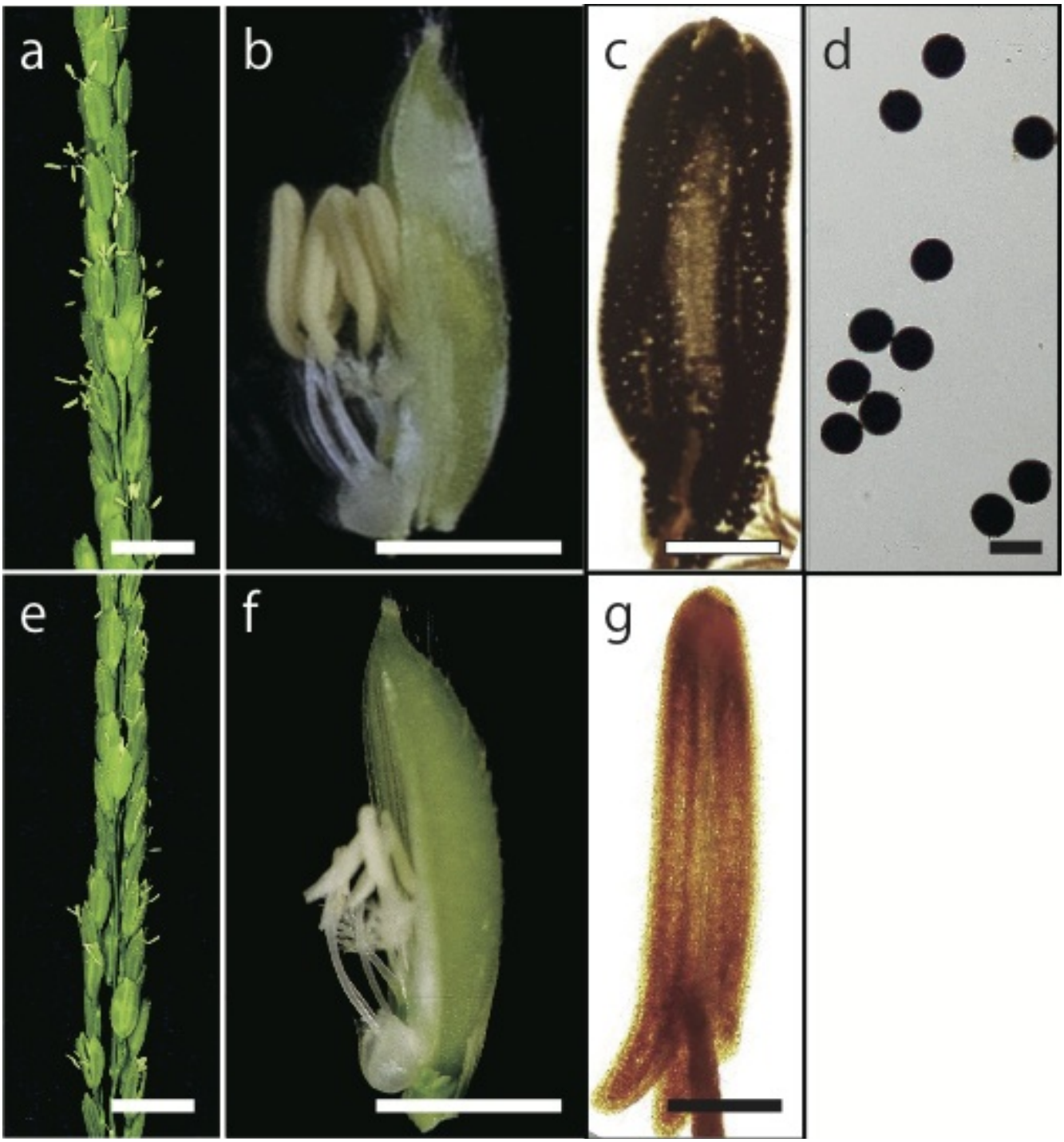


Fig. 1. Comparison of flower morphology between 'Koshihikari' (wild type) and 'C204'.
Panicles (a and e), anthers (b, c, f and g) and pollen grains (d) of 'Koshihikari' (a to d) and 'C204' (e to g). Anthers and pollen grains of c, d, and g were investigated after staining with I_2/KI solution. The bars in the figures indicate 1 cm in (a and e), 3 mm in (b and f), 0.5 mm in (c and g), and 30 µm in (d).

Results

Male sterility of 'C204' and genetic analysis

'C204', a mutant line in which complete male sterile plants segregate in progeny, was selected from several male sterile lines obtained in M_4 of 'Koshihikari' irradiated with gamma-rays. This line was maintained by selfing of heterozygous plants showing normal pollen fertility with progeny test. To investigate stability of male sterility of 'C204' under various environmental conditions, six populations of this line were grown in different seasons in a greenhouse or an open field of Tohoku University, Sendai, Japan (38˚16' N, 140˚52' E). The mutants of 'C204' stably showed complete pollen sterility under average temperatures from 20ºC to 37ºC at the flower development stage in the tests from March to September.

Anthers of 'C204' were smaller and whiter than those of 'Koshihikari' (Fig. 1a, 1b, 1e, 1f). No pollen grains were observed in the anthers of 'C204' (Fig. 1c, 1d, 1g). In 95 F_2 plants obtained from a cross between 'C204' and Akihikari', 70 plants were male fertile and 25 plants were male sterile (χ^2=0.088, d=1, p>0.75), suggesting that the male sterility of 'C204' is

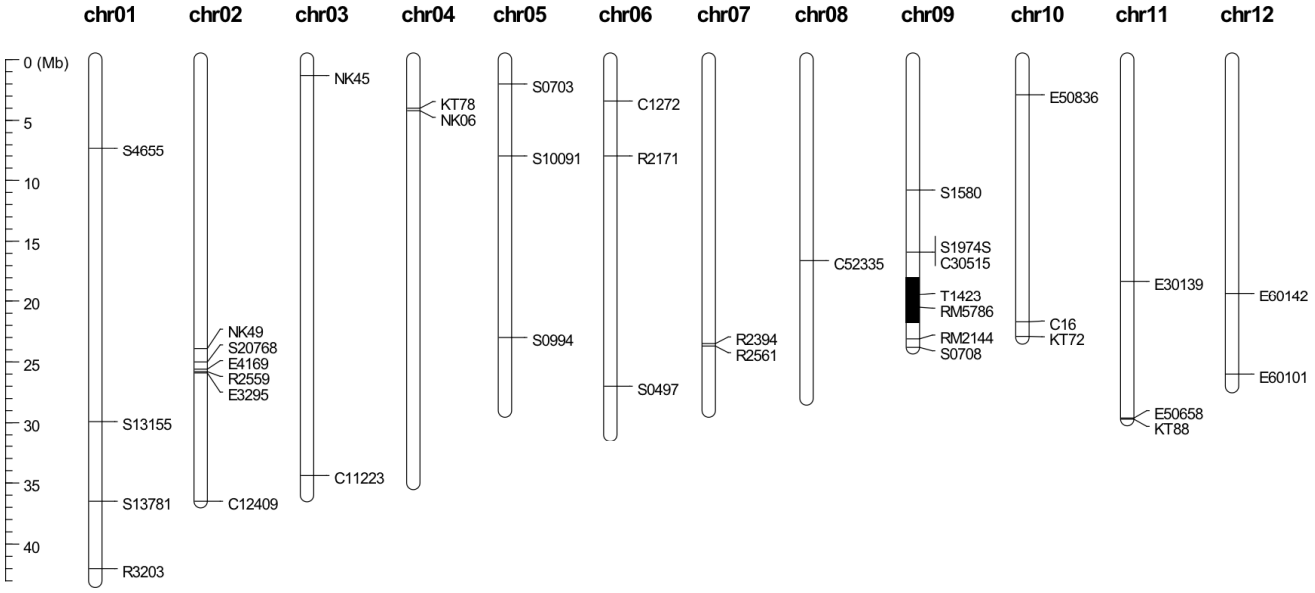


Fig. 2. Position of a chromosomal region linked to the male sterility gene of 'C204' (black bar).
DNA markers used for rough mapping of the male-sterility gene are shown.

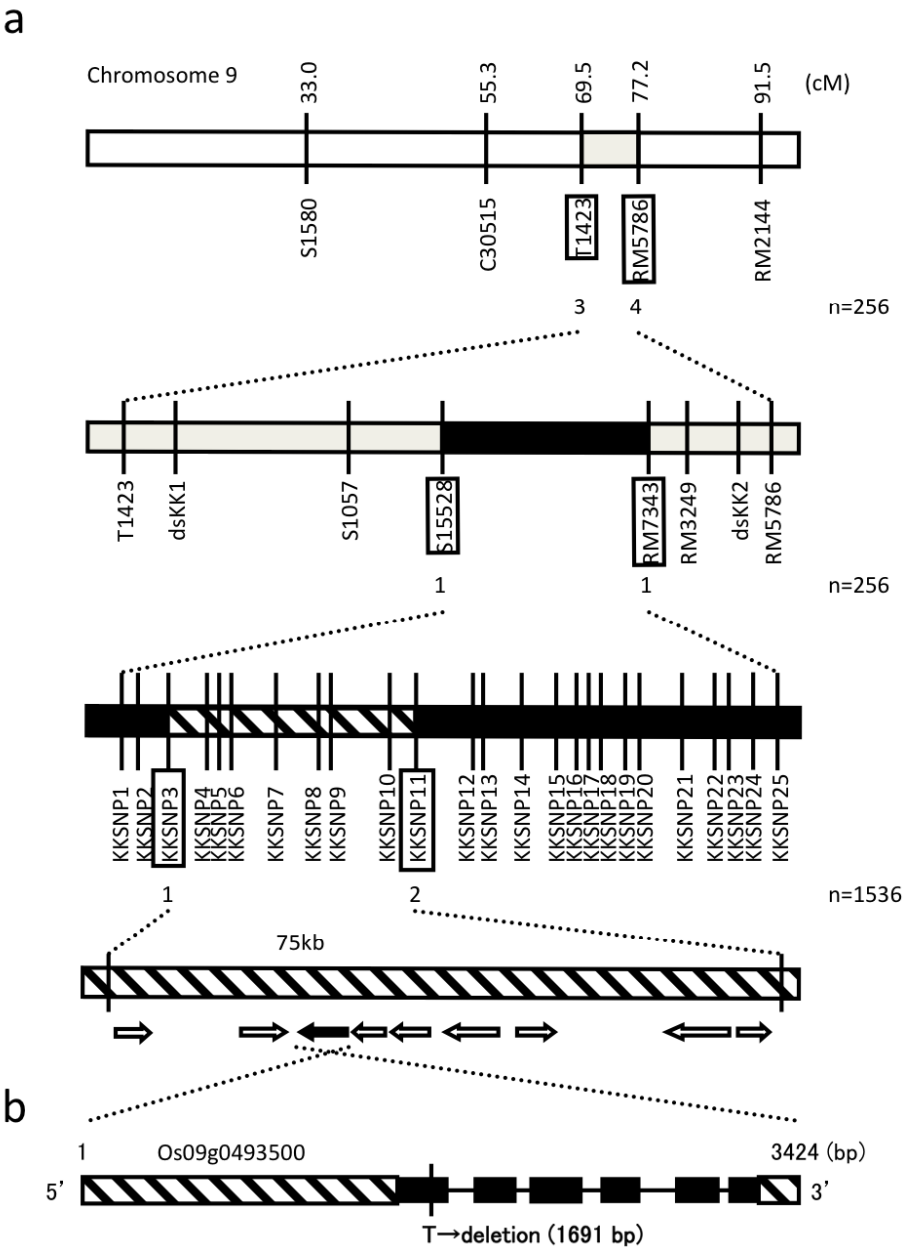
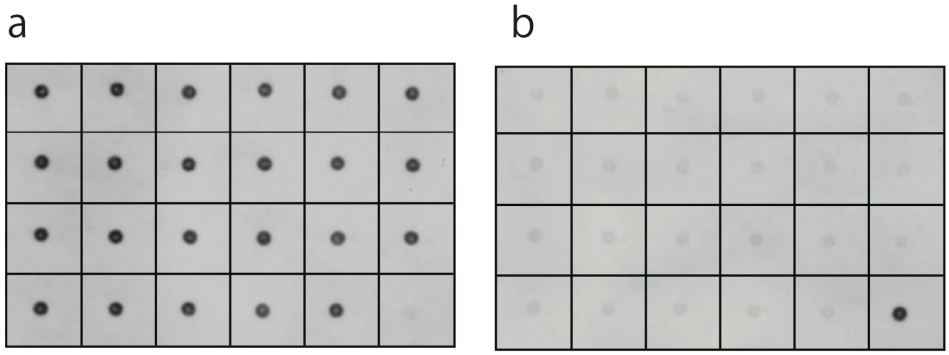


Fig. 3. Fine mapping of the male sterility gene of 'C204'.
(a) Delimitation of a region harboring the male sterility gene using F_2 and F_3 progenies between CSSL228 and 'C204'. The numbers of obtained recombinants between DNA markers and the male sterility gene are shown under DNA markers. (b) The structure of *Os09g0493500*, in which a single-base deletion was identified. Black boxes indicate coding regions, hatched boxes indicate UTR regions, and black bars indicate introns.



Koshihikari	Akihikari	Hinohikari	Kirara 397	Kihou	Nipponbare
Hitomebore	Hatsuboshi	Sasanishiki	Hatsunishiki	Koganebare	Nourin 1
Nourin 6	Nourin 22	Kasalath	IR32	Itadaki	Tohoku 168
Nourin 8	Rikuu 132	Basmati 370	Senshou	Kokoromachi	C204

Fig. 4. Genotyping of 23 rice cultivars with a dot-blot-SNP marker for detection of the single-nucleotide deletion identified in 'C204'.
Genotypes of 23 cultivars were determined using a probe for detecting a wild-type allele (a) and a mutant-type allele (b). Names of 23 cultivars are shown (c). Black box indicates 'C204'.

controlled by a recessive allele of a single gene.

Linkage analysis of a male sterility gene

Since F_1 hybrid plants between 'C204' and 'Kasalath' showed hybrid sterility, F_2 plants derived from a cross between 'C204' and 'Akihikari' were used for rough mapping of the mutated gene responsible for male sterility. Genotyping of the 95 F_2 plants between 'C204' and 'Akihikari' with 38 DNA markers revealed the male sterility gene to be linked to T1423 and RM5786 on chromosome 9 with distances of 5.9 cM and 6.6 cM, respectively (Fig. 2).

For fine mapping of the male sterility gene, 'C204' was crossed with a chromosome segment substitution line having the long arm of chromosome 9 of 'Kasalath' with 'Koshihikari' background, CSSL228, to obtain F_2 population. Using 256 F_2 plants, the male sterility gene was mapped between T1423 and RM5786 at distances 3.1 cM and 3.0 cM from T1423 and RM5786, respectively (Fig. 3). Twenty-four recombinants between T1423 and RM5786 were obtained in the 256 F_2 plants. To obtain more recombinants in this genome region, 1,536 F_3 plants were genotyped with dot-blot-SNP markers dsKK1 and dsKK2, which were developed using nucleotide sequences near T1423 and RM5786, and 145 recombinants between them were selected. These selected recombinant plants were genotyped using two SSR markers, i.e., RM7343 and RM3249, SCAR marker S15528, and CAPS marker S1057, and their pollen fertilities were investigated to delimit a region containing the male sterility gene. For further delimitation of the region, 25 PCR-RF-SSCP markers were designed from the sequences of the genes on the region between dsKK1 and dsKK2 following Shirasawa et al. (2004). These analyses revealed the male sterility gene to be in a region of ca. 75 kb between KKSNP3 and KKSNP11 (Supplementary Fig. 1).

A possible candidate gene of the male sterility gene

Nine genes have been predicted in the 75-kb region between KKSNP3 and KKSNP11 (Rice Annotation Project et al. 2008). Full lengths of the coding regions and 1-kb upstream regions of these nine genes of 'C204' were sequenced and compared with the published 'Nipponbare' genome sequences. In eight genes, i.e., Os09g0493000, Os09g0493400, Os09g0493600, Os09g0493700, Os09g0493800, Os09g0494200, Os09g0494300, and Os09g0496000, the determined nucleotide sequences of 'C204' were completely the same as those of 'Nipponbare'. On the other hand, a single-base deletion was detected in the 1691st "T" from the transcription start site within the first exon of 'C204' allele of *Os09g0493500*, which encodes an NAD-dependent epimerase/dehydratase family protein and has been reported to be specifically expressed in anthers in rice. This single-base deletion results in a frameshift causing a premature stop codon (Fig. 3b).

Development of a SNP marker for the mutation in the possible candidate gene

A dot-blot SNP marker for detection of the single-base deletion in *Os09g0493500* was developed and used for genotyping of 'C204', 'Koshihikari', and 23 cultivars including 19 Japonica cultivars, three Indica cultivars, and one upland rice cultivar. All the cultivars showed the same genotype as that of 'Koshihikari', and the single-base deletion was detected only in 'C204' (Fig. 4), suggesting this single-base deletion to be a mutation induced by gamma-ray irradiation and not a natural variation in rice cultivars.

Using leaf discs of 63 F_2 plants between 'C204' and 'Akihikara' prepared by the leaf punch method as PCR templates, DNAs were amplified for dot-blot SNP analysis of the single-base deletion in *Os09g0493500*. Sixteen of these plants were identified to be homozygotes of the mutant allele, and all of them showed 0% pollen fertility. Fifteen plants homozygous for the wild type allele and 32 heterozygous plants were completely fertile. To confirm F_1 hybrids produced by these male sterile plants to be fertile, the male sterile plants of mutant homozygotes were crossed with 'Koshihikari' pollen. All the F_1 hybrid plants showed normal pollen and seed fertilities.

Discussion

Os09g0493500, the first exon of which was revealed in the present study to have a single-nucleotide deletion in the male-sterile mutant 'C204', has an NAD(P)H-binding domain at the N terminal region and is highly homologous with genes in the dihydroflavonol 4-reductase (DFR) family. A possible function of Os09g0493500 in male organ development has been suggested (Yau et al. 2005). *Os09g0493500* has been identified among 11 clones obtained by anther cDNA subtraction between 'Annong-N' and a TGMS mutant, 'Annong S-1', under high temperature causing male sterility, and has been named *OsDFR2*. Specific expression of *OsDFR2* in anthers at an early stage of microsporogenesis has been detected by *in situ* hybridization, and the gene expression level in 'Annong S-1' has been revealed to be one-fourth of that in

'Annong-N'. The mutated gene causing TGMS, i.e., *tms5*, has been mapped on chromosome 2, not on chromosome 9 harboring *OsDFR2* (Wang et al. 2003; Yang et al. 2007). It has been suggested that *OsDFR2* functions downstream of *tms5* for TGMS of 'Annong S-1'. T-DNA insertion lines, drl1-1 and drl1-2, of *DRL1* (*Dihydroflaconol 4-reductase-like1*; *At4g354200*), which is the ortholog of *OsDFR2* in *Arabidopsis thaliana* and is expressed in tapetum cells, tetrads, and developing microspores and not in mature anthers, have been found to be partially sterile and completely sterile, respectively (Tang et al. 2009). Transformation of these T-DNA insertion lines with endogenous *DRL1* has been found to restore pollen fertility. These previous studies suggest that *OsDFR2* is important in pollen development. Since our genetic analysis of male-sterile line 'C204' revealed only one nucleotide difference in the nine genes predicted in the 75 kb region, i.e., a single nucleotide deletion in the first exon causing a frameshift resulting in premature stop codon, the single nucleotide deletion in *OsDFR2* is considered to be the most probable cause of the male sterility phenotype of the mutant.

Although genetic analysis of GMS mutants has identified several genes participating in the development of male organs (Jung et al. 2005; Han et al. 2006; Jung et al. 2006; Li et al. 2006; Woo et al. 2008), there has been no proposal for F_1 hybrid breeding using the identified mutations as DNA markers so far. Woo et al. (2008) have revealed the *ms-h* gene responsible for a stable GMS mutation to be *UGPase1*, which is also responsible for chalky endosperm. The chalky endosperm trait of brown rice can be used for selection of the seeds of GMS plants, i.e., *ms-h* homozygotes, in F_1 seed production. However, chalky endosperm is also induced by high temperature at the seed ripening stage in most rice cultivars (Tabata et al. 2007; Kobayashi et al. 2008; Yamakawa et al. 2008). Such chalky endosperm caused by high temperature results in selection of fertile plants in the F_1 seed production method using the *ms-h* gene. Furthermore, F_1 hybrid plants having *Ms-h/ms-h* heterozygous genotype produced by this method are considered to set seeds of chalky endosperm at 25%, which lowers the quality of rice. F_1 seed production with no contamination of selfed seeds can be achieved by selection using DNA markers not influenced by environmental factors.

As parental lines for F_1 seed production, Indica cultivars, a combination of which can yield higher heterosis than that of Japonica cultivars, have been commonly used. The combination of an Indica cultivar and a Japonica cultivar provides strong heterosis in vegetative growth, but hybrid sterility of Indica/Japonica hybrids results in low productivity (Ikehashi and Araki 1984; Liu et al. 1996; Zhang et al. 1997). A recent study (Cheng et al. 2007) has identified several combinations of Indica lines and Japonica lines that have super yielding ability, and F_1 hybrids of such combinations have been widely cultivated. A wide-compatibility gene, which overcomes the hybrid sterility between Indica and Japonica, has been isolated (Chen et al. 2008) and has become usable in plant breeding as a DNA marker. 'C204' selected in the present study is a mutant obtained from 'Koshihikari', which has high eating quality. Use of 'C204'as a parent of the F_1 hybrid cultivar may contribute to improvement of the eating quality of F_1 hybrid cultivars. Selection of parental lines used in combination with 'C204' will be important for development of F_1 hybrid cultivars possessing high yield and high eating quality.

In the present study, a dot-blot-SNP marker which can detect the mutation in the candidate gene for GMS was developed, and a simple selection method of GMS plants using the leaf-punch method (Shiokai et al. 2009) was established. Several thousands of plants can be genotyped by dot-blot-SNP analysis at one time (Shirasawa et al. 2006). The selection method for GMS plants developed in the present study requires only low cost and little labor, suggesting the possibility of practical use of this method in rice F_1 hybrid breeding.

Acknowledgment

We wish to thank Mr. T. Sekii and Y. Nakagomi for their technical assistance.

Supplementary Data

Supplementary Fig. 1. Graphical genotypes of recombinants obtained in the F_3 population and their phenotypes.

Black boxes indicate regions homozygous for mutant-type alleles, and white boxes indicate heterozygous regions. Gray boxes are regions having recombination break points. "S" and "F" indicate sterile and fertile, respectively. The numbers to the right are the number of recombinants obtained.

Supplementary Table S1. Sequences of primers and probes used for linkage analysis

Supplementary Table S2. Sequences of primers and probes used for fine mapping

Supplementary Table S3. Sequences of primers used for PCR-RF-SSCP analysis

Supplementary Table S4. Sequences of primers used for sequencing a candidate of the GMS gene

References

Akagi H, Sakamoto M, Shinjyo C, Shimada H, Fujimura T (1994) A unique sequence located downstream from the rice mitochondrial *atp6* may cause male sterility. Curr Genet 25:52-58

Akagi H, Nakamura A, Yokozeki-Misono Y, Inagaki A, Takahashi H, Mori K, Fujimura T (2004) Positional cloning of the rice *Rf-1* gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. Theor Appl Genet 108: 1449-1457

Bentolila S, Alfonso AA, HansonMR (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc Natl Acad Sci USA 99:10887- 10892

Brown GG, Formanová N, Jin H, Wargachuk R, Dendy C, Patil P, Laforest M, Zhang J, Cheung WY, Landry BS (2003) The radish *Rfo* restorer gene of *Ogura* cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. Plant J 35:262-272

Chen J, Ding J, Ouyang Y, Du H, Yang J, Cheng K, Zhao J, Qiu S, Zhang X, Yao J, Liu K, Wang L, Xu C, Li X, Xue Y, Xia M, Ji Q, Lu J, Xu M, Zhang Q (2008) A triallelic system of *S5* is a major regulator of the reproductive barrier and compatibility of indica-japonica hybrids in rice. PNAS 105:11436-11441

Cheng SH, Zhuang JY, Fan YY, Du JH, Cao LY (2007) Progress in research and development on hybrid rice: a super-domesticate in China. Ann Bot 100:959-66

Cui X, Wise RP, Schnable PS (1996) The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. Science 272:1334-1336

Ding J, Lu Q, Ouyang Y, Mao H, Zhang P, Yao J, Xu C, Li X, Xiao J, Zhang Q (2012) A long noncoding RNA regulates photoperiod-sensitive male sterility, an essential component of hybrid rice. Proc Natl Acad Sci USA 109:2654-2659

Ebitani T, Takeuchi Y, Nonoue Y, Yamamoto T, Takeuchi K, Yano M (2005) Construction and evaluation of chromosome

segment substitution lines carrying overlapping chromosome segments of *indica* rice cultivar 'Kasalath' in a genetic background of *japonica* wlite cultivar 'Koshihikari'. Breed Sci 55:65-73

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19: 1349

Han MJ, Jung KH, Yi G, Lee DY, An G (2006) *Rice Immature Pollen 1 (RIP1)* is a regulator of late pollen development. Plant Cell Physiol 47:1457-1472

Ikehashi H, Araki H (1984) Varietal screening of compatibility types revealed in F_1 hybrids of distant crosses in rice. Jpn J Breed 34: 304-313

Jung KH, Han MJ, Lee YS, Hwang I, Kim MJ, Kim YK, Nahm BH, An G (2005) Rice *undeveloped tapetum1* is a major regulator of early tapetum development. Plant Cell 17: 2705-2722

Jung KH, Han MJ, Lee DY, Lee YS, Schreiber L, Franke R, Faust A, Yephremov A, Saedler H, Kim YW, Hwang I, An G (2006) *Wax-deficient anther1* is involved in cuticle and wax production in rice anther walls and is required for pollen development. Plant Cell 18: 3015-3032

Kazama T, Toriyama K (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice. FEBS Lett 544:99-102

Kobayashi A, Genliang B, Shenghai1 Y, Tomita K (2007) Detection of Quantitative Trait loci for white-back and basal-white kernels under high temperature stress in japonica rice varieties. Breed Sci 57:107-116

Komori T, Ohta S, Murai N, Takakura Y, Kuraya Y, Suzuki S, Hiei Y, Imaseki H, Nitta N (2004) Map-based cloning of a fertility restorer gene, *Rf-1*, in rice (*Oryza sativa* L.). Plant J 37:315-325

Kurata N, Miyoshi K, Nonomura K, Yamazaki Y, Ito Y (2005) Rice mutants and genes related to organ development, morphogenesis and physiological traits. Plant Cell Physiol 46: 48–62

Li N, Zhang DS, Liu HS, Yin CS, Li XX, Liang WG, Yuan Z, Xu B, Chu HW, Wang J, Wen TQ, Huang H, Luo D, Ma H, Zhang D (2006) The rice *tapetum degeneration retardation* gene is required for tapetum degradation and anther development. Plant Cell 18:2999-3014

Liu KD, Zhou ZQ, Xu CG, Zhang Q, Saghai Maroof MA (1996) An analysis of hybrid sterility in rice using a diallel cross of 21 parents involving *indica, japonica* and wide compatibility varieties. Euphytica 90:275–280

McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L (2002) Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Res 9: 199-207

Murray MG, WF Thompson (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8: 4321-4325

Pruitt KD, Hanson MR (1991) Transcription of the Petunia mitochondrial CMS-associated *Pcf* locus in male sterile and fertility-restored lines. Mol Gen Genet 227:348-355

Rice Annotation Project (2008) The Rice Annotation Project Database (RAP-DB): 2008 update. Nucleic Acids Res 36 (Database issue): D1028-D1033

Sato Y, Nishio T (2002) Efficient detection of DNA polymorphism in cabbage and rice cultivars by PCR-RF-SSCP (PRS). Plant Cell Rep 21: 276-281

Sato Y, Shirasawa K, Takahashi Y, Nishimura M, Nishio T (2006) Mutant selection of progeny of gamma-ray-irradiated rice by DNA heteroduplex cleavage using *Brassica* petiole extract. Breed Sci 56: 179-183

Shirasawa K, Kishitani S, Nishio (2004) Conversion of AFLP markers to sequence-specific markers for closely related lines in rice by use of the rice genome sequence. Mol Breed 14: 283-292

Shirasawa K, Shiokai S, Yamaguchi M, Kishitani S, Nishio T (2006) Dot-blot-SNP analysis for practical plant breeding and cultivar identification in rice. Theor Appl Genet113: 147-155

Shiokai S, Kitashiba H, Shirasawa K, Nagano K, Nishio T (2009) Leaf-punch method to prepare a large number of PCR templates from plants for SNP analysis. Mol Breed 23:329-336

Shiokai S, Kitashiba H and Nishio T. (2010a) Prediction of the optimum hybridization conditions of dot-blot-SNP analysis using estimated melting temperature of oligonucleotide probes. Plant Cell Rep 29: 829-834

Shiokai S, Shirasawa K, Sato Y and Nishio T (2010b) Improvement of the dot-blot-SNP technique for efficient and cost-effective genotyping. Mol Breed 25: 179-185

Singh M, Brown GG (1991) Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant Cell 3:1349-1362

Tabata M, Hirabayashi H, Takeuchi Y, Ando I, IidaY, Ohsawa R (2007) Mapping of quantitative trait loci for the occurrence of white-back kernels associated with high temperatures during the ripening period of rice (*Oryza sativa* L.) Breed Sci 57:47-52

Tang LK, Chu H, Yip WK, Yeung EC, Lo C (2009) An anther-specific dihydroflavonol 4-reductase-like gene (*DRL1*) is essential for male fertility in Arabidopsis. New Phytol 181: 576-587

Virmani SS, Sun ZX, Mou TM, Jauhar Ali A, Mao CX (2003) Two-line hybrid rice breeding manual. Los Baños (Philippines): International Rice Research Institute. 88 p

Wang YG, Xing QH, Deng QY, Liang FS, Yuan LP, Weng ML, Wang B (2003) Fine mapping of the rice thermo-sensitive genic male-sterile gene *tms5*. Theor Appl Genet 107:917-921

Woo MO, Ham TH, Ji HS, Choi MS, Jiang W, Chu SH, Piao R, Chin JH, Kim JA, Park BS, Seo HS, Jwa NS, McCouch S, Koh HJ (2008) Inactivation of the *UGPase1* gene causes genic male sterility and endosperm chalkiness in rice (*Oryza sativa* L.) Plant J 54: 190-204

Yamakawa H, Ebitani T, Terao T (2008) Comparison between locations of QTLs for grain chalkiness and genes responsive to high temperature during grain filling on the rice chromosome map. Breed Sci 58:337-343

Yang Q, Liang C, Zhuang W, Li J, Deng H, Deng Q, Wang B (2007) Characterization and identification of the candidate gene of rice thermo-sensitive genic male sterile gene *tms5* by mapping. Planta 225:321-330

Yau CP, Zhuang CX, Zee SY, Yip WK (2005) Expression of a microsporocyte-specific gene encoding dihydroflavonol 4-reductase-like protein is developmentally regulated during early microsporogenesis in rice. Sex Plant Reprod 18:65-74

Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D, Long Y, Zhong Y, Liu YG (2006) Cytoplasmic male sterility of rice with boroII cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif

genes via distinct modes of mRNA silencing. Plant Cell 18:676–687

Zhou H, Liu Q, Li J, Jiang D, Zhou L, Wu P, Lu S, Li F, Zhu L, Liu Z, Chen L, Liu YG, Zhuang C (2012) Photoperiod- and thermo-sensitive genic male sterility in rice are caused by a point mutation in a novel noncoding RNA that produces a small RNA. Cell Res 22:649-660

Zhang Q, Liu KD, Yang GP, Saghai Maroof MA, Xu CG, Zhou ZQ (1997) Molecular marker diversity and hybrid sterility in *indica-japonica* rice crosses. Theor Appl Genet 95:112–118