Fine Mapping of a Retarded-Palea2 (REP2) Gene on Chromosome 9 in Rice
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Abstract

Floral organ development influences plant reproduction and crop yield. However, mechanisms underlying the development of floral organs in specific group of species like grasses remain unclear. To understand how palea was formed, we identified a retarded-palea2 (rep2) mutant, which showed that the palea was degenerate, and the lemma was crooked and just like sickle shaped. Genetic analysis confirmed that the rep2 mutant phenotype was due to a single recessive gene mutation. F2 population derived from the rep2 mutant crossed with Oryza sativa subsp. Japonica Taigeng16, was used for molecular mapping of the REP2 gene. Using simple sequence repeats (SSR) and insertion-deletion (Indel) markers, the REP2 gene was fine mapped into a 12.9 kb physical distance on chromosome 9, where two open reading frames were predicted. Sequence analysis indicated that a 10-bp-deletion was found in LOC_Os09g24480 between 8PW33 and the rep2 mutant. The rice RETARDED PLEA1 (REP1) gene was in this locus. Thus, we suspected that a 10-bp deletion in the rep2 mutant caused a frame shift and premature translational termination, and led to the functional alteration of the REP2 gene.

Keywords: Rice (Oryza sativa L. Subsp. indica); Rep2 mutant; Molecular marker; Gene mapping; Floral development

Introduction

The flower is the reproductive organ of angiosperm plants, and its formation occurs through different steps. Firstly, the fate of the floral meristem is specified through the activity of floral meristem genes; secondly, the floral meristem is patterned into the whorls of organ primordia through the activity of floral organ identity genes; thirdly, the floral organ identity genes activate downstream effectors, which specify various tissues and cell types that constitute different floral-organ types [1]. Since some genes involved in flower development have been cloned from the model plants, rapid progress has been made in elucidating the molecular mechanisms regulating flowering [2,3]. Intensive molecular and genetic analyses in those species, notably Arabidopsis thaliana, and snapdragon (Antirrhinum majus), establish the ABC model [4,5].

According to this model, three classes of homeotic genes control the floral organ formation. A-class genes alone specify sepal formation, A- and B-class genes determine petal identity, B-class genes in combination with C-class genes together regulate stamen development, and C-class genes alone specify the innermost whorl, the carpel [6,7]. Although the floral model is proved by some studies in different plants [3,8-10] many questions remain unanswered. For example, the molecular mechanism underlying its floral organ development has not been fully investigated [11], how the target gene is identified by floral homeotic proteins, what is the target of floral homeotic proteins [12,13]. The grasses (Poaceae) is one of the largest monocot families with ~10 000 species, including many important cereal crops such as rice, maize, and barley [14,15]. Grass species have highly specialized flowers that differ from those of eudicots. A typical monocot, such as rice, a single spikelet consists of two pairs of sterile glumes (rudimentary glumes and empty glumes) and one floret, and a single floret is comprised of one lemma, one palea, two lodicules (the equivalent of petals), six stamens, and one pistil [16,17], but the surrounding structures, lodicules, lemma and palea, are unique to grasses [17,18]. In the eudicots, molecular genetic and morphological studies have revealed that lodicules and stamens are organs homologous to petals and stamens [2,19-21]. However, the origin and mechanism of lemma and palea development have long been controversial. Some researchers think of the lemma and palea as sepals, whereas others consider them as additional bracts because of their similar cellular patterns [19]. Hence, it is necessary to identify more mutants related to the development of lemmas and paleas and to isolate these relevant genes. However, to date, there are very few examples of molecular characterization of palea and lemma development in rice. In this study, we analyzed a rice mutant, retarded-palea2 (rep2), which had defects in several floral organ identities, including the palea identity, and in floral meristem determinacy. We also reported the investigation and comparison of the morphological features of the floral organs between the rep2 mutant and WT, genetic analysis of the mutant trait, and mapping of the gene for the rep2 locus to a small physical region. We isolated the REP2 gene by map-based cloning, and the REP2 gene encoded a TCP family transcription factor.

Materials and Methods

Plant materials

The Indica rice 8PW33 and Japonica Taigeng16 were kept in the Rice Research Institute, Fujian Academy of Agricultural Sciences, China. The retarded-palea2 mutant with the background of Indica cultivar 8PW33, was obtained through a screen of a M1 population treated by 60Co γ-ray, and was designated as rep2. About 300 plants in M1 population and 3000 plants in M2 population were grown at Fuzhou Experimental Station in Fujian Academy of Agricultural Sciences in April 2006 and in April 2007, respectively. The Indica cultivar, 8PW33, was derived from a progeny of a cross between Minghui 86 and Dongnanhui 307, which was designated as WT in this paper. In April 2011, the rep2 mutant was crossed with WT, 9311 and Taigeng16 at Sanya Experimental Station in Hainan Province. The F1, seeds, WT and the rep2 mutant were sown at Fuzhou Experimental Station in Fujian Province in June 2011 and all F1 seeds were harvested in October 2011. The F2 seeds, rep2 and 8PW33 were planted at Sanya Experimental...
Molecular mapping of the REP2 gene

The band patterns of the mutant (rep2 rep2) and Taigeng16 (REP2 REP2) were recorded as 1 and 3, respectively, whereas 2 was used to denote the heterozygote (REP2 rep2). In this study, linkage analysis between the rep2 locus and the SSR markers was conducted using MAPMAKER version 3.0 software [26] and map distances were estimated with MapDraw V2 [27]. At the same time, the linkage map was basically the same as reported [28].

Bioinformatics analysis

Candidate genes were predicted according to the available sequence annotation databases (http://rice.plantbiology.msu.edu/; http://www.tigr.org/). DNA and amino acid sequences were used for a complete alignment using Clustal X version 1.81.

Results

Main agronomic characteristics of rep2

To elucidate the genes that control the development of rice flowers, we screen for comparisons of phenotypes between the rep2 mutant and WT. The results showed that the rep2 mutant showed some special traits (Table 1). For example, the rep2 mutant is taller with wider flag leaf, higher with plant height and more spikelets than WT, which shows difference at 0.05 probable levels or significant difference at 0.01 probable levels. Especially, we observe that the seed setting rate of the rep2 mutant (48.4%) is lower than that of WT (92.6%) (Table 1), which shows significant difference at 0.01 probable levels.

Phenotype investigation of the rep2 mutant

WT and the rep2 mutant show indistinguishable phenotypes in their vegetative stage; however, their spikelets are different from booting stage to maturity (Figure 1a-1d). A wild type rice floret consists of a palea, a lemma, two lodicules, six stamens and a pistil with two stigmas, and these organs are all normal. In rep2 floret, the palea is degenerate, and the lemma is crooked, which is just like sickle shaped. Close examination of the base of the spikelet revealed that the degenerate palea in rep2 mutation was formed at the booting stage (Figure 1a). In addition, no abnormality was detected in other spikelet organs such as the pistil and stamens, suggesting that the rep2 mutation specifically affects the palea development. The rep2 mutant flowers were male fertile and female fertile. Microscopy analysis indicated that 95.75% of pollen grains of the mutated plants are normal (Figure 1g). When artificially pollinated with the WT pollens, about 300 germinative seeds were yielded from 400 flowers of 3 panicles on one plant, suggesting that the rep2 mutant was basically female fertile.

Genetic analysis of the gene for the rep2 trait

To determine whether rep2 was controlled by a single gene or multiple genes, the rep2 mutant was crossed with 9311and WT. All F1 hybrids showed normal phenotypes, and all F1 populations showed normal Mendel’s segregation (Table 2). Segregation of WT and the mutant type plants fitted a 3:1 segregation ratio in the two F2 populations ($X^2=0.330-0.688, P>0.05$) (Table 2). So these results indicated that the mutant phenotype was controlled by a single recessive gene.

Preliminary molecular mapping of the REP2 gene

At first, the polymorphisms between the rep2 mutant and Taigeng16 were examined with 324 pairs of SSR primers from the RM series, of which 206 pairs exhibited polymorphism. Using BSA (bulked segregant analysis) method analysis, these 206 primer-pairs, the rep2
The Rep2 gene was preliminarily mapped between molecular markers RM24301 and RM24323 in the terminal region of chromosome 9, at a respective distance of 0.7 cM and 0.4 cM (Figure 2b).

**Fine mapping of the Rep2 gene**

To map the gene to a smaller region, 1808 mutant individuals were identified from the F2 population derived from rep2 × Taigeng16. A higher precision map was constructed using published markers in the region between RM24301 and RM24323 (Figure 2c, Table 3). Seven polymorphic Indels were selected from 16 new InDels (Table 3). The Indel markers were designed from the publicly available rice genome sequences, and the likelihood of detecting polymorphism between the rep2 mutant and Taigeng16 was predicted by comparing sequences from Nipponbare (http://rup.dna.affrc.go.jp/) and the Indica cultivar 93-11 (http://rice.genomics.org.cn/). All recombinants were genotyped using seven polymorphic markers within the above interval. Recombinant screening with nine markers (RM24301, Indel-9-1, Indel-9-3, Indel-9-4, Indel-9-8, Indel-9-12, Indel-9-13, Indel-9-16 and RM24323), which were more internal to the rep2 locus. The Rep2 gene was precisely defined in an 8.0 cM region by Indel-9-4 and Indel-9-12 (Figure 2c). To delimit the gene to a smaller region, new polymorphic molecular markers were developed, and three polymorphic InDels were selected from 8 new InDels (Table 3). Recombinant screening with six other markers (Indel-9-4, Indel-9-17, Indel-9-8, Indel-9-19, Indel-9-21 and Indel-9-12), which were more internal to the rep2 locus, detected two, one, zero, two and six recombinants, respectively (Figure 2d). Thus, the Rep2 gene was precisely defined in a 12.9 cM region by Indel-9-17 and Indel-9-19.

**Candidate genes in the 12.9 kb region**

There are two annotated genes (LOC_Os09g24460 and LOC_Os09g24480) in the 12.9 kb region, according to the available sequence annotation databases (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). In Arabidopsis, an ortholog of Rep2, AT3G18550, encodes a TCP family transcription factor.

**Sequence analyses of the Rep2 gene**

To investigate which gene was responsible for the mutation phenotype, sequencing of two genes in WT and the rep2 mutant revealed that a 10-bp deletion was found in LOC_Os09g24480 between WT and the rep2 mutant (Figure 3), while no difference in the LOC_Os09g24460 between WT and the rep2 mutant was observed. Thus, we concluded that the LOC_Os09g24480 locus corresponded to Rep2. Interestingly, the RETARDED PALEA1 (REPL) gene, encoding TCP gene family members in defining the diversification of floral morphology [33], was in this locus. The phenotype characters of the rep2 were very similar to that of repl (Figure 1a-e). According to the phenotypic resemblance, mapping and sequencing analysis, we suspected that rep2 was probably allelic to repl. The analysis of the ORF region showed that the Rep2 gene (LOC_Os09g24480) had corresponding full length cDNAs of 777 bp (Figure 4). In repl, a 10-bp deletion was found in LOC_Os09g24480, causing a frame shift and premature translational termination (Figure 4). Thus, mutation as such would be expected to significantly alter the functions of the protein.

**Homology analyses of Rep2 genes from other species**

To gain insight into the function of Rep2, we generated homology analyses between rice and Arabidopsis (Figure 5) according to available sequence annotation databases (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). In Arabidopsis, an ortholog of Rep2, AT3G18550,
which belonged to the TCP family, was identified to encode TCP family transcription factor. As shown in Figure 5, REP2 and AT3G18550 had high homology. Interestingly, a 10-bp deletion was found in the rep2 mutant (Figure 4, 5), which caused a frame shift and premature translational termination, and destroyed the conservative region, leading to a functional alteration of the REP2 gene. Thus, we suspected that the phenotype of the rep2 mutant would be caused by the functional alteration of the TCP structural protein.

**Discussion**

**The rep2 mutant exhibits unique qualities**

Facilitated by the recent developments in genome sequencing, molecular markers and bioinformatics, an impressive number of the floral organ identity genes are fine mapped and cloned in the past 20 years, and it is more and more important to identify mutants related to the development of lemmas and paleas in cloning the floral organ genes. In rice, several lemma and/or palea defective mutants have been reported; for example, leafy lemma and calcaroides in barley [29], lsl1 [30], dh1 [31], s1l [32], rep1 [33], mof1 [34], dep [35], pal1 [36], tobi [37], bsl1[2] and slp1 [38] in rice. In the present study, we have characterized and identified the rep2 mutant. The REP2 gene was finally localized to a 12.9 kb region in the BAC clone OJ1294_G08, and the recombinants number between markers and target gene was indicated under the linkage map.
<table>
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<th>Marker type</th>
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<th>Sequence of reverse primer</th>
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**Table 3:** In Del and SSR molecular marker used for fine mapping of the REP2 gene.

**Figure 3:** Sequence comparison between WT and REP2 in LOC_Os09g24480.
The start codon (ATG) and stop codon (TAA) are indicated. Black and gray boxes indicate the ORF region and untranslated region (UTR) of the REP2 gene, respectively. There was a 10-bp deletion at the ORF region of rep2, and arrow showed the mutation site.

**Figure 4:** The structure of the REP2 gene (LOC_Os09g24480) and the mutation site.

**Figure 5:** Alignment of amino acid sequences for TCP family protein domain in plant orthologous group. The positions of two conserved motifs are indicated above the sequences, and the conserved domain is underlined with a straight. Identical and similar amino acids are shaded dark blue. The red arrow indicates deletion position in the rep2 mutant.

**REP2 is a TCP family member**

In this article, using a map-based cloning strategy we isolated the REP2 gene, which encoded a putative protein and belonged to a plant-specific TCP transcription factor family. These TCP family members have previously only been identified in angiosperms and have been shown to be essential in specifying plant morphology [39]. For example, TB1 in maize [40], OsTB1 [41], REP1 [40] in rice, and AtTCP12/BRC1 proteins in Arabidopsis [42] are associated with controlling zygomorphic floral development. However, a key question is whether there are TCP genes that control the diversification of floral asymmetry in grasses. Through genetic and molecular studies, some researchers have addressed this point in rice [33]. Therefore, this finding thereof extended the function of the TCP gene family members in defining the diversification of floral morphology in grasses, and suggested that a common conserved mechanism controlling floral zygomorphy by CYC-like genes existed in both eudicots and monocots [33]. In summary, the rep2, which is allelic to rep1, plays an important role in establishing palea identity and controlling the diversification of floral asymmetry in rice. Therefore, further molecular study on the REP2-like proteins in the relative grass family will help us to facilitate elucidate whether the conserved pathway exists in controlling other grass floral zygomorphy.

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