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Genetic analysis of extra glume and molecular mapping of *eg3* in rice (*Oryza sativa* L.) using SSR markers

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Abstract

The great advances of flower development have been achieved in the past decade by genetic and molecular analysis of floral homeotic mutants in *Arabidopsis thaliana* and *Antirrhinum majus*. But the developmental process of inflorescence and spikelet is still little known in rice, an important crop plant and model monocot plant. In this study, we isolated and characterized a novel extra glume3 (*eg3*) mutant, which produced extra glume-like structure organ on the lemma side of rice spikelet and developed between empty glumes and lemma. To analyze the inheritance of the *eg3*, we generated two segregating F_2 populations by crossing *eg3* mutant with normal cultivar Longdao5 and Qishanzhan. Our results confirmed that a single recessive nuclear gene controls the extra glume trait. Simple sequence repeat (SSR) and bulked segregant analyses of the F_2 population revealed that the *eg3* gene is located on chromosome 4. Using bulked-extreme and recessive-class approach, the *eg3* was mapped between SSR marker RM471 and RM16842. According to the rice annotation project database, the target region is about 64.1 kb from 18,996,727 bp to 19,060,851 bp.

Keywords: rice (*Oryza sativa* L.), extra glume3, fine mapping, *APETALA1 gene*.

Introduction

The formation of a flower is a complicated process marked by conversion of the shoot apical meristem to an inflorescence meristem, and subsequently forming floral meristems from the lateral margins (Coen and Nugent, 1994). The great advances of flower development have been achieved in the past decade. Genetic and molecular mechanisms of floral homeotic mutants have been especially well studied in *Arabidopsis thaliana* and *Antirrhinum majus* to establish the ABC model that control floral development (Weigel and Meyerowitz, 1994). Now, this model has been extended to ABCE model (Theissen, 2001). In contrast to the well-known on the molecular regulation of flower development in eudicots, the genetic control of flower development in monocot grasses is far from clear. The structure of a grass flower differ considerably from those of dicots especially rice (*Oryza sativa* L.) (Itoh et al., 2005). The rice spikelet consists of a single floret because the spikelet meristem is converted into a floret meristem after producing two pairs of sterile glumes (rudimentary glumes and empty glumes). Rice florets comprise lemma, palea and three kinds of organs: two lodicules (petals), six stamens and one pistil constituted by a single carpel (Fig. 1A). Recent studies on transcription factors have revealed that genetic control of inner floral organs appears to be conserved between dicots and grasses, at least to some extent (Itoh et al., 2005). Phylogenetic analyses of angiosperm MADS-box genes suggest that there was four members of *Arabidopsis APETALA1* (*AP1*) in rice genome including *OsMADS14* (Moon et al., 1999; Arora et al., 2007), *OsMADS15* (Moon et al., 1999; Arora et al., 2007), *OsMADS18* (Fornara et al., 2004; Arora et al., 2007) and *OsMADS20* (Moon et al., 1999; Arora et al., 2007). *OsMADS4* shows a significant homology to members in the *PISTILLATA* (*PI*) family and belongs to B gene. *OsMADS3* is highly homologous to the members of the *AGAMOUS* (*AG*) family (C gene) that is essential for the normal development of the internal two whorls (Kang et al., 1998). *OsMADS2* belongs to rice ortholog of the class B gene (Kyozuka et al., 2000). Comparing with the three inner whorls, there is very little information about the outermost whorl (lemma, palea, rudimentary glumes and empty glumes). There were several gene related to rice spikelet organ. *MFS1* gene belongs to an unknown function clade in the APETALA2/ethylene-responsive factor (AP2/ERF) family and plays an important role in the regulation of spikelet meristem determinacy and floral organ identity (Ren et al., 2013). *G1* is a member of a plant-specific gene family that encodes proteins with a previously uncharacterized domain, named here ALOG (Arabidopsis *LSH1* and *Oryza G1*). Its sterile lemma enlarges like the lemma (Yoshida, 2009). *REP1* regulates palea identity and development. It is only expressed in palea primordium during early flower development (Yuan et al., 2009). *EG1* is the first cloned extra glume gene which encoded a putative lipase that specifies empty-glume fate and floral meristem determinacy

Table 1. The polymorphic markers used in this study.

Marker	Repeat Motif	Forward Primer (5'-3')	Reverse Primer (5'-3')
RM471	AG	AGAAATGGATCGGACTGAACATGC	AGACACTCGGACGCACAAGC
RM16842	AGC	AGAGCAGAGGCCACACCATACC	CAAAGCTGCTACTGTTTGTGTTCG
RM3308	AG	CCTCACGCCACTGACATCTGG	GGGAGGAGAGGTGAGGAAGAGAGC
RM16883	AT	TGCCATGATATGATTCCTGTGG	GGTCCTATTACAAGCATGCAGTCC
RM5951	ACC	TCCCATCTCCCGGTACTGATCC	CAAGACGTGTCGTGTGGTGTGG
RM1359	AG	CGACTTGCCAAAGGTCAACG	GATTCTACGGGCCACAAGTCC

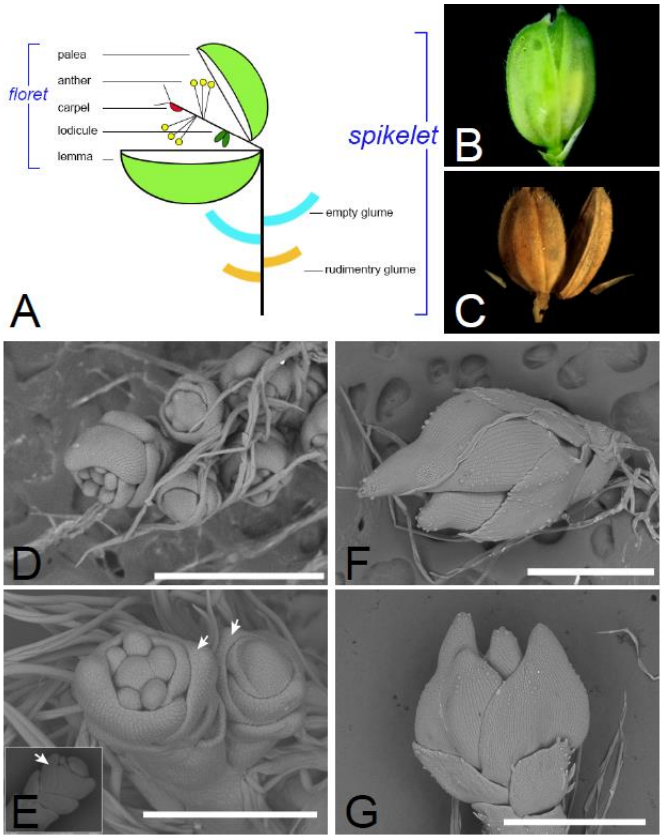

Fig 1. Phenotype and SEM images of the *eg3* mutant in spikelet development. (A) Schematic of a rice spikelet. (B) and (C) Morphology of spikelet in the *eg3*. (D) and (F) SEM images of spikelet at In6-7 in the Akihikari. (E) and (G) SEM images of spikelet at In6-7 in the *eg3*. Arrow in (E) indicate extra glume-like organs. Scale bars = 500 μm in (D), (E), (F) and (G).

(Li et al., 2009). Another extra glume gene *eg2* was mapped on chromosome 6 (Sanchez and Khush, 1998). Although two genes controlling extra glume had been identified, the information about extra glume formation is still limited. In this paper, we report the isolation of one extra glume3 (*eg3*) mutant, which produced extra glume-like structures in the spikelet. We fine mapped *eg3* into 33.9 kb between two SSR markers RM471 and RM16842. These results will provide more information for the better understanding of development rice spikelet.

Results

Phenotypes characteristics of the panicle in the eg3 mutant

The spikelet in wild-type (cv. Akihikari) consists of two pairs of sterile glumes (rudimentary glumes and empty glumes), lemma, palea and three kinds of organs (two petals, six stamens and one pistil constituted by a single carpel) (Fig. 1A). The *eg3* have an extra glume-like structures between lemma and empty glume (Fig. 1B C). And no other organs difference was found in *eg3* spikelet. To further examine the early developmental defects, we observed inflorescence and spikelet development via scanning electron microscopy (SEM) between *eg3* and Akihikari. Akihikari and *eg3* all developed as normal at stage *In1~In5*. And then primary and secondary branch meristems of Akihikari and *eg3* converted into terminal spikelet meristems and formed rudimentary glumes in stage *In6-7* (Fig. 1D E). The *eg3* spikelet development proceeded normally until emergence of the empty glume primordial at the stage *In6*. The spikelet meristem of Akihikari was converted into a floret meristem to produce one lemma and one palea after differentiating a pair of rudimentary glumes and a pair of empty glumes (Fig 1D). In contrast, a glume-like organ could be observed on the lemma side and developed between empty glumes and lemma (Figure 1E). All these information suggested that an additional whorl was formed. At the stage *In8* (rapid elongation of rachis and maturation of reproductive organs), the extra glume structure was observed clearly in *eg3* comparing with Akihikari normal spikelet (Fig. 1F G).

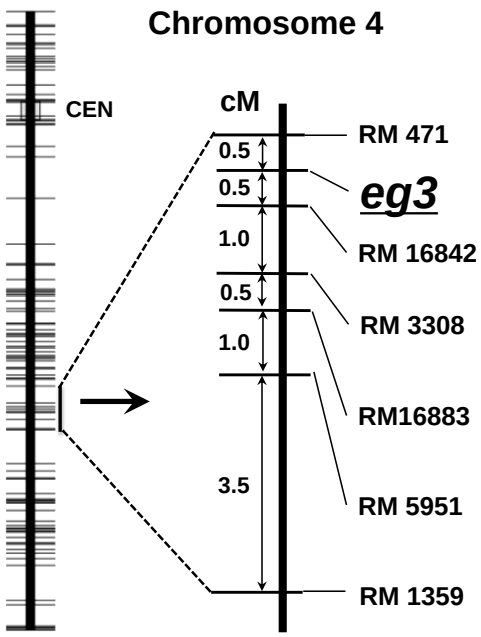

Fig 2. Genetic linkage map of *eg3*.

Phenotypic performance of the segregating population

The extra glume was observed as a recessive trait controlling by one nuclear gene. The F_2 population of Longdao5 (*Japonica* variety) and Qishanzhan (*Indica* variety) were all categorized in two sub-group. Actually, a clear monogenic segregation for extra glume was observed. The 328 F_2 plants derived from the cross between *eg3* and Longdao5 could be divided into 253 normal plants and 75 extra glume plants. And the segregating was 253: 75=3.37: 1.00, fitting well to the 3: 1 ratio ($\chi 2$=0.8470, P>0.05). The segregation ratio of plants with normal spikelet to extra glume individuals was1486: 498=2.98: 1.00 in Qishanzhan population (1984 F_2 individuals), fitting well to 3: 1 ratio ($\chi 2$=3.2842, P>0.05). These results revealed that the extra glume was controlled by a single recessive gene.

Mapping the target gene

Using the bulked-extreme approach, we mapped *eg3* on chromosome 4 in the 75 extra glume plants from F_2 population of Longdao5. But the numbers of recombinants between the polymorphism marker and the target gene was limited in the F_2 population derived from the cross between *eg3* and Longdao5. To increase the polymorphism, we genotyped the *eg3* mutant by using 100 SSR markers covering all the 12 chromosome. The primary mapping location of *eg3* was coming from the *japonica* parent Akihikari, so we selected the Qishanzhan population to map the target gene. To fine map *eg3*, 450 individuals with extra glume were selected in the F_2 segregating from crossing between *eg3* and Qishanzhan. Six markers RM471, RM16842, RM3308, RM16883, RM5951 and RM1359 from 50 markers had polymorphism between two parents. And finally, the *eg3* was mapped between RM471 and RM16842 (Fig 2). The *eg3* region between RM471 (18,996,727 bp) and RM16842 (19,060,851 bp) represents about 64.1 kb of genomic sequence according to Nipponbare genome sequence, and this region covered five genes according to the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/).

Discussion

In this study, we have characterized and fine mapped a novel extra glume gene involved in rice spikelet development. The glume-like organ was observed on the lemma side and developed between empty glumes and lemma. We did not found difference of inner florets organ such as lodicules, stamens, and carpel between *eg3* and Akihikari. Until now, only two mutants have extra glume was reported. One (*EG1*) is come from Zhonghua 11 (ZH11) produced by γ -ray irradiation (Li et al., 2009), the other (*EG2*) is from IR43 protoplast-derived line (Sanchez and Khush, 1998). The extra glume gene *EG1* on chromosome 1 was cloned encoding a putative lipase gene that specifies empty-glume fate and floral meristem determinacy (Li et al., 2009). The *EG2* report by Sanchez and Khush (1998) was be mapped on chromosome 6. The *eg3* found by us was mapped on chromosome 4, it was a new extra glume gene. The most significant finding in our study was narrowed the *eg3* chromosome region to a 64.1 kb. According to the map positions of *eg3*, the delimited regions include one transcriptional factor B3 family protein (*Os04g0386900*), one Floral homeotic protein *APETALA1* (*AP1*) (*MADS C*) gene (*Os04g0387400*), one AT.I.24-6 protein (Fragment) (*Os04g0387900*), one Similar to OSIGBa0148P16.6 protein (*Os04g0386700*) and one Hypothetical conserved gene (*Os04g0387000*). The *Os04g0387400* similar to floral homeotic protein *Arabidopsis AP1* gene have the conservative N terminus structure of *AP1*. It also having DNA binding site, dimerization interface site, protein interaction site and a putative phosphorylation site. In *Arabidopsis*, the *AP1* acts locally to specify the identity of the floral meristem, and to determine sepal and petal development (Mandel et al., 1992). The rice (grass) flower structure differs from that of dicots in that organs that correspond to sepals are lacking (Itoh et al., 2005). The spikelet formed a pair of rudimentary glume primordia firstly, formed a pair of empty glume primordia secondly, and then formed lemma and palea primordium. The *RAP1A* and *RAP1B*, homologs genes of *Os0387400*, expressed in lemma and palea (Moon et al., 1999; Kyozuka et al., 2000). And the SEM analysis showed that the extra glume like organ was on the lemma side and developed between empty glumes and lemma. All these information indicated that *Os0387400* could be a candidate *eg3* gene. But we do not have enough data (sequence polymorphisms or expression levels of these genes by RT-PCR or Quantitative Real-Time RT-PCR) to rule out the possible of the other four candidate genes. Further study is being carried out to clone it.

Materials and methods

Plant materials

The extra glume 3 (*eg3*) mutant was identified from a breeding population produced by crossing Akihikari (*Oryza sativa* L. *Japonica*) with Qishanzhan (*Oryza sativa* L. *Indica*). Akihikari were used as wild-type strains for comparing phenotypes. The maternal parent extra glume line *eg3* was crossed with normal cultivar Longdao5 and Qishanzhan to create a F_2 mapping populations (named Longdao5 population and Qishanzhan population). The Longdao5 population (including 328 individuals) and Qishanzhan population (including 1984 individuals) were used to analysis the genetic base. The

Qishanzhan population was used to fine map the target gene.

Cultivation and morphological analysis

The field experiments were conducted in the rice growing seasons on the experimental farm of Cultivation and Farming Research Institute of Heilongjiang Academy of Agricultural Sciences, Harbin, China (East longitude 126°83′; Northern latitude 45°85′). The sowing date was April 18 and transplanting date was May 21. Field management essentially followed standard agricultural practice. Fertilizers applied were 60, 90, and 90 kg/ha for N, P_2O_5, and K_2O, respectively.
The flowers in wild-type (cv. Akihikari) and *eg3* were observed by using tabletop electron microscope TM-1000 (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from young leaves of a single plant using the CTAB method (Murray and Thompson 1980). All polymorphic markers were chosen from International Rice Genome Sequencing Project (2005) and the report from Zhang et al. (2007). The marker's information was given in Table 1. DNA amplification was performed using a Gene Amp PCR system 9700 thermo cycler (Perkin Elmer Cetus, Norwalk, CT). Each reaction of 15 µl PCR mixture contained 20 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton-X, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 0.2 µM of each primer, 5% (v/v) dimethyl sulfoxide and 0.5 U Taq DNA polymerase (Tiangen Biotech, Beijing, China). Amplification conditions consisted of an initial denaturation at 94 ºC for 5 min, 35 cycles of 94 ºC for 45 sec, 55-60 ºC for 45 sec, and 72 ºC for 1 min, followed by a final extension at 72 ºC for 5 min. To detect polymorphisms of markers, the PCR products were separated on 4% agarose gels.

Molecular mapping of eg3

To perform mapping of target gene, the bulked-extreme and recessive-class approach as described by Zhang et al. (1994) was used to calculate recombination frequencies between the gene and molecular markers in the homozygous recessive plants. Thus, the recombination frequency = $(N_1 + N_2/2)/N$, where N is the total number of recessive plants, N_1 is the number of recessive plants with the marker genotype of dominant parent, and N_2 is the number of recessive plants with heterozygous marker genotype.

Conclusion

A novel gene *eg3* was mapped in 64.1 kb on chromosome 4, which controlled extra glume-like structure organ on the lemma side of rice spikelet.

Acknowledgments

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