Letter

‘Two-floret spikelet’ as a novel resource has the potential to increase rice yield

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Yield in rice (Oryza sativa) is determined by three major components: panicle number per plant, grain weight and grain/spikelet number per panicle (Zhou et al., 2015). Grain number per panicle is one of the main targets and mainly results from the number of spikelets. Traditionally, rice breeders have focused on the improvement of spikelet number per panicle and rarely focused on the number of florets because a normal rice spikelet has one fertile floret and produces one seed. In grass, the spikelet comprises one to 40 florets depending on the species and shows determinacy or indeterminacy. In rice (O. sativa) with a determinate spikelet, the spikelet meristems produced the fixed floral meristems, resulting in the formation of one floret. In wheat (Triticum aestivum) with an indeterminate spikelet, the spikelet meristems produced the variable floral meristems, resulting in the formation of more florets. How to further increase rice yield? If the number of florets in a spikelet could be increased, it may be a new method for rice high production. In our study, we characterized two allelic mutants with two florets within a single spikelet, double floret1-1 (df1-1) and df1-2. We next focused on the df1-1 mutant to investigate the regulation of floret number in rice, and this provided a new perspective for increasing grain number per panicle and yield.

The wild-type rice spikelet has one fertile floret that is flanked by one pair of glumes, which are generated from the spikelet meristem, and one floret per spikelet is strictly regulated in the Oryza genus (Figure 1a–d). The floret comprises the lemma, palea, lodicule, stamen and pistil (Figure 1a–d).

The 15%–20% spikelets developed two florets inside one pair of sterile lemma which randomly distributed in the df1-1 mutant (Figure 1g–h). Rarely, three florets were also observed (Figure 1c). In the df1-1 mutant, we observed four whorls of floral organs within each single floret (Figure 1g–j). Each lemma and palea had a similar histological texture and vascular bundles as the wild type, respectively (Figure 1c,i). To confirm the identity of the organs in each single floret, we investigated the expressions of OsMADS1, OsMADS14, OsMADS15, OsMADS6 and DL responsible for the lemma and/or palea identity. No differences in gene expression were found within each single spikelet between the wild type and df1-1 mutant. We next investigated the expression of the OSH1 gene, which is essential for floral meristem activity. OSH1 showed a much higher expression in the young panicles of the df1 mutant. These results suggested that each floret in the df1-1 mutant has a normal lemma and palea, and two florets form within a single spikelet. At maturity, the seed setting rate, grain size and weight of the normal and original florets in the df1-1 mutant were comparable with those in the wild type, respectively. The seed setting rate of the secondary florets was lower, and the grain size and weight were variable in the df1-1 mutant. These results suggested that DF1 has the potential for increasing the grain number per panicle and yield.

We next investigate early spikelet development. Compared with the wild type, we found two pairs of the lemma and palea in a single df1-1 spikelet at the Sp4 stage, which were generated from different floral meristems (Figure 1m,q). At the Sp5 and Sp6 stages, two florets within a single spikelet developed normal stamen primordia which exhibited a similar developmental process with that of the wild-type floret (Figure 1c,j,n,r). At the Sp7 stage, with formation of the pistil, the stamen primordia of
the original floret differentiated into quadrangular anthers, whereas the secondary floret displayed hemispherical stamen primordial (Figure 1o,s). At the Sp8 stage, two apparent rudiments of florets were observed within the single spikelet (Figure 1d,j,p,t). Taken together, the formation of two florets in a single spikelet revealed that the two floret meristems were generated from one spikelet meristem.

Then, we detected the expression of several marker genes by in situ hybridization. OsMADS6 expression is uniformly found in the floral meristem of the wild-type spikelet. We detected two independent OsMADS6 signals within a single spikelet in the df1-1 mutant at the Sp4 stage. During stages Sp5 to Sp8, OsMADS6 expression was strongly visible in two mrps of the wild-type floret. In the df1-1 mutant, their signals were induced in four mrps of two florets in a single spikelet. At stages Sp4-Sp8, DL was strongly expressed in the lemma of the wild type. However, DL signals were observed in the lemma of the secondary floret in addition to the lemma of the original floret. OSH1 is the marker gene for indeterminate meristems, and abundant OSH1 transcripts were observed throughout the early floral meristem in the wild-type floret. Interestingly, at the Sp4-Sp6 stage, two strong, independent OSH1 signals were visible inside one pair of sterile lemma in the df1-1 mutant, implying that two floret meristems were present in a spikelet. In the wild-type spikelet, G1 signal was found in a palea. Two independent G1 signals were detected inside a pair of sterile lemma in the df1-1-mutant spikelet, implying that two palea were initiated in the single spikelet. These findings revealed that the df1-1-mutant developed two floral meristems from one spikelet meristem and formed two independent florets in a single spikelet.

The DF1 locus was narrowed to a 56-kb region. A single-nucleotide substitution from G to T in the df1-1 mutant and C to T in the df1-2 mutant was found within a predicted lipase gene (Os01g0900400). The complementation test showed that the

Figure 1 Phenotypes of spikelets in the wild type and the df1-1 mutant. (a–b) Wild-type spikelet. (c) Transverse section of the wild-type spikelet. (d) Longitudinal section of the wild-type spikelet. (e–f) Wild-type seed. (g–h) df1-1-mutant spikelet. (i) Transverse section of the df1-1-mutant spikelet. (j) Longitudinal section of the df1-1-mutant spikelet. (k–l) df1-1-mutant seed. (m–n) Early spikelet development in the wild-type and the df1-1 mutant. (o–p) Wild-type spikelet. (q) Sp4, (r) Sp5-6, (s) Sp7, (t) Sp8. (q–t) df1-mutant spikelet. (u) Sp4, (v) Sp5-6, (w) Sp7, (x) Sp8. sl, sterile lemma; le, lemma; pa, palea; leo, lemma of the original floret; les, lemma of the secondary floret; po, palea of the original floret; ps, palea of the secondary floret; bop, body of the palea; mrp, marginal regions of the palea; lo, lodicule; st, stamen; pi, pistil; fm, floral meristem. Red stars indicate vascular bundles. Regions surrounded by red and yellow lines indicate different florets in (i). Bars = 1000 μm in (a), (b), (g), and (h); 100 μm in (c), (i) and (m–t); 100 μm in (d) and (j); 1 cm in (f) and (l).
df1-1 phenotypes were rescued, and the Cas9-DF1 mutant produced two florets and seeds, resembling that of the df1 mutants. These results confirmed that DF1 was Os01g0900400. Strong GUS signals from proDF1-GUS and DF1 transcripts were specifically detected in the spikelets and panicles. In situ hybridization revealed that DF1 transcripts were strongly expressed in the spikelet meristem, floral meristem, floral organs. Analysis of enzyme activity revealed that purified DF1 protein had lipase activity (3.85 U/mg protein), validating that DF1 encodes a lipase.

Here, the df1-1 spikelets produced two fertile florets, suggesting that the spikelet determinacy was lost. In the mfs1, tob1 and snb mutants, and snb/osids1 double mutant, some spikelets developed an additional lemma-like organ, implying that these genes regulate the spikelet meristem determinacy and the timing of the transformation from the spikelet meristem to the floral meristem (Lee and An, 2011; Lee et al., 2006; Ren et al., 2013; Tanaka et al., 2012). Unlike the df1-1 mutant, extra florets of mfs1, tob, snb and snb-osids1 spikelets bore a vestigial secondary floret that only possessed a lemma-like organ, and failed to produce extra seeds. Particularly in the mfs1 mutant, some spikelets also formed two paleae and lemmas, suggesting that these spikelets were prone to produce two florets. In Ehrhartioideae, Ehrharta has one terminal floret and two lateral florets that only contained unReduced lemmas inside a pair of large glumes (Kellogg, 2009; Lin et al., 2014). In contrast, the df1-1 spikelets contained two florets: a terminal floret and a secondary floret, which consisted of four whorls of floral organs and produced two seeds within each spikelet. Moreover, the spikelet meristem is indeterminate and also induces two to six florets in wheat, which results in the formation of more seeds within a single spikelet. These findings revealed that the presence of two or three florets within a single spikelet is possible in the genus *Oryza*.

A series of genes related to panicle branching have been cloned, such as DEP1, GN1a/OsCKX2, IPA1 and NAL1, which mainly improve rice yield by increasing the number of panicle branches and controlling the arrangement of spikelets. However, rice breeders have not focused on the number of florets because the spikelets of *Oryza* produce a fixed number of florets, implying that the spikelet meristem is determinate. Our findings revealed that DF1 plays a key role in the regulation of spikelet determinacy. Inducing a switch to indeterminacy in the spikelet meristem with mutated *DF1* or prolonging the activity of the spikelet meristem with mutated *DF1*, *MFS1*, *TOB* and *SNB* may provide a new means to develop rice cultivars with multiflorets spikelet for increasing grain number per panicle. According to the present study, it is possible to breed a multiflorets spikelet by designing and mining those genes which regulate the determinacy and indeterminacy of the spikelet meristem in rice. In summary, two-/three-floret spikelet as a novel resource has the potential to further increase rice yield.

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**Conflict of interest**

The authors have no conflict of interests.

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