

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

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## 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 photo-thermo 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, *h<sub>2</sub>s*, which exhibited small, white anthers and failed to produce mature pollen. Unlike CMS, the *h<sub>2</sub>s* 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 *h<sub>2</sub>s* cytoplasm may show promise for the improvement of rice yield. Genetic analysis suggested that the phenotype of *h<sub>2</sub>s* was controlled by a single recessive locus. We mapped *h<sub>2</sub>s* 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 *h<sub>2</sub>s*. Sequencing analysis showed a 12 bp deletion in the sixth exon of *Loc\_Os06g40550* in *h<sub>2</sub>s* in comparison to wild type, suggesting that *Loc\_Os06g40550* is the best candidate gene. These results lay a strong foundation for cloning of the *H<sub>2</sub>S* gene to elucidate the molecular mechanism of male reproduction.

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## 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 *IR19661-283-3-2* has a significant negative effect on both yield and days to flowering but does not affect plant height. The male sterile cytoplasm of *IR46828*, *IR46831*, *IR48483*, *IR54752*, *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 cytoplasm 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 F<sub>1</sub> hybrid. Genetic interaction limits the flexibility of germplasm selection and increases pro-

duction 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 (*h<sub>2</sub>s*) 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, Peiai64s and Y58s [6]. A spontaneous point mutation in *PMS3* (*photoperiod-sensitive male sterility3*) [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 *PTC1* (*persistent tapetal cell1*) [16]. All these genes were found to be related to lipid metabolism and to be critical to anther and pollen exine development. The corresponding mutants show defective anthers and/or pollen development, causing MS 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 *H<sub>2</sub>S* gene and for elucidating the molecular mechanism of anther and pollen development.

## Materials and Methods

### Plant Materials

The *h<sub>2</sub>S* mutant (a spontaneous mutant identified from an *indica* maintainer *Chuannong H<sub>2</sub>S*) and F<sub>2</sub> mapping populations generated by respectively crossing *h<sub>2</sub>S* 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 (*h<sub>2</sub>S*+/-) as male with Zhenshan97A, D702A, G46A, K18A and XieqingzaoA and then backcrossing 8 times using *h<sub>2</sub>S*+/- as the recurrent parent to substitute the nuclei of Zhenshan97A, D702A, G46A, K18A, and XieqingzaoA with the *h<sub>2</sub>S* nucleus. The *h<sub>2</sub>S* 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 F<sub>1</sub>s. For

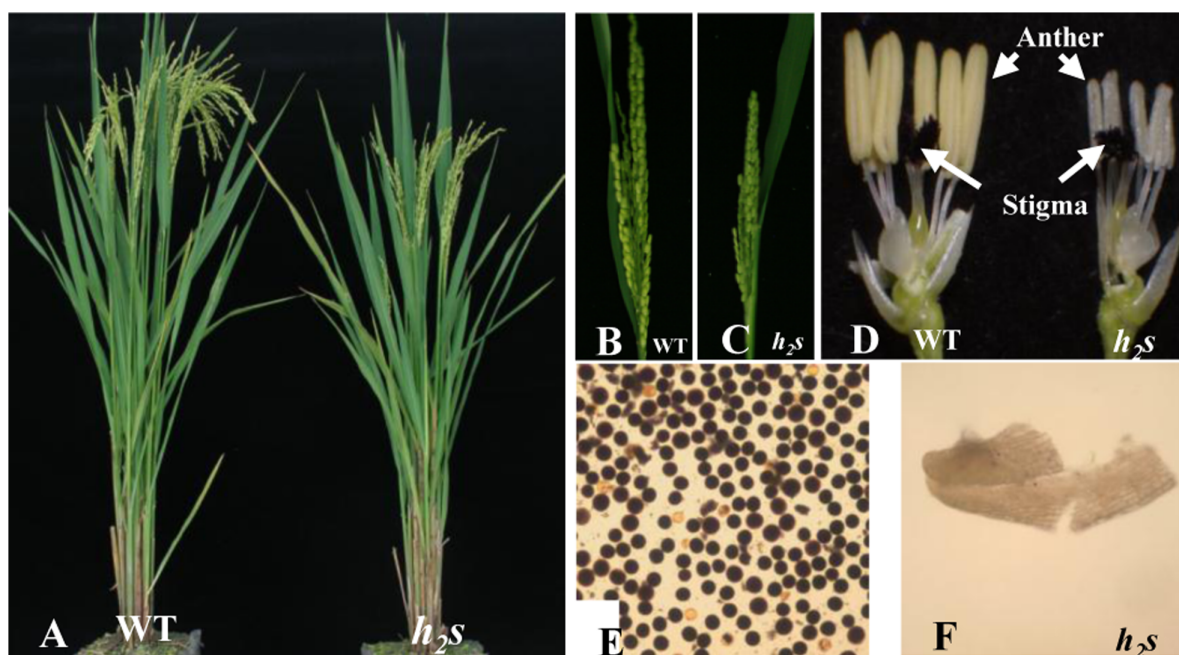
convenience, we defined A1–A6 as parent 1 (P1) and R1–R5 as parent 2 (P2).

### Cytological Characterization of *h<sub>2</sub>S*

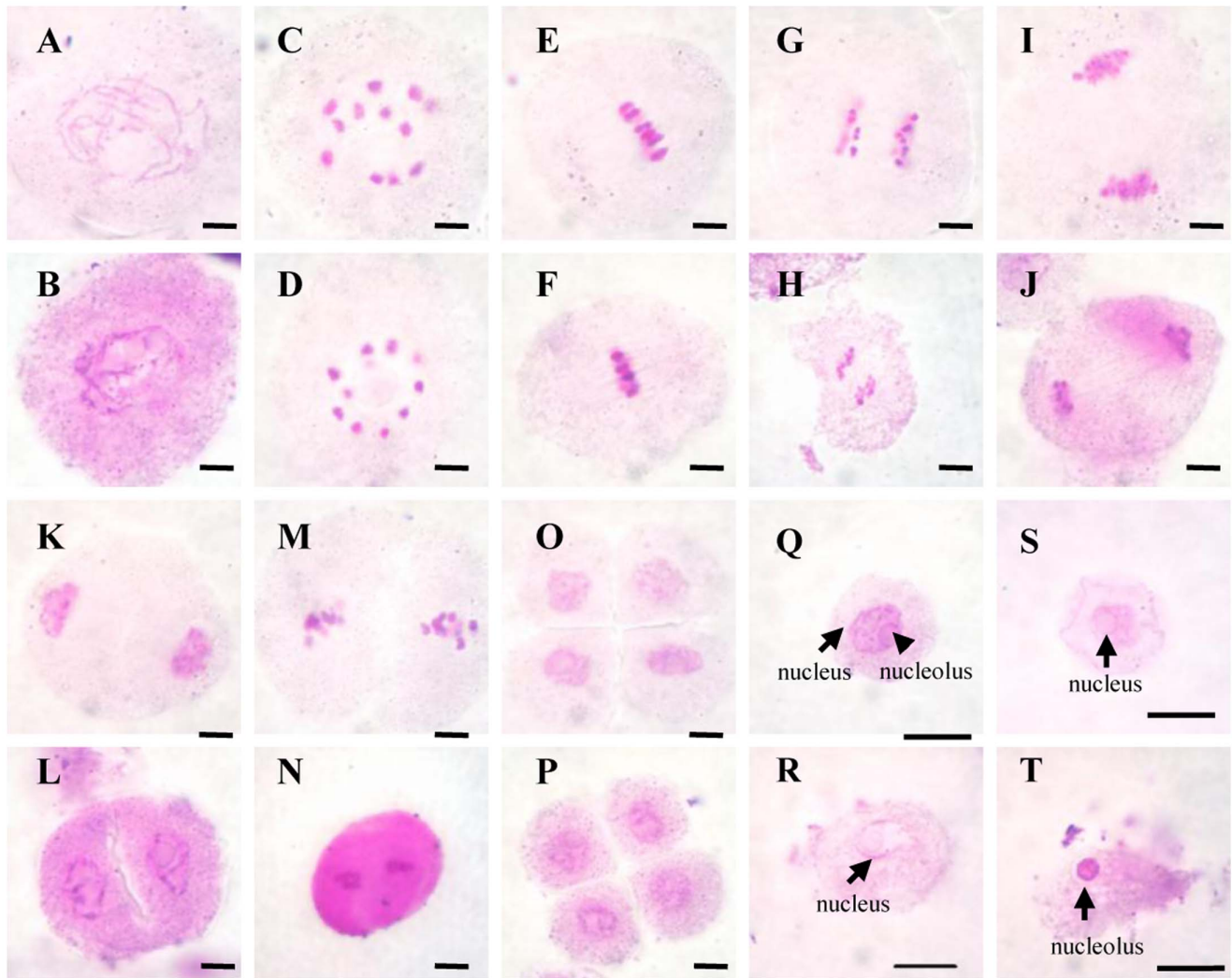
The wild type and the *h<sub>2</sub>S* mutant were cytologically examined for anthers at the meiosis, post-meiosis, young microspore, and vacuolated pollen stages. The preparation for chromosome spreads followed the method described in Chen et al. [17]. Anthers at various stages were fixed overnight 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 F<sub>1</sub>s 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



**Figure 1. Phenotypic comparison of wild type (WT) and *h<sub>2</sub>S*.** Comparison between WT (left) and *h<sub>2</sub>S* (right) for heading plant (A), headed panicle (B, C), flower (D) and pollen of WT (E) and anther of *h<sub>2</sub>S* (F). doi:10.1371/journal.pone.0061719.g001



**Figure 2. DAPI staining analysis for the meiosis of microspore mother cells and mitosis of young microspores between the wild type (WT) and *h<sub>2s</sub>*.** (A–I) and (K–S) for WT, (B–J) and (L–T) for *h<sub>2s</sub>*; (A) and (B) Pachytene, (C) and (D) diakinesis, (E) and (F) metaphase I, (G) and (H) anaphase I, (I) and (J) telophase I, (K) and (L) prophase II, (M) and (N) metaphase II, (O) and (P) tetrad, (Q) and (R) young microspore stage, and (S) and (T) vacuolated pollen stage. No difference in the process of meiosis was observed between WT and *h<sub>2s</sub>*. Bars = 10  $\mu$ m. doi:10.1371/journal.pone.0061719.g002

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 A1–A6 GCA was calculated by the difference between a mean over all combinations for a line A such as A1 to be a parent with all restores (R1–R5) and the overall mean for a trait. The effects of *h<sub>2s</sub>* cytoplasm on plant height and yield-related traits were estimated as follows: [*h<sub>2s</sub>* (A1)/R (R1–R5)] F<sub>1</sub>–[isonuclear alloplasmic line (A2–A6)/R (R1–R5)] F<sub>1</sub>.

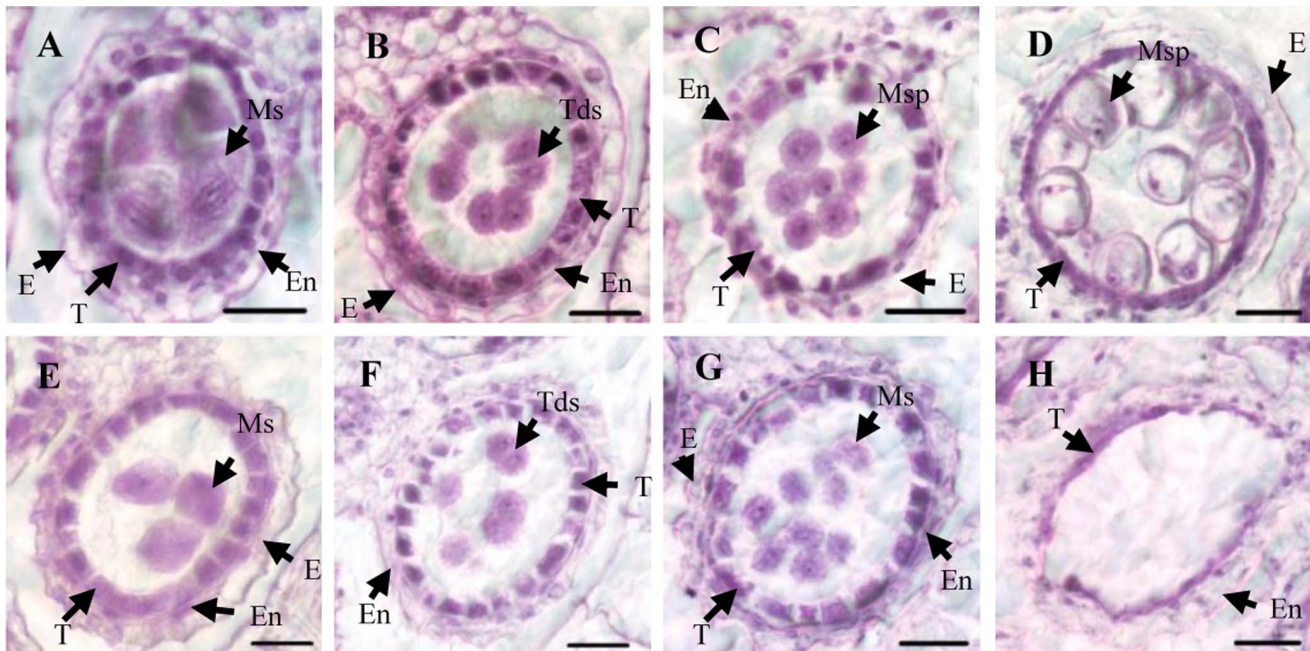
### Molecular Mapping

The *h<sub>2s</sub>* gene was mapped using a procedure described previously by Chen et al. [19]. Two F<sub>2</sub> populations were

generated by crossing *h<sub>2s</sub>* as a female with Zhenshan97B and *Nipponbare*, respectively. We used 342 SSR markers to examine polymorphism between *h<sub>2s</sub>* and Zhenshan97B. Preliminary mapping was based on 1532 male sterile F<sub>2</sub> individuals from the cross between *h<sub>2s</sub>* and Zhenshan97B. For fine mapping, 35 SSRs and 74 indel markers were used to examine polymorphism for the target region between *h<sub>2s</sub>* and *Nipponbare*, and 2400 male sterile F<sub>2</sub> individuals produced from this cross were analyzed. Among the 74 indel markers, the closest marker to the mutation locus was W11 (F: TTGGTCCCACAAATAAGTCATG; R: TTGGAGCAACTGAAGCAAGGAA). 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/efp/efpWeb.cgi>) and the rice anther expression database [21].

### Expression Analysis for Candidate Gene

Total RNA was isolated using a Qjagen RNA plant mini kit with on-column DNase digestion (Qjagen). Two micrograms of



**Figure 3. Section analysis between the wild type (WT) and  $h_{2s}$ .** (A–D) for WT, (E–G) for  $h_{2s}$ . (A) and (E) Meiosis, (B) and (F) post-meiotic stage, (C) and (G) young microspore stage. The young microspores in  $h_{2s}$  exhibited defects at the post-meiotic stage (D) and vacuolated pollen stage (H). However, no microspore was observed at the vacuolated pollen stage. E: epidermis; En: endothecium; T: tapetum; Ms: microsporocyte; Msp: microspore; Tds: tetrad; bars = 15  $\mu$ m.

doi:10.1371/journal.pone.0061719.g003

RNA was used for reverse transcription using M-MLVRT (Promega) with oligo (dT18) primer, and 1  $\mu$ L RT product diluted with 20  $\mu$ L ddH<sub>2</sub>O 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: ATGCCGCCCGCCCTATCCTTCCCTCA) and *OsActin1* (F: ACGGCGATAACAGCT CCTCTT; R: CCTCTTCCAGCCTTCCCTTCAT).

## Results

### Identification and Characterization of $h_{2s}$

The  $h_{2s}$  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  $h_{2s}$  mutant failed to produce mature pollen (Figure 1E and F). To characterize defects during anther development in the  $h_{2s}$  mutant, we performed 4', 6-diamidino-2-phenylindole staining. No abnormal activity was observed in  $h_{2s}$  during meiosis (Figures 2A–P). However, the cytoplasm of the young  $h_{2s}$  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 $\times$ P2               | 20  | 11.92*       | 1.60                  | 1.21                | 3.40**             | 4.67**        | 5.44**         | 3.3**   |
| P1 $\times$ Year             | 5   | 2.44         | 5.60**                | 1.554               | 4.27**             | 0.48          | 0.90           | 4.51*   |
| P2 $\times$ Year             | 4   | 2.08**       | 4.56**                | 6.07**              | 1.32               | 25.54**       | 12.03**        | 11.32** |
| P1 $\times$ P2 $\times$ 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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**Table 2.** *h<sub>2s</sub>* 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       | 0/0.03             | 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. doi:10.1371/journal.pone.0061719.t002

tetrads (Figure 2Q and R). 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 *h<sub>2s</sub>*

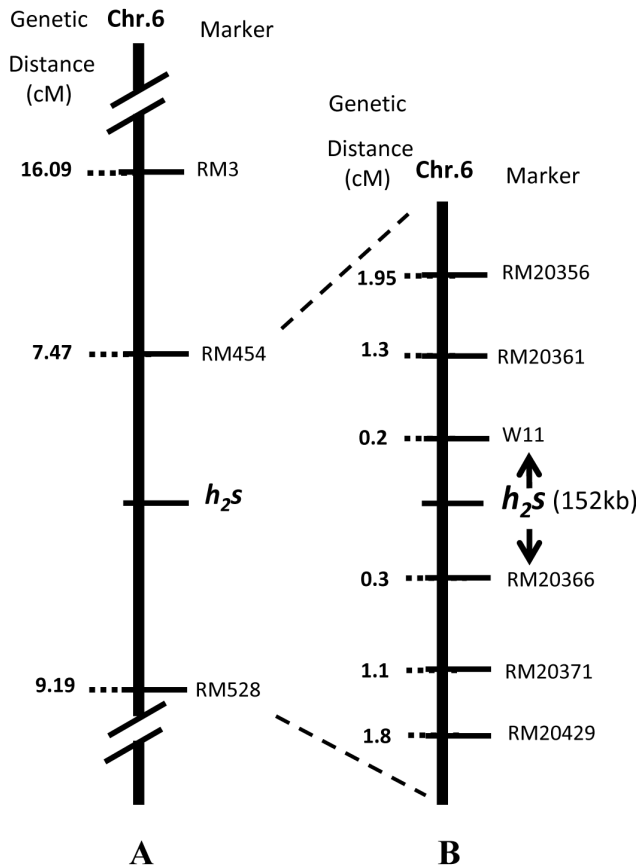
mutant occurred after the release of young microspores from tetrads.

To obtain more information regarding the morphological defects of *h<sub>2s</sub>* anthers, we examined thin sections of wild-type and *h<sub>2s</sub>* anthers. At the meiosis stage, as with 4', 6-diamidino-2-phenylindole staining, no obvious difference between the wild type and the *h<sub>2s</sub>* 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 *h<sub>2s</sub>* 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 *h<sub>2s</sub>* microspores appeared degraded. The microspores in *h<sub>2s</sub>* seemed to lack exine, so the microspore degradation might be due to the absence of exine. At the vacuolated pollen stage, the microspores in *h<sub>2s</sub>* were completely degraded and formed empty anther locules.

**Table 3.** GCA effects on seed set rate, weight per panicle and yield among cytoplasm of *h<sub>2s</sub>* (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/0b          | –0.01bc/–0.01b  |
| A3      | –0.75b/–1.08b     | –0.05b/–0.02b      | –0.01bc/–0.01b  |
| A4      | –1.13b/–1.75b     | 0.03b/0b           | –0.01bc/–0.02b  |
| A5      | –0.11b/0.12b      | –0.03b/–0.01b      | –0.03c/–0.02b   |
| A6      | –0.79b/–1.16b     | –0.07b/–0.08b      | 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  $\alpha=0.05$ . doi:10.1371/journal.pone.0061719.t003



**Figure 4. Linkage mapping of the *h<sub>2s</sub>* gene.** (A) Preliminary mapping results using a population of *h<sub>2s</sub>*/Zhensan97B and (B) fine mapping results using a population of *h<sub>2s</sub>*/Nipponbare. Genetic distance between a marker and the *h<sub>2s</sub>* gene is given on the left in centimorgans (cM).D. doi:10.1371/journal.pone.0061719.g004

**Genetic Analysis of the *h<sub>2s</sub>* Mutant**

All F<sub>1</sub> plants from the cross between *h<sub>2s</sub>* and *9311* produced normal mature pollen and seed sets. Among 120 F<sub>2</sub> 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<sub>2s</sub>* mutant. *h<sub>2s</sub>* also showed stable MS in both Sichuan (E103°82', N30°97') and Hainan (E110°05', N18°28'), suggesting that *h<sub>2s</sub>* was not a PTMS.

**Cytoplasmic Effects of *h<sub>2s</sub>* on Plant Height and Yield-related Traits**

No differences in genetic background among the six isonuclear alloplasmic lines (A1–A6) were detected (Figure S1), indicating that the nuclei of Zhenshan97A, D702A, G46A, K18A, and XieqingzaoA were successfully substituted by the *h<sub>2s</sub>* nucleus. This allowed reliable comparison of the cytoplasmic effects among A1–A6.

The combined ANOVA for the data collected in two years (Figures S2, S3, S4, S5, S6, S7, and S8) showed that the cytoplasm (P1) had significant effects on all investigated traits except effective panicle number (Table 1). The year effect was significant on all traits except the 1000-grain number and grain number per panicle. The interaction between year and cytoplasm showed a significant effect on effective panicle number, grain number per panicle, and yield. The cytoplasmic effects of *h<sub>2s</sub>* on plant height

**Table 4. Candidate genes in the 154 kb region on chromosome 6 where *h<sub>2s</sub>* locus was fine mapped.**

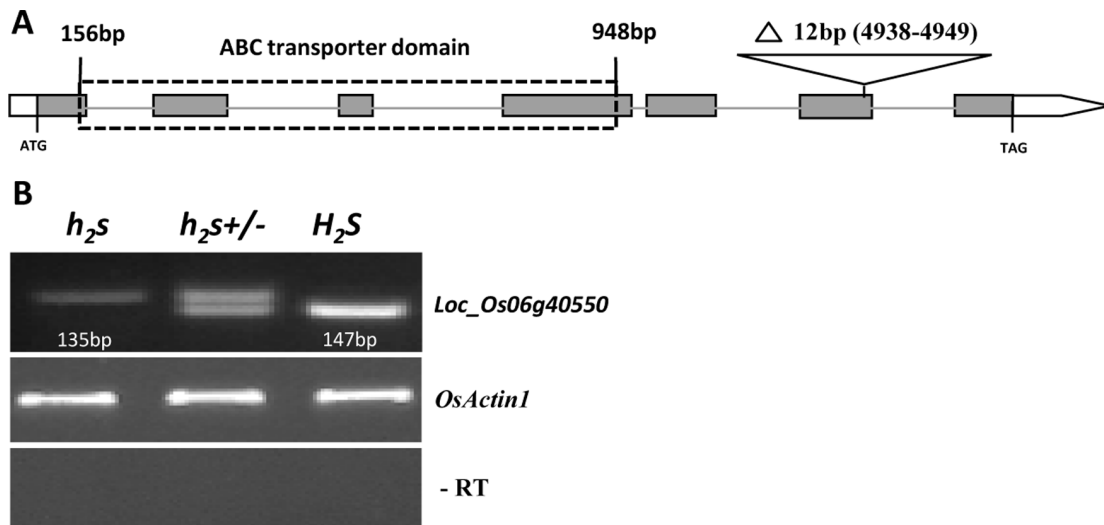
| Gene ID         | Putative function                                |
|-----------------|--|
| LOC_Os06g40480  | retrotransposon protein                          |
| LOC_Os06g40490* | glycosyl hydrolases family 17                    |
| LOC_Os06g40500* | Unknown  |
| LOC_Os06g40510  | CRAL/TRIO domain containing protein              |
| LOC_Os06g40520  | TNP1 domain containing protein                   |
| LOC_Os06g40530  | transposon protein CACTA, En/Spm sub-class       |
| LOC_Os06g40540* | retrotransposon protein                          |
| LOC_Os06g40550* | ABC-2 type transporter domain containing protein |
| LOC_Os06g40560  | 26S protease regulatory subunit S10B             |
| LOC_Os06g40570  | GRAM and C2 domains containing protein           |
| LOC_Os06g40580* | Unknown  |
| LOC_Os06g40590  | Unknown  |
| LOC_Os06g40600  | elongation factor                                |
| LOC_Os06g40609* | Unknown  |
| LOC_Os06g40620* | SNF7 domain containing protein                   |
| LOC_Os06g40630* | SFT2 domain containing protein                   |
| LOC_Os06g40640  | fructose-bisphosphate aldolase isozyme           |
| LOC_Os06g40650* | copine-1   |
| LOC_Os06g40660* | retrotransposon protein                          |
| LOC_Os06g40670  | unknown  |
| LOC_Os06g40680  | unknown  |
| LOC_Os06g40700  | OsSub50 - Putative Subtilisin homologue          |

\*expressed in inflorescence. doi:10.1371/journal.pone.0061719.t004

and yield-related traits were estimated by comparing two hybrids with the same male parent. Out of 25 paired comparisons, all but two showed that *h<sub>2s</sub>* cytoplasm had a positive effect on yield. These positive effects were significant in 12 (48%) and 10 (40%) paired comparisons in 2006 and 2008, respectively (Table 2). Similarly, all but two paired comparisons showed that *h<sub>2s</sub>* cytoplasm had positive effects on the seed set rate. Thirteen (52%) and fourteen (56%) of such comparisons were found to be significant in 2006 and 2008, respectively. Six (24%) and seven (28%) paired comparisons showed significant positive cytoplasmic effect on weight per panicle in 2006 and 2008, respectively. For plant height, effective panicle number, and 1000-grain weight, there were fewer comparisons with significant positive cytoplasmic effects, even though the high proportion of paired comparisons showed positive cytoplasmic effects (Table S2). In contrast, 4 and 9 paired comparisons showed significant negative cytoplasmic effects on grain number per panicle in 2006 and 2008, respectively (Table S2). In conclusion, the cytoplasm of *h<sub>2s</sub>* showed a significant and positive effect on yield, seed set rate, weight per panicle, 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.

**Cytoplasmic Effects of *h<sub>2s</sub>* 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<sub>2s</sub>* showed significant positive cytoplasmic effects on GCA with respect to



**Figure 5. Sequencing analysis of candidate gene *Loc\_Os06g40550*.** (A) Schematic structure of gene *Loc\_Os06g40550*, 12 bp deletion was detected at the predicted sixth exon. White and grey boxes for UTR regions and exons, respectively. The gray lines for introns, and the black dash box for ABC transporter domain region. (B) RT-PCR examination of 12 bp deletion in *Loc\_Os06g40550* RNA. The deletion was detected in the RNA of heterozygous and homozygous plants. doi:10.1371/journal.pone.0061719.g005

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 *h<sub>2s</sub>* in either year (Table S3). The significant and positive cytoplasmic effect on GCA, especially with respect to yield, suggests that the *h<sub>2s</sub>* mutant is a good parent for rice breeding.

### Molecular Mapping of the *h<sub>2s</sub>* Mutation

Although cytoplasmic analysis of agronomic traits and GCA indicated that *h<sub>2s</sub>* had considerable potential for the improvement of rice yield, the male sterility of *h<sub>2s</sub>* severely restricts its utility in the production of hybrid seeds. This is because *h<sub>2s</sub>* is a non-photoperiod/thermo-sensitive genic male sterile mutant. However, one previously described approach provides a possibility to maintain the MS of recessive GMS, so that *h<sub>2s</sub>* 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 *H<sub>2S</sub>* gene using a mapping-based approach.

A  $F_2$  population was generated from a cross between *h<sub>2s</sub>* and Zhensan97B to map the *h<sub>2s</sub>* gene. A bulk segregant analysis was conducted using 52 polymorphic SSR markers between *h<sub>2s</sub>* and Zhensan97B with two DNA bulks, each with ten sterile  $F_2$  plants and ten fertile  $F_2$  plants. Of the 52 SSRs, only RM3 on chromosome 6 was polymorphic between the two DNA bulks, indicating that the putative *h<sub>2s</sub>* gene was located on chromosome 6. We then examined 23 SSRs flanking RM3 for polymorphisms between *h<sub>2s</sub>* and Zhensan97B, and the polymorphic SSRs were used to analyze 612 male sterile  $F_2$  individuals. Both RM454 (located at 27832 kb) and RM528 (located at 26544 kb) were found to be linked with the *h<sub>2s</sub>* locus, with 7.47 and 9.19 cM genetic distance, respectively (Figure 4A). Because no other SSRs were found to be polymorphic between *h<sub>2s</sub>* and Zhensan97B for the region between RM454 and RM528, 35 SSRs and 74 indel markers were examined for polymorphism between *h<sub>2s</sub>* and *Nipponbare*. The identified polymorphic markers were used to analyze 2400 male sterile  $F_2$  individuals in the cross between *h<sub>2s</sub>* and *Nipponbare*. The *h<sub>2s</sub>* 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 *h<sub>2s</sub>* phenotype. This result suggests that *H<sub>2S</sub>* 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\_Os06g40490* encodes a glycosyl hydrolase that is likely to be involved in carbohydrate metabolic processes [24]. *Loc\_Os06g40550* encodes a protein with an ATP binding cassette (ABC) transporter domain. Its homolog in *Arabidopsis*, *ABCG26/WBC27*, has been reported to be involved in pollen exine formation, and the *abcg26/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\_Os06g40650* 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\_Os06g40609*) are unknown at present. Among the ten candidate genes, *Loc\_Os06g40550* 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*, *abcg26/wbc27*, also exhibits MS, and the spatial and temporal expression of *Loc\_Os06g40550* was consistent with *h<sub>2s</sub>* 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 *h<sub>2s</sub>*

genomic DNA and RNA by sequencing and RT-PCR analysis (Figure 5A and B). However, the deletion did not change the reading frame. These results suggest that the deletion in *Loc\_Os06g40550* may be responsible for the *h<sub>2s</sub>* 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 *h<sub>2s</sub>* on yield and GCA of yield were positive and significant in both years, suggesting that *h<sub>2s</sub>* 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 *h<sub>2s</sub>*. The positive-cytoplasmic effects on yield in the GMS should be seriously considered in rice breeding.

Although the GMS mutant *h<sub>2s</sub>* shows promise for rice breeding, the challenge of maintaining the MS of *h<sub>2s</sub>* for the production of hybrid seeds limits its practical application. In order to utilize the positive-cytoplasmic effects of *h<sub>2s</sub>* and resolve the challenge, we could generate a PTMS line with both *h<sub>2s</sub>* cytoplasm and PTMS nuclei using nuclear substitutions. However, the fact that the fertility of PTMS line with *h<sub>2s</sub>* cytoplasm is easily affected by environment also restricts the wide application of positive cytoplasmic effects of *h<sub>2s</sub>*. The approach described by Marc et al. [7] has been proposed to maintain the male sterile condition of recessive genic MS and provide an alternative way of utilizing the positive-cytoplasmic effects of *h<sub>2s</sub>* in rice breeding. This approach requires cloning the gene responsible for the *h<sub>2s</sub>* phenotype. Our mapping results showed that no gene in the fine-mapping region has been reported to be responsible for *h<sub>2s</sub>* phenotype, suggesting that *h<sub>2s</sub>* is a novel gene essential to male reproductive development. Sequencing and bioinformatics analysis of the candidate genes suggested that *Loc\_Os06g40550*, encoding a protein with ABC transporter domain, was the best candidate. This lays a strong basis for cloning the *H<sub>2S</sub>* gene and for using the *h<sub>2s</sub>* mutant in rice breeding. Because the *h<sub>2s</sub>* 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.**

PPTX

**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.**

PPTX

**Figure S3 Mean grain number per panicle and CV (coefficient of variation) of 30 combinations of 6 iso-**

**nuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.**

PPTX

**Figure S4 Mean 1000-grain weight and CV (coefficient of variation) of 30 combinations of 6 isonuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.**

PPTX

**Figure S5 Mean effective panicle number and CV (coefficient of variation) of 30 combinations of 6 isonuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.**

PPTX

**Figure S6 Mean plant height and CV (coefficient of variation) of 30 combinations of 6 isonuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.**

PPTX

**Figure S7 Mean yield and CV (coefficient of variation) of 30 combinations of 6 isonuclear alloplasmic lines (A1–A6) with 5 restorers (R1–R5) during both years.**

PPTX

**Figure S8 Mean weight 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.**

PPTX

**Table S1 Isonuclear alloplasmic lines used in cytoplasm effects analysis.**

(DOCX)

**Table S2 *h<sub>2s</sub>* cytoplasmic effects on seed set rate, weight/panicle and yield in comparison with Zhenshan97A (A2), D702A (A3), G46A (A4), K18A (A5) and XieqingzaoA (A6).**

(DOCX)

**Table S3 GCA effects on seed set rate, weight per panicle and yield among cytoplasm of *h<sub>2s</sub>* (A1), Zhenshan97A (A2), D702A (A3), G46A (A4), K18A (A5) and XieqingzaoA (A6).**

(DOCX)

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## Author Contributions

Conceived and designed the experiments: SL YW BM. Performed the experiments: PQ YW YL. Analyzed the data: PQ YW SL. Contributed reagents/materials/analysis tools: YW BM SL. Wrote the paper: PQ YW SL.

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