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Fine Mapping and Candidate-Gene Analysis of *an open glume multi-pistil 3 (mp3)* in Rice (*Oryza sativa* L.)

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Abstract: The rice mutant mp3 was derived from an indica–japonica cross between Rejing35 and XieqingzaoB, producing an inconstant number of pistils ranging from one to four pistils in a floret at heading stage, which also developed an open-glume with one or two seeds and twin seedlings at mature and seedling stage. Several altered characteristics, including filling grain panicle^{-1} (62.90), grain-setting rate (60.48%) and grain yield plant^{-1} (13.42 g), decreased but an increase in 1000-grain weight (36.87 g) was observed. Genetic analysis revealed that the mp3 mutant phenotype was controlled by a single recessive gene. Using a chromosome walking strategy in the F_2 population of 02428/mp3, the mp3 gene was fine mapped between L3-135 and RM7576, with a physical distance of 30.617 kb on rice chromosome 3. Four candidate genes were found in this region referred to the rice genome annotations. LOC_Os03g11614/OsMADS1 corresponded with the mutant mp3 phenotype. Sequencing showed no sequence alterations in the coding and promoter sequence of the LOC_Os03g11614/*OsMADS1* of mp3. The mp3 gene may be an allelic gene with three previously reported genes but controlled different mutant floral organ phenotypes in rice. Therefore, this mp3 gene provided a novel perspective on the biological function of *OsMADS1* in the development of rice floral organ.

Keywords: open glume multi-pistil; floral organ development; genetic analysis; gene mapping; candidate-gene analysis; rice

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1. Introduction

Rice (*Oryza sativa* L.) is a typical monocot plant that serves as the staple food for half of the global population [1,2]. In the whole life cycle of rice, floral organ formation is the most important transformation period of rice, from vegetative growth to reproductive growth for reproduction and seed dispersal. It also plays an important role in determining grain yield and quality in rice production, so it has always been the focus and hotspot of biology research. Generally, wild-type rice exhibits a constant number of floral organs and has only one carpel in a floret, including a pair of lemma and palea, two small oval-shaped lodicules, six stamens, and one pistil [3,4]. Rice seed is protected with hull, a hard cover structure composed largely of lemma and palea. About two-thirds of the total surface of a rice seed is generally covered with the lemma due to its larger size than the palea, in which two lemmas enclose the edges of the palea from outside, so that they do not separate easily [5]. Over the last two decades, many rice floral organ mutants that cause variations in the structure and number of flower organs have been involved in elucidating the development mechanism of flower organs [6].

Many mutants that cause abnormal floral organ structure in rice have been fine mapped and even functionally characterized. For example, the open-hull and male sterile 1 (ohms1) and open hull semi-sterile mutant (ohss(t)) control an open glume male sterile,

and an open glume semi-sterility of rice is located repeatedly on chromosome 3 [7,8]. Meanwhile, the mutant gene controlling an open hull sterile phenotype is located on the short arm of chromosome 3 [9]. RTS regulates the development of male fertility in rice [10]. MSP1 encodes a Leu-rich repeat receptor-like protein kinase and controls early sporogenic development in rice [11]. Aid1 encodes a single MYB domain protein and regulates anther development in rice [12]. OsYAB1 regulates the meristem development and maintains the stamens and carpels in rice [13]. OsMADS1 controls the differentiation of specific cell types in the lemma and palea and regulates the inner floral organs in rice [14]. Udt1 regulates the tapetum development in the early meiosis of rice [15]. Tdr regulates the development and degeneration of tapetum in rice [16]. OsMADS50 regulates the development of flowering time in rice [17]. EMF1 regulates the floret opening time by mediating lodicule cell-wall formation in rice [18]. Meanwhile, several mutants causing an inconsistent number of floral organs in rice have been fine mapped, and even their functions determined. For example, the multiple pistil 1 (mp1) and multiple pistil 2 (mp2) underlying the number of stamen and carpel primordial in rice are located on chromosomes 1 and 3, respectively [19,20]. Fon2-1 and fon2-2 affect the number of stamens and pistils of rice [21]. Fon3 controls the number of pistils in rice [22]. Fun(t) affects the number of stamen and pistil and displays open hull phenotypes in rice [23]. TOR affects the number of pistils, stigma, and ovaries in rice [24]. ISM(t) affects the number of stigma and pistils in rice and is located on the long arm of chromosome 1 [25]. Afon1 is located on chromosome 1 and controls the development of floral organ numbers in rice [26]. Mf2 is located on the long arm of chromosome 1 and affects the number of glume, lodicule, stamens, and pistil in rice [27]. FON1 has been cloned on the long arm of chromosome 6 of rice and functionally characterized as a leucine-rich repeat kinase [28,29]. FON4 increases the number of pistils, ovaries, and primary rachis branches in rice and is cloned on the long arm chromosome 11 [30–32]. LF1 encodes a class III homeodomain–leucine zipper protein, induces the development of the three-florets spikelet in rice, and is cloned on the short arm chromosome 3 [33].

Most genes related to floral organ structure and floral organ number have been identified and are found to be involved in elucidating the molecular mechanism of flower organ in rice, even if the flower development of monocots is more complex than that of dicots. In the present study, we identified *a novel open glume multi-pistil 3* (mp3) of rice, which developed two or four pistils in a floret but normal stamen in the spikelet at heading stage and developed two seeds in a floret at mature stage. The mutant mp3 germinated twin seedlings at germination stage. Genetic analysis revealed that the mp3 phenotype was controlled by a single recessive nuclear gene. The mp3 gene was located on the short arm of rice chromosome 3, with a physical distance of 30.617 kb. Our results provided a novel perspective on the biological function of OsMADS1-MADS in the development of an *open glume* and *multi-pistil* rice.

2. Materials and Methods

2.1. Plant Materials

The rice mutant mp3 was a descendant of the F_2 population of "Rejing35×XieqingzaoB" After being selfish for 10 generations, it exhibited an open glume and multi-pistil with a wide range of two to four pistils in a floret. The rice Rejing35 (RJ35) was of the *japonica* variety, cultivated by the Chongqing Academy of Agricultural Sciences (CAAS), China, and provided by Professor Xianyong Li. The rice Xieqingzao B (XQZB) is an *indica* maintainer line of the "three line" hybrid rice variety, cultivated by Anhui Guangde Agricultural Science Institute, China. The mutant mp3 was selected as the male parent to cross the wide-compatibility rice variety 02428 (*O. sativa* ssp. *japonica*) and the sequencing rice variety Nippobare (*O. sativa* ssp. *japonica*), thereby producing two F_1 generations. The two F_1 generations were self-crossed, and two F_2 populations of 5785 and 472 individuals were developed in Lingshui County, Hainan Province, China. The F_1 and F_2 populations and their biparent plants were planted at the Biotechnology Testing Station of Chongqing

Normal University, Chongqing (29°32′ N, 106°32′ E), Southwest China for the genetic analysis and gene mapping of mp3.

2.2. Survey of Agronomic Traits in Mutant mp3 and Wild-Type Rice

A random sample of five plants per wild-type rice (XQZB and RJ35) and mutant mp3 was collected to measure the following phenotypic values according to the method described by Shen [34]: HD, days to heading; PH, plant height (cm); PP, panicles plant^{-1}; PL, panicle length (cm); FGP, filling grains panicle^{-1}; EGP, empty grains panicle^{-1}; SP, spikelet panicle^{-1}; 1000-GW, 1000-grain weight (g); GYMP, grain yield of major panicle (g); and GYP, grain yield plant^{-1} (g). The grain shape traits included the following: GL, grain length (mm); GW, grain width (mm); and GT, grain thickness (mm). They were measured with a Mitutoyo absolute digimatic caliper (model 500-173). Three derived traits were calculated including GSR, grain-setting rate (%); GSD, grain-setting density; and LWR, length-to-width ratio. The phenotypic data for each trait in five plants within each wild-type rice and mutant mp3 with three replicates were calculated for statistical analysis using SPSS Statistics version 25#.

During the flowering stage, the phenotypic characteristics of wild-type rice and mutant mp3 were observed at three different stages of heading, germinating, and maturing by using a stereoscopic microscope (Nikon SMZ18, Japan). The pollen fertility of wild-type rice and mutant mp3 was investigated using a stereoscopic microscope (Eclipse 80i, Nikon, Japan) after being stained with 1% I_2-IK solution. At the germination stage, twin seedlings of the mutant mp3 were observed after six days of germination.

2.3. DNA Isolation

The total genomic DNA of the biparents and the recessive multi-pistil F_2 individuals at tillering stage were extracted using the method of SDS with some modifications [35]. Powdered rice leaves were first incubated with the extraction buffer (100 mM Tris-HCL, 50 mM EDTA, 0.5 M NaCl, 1.5% SDS, pH 8.0) at 65 °C followed by chloroform–isoamyl alcohol (24:1, v/v) extraction, anhydrous ethanol precipitation, 75% alcohol cleaning, anhydrous ethanol drying, and final ddH_2O solution for PCR application.

2.4. Marker Development

The sequences of all published simple sequence repeat (SSR) markers used in this study were downloaded from the publicly available rice genomic database (http://www.gramene.org, and accessed on 5 June 2018). A total of 600 pairs of published simple sequence repeat (SSR) markers were used in this study. A total of 141 pairs of additional (L3#) primers within the target chromosome region were developed and evaluated using the primer-blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast, and accessed on 20 April 2020) (Supplementary Table S1). Repeat motifs of potential SSR markers were searched using the identification tool of SSR Hunter version 3.0 software [36] based on the existing genomic sequence of the *japonica* cultivar, cv. Nipponbare (http://rgp.dna.affrc.go.jp (accessed on 20 April 2020), the indica cultivar, cv. 93-11 (http://rice.Genomics.org.cn (accessed on 20 April 2020), and the indica maintainer line, cv. XieqingzaoB sequenced by BGI (https://www.genomics.cn/ (accessed on 20 April 2020). The gap sequence of potential insertions/deletions (InDels) markers of between Nipponbare and 93-11, and Nipponbare and XieqingzaoB, were searched using the MegAlign tool of Lasergene7.0 software. All primers were synthesized by the Shanghai Invitrogen Co., China.

2.5. PCR Analysis

Localizing PCR amplification was performed in a final mixture of 20 µL comprising 2.5 µL (50 ng) of template DNA, 2 µL of 10×PCR buffer (Mg^{2+} plus), 2 µL of 20µmol/primers (primer 1#, 1 µL; primer 2#, 1 µL), 1 µL of 2.5 mM dNTPs mixture, 0.5 U of rTaq DNA polymerase (5 U/µL), and 12 µL of ddH_2O using a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR amplification profile was as follows: 94 °C for 2 min; 35 cycles of 30

s at 94 °C, 30 s at 55 °C, 1 min at 72 °C; followed by a final extension of 5 min at 72 °C. The PCR amplification products were separated on 8% PAGE gels and visualized by the silver-staining method for gene analysis [37].

Sequencing of PCR amplification was performed in a final mixture of 50 μL comprising 3 μL (50 ng) of template DNA, 5 μL of 10× PCR buffer for KODFXNeo-201 (with 1.5 mM Mg^{2+}), 5 μL of dNTPs (2 mM) mixture, 2 μL of $MgSO_4$ (2 mM), 2 μL of 20μmol/Lprimer (primer 1#1μL, primer 2#1μL), 1 U of KOD-Plus-201 (1 U/1 μL), and 32 μL of ddH_2O using a T100 thermal cycler (Bio-Rad, USA). The PCR amplification profile was as follows: 94 °C for 2 min; 35 cycles of 10 s at 98 °C, 30 s at (Tm) °C, 68 °C 1 min/kb; followed by a final extension of 8 min at 68 °C. The PCR amplification products were separated on 2% agarose electrophoresis imaging system for candidate-gene sequencing (Sangon Biotech (Shanghai, China) Co., Ltd.).

2.6. Molecular Mapping and Linkage-Map Construction

A small mp3 recessive F_2 mapping population with 22 recessive individuals was randomly selected to localize the target gene. The polymorphic markers between 02428 and mp3 were selected to identify the genetic linkage marker associated with the mp3 gene. Among the recessive 22 individuals, the number of bands amplified by polymorphic markers similar to the recessive parent mp3 were more than 14 and displayed a probability of linkage to the mp3 gene. The linkage markers relate to the mp3 gene selected from the small population were further utilized to screen the large mp3 recessive F_2 population individuals.

The band pattern of the 02428 and mutant mp3 were coded "A" and "B," respectively, and H was designated as heterozygote. The linkage relationship was analyzed using QTLIciMapping 4.10 [38]. The relative genetic distances between the mp3 locus and the linkage markers were noted by referring to the number of recombinants. The recombination rate was transformed into genetic distance (cM) by using the Kosambi mapping function. We constructed a highly saturated physical map based on the rice genome–sequence information contained in the Gramene data database (http://www.gramene.Org (accessed on 20 May 2021).

2.7. Development of Sequencing Primers and Candidate-Gene Analysis

Based on the linkage-marker information on target gene, we searched the gene information on predictable candidate genes within the target genomic interval through the MSU gene website (https://www.gramene.org/ (accessed on 20 June 2021). We downloaded the full-length genomic sequence of the candidate gene including 2 kb DNA sequence at promoter and terminator with PLAZA3.0 Monocot families (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_monocots/genes (accessed on 25 June 2021). The forward primer of sequencing primer of the candidate gene was developed within the range of 100 bp at promoter (ATG). The reverse primer of the sequencing primer was developed within the range of 100 bp at terminator (TAG, TAA, and TGA). The genomic sequence of the candidate gene was submitted to the Primer-BLAST website for the development of sequencing primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (accessed on 25 June 2021). All sequencing primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. The PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. Multiple sequence–alignment analysis was performed using the function of ClustaIM multiple alignment of BioEdit 7.2 version software.

3. Results

3.1. Morphological Analysis of Mutant mp3

We identified a recessive mutant mp3 associated with the development of the rice floral organ. The floral morphologies of the mutant mp3 and wild-type rice were observed at three different stages of heading, mature, and seedling, respectively (Figures 1A–I and 2A,B). At heading stage, the pollen fertility of wild-type rice (RJ35, XQZB, 02428, and

02428×mp3 F_1 individual) and mutant mp3 developed normally and displayed normal fertility. Pollens of the wild-type spikelet and mutant mp3 were fully fertile, showing that the characteristic round and filled appearance was stained dark with 1% I_2-KI solution (Figure 1A–E). Generally, the floret of wild-type spikelets with only one pistil was enclosed within the palea and lemma (Figure 1F). However, the inconsistent number of pistils in mutant mp3 ranged from two to four pistils in one floret. The floral glume in mutant mp3 was unclosed and developed an inconsistent number of pistils ranging within two from four in a floret, whereas the mutant mp3 exhibited multi-pistils ranging from six to twelve anthers (Figure 1G,H). At mature stage, the mutant mp3 developed one or twin seeds when two or more pistils were produced in a floret (Figure 1D). In particular, the twin seeds of the mutant mp3 were covered by two lemmas and two paleas, respectively. The twin seeds of the mutant mp3 formed an angle of 30°–45° (Figure 1I). Furthermore, three or four seeds in a floret of mutant mp3 were not observed at mature stage due to the dysfunctional multi-pistil. Interestingly, the twin seeds of mutant mp3 germinated twin-seedlings at the seedling stage compared with the wild-type rice (Figure 2A,B).

Figure 1. Phenotype of wild-type rice and mutant mp3. (**A**–**C**) Pollen fertility of wild-type rice. (**D**,**E**) Pollen fertility of mutant mp3 and 02428×mp3 F_1 individual. (**F**) Wild-type rice (XQZB) at heading stage. (**G**,**H**) Mutant mp3 at heading stage. (**I**) Twin seeds of mutant mp3.

Figure 2. Mutant mp3 and wild-type rice at seedling stage. (**A**) Wild-type rice at seedling stage. (**B**) Mutant mp3 at seedling stage.

3.2. Analysis on Agronomical Traits of Mutant mp3

Compared with the wild-type rice of RJ35 and XQZB (Figure 3A,B; Table 1), the mutant mp3 exhibited similar phenotype values on heading date (HD; day), plant height (PH; cm), spikelets panicle^{-1} (SP), grain-setting density (GSD), and grain yield main panicle (GYMP; g) with the male parent of XQZB, but it had significantly lower phenotype values than those of the female parent of RJ35. The phenotype values on panicle plant^{-1} (PP) and panicle length (PL; cm) of mutant mp3 exhibited similar phenotype value to those of its biparents. In particular, the mutant mp3 displayed significantly lower phenotype values on filling grain panicle^{-1} (FGP, 62.90), grain setting rating (GSR, 60.48%), and grain yield plant^{-1} (GYP, 13.42 g) than those of its biparents. However, the phenotype values on empty grain panicle^{-1} (EGP, 41.10) and 1000-grain weight (1000-GW, 36.87 g) of mutant mp3 were significantly higher than those of RJ35 and XQZB. In particular, the mutant mp3 exhibited a significantly lower phenotype value on FGP (62.90), GSR (61.27%), and GYP (13.42 g) than those of its biparents. In summary, the mutant mp3 exhibited significantly lower phenotype values on FGP (62.90), GSR (60.48%), and GYP (13.42 g), but it had significantly higher phenotype value on 1000-GW (36.87 g) compared with that in wild-type rice. These results demonstrated that the mp3 gene may affect the genetic expression of grain yield related to FGP, GSR, and GYP, as well as the open-hull and multi-pistil trait mutant mp3 of rice.

Figure 3. Plant morphology of XQZB and mutant mp3 at heading stage. (**A**) Plant morphology of mutant mp3. (**B**) Plant morphology of wild-type rice. (**C**) Panicle of wild type. (**D**) Panicle of mutant mp3.

Table 1. Agronomic traits of mutant mp3 and wild-type rice.

Genotypes	HD	PH	PL	PP	FGP	EGP	SP	GSR	GSD	1000-GW	GYMP	GYP
	d	cm			n			%			g	
RJ35	124.40 ± 1.02 A	91.43 ± 1.00 A	19.95 ± 0.64 A	8.34 ± 0.33 A	208.12 ± 4.84 A	15.99 ± 1.89 B	247.16 ± 26.66 A	85.12 ± 9.15 A	12.40 ± 1.42 A	22.90 ± 0.37 C	3.93 ± 0.11 A	26.86 ± 0.27 A
XQZB	82.00 ± 1.41 B	74.92 ± 0.65 B	20.45 ± 1.01 A	8.04 ± 0.64 A	97.28 ± 7.24 B	10.72 ± 0.74 C	104.00 ± 1.41 B	93.47 ± 5.76 A	5.10 ± 0.24 B	26.70 ± 0.57 BC	1.98 ± 0.12 B	24.16 ± 0.20 A
mp3	84.40 ± 1.02 B	74.70 ± 0.75 B	19.42 ± 0.47 A	7.60 ± 1.02 A	62.90 ± 1.69 C	41.10 ± 1.28 A	104.00 ± 1.41 B	60.48 ± 1.48 B	5.36 ± 0.13 B	36.87 ± 0.31 A	1.88 ± 0.05 B	13.42 ± 0.18 B

Note: significance difference marked with a capital letter from A to D for $p < 0.05$.

3.3. Analysis on Grain Shapes of Mutant mp3

Compared with the wild-type rice (RJ35, XQZB and 02428) and the 02428×mp3 F_1 individual (Figure 4A,B; Table 2), the mutant mp3 exhibited significantly lower phenotype value on grain length (GL, 8.02 mm) with the *indica* rice XQZB (9.85 mm), but it had similar phenotype value to wild-type rice (RJ35 and 02428) and the 02428×mp3 F_1 individual. Mutant mp3 exhibited the maximum phenotype value on grain width (GW 5.91 mm) and significantly higher phenotype values than that of wild-type rice (RJ35, XQZB, 02428, and 02428×mp3 F_1) due to its unclosed glume. In particular, the phenotype value on GW (3.67 mm) of the 02428×mp3 F_1 individual exhibited similar phenotype value to 02428 (3.87 mm) owing to the recessive genes of the open glume and multi-pistil in mutant mp3. The phenotype value on grain thickness (GT) in mutant mp3 (2.30 mm) was similar

to RJ35 and the 02428×mp3 F_1 individual. However, the mutant mp3 (1.36) displayed significantly lower phenotype value on LWR than that of wild-type rice and the 02428×mp3 F_1 individual due to its 30–45°angle between twin seeds. Especially for 1000-GW, mutant mp3 exhibited the maximum phenotype value on 1000-GW (36.87 g) due to its twin seed in a floret. Interestingly, mutant mp3 rice displayed *S*-curve rice and significantly differed from wild-type rice and the 02428×mp3 F_1 individual (Figure 4B).

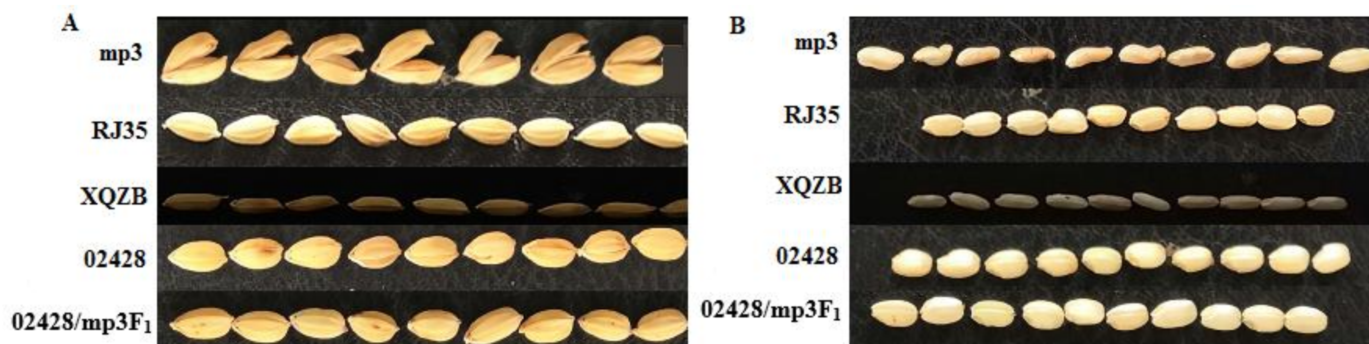

Figure 4. Grain shapes of mutant mp3 and wild-type rice. (**A**) Grain shapes of mutant mp3 and wild-type rice. (**B**) Brown rice of mutant mp3 and wild-type rice.

Table 2. Grain shape of mutant mp3 and wild-type rice.

Genotypes	GL	GW	GT	LWR	1000-GW
		mm			g
RJ35	7.23 ± 0.24 C	3.23± 0.13 C	2.28 ± 0.13 B	2.21 ± 0.08 B	22.90 ± 0.37 C
XQZB	9.85 ± 0.14 A	2.55 ± 0.06 D	2.06 ± 0.05 C	3.86 ± 0.13 A	26.70 ± 0.57 BC
mp3	8.02 ± 0.30 B	5.91 ± 0.46 A	2.30 ± 0.07 B	1.36 ± 0.06 D	36.87 ± 0.31 A
02428	7.34 ± 0.21 C	3.87 ± 0.08 B	2.52 ± 0.07 A	1.90 ± 0.07 C	26.07 ± 0.61 BC
02428/mp3 F_1	7.63 ± 0.12 BC	3.67 ± 0.16 BC	2.28 ± 0.10 B	2.08 ± 0.12 BC	27.47 ± 0.52 B

Note: significance difference marked with a capital letter from A to D for $p < 0.05$.

3.4. Genetic Analysis of Multi-Pistils in Mutant mp3

Mutant mp3 exhibited the stable multi-pistil in one floret across Hainan province and Chongqing Municipality, China. All F_1 plants from 02428×mp3 and Nippobare×mp3 crosses exhibited the normal number of floral organs, indicating a recessive inheritance of the multi-pistil in mutant mp3 (Table 3). Among the two F_2 populations, 5785 and 472 plants grew in Chongqing Municipality, China, respectively. The segregation ratios were investigated during the flowering stage. A total of 4368 plants showed a normal number of floret organs, and 1417 plants revealed multi-pistils in one floret in the cross of the 02428×mp3. Moreover, 355 plants showed the normal number of floret organs, and 117 plants revealed multi-pistils in one floret in the cross of the Nippobare×mp3. Genetic segregations further showed a good fit to the 3:1 ratio in 02428×mp3 (x^2 = 0.79) and Nippobare×mp3 (x^2 = 0.03), with chi-square values (x^2) for the multi-pistil trait in mutant mp3 being significantly lower than the threshold value $x^2_{0.01(3:1)} =$ 3.84 (Table 3). All these results demonstrated that the multi-pistil trait in mutant mp3 was controlled by a single recessive gene. Consequently, the strategy of map-based cloning was suitable for mp3 positional cloning.

Table 3. Genetic analysis of the multi-pistil trait in mutant mp3.

Cross Combinations	F_1 Plant	F_2 Population			$x^2{}_{3:1}$	$x^2{}_{0.05}$
		Total	Normal Plant	Mutant Plant		
02428×mp3	Normal	5785	4368	1417	0.79	3.84
Nippobare×mp3	Normal	472	355	117	0.03	

3.5. Gene Mapping of mp3

Altogether, 600 pairs of (SSR and InDel#) primers were distributed throughout the whole rice genome and were selected to screen the genetic polymorphism between 02428 and mutant mp3. Altogether, 137 pairs of polymorphic markers were used to identify the molecular markers linked to the mp3 gene. Through the strategy of chromosome walking in the F_2 population of 02428×mp3, a small F_2 population comprising 22 recessive multi-pistil individuals was randomly selected to localize the target gene of mp3. The number of bands amplified by polymorphic markers similar to the recessive parent mp3 was more than 14 and displayed a probability of linkage to the mp3; the linkage markers selected from the small population were further utilized to screen the large mp3 recessive F_2 population individuals. The mp3 gene was tightly linked to the SSR marker RM3467 (6,003,496 bp) and RM7197 (9,888,524 bp) on the short arm of chromosome 3 (Figure 5A,B). Therefore, these two markers were used to survey 1417 recessive F_2 individuals, and the mp3 gene was primarily located between RM3467 and RM7197 on the short arm of rice chromosome 3. The SSR marker RM3467 had six recombinant plants, and RM7197 was used to survey 44 recombinant plants. The marker interval of RM3467-RM7197 covered a ~885.028 kb physical segment on the short arm of rice chromosome 3.

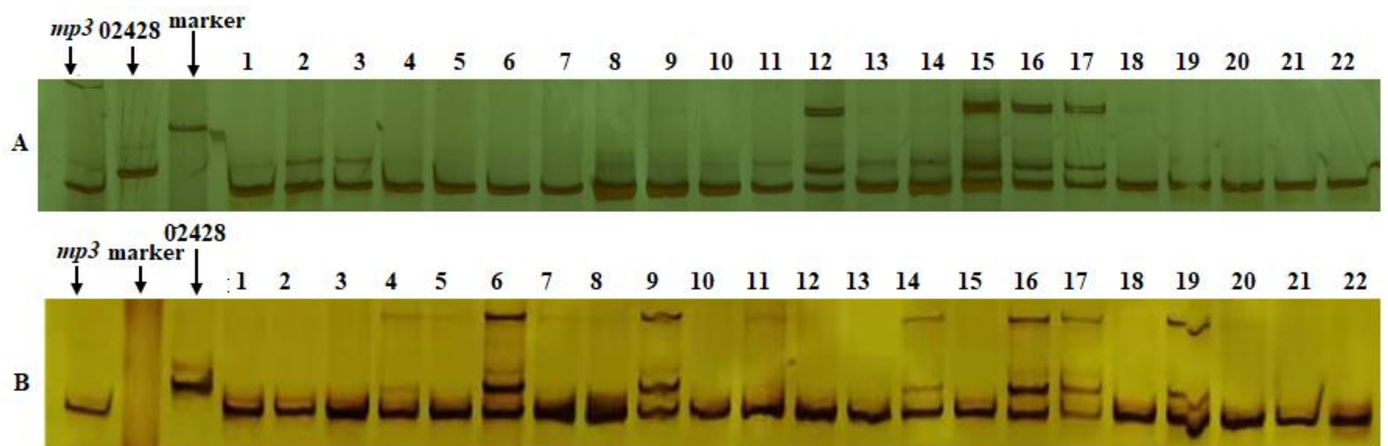

Figure 5. Segregation of the SSR **RM3467** and **RM7197** markers in the F_2 population derived from the cross of 02428 and mp3. Marker. DL-2000 plus DNA marker; (**A**) Male parent mp3; female parent 02428. Marker.1-22: PCR amplification of **RM3467** with 22 multi-pistil individual plants (homozygote or heterozygote) in the F_2 population. (**B**) Male parent mp3; female parent 02428. Marker.1-22: PCR amplification of **RM7197** with 22 multi-pistil individual plants (homozygote or heterozygote) in the F_2 population.

3.6. Fine Mapping of mp3

The mp3 gene was roughly mapped to the marker interval between RM3467 (6,003,496 bp) and RM7197 (9,888,524 bp) on the short arm of rice chromosome 3 by using a small F_2 population from 02428×mp3. To further localize the position of the mp3 gene, a total of 141 additional (L3#) pairs of (SSR or InDel#) primers between RM3467 and RM7197 were developed (Supplementary Table S1). Among them, 51 pairs of (SSR or InDel#) primers were found to exhibit significant polymorphism between 02428 and mutant mp3 (Figure 6; Table 4). These primers were further used to survey a total of 1417 multi-pistil

recessive individuals in the F_2 population, and they were all verified to be linked to the mp3 gene (Table 4). Among them, one marker designated as L3-135 was utilized to survey only three recombinants. Meanwhile, RM7576 was used to survey only one recombinant (Supplementary Table S2). Finally, the mp3 gene was narrowed down to the genomic region and flanked by the InDel# marker L3-135 (6,048,599 bp) and SSR marker RM7576 (6,078,216 bp), a physical distance of approximately 30.617 kb in length (Figure 6).

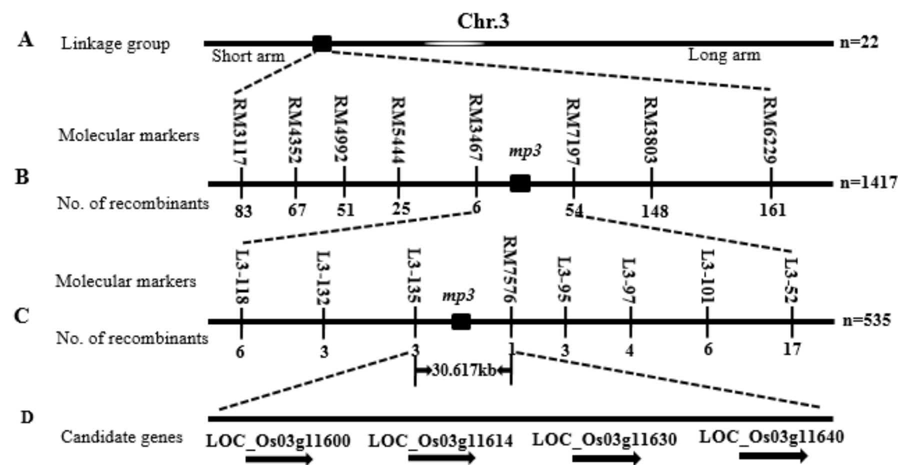

Figure 6. Localization of mp3 gene and the candidate-gene analysis. (**A**) Linkage group of mp3 gene established on the short arm of rice chromosome 3 using 22 recessive F_2 individuals. (**B**) Primary mapping of mp3 flanked by both **RM3467** and **RM7197** using 1417 recessive individuals. (**C**) The mp3 was fine mapped to a 30.617 kb genomic interval using 44 recombinant individuals. (**D**) Four putative candidate genes were annotated in the 30.617 kb genomic region.

Table 4. Primers used in this study.

Primers	GP (bp)	Forward 5′-3′	Reverse3′-5′	Purpose
RM3467	6003496	ATAATGGCAGGGTTGTCTCG	CTCGGTGAGCCTCCTACAAC	Fine mapping
L3-118	6012517	GAATTGGGAACTCCCGCCTA	TCATGACACTATCCTGCACCA	Fine mapping
L3-132	6043090	TTGCCTGTTTTCCGAGTTGAC	AGCTTCCAGCTCTAGCACATT	Fine mapping
L3-135	6048599	CGGAGCTTCTGTTCATGTGTC	GACTGCTTCTTGTACGACGAC	Fine mapping
RM7576	6078216	CTGCCCTGCCTTTTGTACAC	GCGAGCATTCTTTCTTCCAC	Fine mapping
L3-95	6127475	TCGAGGAATGATTTCACTTTCCCA	TGCAGCTAGAGAATGGTCGAT	Fine mapping
L3-97	6239662	TAACGGCCAGTCACTCTCCA	CGATTCCGACGACAAGGAGT	Fine mapping
L3-101	6240244	GCACGATTGATCACAGCTCG	TGCATGAAACCGACACCGAT	Fine mapping
L3-52	6812449	GGCAGCCCACTACAAGTCTA	ACGAACGGGAAGTGCAAGAA	Fine mapping
RM7197	9888524	AACGTGGGAATTTCTAGCCC	GTTTTGGGCCTAAACGAGTG	Fine mapping
GS2	6066335-6069291	ACAGCCGAGGGCAAGATAAG	CACTTGGGTCGTTGCTACAAA	LOC_Os03g11630
GS5	6071382-6073151	CAAATTTGTGCAGGCAGCCA	GCGTGGAACTTTGTTGCTCC	LOC_Os03g11640
GS6	6041245-6048687	GCTAGGGCTAGCTTGCTTGT	TGGCCTGGAATCTGTTCCAAA	LOC_Os03g11600
GS7	6041245-6048687	TGAAGGGTCTCTCTGTTTCCAG	AATTTGGTACTCTCCCCAGTG	LOC_Os03g11600
GS18	6041245-6048687	ATGCATGGCAAGCGTTTGAG	ACAGTGTCTTGGCTAGGATTCG	LOC_Os03g11600
GS9	6052750-6061369	CTGAGTGTCAATCCCCACCTTT	GGACACTGTTTGCATTGGCTT	LOC_Os03g11614
GS47	6052750-6061369	TTAAGTGTGTGGACGAGCGA	ACATTGTCCTAAACGACTTCCT	LOC_Os03g11614
GS52	6052750-6061369	CTCCAATTCTACCGCTGGCT	CCGATGCATGCAGGACTAGC	LOC_Os03g11614
GS64	6050750-6052750	AGATGTGCAAAATAATGCTGGA	ACGTACCATGATGAGCTGGA	LOC_Os03g11614 (Promoter)

GP: Genomic position (bp) reference to the genome sequence of Nipponbare.

3.7. Gene Annotation and Sequence Analysis of the Predicated Genes of mp3

According to the gene-annotation information provided by the Gramene website (http://www.gramene.org/ (accessed on 20 August 2021) (Table 5), in the 30.617 kb genomic interval of the Nipponbare genome, we found a total of four putative genes: LOC_Os03g11600 encoding a putative YABBY domain containing protein; LOC_Os03g11614 encoding a OsMADS1-MADS-box family gene with MIKCc type-box; LOC_Os03g11630 encoding a putative transposon protein; and LOC_Os03g11640 encoding a hypothetical protein. Among them, LOC_Os03g11614 is a MADS box gene related to floral organ development in rice and has the function of a Class E gene, which may be involved in attributing to the determination and development of an open-hull and multi-pistil rice [39]. On this basis, the putative candidate gene of mp3 was designated as *OsMADS1* [7–9,14,40].

Table 5. Predicted genes located at the mp3 locus.

TIGR Rice Locus	Putative Function
LOC_Os03g11600	YABBY domain-containing protein, putative, expressed
LOC_Os03g11614	OsMADS1-MADS-box family gene with MIKCc type-box, expressed
LOC_Os03g11630	Transposon protein, putative, Mutator sub-class, expressed
LOC_Os03g11640	Hypothetical protein

Annotated by https://rapdb.dna.affrc.go.jp/ (accessed on 20 August 2021).

The LOC_Os03g11614/OsMADS1 gene comprised eight exons and seven introns (Figure 6D) [7,14,40]. The full length of OsMADS1 gene was 8620 bp, and the coding region was 774 bp, encoding a product of 257 amino acids (https://rapdb.dna.affrc.go.jp/ accessed on 20 August 2021) [14,40]. By sequencing the OsMADS1 gene in wild-type rice (93-11, RJ35, XQZB and Nipponbare) and mutant mp3, no sequence change in the coding and promoter sequence of LOC_Os03g11614/OsMADS1 of the mutant mp3 gene was detected between wild-type rice and mutant mp3 by using four sequencing primers, namely, GS9, GS47, GS52, and GS64 (Figure 7A,B; Table 4). Only a series of different bases between indica rice (93-11, XQZB and mp3) and japonica rice (RJ35 and Nipponbare) were detected in the coding region and promoter region of the LOC_Os03g11614/Os MADS1 gene. Further sequencing is in progress. ‘

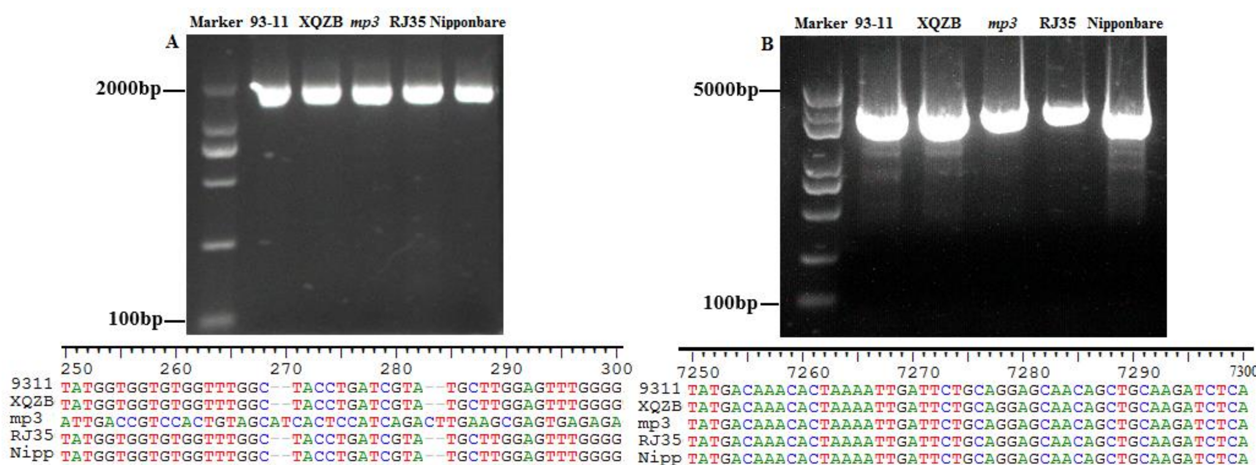

Figure 7. Sequencing of the promoter and coding sequence of OsMADS1/ LOC_Os03g11614 in mutant mp3 and wild-type rice. (**A**) Sequencing of the promoter sequence of OsMADS1/ LOC_Os03g11614 in mutant mp3 and wild-type rice by GS64 amplified. (**B**) Sequencing of the coding sequence of OsMADS1/ LOC_Os03g11614 in mutant mp3 and wild -type rice by GS9 amplified.

4. Discussion

We identified and characterized an open glume and multi-pistil gene mp3 of rice. The mp3 gene was flanked by L3-135 and RM7576 and located in the genomic region of 30.617 kb on the short arm of rice chromosome 3. Four candidate genes including LOC_Os03g11600, LOC_Os03g11614, LOC_Os03g 11,630, and LOC_Os03g11640 were found in this region referred to the rice genome annotations.

Currently, a number of flower organ mutant genes have been mapped or even cloned in rice [3,23,28]. For example, ohss(t) and ohms1 have been repeatedly located on the short arm of rice chromosome 3. Ohms1 was located between markers KY2 (6,079,345 bp) and KY29 (6,037,325 bp) in the genomic region of 42.020 kb in length. Three candidate genes including LOCOs03g11614, LOCOs03g 11,630, and LOCOs03g11640 that were found in this region referred to the rice genome annotations [7], whereas ohss(t) was located between markers InDel6043 (6,043,122 bp) and InDel6070 (6,070,764 bp) in the genomic region of 27.642 kb in length. Three putative genes including Os03t0215200-01, Os03t0215400-01, and Os03t02152600-010 that were found in this region referred to the rice genome annotations [8]. Meanwhile, the mutant gene controlling an open-hull sterile phenotype was located between STS marker 105732-3 (6,048,694 bp) and 105732-8 (6,079,347 bp) in the genomic region of 30.6 kb in length on the short arm of chromosome 3, three candidate genes including LOCOs03g11614, LOCOs03g11630 and LOCOs03g 11,640 found in this region referred to the rice genome annotations [9]. The three previously reported genes were located at the same genomic region of mp3. In particular, the LOCOs03g11614/OsMADS1 appears to be the most likely candidate responsible for the rice organ mutant phenotype. Therefore, we claimed that mp3 was allelic with the three previously reported genes on the short arm of rice chromosome 3. However, the three previously located genes controlled different mutant phenotypes on floral organs from the mutant mp3. The ohms1 gene controlled an open-hull male sterile of rice, whilst the mutant ohms1 displayed open-hull phenotype, male sterility, and three "triodia-like" flower glumes. The mutant ohms1 exhibited complete self-sterility, even pollen fertility was 60%–70%, and induced by the ^{60}Co-γ-treated indica restorer line Zhonghui8015$^{\#}$. The ohss(t) gene controlled an open-hull semi-sterility of rice and exhibited significantly lower phenotype value on pollen fertility and seed-setting rate than those of wild-type rice and induced by the spaceflight of Hanhui7$^{\#}$. The open-hull sterile mutant exhibited narrow palea, decreased grain size, 19% pollen sterility, and was induced by N-methyi–N -nitrosourea treatment on Sinsunchalbyeo rice, a japonica type. The mutant mp3 originated from the indica–japonica of RJ35/XQZB cross and exhibited a wide range from two to four pistils in one floret, as well as significantly lower phenotype values on FGP, GSR, and GYP than those of its biparents. Conversely, it had significantly higher phenotype values on EGP, GW, and 1000-GW. The mutant mp3 exhibited unclosed glume and developed twin seeds at mature stage when two or more pistils were produced in a floret at heading stage. Interestingly, the mutant mp3 germinated twin seedlings at germination stage. In summary, the mutant mp3 exhibited distinct mutant phenotype on floral organ from the three previously reported genes.

Three previously reported floral organ mutant genes were located at the same genomic region of the mutant mp3 gene, suggesting that an important mutant gene controlling multiple different mutant phenotypes on floral organ was distributed on the short arm of chromosome 3. Consequently, we predicted that the putative candidate of LOC_Os03g11614/OsMADS1 probably corresponded with ohms1 and mp3 and the open-hull sterile mutant, which encoded a MADS-box gene allelic with OsMADS1, comprising eight exons and seven introns. The full length of the OsMADS1 gene was 8620 bp, and the coding region was 774 bp, encoding a product of 257 amino acids. Furthermore, OsMADS1 gene is from the MADS-box gene family. OsMADS1 knockdown perturbs the differentiation of specific cell types in the lemma and palea, creating glume-like features, with severe derangements in lemma differentiation. Conversely, ectopic OsMADS1 expression suffices to direct lemma-like differentiation in the glume. Strikingly, in many OsMADS1 knockdown florets, glume-like organs occupy all the inner whorls. Such effects in the

second and third whorl are unexplained. Meanwhile, OsMADS1 play a novel role as an early-acting regulator of second and third whorl organ fate [14]. More interestingly, ectopic expression of OsMADS1 in rice results in dwarfism. Additionally, in spikelets, the out rudimentary glumes are transformed to a lemma/palea-like organ. Transgenic OsMADS1 plants presented serious dwarfism and floral organs by different influences, such as elongated lemma and palea; the number of stamen induced and transformed to lodcule; the number of pistil increases and arise two stigma; and the number of lodicule changed and transformed to leaf-like or lemma/palea-like organs [40]. All in all, OsMADS1 play an important role in the development of flower organ in rice. However, ohms1 had a single nucleotide transformation (A to G of 7269th base) at the bottom of the fifth intron as revealed by sequencing analysis [7]. However, the ohss(t) gene displayed an enclosed panicle and abnormal florets at the reproductive stage, although no mutation occurred in the coding and promoter sequence of LOC_Os03g11614/OsMADS1 of mutant ohss(t), whereas changes in gene-expression pattern were strong [8]. The mutant gene controlling an open hull sterile phenotype was located on the short arm of chromosome 3, but no sequence alterations were found in the promoter and coding sequences [9]. The multi-pistil of the mutant mp3 gene was fine mapped on the short arm of chromosome 3 at a physical distance of 30.617 kb, but no sequence alterations were detected in either the promoter or coding sequence of the LOC_Os03g11614/OsMADS1 gene of mutant mp3. The present mp3 may be an allelic gene with the three previously reported genes but controlled different mutant phenotypes on floral organ in rice.

The present study described the phenotypic observation, genetic analysis, fine mapping, and sequencing of the open-hull and multi-pistil mutant on the short arm of chromosome 3. Our results can help expand the knowledge of an open-hull and multi-pistil mutant trait of rice, especially for the map-based cloning of the open hull and multi-pistil mutant gene, which can provide more insights into understanding the mutant-gene function and molecular mechanism of LOC_Os03g11614/OsMADS1 in future developmental biology projects.

5. Conclusions

The present study reported the rice mutant mp3 that derived from an indica–japonica cross between Rejing35 and XieqingzaoB, the mutant mp3 plant produced an inconstant number of pistils ranging from one to four pistils in a floret at heading stage, which also developed an open glume with one or two seeds and twin seedlings at mature and seedling stage. Several altered characteristics including both filling grain panicle^{-1} (62.90) and grain-setting rate (60.48%) decreased but an increase in 1000-grain weight (36.87 g) was further observed. The mp3 gene controlled by a single recessive gene was located between L3-135 and RM7576 in the genomic region of 30.617 kb on the short arm of rice chromosome 3 using the F_2 population of 02428/mp3. LOC_Os03g11614/OsMADS1, corresponding with the mutant mp3 phenotype, was found in this region referring to the rice genome annotations. Sequencing showed no sequence alterations in the coding and promoter sequence of the LOC_Os03g11614 of mp3. The mp3 gene may be an allelic gene with the three previously reported genes but controlled different mutant floral organ phenotypes in rice. In summary, this mp3 gene provided a novel biological function of OsMADS1 in the development of floral organ in rice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12101731/s1, Table S1: Polymorphic marker between 02428 and mp3 screened in this study; Table S2: Recombinants for mp3 fine mapping in rice.

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Abbreviations

Ohms1	Open-hull and male sterile 1
Ohss(t)	Open-hull semi-sterility mutant
MSP1	Multiple sporocyte 1
Aid1	Anther indehiscence 1
Udt1	Undeveloped tapetum 1
Tdr	Tapetum degeneration retardation
EMF1	Early morning flowering 1
Mp1	Multiple pistil 1
Mp2	Multiple pistil 2
Fon2-1	Floral organ number 2-1
Fon2-2	Floral organ number 2-1
Fon3	Floral organ number 3
Fon(t)	Floral–organ–number mutant
TOR	Twin–ovary mutant
ISM(t)	Increased stigma mutant
Afon1	Abnormal floral organ number 1
Mf2	Multi-floret 2
Fon1	Floral organ number 1
FON4	Floral organ number 4
LF1	Lateral floret 1
Mp3	multi-pistil 3
HD	Days to heading
PH	Plant height
PP	Panicles plant^{-1}
PL	Panicle length
FGP	Filling grains panicle^{-1}
EGP	Empty grains panicle^{-1}
SP	Spikelets panicle^{-1}
1000-GW	1000-grain weight
GYMP	Grain yield of major panicle
GYP	Grain yield plant^{-1}
GL	Grain length
GW	Grain width
GT	Grain thickness
GSR	Grain-setting rate
GSD	Grain-setting density
LWR	Length-to-width ratio

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