Analysis of Cytoplasmic Effects and Fine-Mapping of a Genic Male Sterile Line in Rice

Peng Qin1,2,*, Yuping Wang1,2,*, Yuanyuan Li1, Bingtian Ma1, Shigui Li1,2,*

1 Rice Research Institute of Sichuan Agricultural University, Chengdu Wenjiang, Sichuan, China, 2 State Key Laboratory of Hybrid Rice, Sichuan Agricultural University, Chengdu Wenjiang, Sichuan, China

Abstract
Cytoplasm has substantial genetic effects on progeny and is important for yield improvement in rice breeding. Studies on the cytoplasmic effects of cytoplasmic male sterility (CMS) show that most types of CMS have negative effects on yield-related traits and that these negative effects vary among CMS. Some types of genic male sterility (GMS), including photothermo-sensitive male sterility (PTMS), have been widely used in rice breeding, but the cytoplasmic effects of GMS remain unknown. Here, we identified a GMS mutant line, h2s, which exhibited small, white anthers and failed to produce mature pollen. Unlike CMS, the h2s had significant positive cytoplasmic effects on the seed set rate, weight per panicle, yield, and general combining ability (GCA) for plant height, seed set rate, weight per panicle, and yield. These effects indicated that h2s cytoplasm may show promise for the improvement of rice yield. Genetic analysis suggested that the phenotype of h2s was controlled by a single recessive locus. We mapped h2s to a 152 kb region on chromosome 6, where 22 candidate genes were predicted. None of the 22 genes had previously been reported to be responsible for the phenotypes of h2s. Sequencing analysis showed a 12 bp deletion in the sixth exon of Loc_Os06g40550 in h2s in comparison to wild type, suggesting that Loc_Os06g40550 is the best candidate gene. These results lay a strong foundation for cloning of the H2S gene to elucidate the molecular mechanism of male reproduction.

Introduction
Cytoplasmic male sterility (CMS) is essential to the exploitation of heterosis in three-line hybrid rice breeding programs. CMS cytoplasm has been reported to have substantial genetic effects on various traits of hybrid rice [1,2,3]. Studies of the cytoplasmic effects of CMS show that the effects within a type of cytoplasm vary across different agronomic traits in hybrid rice and that the effects on one agronomic trait vary among different types of cytoplasm. For example, the male sterile cytoplasm of IR46828, IR46831, IR40483, IR54732, IR17492-18-10-2-2-3, IR54753, IR19661-283-1-3-2, IR54758, and IR54756 have been found to have different effects on grain yield [4]. Most CMS cytoplasms have been reported to have negative effects on yield-related traits, and these effects vary among different sources of cytoplasm [5]. These studies provide practical information for breeding CMS lines and for selecting appropriate CMS line to mate with certain restorers to improve rice yield.

In the three-line system of hybrid rice, male sterility (MS) and its restoration are governed by genetic interaction between male sterile cytoplasm and the nucleus. The interaction maintains the MS, allows the regeneration of the MS line, and restores MS for enjoyment of yield advantage in the F1 hybrid. Genetic interaction limits the flexibility of germplasm selection and increases production costs [6]. Genic male sterility (GMS) is solely determined by the nucleus, thus has a wider spectrum of restorers than CMS and facilitates the widespread use of hybrid vigor. GMS can also be used to simplify rice breeding by creating a two-line breeding system. This is because the male sterility caused by recessive GMS can be maintained and restored by introducing a hemizygotic construct into the male sterile plant. This construct comprises a gene whose product is exclusively fatal to pollen and a corresponding restoration gene [7]. Although GMS is useful in rice breeding, the cytoplasmic effects of GMS have not received as much attention as those of CMS. Here, we used a GMS mutant line (h2s) identified in our breeding program to evaluate cytoplasmic effects on plant height, yield-related traits, and general combining ability (GCA) for these traits.

GMS includes photoperiod-sensitive male sterility (PMS), temperature-sensitive male sterility (TMS), photo-thermo-sensitive male sterility (PTMS), and non-photo-thermo-sensitive genic male sterility. The MS in PMS, TMS, and PTMS can be maintained and reproduced under certain light and temperature conditions. PMS and PTMS have been successfully applied to the production of hybrid seeds, such as Nongken58s, Pehaitans, and Y58s [6]. A spontaneous point mutation in PMS (photoperiod-sensitive male sterility) [8] (also called P/TMS12-1) [Photoperiod-and-thermosensitive genic male sterility] [9]) causes PMS in japonica background and TMS in indica background. Recently, a number of non-photo-thermo-
sensitive genic MS genes have been identified in rice, including WDA1 (wax-deficient anther1) [10], CYP704B2 [11], TDR (tapetum degeneration retardation) [12], GAMYB [13,14], DPW (defective pollen wall) [15], and PTCl (persistent tapetal cell) [16]. All these genes were found to be related to lipid metabolism and to be critical to anther and pollen development in rice. However, the molecular mechanism of anther and pollen development in rice remains unknown. In this study, we identified a male sterile mutant and fine mapped the mutation to a 152 kb region on chromosome 6. Our results lay the groundwork for cloning the H2S gene and for elucidating the molecular mechanism of anther and pollen development.

Materials and Methods

Plant Materials

The h2s mutant (a spontaneous mutant identified from an indica maintainer Chuannong H2S) and F2 mapping populations generated by respectively crossing h2s as female with Zhenshan97B and Nipponbare were planted in a paddy field at Sichuan Agricultural University (Chengdu, Sichuan, China) and at the Hainan rice station (Lingshui, Hainan, China). Five isonuclear alloplasmic lines were generated by crossing heterozygote (h2s+/−) as male with Zhenshan97A, D702A, G46A, K18A and XieqingzaoA and then backcrossing 8 times using h2s+/− as the recurrent parent to substitute the nuclei of Zhenshan97A, D702A, G46A, K18A, and XieqingzaoA with the h2s nucleus. The h2s mutant was named A1, and the resultant isonuclear alloplasmic lines were A2, A3, A4, A5, and A6, respectively (Table S1). The nuclear background in A1–A6 was checked using 30 ISSR (inter-simple sequence repeat) and 371 SSR markers were used to check the nuclear background of each isonuclear alloplasmic line (A1–A6). A1, A2, A3, A4, A5, and A6 were respectively crossed as female parents with five major restorer lines (Shuhui527 (R1), Minghui63 (R2), Guanghui128 (R3), CDR22 (R4), and Mianhui725 (R5)) to generate 30 F1s. For convenience, we defined A1–A6 as parent 1 (P1) and R1–R5 as parent 2 (P2).

Cytological Characterization of h2s

The wild type and the h2s mutant were cytologically examined for anthers at the meiosis, post-meiosis, young microspore, and vacuolated pollen stages. The preparation for chromosome spreads followed the method described in Chen et al. [17]. Anthers at various stages were fixed overnight in Carnoy’s solution (ethanol/glacial acetic acid = 3:1) and then rinsed twice with water and twice with 10 mmol/L citrate buffer (pH 4.5), and finally washed with citrate buffer. Treated anthers were placed on a slide with 60% acetic acid and gently pressed with cover glass to release microspore mother cells (MMC). They were then stained with 4′,6-diamidino-2-phenylindole (1 μg/ml DAPI in a buffer containing 50% glycerol and 10 mM citrate, pH 4.5). Paraffin embedding and sectioning were performed using a previous protocol described by Feng et al. [18]. Briefly, the spikelets were fixed overnight in 2.5% glutaraldehyde (pH 6.8) at 4°C and washed twice using 0.1 M phosphate buffer (pH 6.8). They were then dehydrated in an ethanol series and embedded in paraffin (Segma) for polymerization at 45°C for 4 hours. Sections were made using a rotary microtome (Leica, RM2235).

Experimental Design and Statistical Analysis

Thirty F1s and their parents were sown on April 4 and transplanted on May 20, 2006 in the Sichuan Agricultural University paddy (Chengdu, Sichuan, China) to evaluate cytoplasmic effects. A randomized complete block design with three replications was used. For each replication, three rows with 12 plants in each row were planted in plots 2.12 m×0.8 m in size. Rows were 26.6 cm apart and plants within rows were 17.7 cm apart. Ten randomly selected plants from each plot were measured for plant height (cm), effective panicle number, 1,000-grain weight (g), grain number per panicle, seed set rate (%), and...
weight per panicle (g). Mean values were used for analysis of variance (ANOVA). Most of the plants from each plot were bulk harvested for grain yield, but one plant from each end of each row was omitted to exclude any border effect. This experiment was repeated in the summer of 2008, with the same design. A fixed model with type III sum of squares in SPSS software (Version 21.0. Armonk, NY, U.S.: IBM Corp) was adopted and used to perform an ANOVA. Least significant difference (LSD) was estimated for mean comparison of general combining ability (GCA) effects. The $A_1$–$A_6$ GCA was calculated by the difference between a mean over all combinations for a line $A$ such as $A_1$ to be a parent with all restores ($R_1$–$R_5$) and the overall mean for a trait. The effects of $h_{2s}$ cytoplasm on plant height and yield-related traits were estimated as follows: $[h_{2s}/A_1/R_1/R_5]_{F_1}$ – [isonuclear alloplasmic line ($A_2$–$A_6$)/$R_1/R_5]_{F_1}$.

**Molecular Mapping**

The $h_{2s}$ gene was mapped using a procedure described previously by Chen et al. [19]. Two $F_2$ populations were generated by crossing $h_{2s}$ as a female with Zhenshan97B and Nipponbare, respectively. We used 342 SSR markers to examine polymorphism between $h_{2s}$ and Zhenshan97B. Preliminary mapping was based on 1532 male sterile $F_2$ individuals from the cross between $h_{2s}$ and Zhenshan97B. For fine mapping, 35 SSRs and 74 indel markers were used to examine polymorphism for the target region between $h_{2s}$ and Nipponbare, and 2400 male sterile $F_2$ individuals produced from this cross were analyzed. Among the 74 indel markers, the closest marker to the mutation locus was W11 (F: TTGGTCCCAAAATAGTCATG; R: TTGGAGCAACTGAAAGCAAGGAA). Genetic distance was estimated using Mapmaker 3.0 software [20]. The expression patterns of candidate genes were analyzed using Rice eFP Browser (http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi) and the rice anther expression database [21].

**Expression Analysis for Candidate Gene**

Total RNA was isolated using a Qiagen RNA plant mini kit with on-column DNase digestion (Qiagen). Two micrograms of...
RNA was used for reverse transcription using M-MLV RT (Promega) with oligo (dT18) primer, and 1 μL RT product diluted with 20 μL ddH2O was used as a template for PCR. The RNA used for RT-PCR was from wild type and mutant anthers at the post-meiosis stage, and the primer pair for **Loc_Os06g40550** was W70 (F: CACCACCTACAGTCCCTGCCTCAAA; R: ATGCCGCCCCGCCTATCCTTCCTCA) and **OsActin1** (F: ACGGCGATAACAGCCTCCTT; R: CCTCTTCCAGCCTCTCTCA).

**Results**

**Identification and Characterization of h2s**

The h2s mutant showed shorter spikelets with smaller anthers than the wild type (Figure 1B, C, D) but exhibited normal pistil and vegetative development with only slightly shorter plant height (Figure 1A). The h2s mutant failed to produce mature pollen (Figure 1E and F). To characterize defects during anther development in the h2s mutant, we performed 4′, 6-diamidino-2-phenylindole staining. No abnormal activity was observed in h2s during meiosis (Figures 2A–P). However, the cytoplasm of the young h2s microspores collapsed after they were released from

**Table 1.** Variance analysis of yield-related traits and plant height (F value) for experiments conducted in 2006 and 2008 in Chengdu, China.

| Source of variation | Df | Plant height | Effective panicle No. | 1,000-grain weight | Grain No./panicle | Seed set rate | Weight/panicle | Yield |
|---------------------|----|--------------|----------------------|--------------------|-------------------|--------------|---------------|-------|
| Replication         | 2  | 3.02         | 1.95                 | 1.03               | 2.53              | 0.30         | 1.06          | 0.58  |
| P1(A1–A6)           | 5  | 1.82*        | 1.52                 | 2.15*              | 3.99**            | 5.52**       | 4.97**        | 11.16** |
| P2(R1–R5)           | 4  | 19.74**      | 16.19**              | 71.00**            | 8.25**            | 5.13**       | 65.70**       | 32.48** |
| Year                | 1  | 34.84**      | 8.15*                | 3.20               | 3.13              | 9.15*        | 11.48**       | 10.73*  |
| P1 × P2             | 20 | 11.92*       | 1.60                 | 1.21               | 3.40**            | 4.67**       | 5.44**        | 3.3**   |
| P1 × Year           | 5  | 2.44         | 5.60**               | 1.554              | 4.27**            | 0.48         | 0.90          | 4.51*   |
| P2 × Year           | 1  | 2.08**       | 4.56**               | 6.07**             | 1.32              | 25.54**      | 12.03**       | 11.32** |
| P1 × P2 × Year      | 20 | 7.47**       | 0.67                 | 0.18               | 8.23**            | 5.38*        | 1.12          | 3.14    |
| Error (mean square) | 118| 806          | 54                   | 67                 | 4383              | 1124         | 2.60          | 0.34    |

*and **Significant at 0.05 and 0.01 probability level, respectively.
P1: Isonuclear alloplasmic lines (A1–A6), represents the cytoplasmic effect.
P2: Restorer lines (R1–R5).
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At the vacuolated pollen stage, the microspores were completely collapsed and only nuclei were left with some residual cytoplasm in the locule (Figure 2S and T). These observations suggest that the developmental defect in the h2s mutant occurred after the release of young microspores from tetrads.

To obtain more information regarding the morphological defects of h2s anthers, we examined thin sections of wild-type and h2s anthers. At the meiosis stage, as with 4',6-diamidino-2-phenylindole staining, no obvious difference between the wild type and the h2s mutant was observed (Figure 3A and E). At the post-meiotic stage, MMC went through meiosis and formed tetrads in the wild type (Figure 3B). In this way, young microspores in tetrads were released in the anther locule at the young microspore stage (Figure 3C). At the vacuolated pollen stage, the microspore nucleus was pressed near the cytoplasmic membrane by the vacuole and underwent mitosis twice to form mature pollen grains. The tapetum became condensed and degraded (Figure 3D). In contrast, at the post-meiotic stage, the h2s mutant showed young microspores but there was no clear boundary around them (Figure 3F). At the young microspore stage, as with 4',6-diamidino-2-phenylindole staining, the young h2s microspores appeared degraded. The microspores in h2s seemed to lack exine, so the microspore degradation might be due to the absence of exine. At the vacuolated pollen stage, the microspores in h2s were completely degraded and formed empty anther locules.

**Table 2.** h2s cytoplasmic effects on seed set rate, weight/panicle and yield in comparison with Zhenshan97A (A2), D702A (A3), G46A (A4), K18A (A5) and XieqingzaoA (A6).

| Paired comparison | Seed set rate (%) | Weight/panicle (g) | Yield (kg/plot) |
|-------------------|-------------------|--------------------|-----------------|
| A1/R1–A2/R1       | 3.94**/4.24**     | 0.34*/0.30*        | 0.10*/0.15*     |
| A1/R1–A3/R1       | 3.64*/6.19**      | 0.06/−0.05         | 0.12*/0.13*     |
| A1/R1–A4/R1       | 5.91**/8.15**     | 0.21/0.10          | 0.15**/0.21**   |
| A1/R1–A5/R1       | 4.05*/6.82**      | 0.46*/0.42**       | 0.16*/0.23**    |
| A1/R1–A6/R1       | 3.61*/6.35**      | 0.24/0.07          | 0.12*/0.20**    |
| A1/R2–A2/R2       | 3.94*/5.01**      | 0.22/0.20          | 0.07/0.09*      |
| A1/R2–A3/R2       | 3.25*/3.54*       | 0.24/0.14          | 0.07/0.05       |
| A1/R2–A4/R2       | 1.07/−1.21        | 0.25/0.34*         | 0.05/0.08       |
| A1/R2–A5/R2       | 0.76/3.36         | 0.02/−0.06         | 0.06/0.04       |
| A1/R2–A6/R2       | 2.52/6.29         | 0.24/0.35*         | −0.04/−0.04     |
| A1/R3–A2/R3       | −0.8/1.02         | 0.08/0.02          | 0.08/0.07       |
| A1/R3–A3/R3       | 0.58/1.58         | −0.10/−0.06        | 0.10*/0.08*     |
| A1/R3–A4/R3       | 1.29/4.77*        | −0.07/0.07         | 0.12*/0.15**    |
| A1/R3–A5/R3       | 1.76/0.25         | 0.09/0.11          | 0.10*/0.07      |
| A1/R3–A6/R3       | −0.67/−0.40       | 0.03/0.03          | 0.06/0.06       |
| A1/R4–A2/R4       | 5.54*/12.42**     | 0.15/−0.03         | 0.10*/0.05      |
| A1/R4–A3/R4       | 8.00*/12.51**     | 0.41*/0.44**       | 0.12*/0.09*     |
| A1/R4–A4/R4       | 8.07*/12.77**     | 0.09/0.09          | 0.07/−0.02      |
| A1/R4–A5/R4       | 7.01*/13.82**     | 0.39*/0.41**       | 0.10*/0.09*     |
| A1/R4–A6/R4       | 10.01*/13.41**    | 0.43*/0.44**       | 0.09/0          |
| A1/R5–A2/R5       | 0.88/−2.09        | −0.02/0.09         | 0.05/0.02       |
| A1/R5–A3/R5       | 2.00/0.57         | 0.33*/0.20         | −0.01/0         |
| A1/R5–A4/R5       | 3.06*/3.22*       | 0.08/0             | 0.01/0          |
| A1/R5–A5/R5       | 0.69/−1.90        | −0.09/−0.24        | 0.07/0.03       |
| A1/R5–A6/R5       | 2.20/−0.88        | 0.15/0.09          | 0.06/0.05       |

Percentage of comparisons with significant positive effect (%): 52/56 24/28 48/40

The numbers separated by slashes represent data from different years, 2006 (left) and 2008 (right). * and ** Significant at 0.05 and 0.01 probability level, respectively.

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**Table 3.** GCA effects on seed set rate, weight per panicle and yield among cytoplasms of h2s (A1), Zhenshan97A (A2), D702A (A3), G46A (A4), K18A (A5) and XieqingzaoA (A6).

| Parents Seed set rate (%) | Weight/panicle (g) | Yield (kg/plot) |
|---------------------------|--------------------|-----------------|
| A1 2.74a/3.79a            | 0.14a/0.11a        | 0.07a/0.06a     |
| A2 0.04b/0.07b            | −0.01b/0.01b       | −0.01b/−0.01b   |
| A3 −0.75b/−1.08b          | −0.05b/−0.02b      | −0.01bc/−0.01b  |
| A4 −1.13b/−1.75b          | 0.03b/0.06b        | −0.01bc/−0.02b  |
| A5 −0.11b/0.12b           | −0.03b/−0.01b      | −0.03c/−0.02b   |
| A6 −0.79b/−1.16b          | −0.07b/−0.09b      | 0.01b/0.01b     |

The numbers separated by slashes represent the data from different years, 2006 (left) and 2008 (right). Values followed by the same letter in a column within a year are not significantly different at α = 0.05.

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Genetic Analysis of the \( h_{2s} \) Mutant

All F\(_1\) plants from the cross between \( h_{2s} \) and 9311 produced normal mature pollen and seed sets. Among 120 F\(_2\) plants, 87 had normal pollen and 33 had no pollen, \((\chi^2[3:1] = 0.259; P > 0.5)\), indicating a single recessive mutation in the spontaneous \( h_{2s} \) mutant. \( h_{2s} \) also showed stable MS in both Sichuan (E103\( u \), N30\( u \)) and Hainan (E110\( u \), N18\( u \)), suggesting that \( h_{2s} \) was not a PTMS.

Cytoplasmic Effects of \( h_{2s} \) on General Combining Ability (GCA)

Studies on GCA have provided information on the potential of specific rice lines as parents in breeding programs. Compared with isonuclear alloplasmic parents A2, A3, A4, A5, and A6, \( h_{2s} \) showed significant positive cytoplasmic effects on GCA with respect to plant height, effective panicle number, and 1000-grain weight, and a significant and negative effect on the grain number per panicle, but plant height, 1000-grain weight, and grain number per panicle were less affected.
yield-related traits (seed set rate, weight per panicle, and yield) (Table 3) and plant height (Table S3) in both years. However, no cytoplasmic effect on GCA with respect to effective panicle number or grain number per panicle was observed for h2s in either year (Table S3). The significant and positive cytoplasmic effect on GCA, especially with respect to yield, suggests that the h2s mutant is a good parent for rice breeding.

**Molecular Mapping of the h2s Mutation**

Although cytoplasmic analysis of agronomic traits and GCA indicated that h2s had considerable potential for the improvement of rice yield, the male sterility of h2s severely restricts its utility in the production of hybrid seeds. This is because h2s is a non-photoperiod thermo-sensitive male sterile mutant. However, one previously described approach provides a possibility to maintain the MS of recessive GMS, so that h2s could be used to produce hybrid seed in breeding practices [7]. This approach requires cloning the gene responsible for MS. For this reason, we tried to clone the H2S gene using a mapping-based approach.

A F2 population was generated from a cross between h2s and Zhensan97B to map the h2s gene. A bulk segregant analysis was conducted using 52 polymorphic SSR markers between h2s and Zhensan97B with two DNA bulks, each with ten sterile F2 plants and ten fertile F2 plants. Of the 52 SSRs, only RM3 on chromosome 6 was polymorphic between the two DNA bulks, indicating that the putative h2s gene was located on chromosome 6. We then examined 23 SSRs flanking RM3 for polymorphisms between h2s and Zhensan97B, and the polymorphic SSRs were used to analyze 612 male sterile F2 individuals. Both RM454 (located at 27832 kb) and RM528 (located at 26544 kb) were found to be linked with the h2s locus, with 7.47 and 9.19 cM genetic distance, respectively (Figure 4A). Because no other SSRs were found to be polymorphic between h2s and Zhensan97B for the region between RM454 and RM528, 35 SSRs and 74 indel markers were examined for polymorphism between h2s and Nipponbare. The identified polymorphic markers were used to analyze 2400 male sterile F2 individuals in the cross between h2s and Nipponbare. The h2s locus was mapped to a 152 kb region between indel W11 (located at 24044 kb) and RM20366 (located at 24196 kb) with 0.2 and 0.3 cM genetic distance, respectively (Figure 4B). A total of 22 genes were predicted in this mapped region [22] (Table 4), and none of them have been previously reported for the h2s phenotype. This result suggests that H2S is a novel gene controlling post-meiotic anther and pollen development.

**Analysis of Candidate Genes**

Analysis of expression patterns for the 22 candidate genes showed that ten are expressed in the inflorescence [23] (Table 4). Among the ten candidates, **Loc_Os06g40550** encodes a glycosyl hydrolase that is likely to be involved in carbohydrate metabolic processes [24]. **Loc_Os06g40540** encodes a protein with an ABC transport domain. Its homolog in *Arabidopsis*, **ABC26/WBC27**, has been reported to be involved in pollen exine formation, and the **absc26/wbc27** mutant exhibits impaired pollen wall formation that causes MS in *Arabidopsis* [25,26,27]. The homolog of **SNF7** (sucrose non-fermenting 7) protein encoded by **Loc_Os06g40620** in *Arabidopsis* is a subunit of the ESCRT III complex. It may be involved in vesicle-mediated transport [28]. **Loc_Os06g40630** encodes a protein containing a SFT2 domain, its homolog in yeast is involved in trafficking to the Golgi complex [29]. **Loc_Os06g40630** encodes copine-1, whose homolog in *Arabidopsis* (ring domain ligase 2) negatively regulates drought stress response [30]. Two genes (**LOC_Os06g40540** and **LOC_Os06g40660**) are predicted to be retro-transposons, and the genetic functions of three other genes (**LOC_Os06g40500**, **LOC_Os06g40580**, and **LOC_Os06g40690**) are unknown at present. Among the ten candidate genes, **LOC_Os06g40530** is the only one to be expressed in anthers around the tetrad stage [21]. Considering that a mutant of the **LOC_Os06g40550** homolog in *Arabidopsis*, **abc26/wbc27**, also exhibits MS, and the spatial and temporal expression of **LOC_Os06g40550** was consistent with h2s phenotype, we chose **LOC_Os06g40550** as a top candidate gene for further study.

A twelve-nucleotide (GCCACCCTCCTC) deletion in the predicted sixth exon of **Loc_Os06g40550** was detected in the h2s
genomic DNA and RNA by sequencing and RT-PCR analysis (Figure 3A and B). However, the deletion did not change the reading frame. These results suggest that the deletion in Loc_Os06g40530 may be responsible for the h2s phenotype, but a complementary experiment is needed.

Discussion

In CMS, cytoplasmic effects on most of the agronomic traits are either negative or not actively favorable to the achievement of high grain yield. Our study showed that the cytoplasmic effects of h2s on yield and GCA of yield were positive and significant in both years, suggesting that h2s may be more useful in improving rice yield than CMS. GMS does not have male sterile cytoplasm which is responsible for negative cytoplasmic effect on agronomic traits in the CMS [1,5,31]. For this reason, the cytoplasmic effects are probably positive in the GMS as h2s. The positive-cytoplasmic effects on yield in the GMS should be seriously considered in rice breeding.

Although the GMS mutant h2s shows promise for rice breeding, the challenge of maintaining the MS of h2s for the production of hybrid seeds limits its practical application. In order to utilize the positive-cytoplasmic effects of h2s and resolve the challenge, we could generate a PTMS line with both h2s cytoplasm and PTMS nuclei using nuclear substitutions. However, the fact that the fertility of PTMS line with h2s cytoplasm is easily affected by environment also restricts the wide application of positive cytoplasmic effects of h2s. The approach described by Marc et al. [7] has been proposed to maintain the male sterile condition of recessive génic MS and provide an alternative way of utilizing the positive-cytoplasmic effects of h2s in rice breeding. This approach requires cloning the gene responsible for the h2s phenotype. Our mapping results showed that no gene in the fine-mapping region has been reported to be responsible for h2s phenotype, suggesting that h2s is a novel gene essential to male reproductive development. Sequencing and bioinformatics analysis of the candidate genes suggested that Loc_Os06g40530, encoding a protein with ABC transporter domain, was the best candidate. This lays a strong basis for cloning the H2S gene and for using the h2s mutant in rice breeding. Because the h2s mutant showed defective rice anther and pollen wall development, our work may also be helpful for understanding the molecular mechanisms underlying the development of these structures.

Supporting Information

Figure S1  Two representative results of checking A1–A6 background using ISSR primers and SSRs.

Figure S2  Seed set rate and CV (coefficient of variation) of 30 combinations of 6 isonuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.

Figure S3  Mean grain number per panicle and CV (coefficient of variation) of 30 combinations of 6 isonuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.

References

1. Chandra-Shekara AC, Prasanna BM, Singh BB, Unnikrishnan KV, Seetharam A (2006) Effect of cytoplasm and cytoplasm-nuclear interaction on combining ability and heterosis for agronomic traits in pearl millet {Pennisetum glaucum} (L) Br. R. Euphytica 153: 15–26.

2. Tao D, Xu P, Zhou J, Deng X, Li J, et al. (2011) Cytoplasm affects grain weight and filled-grain ratio in indica rice. BMC Genomics 12: 53.
6. Yang S, Chen B, Shen W, Xia J (2009) Progress of application and breeding on two-line hybrid rice in China. Hybrid Rice 24: 5–9.
7. Marc A, Timnohy F, Howard H, Gary H, Youngshong W (2006) Nucleotide sequences mediating plant male sterility and method of using same. International Patent International application number: PCT/US2006/024273.
8. Ding J, Liu Q, Ouyang Y, Mao H, Zhang P, et al. (2012) A long noncoding RNA regulates photoperiod-sensitive male sterility, an essential component of hybrid rice. Proc Natl Acad Sci USA 109: 2654–2659.
9. Zhou H, Liu Q, Li J, Jiang D, Zhou L, et al. (2012) Photoperiod- and thermo-sensitive gene male sterility in rice are caused by a point mutation in a novel noncoding RNA that produces a small RNA. Cell Res.
10. Jung KH, Han MJ, Lee Dy, Lee YS, Schreiber L, et al. (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.
11. Li H, Pinot F, Sauveplane V, Weck-Reichart D, Diehl P, et al. (2010) Cytochrome P450 family member CYP704B2 catalyzes the hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice. Plant Cell 22: 173–190.
12. Li N, Zhang DS, Liu HS, Li Xx, et al. (2006) The rice tapetum degeneration retardation gene is required for tapetal degradation and anther development. Plant Cell 18: 2999–3014.
13. Aya K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, et al. (2009) Gibberellin modulates anther development in rice via the transcriptional regulation of GAMYB. Plant Cell 21: 1453–1472.
14. Liu Z, Bao W, Liang W, Yin J, Zhang D (2010) Identification of gamy-b4 and analysis of the regulatory role of GAMYB in rice anther development. J Integr Plant Biol 52: 670–678.
15. Shi J, Tan H, Yu XH, Liu Y, Liang W, et al. (2011) Defective pollen wall is required for anther and microspore development in rice and encodes a fatty acyl carrier protein reductase. Plant Cell 23: 2225–2246.
16. Li H, Yuan Z, Vazcay-Barrena G, Yang C, Liang W, et al. (2011) PERSISTENT TAPETAL CELL1 encodes a PHD-Finger protein that is required for tapetal cell death and pollen development in rice. Plant Physiol 156: 615–630.
17. Chen C, Xu Y, Ma H, Wang K (2005) Cell biological characterization of male meiosis and pollen development in rice. J Integr Plant Biol 47: 734–744.
18. Feng J, Lu Y, Lu X, Xue X (2001) Pollen development and its stages in rice (Oryza sativa L.). Chinese J Rice Sci 15: 21–28.
19. Chen JB, He F, Qiu P, Wang YP, Xu J, et al. (2009) Genetic analysis and gene mapping of a rice recessive male sterile mutant. Plant Breeding 129: 313–317.
20. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, et al. (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1.
21. Huang MD, Wei EJ, Wu CC, Hsing YIC, Huang AHC (2006) Analyses of advanced rice anther transcriptomes reveal global tapetum secretory functions and potential proteins for lipid exine formation. Plant Physiol 149: 694–707.
22. Ouyang S, Zhu W, Hamilton J, Lin H, Campbell M, et al. (2007) The TIGR Rice Genome Annotation Resource: improvements and new features. Nucleic Acids Res 35: D883–887.
23. Baxter I, Vinegar B, Winter D, Nahal H, Ammar R, et al. (2007) An “Electronic Fluorescent Pictograph” Browser for Exploring and Analyzing Large-Scale Biological Data Sets. PLoS ONE 2: e718.
24. Lopez-Casado G, Urbanowicz BR, Damasceno CM, Rose JK (2008) Plant glycosyl hydrolases and biofuels: a natural marriage. Curr Opin Plant Biol 11: 329–337.
25. Choi H, Jin J-Y, Choi S, Hwang J-U, Kim Y-Y, et al. (2011) An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. Plant J 65: 181–193.
26. Quilichini TD, Friedmann MC, Samuels AL, Douglas CJ (2010) ATP-Binding Cassette Transporter G26 is required for male fertility and pollen exine formation in Arabidopsis. Plant Physiol 154: 678–690.
27. Dou X, Yang K, Zhang Y, Wang W, Liu X, et al. (2011) WBC27, an Adenosine Triphosphate-binding Cassette protein, controls pollen wall formation and patterning in Arabidopsis. J Integr Plant Biol 53: 74–88.
28. Bil V, Ciaszar E, Schlager N, Neubert S, Spitzer C, et al. (2012) Interactome of the plant-specific ESCRT-III component AtVPS2.2 in Arabidopsis thaliana. J Proteome Res 11: 397–411.
29. Conchon S, Cao X, Barlowe C, Pelham HR (1999) Got1p and Sft2p: membrane proteins involved in traffic to the Golgi complex. EMBO J 18: 3934–3946.
30. Cheng MC, Hsich EJ, Chen JH, Chen HY, Lin TP (2012) Arabidopsis RGL2, functioning as a RING E3 ligase, interacts with AtERF53 and negatively regulates the plant drought stress response. Plant Physiol 158: 363–375.
31. Tao D, Hu F, Yang J, Yang G, Yang Y, et al. (2004) Cytoplasm and cytoplasm-nucleus interaction affect agronomic traits in japonica rice. Euphytica 135: 129–134.