Rice Science, 2006, 13(4): 227-233
http://www.ricesci.cn; http://www.ricescience.org

Morphogenesis, Anatomical Observation and Primary Genetic Analysis of a Multi-glume Floral Organ Mutant in Rice

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Abstract: A multi-glume (*mg*) mutant was obtained by screening the T-DNA inserted mutant pool. Anatomical observation revealed that the florets of the mutant showed elongated leafy paleas/lemmas and palea/lemma-like structures, just like multi-glumes. Among the 215 observed florets of the mutant, 14.27% were failed to produce pistil and stamens, 23.72% showed extra floret generated on the same rachilla, while 62.01% consisted of one to nine stamens and one to three pistils in a single floret. On the other hand, in some cases the transparent bulged vesile-like tissue could be observed at the basis of filament. The mutant showed glumaceous lodicules, which prevented the florets from opening in natural conditions, while the absolute male and female sterility was an obvious character of the current mutant. Observation on the process of floral organ morphogenesis by a scanning electron microscopy (SEM) indicated that no phenotype difference in floret primordia was found between the wild-type and the mutant. Meanwhile, for the mutant, the beginning of stamen and pistil primordial differentiation was later than the wild type and the palea/lemma-like structure continued to differentiate after the formation of normal palea and lemma. Furthermore, in the mutant the asymmetrical division of floral primordial caused variation in the number of stamens and pistils. Therefore, the genetic analyses indicated that the mutation phenotype was a recessive trait controlled by a single gene and co-segregated with the T-DNA. Based on the phenotypic characteristics, it could be deduced that the mutant was the result of homeotic conversion from the function of the class E genes in ABCD model.

Key words: rice (*Oryza sativa*); multi-glume mutant; anatomical structure; morphogenesis; genetic analysis

The floral development is one of the pivotal characteristics of the transition from vegetative to reproductive phase in plant, while the generated seeds are the important source of propagation and population dispersal. Furthermore, it serves as the foundation of grain yield and quality in crop. Therefore, the floral development and its regulation becomes current research hotspot in plant molecular biology. Previously, some genes controlling flower development have been cloned in *Snapdragon* and *Arabidopsis*. The 'ABC' model [1] in floral development has been proposed and extended to 'ABCD' and 'ABCE' model subsequently, which illuminated gene interactions and flower morphogenesis in floral development process and partially revealed the arcanum of flower development [2-4]. Although the mechanism of floral development and regulation in monocotyledon is similar to dicotyledon [5-7], some characters of floral organs and development in monocotyledon are different from dicotyledon. Presently, the research work on floral development and regulation in monocotyledon and dicotyledon is in progress. As an ideal model monocotyledonous plant rice can be used to study floral inheritance and development. Presently, tens of MADS-box genes involved in floral development of rice have been cloned, among which *OSH1* belongs to the Kn1 class, *OsMADS6*, *OsMADS14*, *OsMADS15* belong to the AP1/AG family, *RAP1A*, *RAP1B* to class A, *OsMADS2*, *OsMADS4*, *OsMADS16* to class B, *OsMADS3* to class C, *OsMADS13* to class D and *OsMADS1* to class E [8-12]. However, the majority of

Received: 11 May 2006; **Accepted:** 10 July 2006
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these genes were isolated using the conserved sequences of the known homogenous genes as probes, not by identifying from rice mutants directly. Therefore, their genetic functions could not be confirmed due to lack of direct connection between the traits and the genes.

A multi-glume (*mg*) mutant was obtained by screening the T-DNA inserted mutant pool constructed by our research group. By analyzing of anatomical observation and scanning electron microscopy (SEM) the particular mutation phenotype and a morphogenesis process was acquainted. In addition, we also analyzed the heredity behavior of the mutant in segregated population and believed that the *mg* is an ideal mutant material to study the mechanism of rice floral development and regulation.

MATERIALS AND METHODS

Test materials

Several mutants were selected from a T-DNA-tagged line (01Z15AF20) in T_1 generation by screening the T-DNA inserted mutant pool (detailed in reference [13]). The florets of the mutant plant displayed long, leafy paleas/lemmas and palea/lemma-like structures. The mutant was sterile, and the mutant phenotype was steadily inheritable during the asexual propagation, therefore, the same mutant phenotype could be observed in T_2 and T_3. The mutant was named as the multi-glume (*mg*) mutation based on the phenotype.

Test methods

Observation of anatomy and sterility

During the experiment two hundred and fifteen spikelets obtained from panicles of various mutants were observed routinely with an anatomical microscope (LEICAM26), and the photographs were taken to further analyze the mutant phenotype.

Anthers of the mutant were placed on a glass slide, broke with the help of forceps and then dyed with I_2-KI. The percentage of fertile pollens was estimated under the visual field of a microscope according to pollen shape, size and dyed degree.

Morphogenesis observation of the mutant

Conventional tissue preparation method for SEM analysis was adopted. Spikelets were sampled at various stages of panicle differentiation and fixed in a fixative solution of buffered glutaraldehyde (3%), and then rinsed by distilled water three times (10 min each), dehydrated orderly in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 20 min each, and 100% for 2 times) and incubated in an ethanol-isoamyl acetate mixture for 1 h. Samples were dried in liquid carbon dioxide, and mounted on copper stub and sputtered with gold. The mounted specimens were examined, and photographed with a SEM (XL30-ESEM).

Genetic analysis

In T_1 generation of 2003, 20 plants of a T-DNA-tagged line 01Z15AF20 were grown, and the *mg* mutation was observed. Moreover, the proportion of the mutant to the wild type was evaluated. At the same time, all plants were tested by PCR based on special T-DNA segment to estimate the co-segregation between the mutant phenotype and T-DNA insertion. The forward and reverse primers were as follows: 5'-GGCATCGGTAAACATCTGCT- 3' and 5'-GCCT CAAGAAGCTCAAGTGC-3'. The length of PCR product was 0.611 kb (reaction conditions was followed by the ones described in reference [13]). The T_2 generation was grown in 2004 to evaluate the segregation proportion and analyze the co-segregation.

Direct hybridization was not possible due to the absolute sterility of the mutant. Hybridization was carried out by heterozygote selection (natural phenotype but carried T-DNA) as female and Zhonghua 15 as male, then phenotype and segregation proportion were observed.

RESULTS

Phenotype of the *mg* mutant

The glumes of *mg* mutant were about 50% longer than those of the wild type, furthermore, the mutant spikelets were not completely closed (Fig.1-a). The palea/lemma of *mg* mutant was elongated, while some palea/lemma-like structures appeared from the base glume, exhibited multi-glume and rudimentary palea individually (Fig. 1-b). Furthermore, an extra sterile

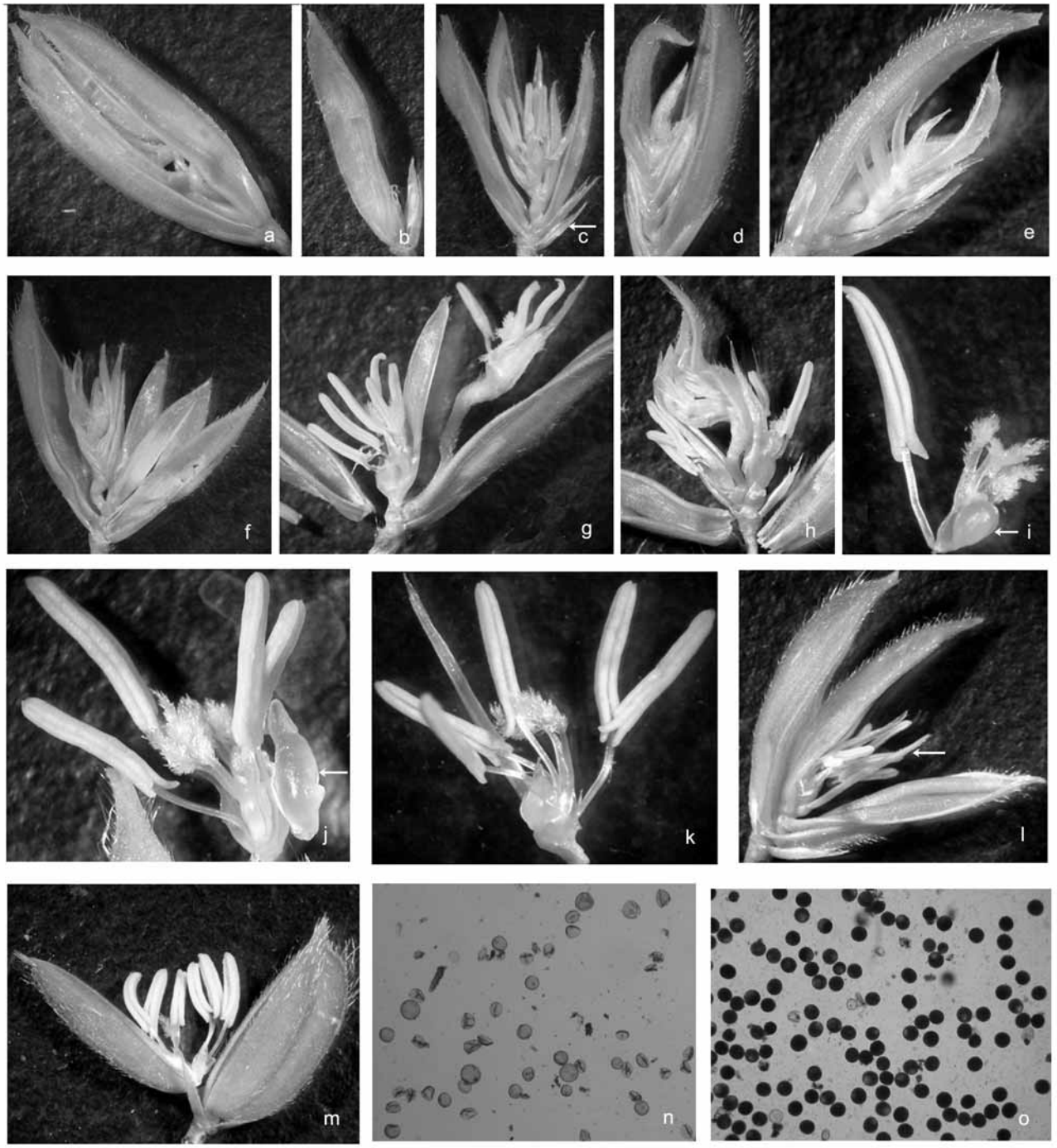

Fig. 1. Partial phenotype of *mg* mutant.

a, Mutant florets in natural conditions; b, Absence of palea; c, An extra sterile lemma; d to f, Failed to form pistil and stamens and showed the palea/lemma-like structures to different extent; g to h, Extra florets on the same rachilla; i to k, Variation of the number of pistils and stamens and distinguished bulged tissue (arrowhead); l, Glumaceous lodicules (arrowhead); m, Normal floret of the wild type; n, Pollen fertility of the mutant; o, Pollen fertility of the wild type.

lemma was also grown (Fig. 1-c). The wild-type floret was made of 6 organs and 13 components, comprising a pair of sterile lemma, 1 palea, 1 lemma, 2 lodicules, 6 stamens, 1 pistil (Fig. 1-m). The anatomical observation of the *mg* mutant revealed that the number of organs and components of floret varied in 3 types, such as: (1) 14.27% failed to form pistils and stamens (Fig.1-d, e, f). (2) 23.72% showed an extra floret on the same rachilla (Fig. 1-g), with incompletely developed palea/lemma and abnormal number of pistils and stamens. Moreover, the number of stamens mainly ranged from 1 to 5, while the number

of pistils varied from 0 to 3. Three florets generated on the same rachilla occasionally (Fig. 1-h). (3) 62.01% of single floret consisted of one to nine stamens and one to three pistils (Fig. 1-i, j, k). Statistical results showed that the florets containing one, two, three, four and five stamens accounted for 1.23%, 10.47%, 15.58%, 19.88%, and 9.77%, respectively. Percentage of the florets with normal 6 stamens was 2.31%, and with 7-9 stamens was 2.77%. The number of pistils in a floret was one in most of the cases, but 4.18% of florets consisted of 2 pistils or an embedded pistil with 3 to 6 members. Some florets still had filaments but tipped by bulged tissue on the base (Fig. 1-i, j). However, no natural floret with normal number of pistils and stamens was observed.

The glumaceous lodicules (Fig.1-l) of the *mg* mutant lost their native function and as a result the opening floret was hard to be observed. The anthers of the *mg* mutant were so inferior for pistils that the spikelet could not get mature. With I_2-KI staining method and observation with a microscope, it might be concluded that most of the pollen granules of the *mg* mutant were typical-abortive. The mutant exhibited absolute male sterility, while the pollen fertility was over 90% in the wild type (Fig. 1-n, o). Though the *mg* mutant was pollinated with natural pollens, it exhibited an absolute female sterility, which might correlate with the aberrance of pistils.

Morphogenesis of the mg mutant

The process of floral organ morphogenesis was investigated using a scanning electron microscopy (SEM). The results showed no phenotype difference between wild type and the mutant for the floral primordium (Fig. 2-a, d). Later the stamen and pistil of the wild type began to shape. The sixth stamen close to lemma formed later, but one to palea earlier, at last, 6 uniform stamens array around pistil with concentric circularity formed during this process (Fig. 2-b, c); but in the mutant, the beginning of stamen and pistil primordium differentiation was later than that in the wild type and the palea/lemma-like structure continually differentiated after the formation of normal palea and lemma (Fig. 2-e, f, l). The division of floral primordium in the *mg* mutant was asymmetrical and different from the wild-type, therefore, the number of stamen and pistil primordium was different and arranged irregularly (Fig. 2-g, h, i, j). During process of floral primordium differentiation in the *mg* mutant, chimeric pistil and 2 florets generated on the same rachilla could be observed.

Primary genetic analysis of the mg mutant

The *mg* mutant was obtained from T_1 generation of T-DNA-tagged line. Four mutants and 16 wild-type plants were segregated from 20 plants in T_1 generation at 3∶1 ratio in the wild-type to *mg* mutant (Table 1), which indicated that the *mg* mutant was governed by a single recessive gene. To estimate whether the loss-of-function mutation was induced by insertion of T-DNA, plants of T_1 generation were tested by PCR using primers amplifying specific T-DNA segment. The results of the four tested mutants were positive, while the 16 wild-type plants showed the segregation at the ratio of 10∶6 in positive to negative reaction, indicating that T-DNA co-segregated with mutant phenotype (Fig. 3) and the loss-of-function could be induced by insertion of T-DNA.

During the experiments the hypothesis was tested in two ways: (1) seeds from the wild-type plants were grown to investigate the phenotype of T_2 generation. No mutant did appear in 3 lines with negative PCR,

Table 1. Segregation ratio of the mutants in different generations or in the generation of cross for the T-DNA tagged line.

Parent/cross	Generation	Normal phenotype plants	Mutation phenotype plants	Ratio	x^2 value
Zhonghua 15 (Mutant pool)	T_1	16	4	3:1	0.061
	T_2	62	18	3:1	0.150
T_1 Heterozygote/ Zhonghua 15	F_1	37	0	—	—
(CK)	F_2	209	80	3:1	0.969

$x^2_{0.05}$=3.84.

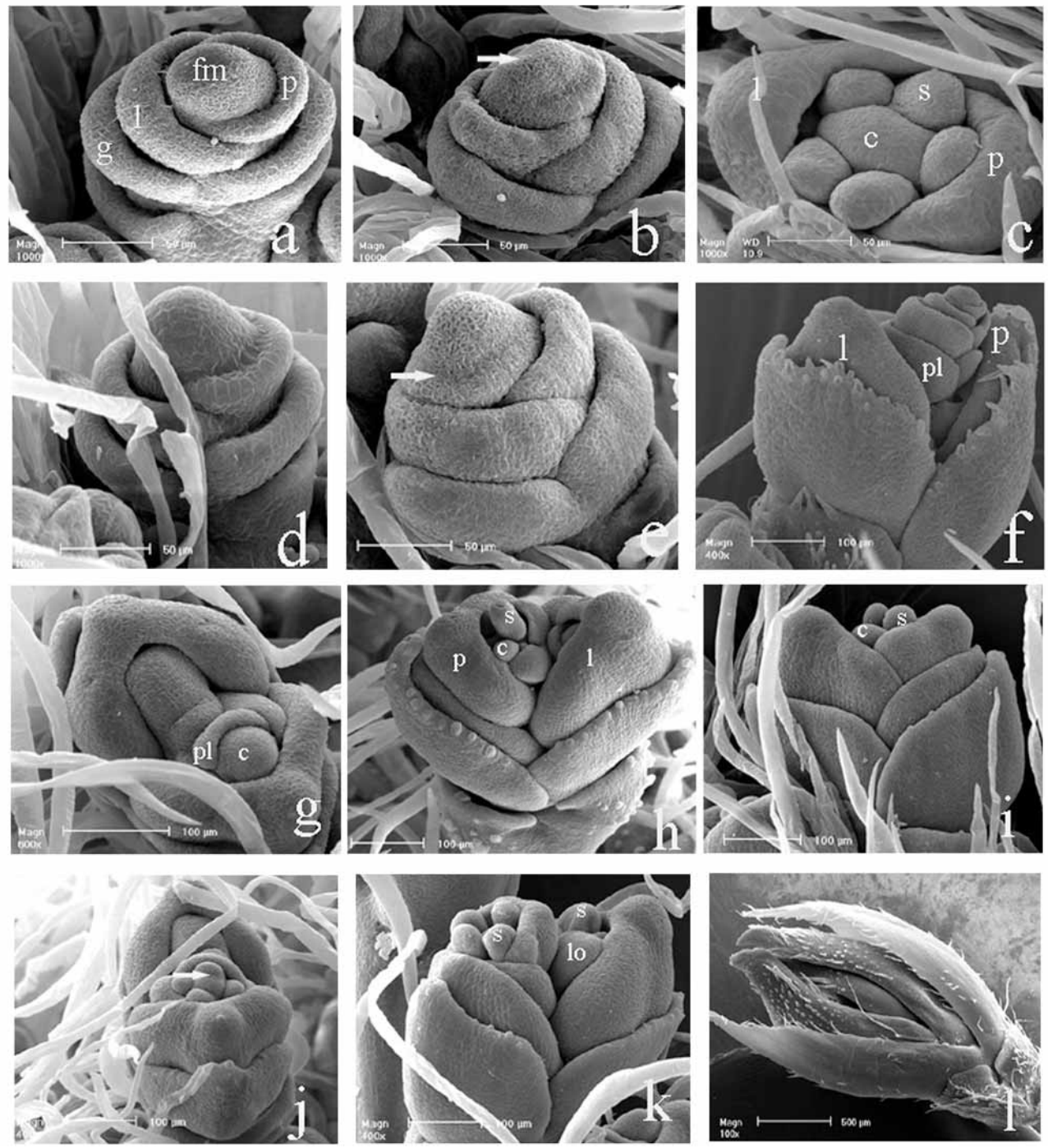

Fig. 2. Morphogenesis process of the *mg* mutant.

a to c, Primordium of normal florets for wild type; d to l, Primordium of florets for the mutant. a and d, No difference was observed between the mutant and wild-type at the floret primordial differentiation stage; b and c, Floret primordial divided symmetrically for the wild-type (arrowhead); e, f and l, Floret continually differentiated and formed palea/lemma-like structure (arrowhead) in the mutant; g to j, Floret primordial divided un-symmetrically and caused the variation in number of stamens and pistils (arrowhead shows the chimera pistil) in the mutant; k, Extra floret differentiated on the same rachilla in the mutant.

fm, Floral primordium; p, Palea; l, Lemma; sl, Sterile lemma; c, Carpel; pl, Palea-like structures; s, Stamen; lo, Lodicules.

but the *mg* mutant was found in 4 lines with positive PCR. Moreover, 18 mutant plants appeared in all of the 80 plants with ratio of 3∶1(Table 1). The 18 mutant plants showed positive results in PCR, and co-segregated still. (2) the *mg* mutant was not hybridized due to complete male/female sterility. Plant

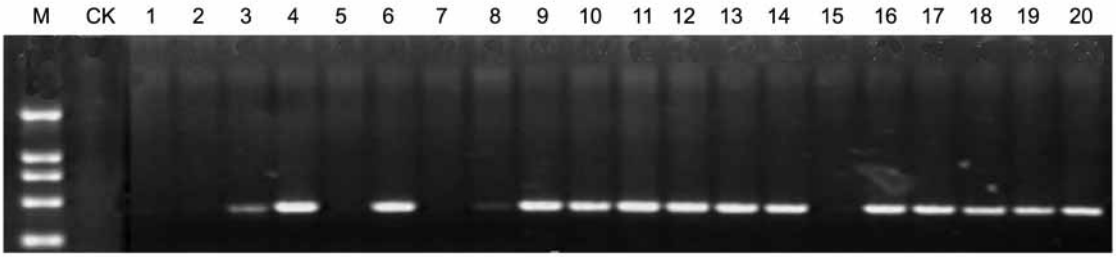

Fig. 3. PCR co-segregation analyses of the T-DNA tagged line in T_1 generation.

M, Ladder marker; CK, Negative control; Lanes 1 to 16, Normal phenotype plants (PCR results based on inserted T-DNA sequence showed the segregation of 10 positive to 6 negative); Lanes 17 to 20, Mutants (Specific PCR detection all showed positive).

with natural phenotype and positive PCR result (If this mutant was induced by insertion of T-DNA, it was heterozygote theoretically) was hybridized with wild-type Zhonghua 15, which served as the male parent. All the 37 plants in F_1 generation displayed natural phenotype. The PCR result indicated that 17 plants were PCR positive and 20 were PCR negative, which fits the ratio of 1∶1(χ^2 =0.108). The same mutants still appeared in F_2 generation generated from natural plants with positive PCR result. Moreover, the segregation ratio was 3∶1 in F_2 generation, which confirmed that the *mg* mutant was induced by insertion of T-DNA.

DISCUSSION

Previously, a few mutants related to floral development was obtained in rice such as, *fon1*, *fon2*, *mp1*, *mp2*, *ops*, *nsr*, *lhs1*, *lh*, *lrs*, and *srs* [14-22]. Among these mutants, *lhs1*, *lh*, *lrs* and *srs* exhibited elongated and lobated paleas/lemmas, longer paleas than lemmmas, extra rachilla, less seed setting percentage. In the *mg* mutant, the elongated and lobated paleas /lemmas were observed. Moreover, the palea/lemma-like structure continually differentiated after the formation of the normal palea and lemma, multiple glumes, decreased number of stamens; rachillas mostly without stamens and pistils, two or more florets on one rachilla, and absolute male/female sterility were found also. We have noted that the phenotype of *mg* mutant was partially similar to that of *lhs1*, *lh*, and *lrs*. Compared with those known mutants, the *mg* mutant did not exhibit longer paleas than lemmas. Furthermore, in the mutant complete male/female sterility and phenotypic characters of degenerative stamen/pistil appeared, which didn't appeared in majority of the above-mentioned mutants. Therefore, we deduced that *mg* mutant was a novel mutant different from *lhs1*, *lh*, *lrs*, and *srs*.

The 'ABC' model of floral development for dicotyledon proposed by Coen and Meyerowitz showed that the class A genes were required for sepal formation in the first whorl, while the combination of class A and B genes resulted the formation of petals in the second whorl. Moreover, Class B and C genes together defined stamen identity and Class C genes were responsible for the carpel formation [1]. *FBP7* and *FBP11* regulating ovule development in petunia were found by Colombo and Angenent, which were named as class D gene and 'ABCD' model on floral development was shaped [2-3]. Subsequently, Pelaz found that the class E genes (*SEP1/2/3*) [4] regulated the development of petals, stamens and carpels in the 2nd, 3rd, and 4th whorl in *Arabidopsis*, and extended the model of floral development. However, the class E genes responsible to regulate floral development and their interaction with other genes remained to be investigated.

The stamens and pistil as the 2nd whorl organs of monocotyledon were as conservative as those of dicotyledon, while the outer floral organs (paleas, lemmas, lodicules) were clearly distinguishable from that of dicotyledon (calyces and petals) [23]. The lodicules of rice corresponded to the petals of dicotyledon was previously confirmed in some experiments [6, 24-25]. For the *mg* mutant, paleas/ lemmas existed in the majority of floret and the mutant phenotype showed multi-glume owing to glumaceous lodicules, while decreased number of

stamens and rudimentary pistils were observed in the majority of floret. Therefore, it can be assumed that the phenotype of mutant related with development of the 2nd, 3rd, 4th whorl organs in floret. However, the phenotypes of *mg* and *lhs1* mutant were partially similar. The *lhs1* was the homeotic mutation, which contained two missense mutations in the *OsMADS1* MADS domain [17]. *OsMADS1* was classified into class E genes in rice [12]. Based on above results, it can be concluded that the *mg* is a function mutant aroused by class E genes.

From the primary genetic analysis it is clear that *mg* is a recessive mutant of monogene and co-segregated with T-DNA, showing that it might be a T-DNA insertion mutation. This indicated that T-DNA tagging was a high throughput method of gene identification [26-27], which would lay the foundations to isolate *mg* based on getting flanking sequence and co-segregation of T-DNA and mutant phenotype.

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