The pleiotropic ABNORMAL FLOWER AND DWARF1 affects plant height, floral development and grain yield in rice

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Abstract Moderate plant height and successful establishment of reproductive organs play pivotal roles in rice grain production. The molecular mechanism that controls the two aspects remains unclear in rice. In the present study, we characterized a rice gene, ABNORMAL FLOWER AND DWARF1 (AFD1) that determined plant height, floral development and grain yield. The afd1 mutant showed variable defects including the dwarfishm, long panicle, low seed setting and reduced grain yield. In addition, abnormal floral organs were also observed in the afd1 mutant including slender and thick hulls, and hull-like lodicules. AFD1 encoded a DUF640 domain protein and was expressed in all tested tissues and organs. Subcellular localization showed AFD1-green fluorescent fusion protein (GFP) was localized in the nucleus. Meantime, our results suggested that AFD1 regulated the expression of cell division and expansion related genes.

INTRODUCTION

Angiosperms produce diverse forms of flowers, but the genetic mechanism directing floral development is fundamentally conserved. Recently, with a growing number of genes involved in floral development have been isolated, floral organ specification is explained by the robust ABCDE model and temporally expression patterns of the five groups of genes and the complicated interaction patterns of their encoded proteins, which determine floral organ identity and patterning (Ohmori et al. 2009; Sang et al. 2012). However, many questions remain unanswered, including the development of floral organs and flower-associated structures distinctive to a group of plant species remaining poorly understood.

The grasses (Poaceae), one of the largest monocot families, include many agronomically and economically important crops including rice (Oryza sativa), maize (Ze a mays), wheat (Triticum aestivum) and barley (Hordeum vulgare), and have floral architectures that are distinct from those of eudicots (Arabidopsis thaliana, Antirrhinum majus and Petunia hybrid). The structural units of grass flowers are spikelets and florets. In rice, the floret has unique floral organs of the lemma, palea and lodicule, as well as the stamen and pistil.

Molecular genetic and morphological studies have revealed that lodicules and stamens are organs homologous to petals and stamens in the eudicots. B and C genes specify the petal/lodicule and the stamen individually, and are both conserved in monocots and dicots (Xiao et al. 2003; Whipple et al. 2004; Yamaguchi et al. 2006; Yadav et al. 2007; Yao et al. 2008; Li et al. 2011a). However, A class genes that control the origin and the mechanism of lemma and palea (the outer whorl floral organs) development have long been controversial and still remain unclear (Schmidt and Ambrose 1998; Ferrario et al. 2006; Wang et al. 2010). Using map-based cloning strategy, many lemma and/or palea defective mutants or quantitative trait loci (QTLs) have been characterized in rice. LEAFY HULL STERELE1 (LHs1/OsMADS1), DEPRESSED PALEA1/PALEALESS1 (Dp1/pa1), RETARDED PALEA1 (Rep1), DEGENERATIVE PALEA1 (Dep1/OsMADS15), GRAIN WIDTH2 (Gw2), GRAIN WIDTH5 (Gw5), GRAIN WIDTH8 (Gw8), and grain length QTL (qGL3) have been cloned to control the hull (lemma and palea) development and grain size in rice (Prasad et al. 2001; Luo et al. 2005; Song et al. 2007; Shomura et al. 2008; Guo et al. 2008; Yuan et al. 2009; Wang et al. 2010; Jin et al. 2011; Zhang et al. 2012; Qi et al. 2012; Wang et al. 2012). Nevertheless, our understanding of the hull development is not sufficient and more work needs to...
be done to fully reveal the molecular mechanism of floral development and grain traits in rice.

Plant height plays important roles in determining the ultimate production and economic value. Dwarfism is one of the most important agronomic traits in crop breeding programs because moderate dwarf (semi-dwarf) cultivars are more resistant to lodging and increase the grain yield of cereal crops (Sasaki et al. 2002). To date, numerous dwarf mutants have been reported and characterized such as dwarf mutant1 (d1), dwarf mutant10 (d10), dwarf mutant14 (d14), dwarf mutant27 (d27), dwarf mutant50 (d50), dwarf and low tillering (dlt), high tillering dwarf1 (hdt1) and so on. These dwarf mutants present pleiotropic phenotypes including small grain, variable tillers and deformed leaf as well as plant height, which are related to biosynthesis or signaling pathways of phytohormones, such as gibberellin (GA) and brassinosteroid (BR) (Yamamuro et al. 2000; Sasaki et al. 2002; Hong et al. 2003, 2005; Fujikura and Yokota 2003; Itoh et al. 2004; Sakamoto et al. 2004; Tanabe et al. 2005; Nakamura et al. 2006). However, it is reported that some dwarf mutants are insensitive to BR and GA, and exhibit varied phenotypes that are distinct from those of GA and BR-defective mutants. Brittle Culm 12 (BC12) encodes a kinesin-4 protein and controls cell cycle progression and wall properties in rice (Zhang et al. 2010; Li et al. 2011b). Super Apical Dormant1 (SAD1) and Dwarf and Deformed Flower1 (DDF1) encodes a RNA polymerase I and an F-box protein, respectively (Duan et al. 2012; Li et al. 2015). The sad1 and ddf1 mutants display a variety of defects, including dwarfism, poor root growth, narrow leaf, and abnormal floral organs, suggesting that SAD1 and DDF1 play critical roles in vegetative and reproductive development in rice.

In the present study, we characterized a recessive mutant designated as abnormal flower and dwarf1 (afd1) and AFD1 encoded a DUF640 domain protein. The afd1 mutant exhibited variable characteristics including the dwarfism, long panicles, low seed setting, small grains, slender and thick hulls, and elongated lodicules. These results suggest that AFD1 plays an important role in the regulation of size and identity of organs and grain yield. Our findings also reveal that AFD1 regulates the expression of cell proliferation and expansion related genes. This work would facilitate understanding of the molecular mechanism of vegetative and reproductive development, and provide insight into the functions of AFD1 gene.

RESULTS

Dwarfism phenotype in the afd1 mutant

The afd1 mutant was discovered from ethylmethane sulfonate (EMS)-treated Zhong Hua (ZH11). The afd1 plant was much shorter than the wild type and the difference was obvious in the whole life cycle. Compared with the wild type, plant height of the afd1 mutant reduced by 27%, 20% and 14% at the seedling stage, tillering stage and maturation stage, respectively. (Figures 1A–D, S1). When rice plants reach maturity, the 2nd, 3rd, 4th and 5th internodes showed no differences, but the 1st

Figure 1. Phenotypes characterization in the wild type and afd1 mutant

(A–C) Wild type and afd1 mutant plants at seedling stage, tillering stage and heading stage. (D, E) Internode length of the main culm in the wild type and afd1 mutant. (F) Longitudinal sections of the 1st internode in the wild type and afd1 mutant. wt, wild type. Bars = 10 cm in (A) and (D), 20 cm in (B), 30 cm in (C), and 100 μm in (F).
internode was distinctly shorter in the *afd1* mutant (Figure 1D, E). To reveal the cause of dwarfism phenotype in the *afd1* mutant, we performed histocytological analysis on the internal structure of internodes by paraffin section. The result showed that cell sizes were significantly reduced in *afd1* internodes, suggesting that the cell size was disturbed in the *afd1* mutant (Figure 1F). Meantime, GA treatment exhibited that no differences were observed in the shoot elongation and the endosperm a-amylase activity between the wild type and *afd1* mutant, and BR treatment showed similar growth tendency in roots, coleoptiles and second leaf sheath between the wild type and *afd1* mutant (data not showed), indicating that the dwarfism phenotype of *afd1* mutant was not dependent on GA and BR biosynthesis pathway and signal transduction.

**Floral development defects in the *afd1* mutant**

During heading stage, significant abnormalities were observed in the *afd1* mutant. The panicles (inflorescences) of *afd1* mutant were 11% longer than those of wild type but the primary branches, secondary branches and spikelets number were not changed (Figures 2A, S3). At anthesis, the hull (i.e. lemma and palea) of *afd1* flowers seemed not open and the stamens were enclosed in the lemma and palea (Figure 2B). Meantime, a conspicuous defect of lemma and palea was observed (Figure 2B). We also investigated the defects of the wild type and the *afd1* mutant. Simultaneously, we also investigated the defects of known floral organ development genes using quantitative reverse transcription-PCR (qPCR). **MOSAIC FLORAL ORGANS1** (*MFO1/OsMADS6*) and **CHIMERIC FLORAL ORGANS1** (*CFO1/OsMADS32*) determined the marginal regions of the palea of *afd1* flowers (Figure 3E). Histological analysis of wild type spikelet. (A, B) Panicles of wild type and *afd1* mutant. (C–E) Wild type spikelet. (F) Lodicule in the wild type floret. (G) Histological analysis of wild type lodicule in (G). (I) Epidermal surface of wild type lodicule in (G). (J–L) *afd1* spikelet. (M) Histological analysis of *afd1* spikelet. (N) Lodicule in the *afd1* floret. (O) Histological analysis of *afd1* lodicule in (N). (P) Epidermal surface of *afd1* lodicule in (N). wt, wild type; le, lemma; pa, palea; lo, lodicule; st, stamen; pi, pistil; bop, body of palea; mrp, marginal region of palea; an, awn; elo, epidermal surface of lodicule. Black lines represent epidermal surface of lodicule in (H) and (O). Bars = 5 cm in (A) and (B), 2,000 μm in (C, D, J, K), 1,000 μm in (E) and (L), 500 μm in (G, I, N, P) and 100 μm in (F, H, M, O).

mutated organs in *afd1* florets, we detected the mRNA levels of known floral organ development genes using quantitative reverse transcription-PCR (qPCR). **MOSAIC FLORAL ORGANS1** (*MFO1/OsMADS6*) and **CHIMERIC FLORAL ORGANS1** (*CFO1/OsMADS32*) determined the marginal regions of the palea (mrp) identity, whereas *DP1/PAL1* was involved in regulation of the bop development. In the *afd1* lemma and palea, the expression levels of *MFO1/OsMADS6* and *CFO1/OsMADS32*...
seemed to be close to that of wild type, but DP1/PAL1 expressions were downregulated (Figure 4A). Next, the investigations of organs (lemma, bop and mrp) sizes showed that the lemma and bop were reduced but the mrp was not affected in the afd1 mutant (Figure S2). These results revealed that AFD1 was involved in regulation of the bop and lemma but not the mrp. In view of the afd1 lodicules with rough, crumpled outer epidermis and green features, which were only present in the wild type hulls, we examined the expression levels in A class genes OsMADS14 and OsMADS15, and B class genes OsMADS2, OsMADS4 and OsMADS16, and E class gene OsMADS1. In afd1 lodicules, OsMADS1, OsMADS14 and OsMADS15 transcripts were expressed but not in wild type lodicules (Figure 4B); OsMADS2, OsMADS4 and OsMADS16 mRNA levels were downregulated (Figure 4B). Taken together, we concluded that the lodicule was prone to transformation of the lodicule into the hull and has acquired the hull identity in the afd1 mutant, and that the hull-like lodicules and the thickened hulls confined the stamens in the space bounded by the hulls and for better pollination. Additionally, 9% of afd1 florets generated awns at the top of the lemma and the epidermal cells of awns had lots of trichomes, and no awns were formed in the florets of the wild type (Figures S3, S4A).

afd1 mutant affects grain yield
We found that the grain shape and size of afd1 mutant were significantly different from those of wild type (Figure 5A–C, G–I). The grain and brown grain in size, 1000-grain weight and weight of 1000 brown grain were markedly decreased in the afd1 mutant (Figure S5). To determine whether the small grains were caused by the deformed hulls, we removed parts of the hulls in the wild type and afd1 spikelets before pollination (Figure 5D, J). Interestingly, no differences were observed in the brown grain size and weight between the wild type and afd1 mutant (Figures 5E, K, S5). Together with the thicker afd1 hulls, the results indicated that the abnormal hulls limited the brown grain size and weight in the afd1 mutant. The information perhaps suggests the reason that the afd1 hulls were open at maturation stage. Additionally, the lower setting percentage (i.e. low seed setting) was also observed in the afd1 mutant (Figures 5A, G, S5). Compared with the wild type, the setting percentage in the afd1 mutant and afd1 mutant with removed upper parts of all abnormal hulls reached about 65% and 74%, respectively (Figure S5). Therefore, we focused on the fertility of stamens. Under normal conditions, pollen viability of the wild type stamens was 98%, and the spikelet fertility (i.e. the setting percentage) was 90% (Figure S5). The pollen viability was 82% in the afd1 mutant (Figure 5F, L). To further find out the low spikelet fertility, we hand-pollinated the afd1 mutant with the wild type pollens when the upper parts of all abnormal hulls were removed, and the spikelet fertility reached 88% (Figure S5). These results suggested that the low setting percentage in the afd1 mutant was due to both inefficient pollination (i.e. the thicker hulls limited the pollination in the afd1 mutant) and defective pollen grains.

Figure 3. Hull thickness in the wild type and afd1 mutant (A, D) Freehand section analysis in the wild type and afd1 mutant, respectively. (B, E) Paraffin section analysis in the wild type and afd1 mutant, respectively. (C, F) SEM analysis in the wild type and afd1 mutant, respectively. (G) Hull (lemma and palea) thickness. Le, lemma; Pa, palea. Bars = 250 μm in (A) and (D), 100 μm in (B) and (E), and 500 μm in (C) and (F). Error bars indicate SD.

Figure 4. Relative expression levels of floral organ identity genes in the wild type and afd1 floral organs le, lemma; pa, palea; lo, lodicule. Error bars indicate SD.
Early development of floral organs in the \textit{afd1} mutant

To further analyze the developmental defects in the \textit{afd1} mutant, we investigated young florets in the wild type and \textit{afd1} mutant at different developmental stages by SEM. At the spikelet 4 stage (Sp4), lemma and palea primordia in the wild type flower started to develop and the lemma was larger than the palea (Figure 6A). During Sp5 and Sp6, six spherical stamen primordia were observed in the wild type flower and the development of the stamen primordium on the lemma side was retarded (Figure 6B). During the Sp7 stage and Sp8, the lemma and palea were enlarged, and the carpel primordium was formed in the wild type (Figure 6C, D). No significant differences were observed at the Sp4-Sp7 stages (Figure 6E–G). However, the \textit{afd1} lemma and palea differed significantly from that of the wild type and the \textit{afd1} florets showed the slender lemma and palea than that of the wild type at the Sp8 (Figure 6H). Meantime, the awn was observed at the top of the \textit{afd1} lemma primordium but no awn was found in the wild type (Figure 6D, H). These results suggested that AFD1 affected the enlargement of the hulls and restrained the formation of the awns at later stages, but seemed not to influence floral organ initiation.
Isolation of the AFD1 gene

To isolate the gene responsible for the afd1 mutant, we carried out a cross between afd1 mutant and Nan Jing 6 (NJ6). All F1 plants displayed the wild type phenotype. Among the randomly selected 242 plants from the F2 population, 190 and 52 plants showed the normal or afd1 mutant phenotypes, respectively. The segregation rate fitted the ratio of 3:1, suggesting that the mutational phenotypes were controlled by a single recessive nuclear gene. Among the 215 simple sequence repeat markers (SSR) markers distributed evenly on 12 chromosomes, 130 markers were polymorphic between the two parental lines. Then the polymorphic markers were analyzed within both the wild type and afd1 mutation DNA pools, and the results displayed that the AFD1 was mapped to the long arm of chromosome 2, flanked by the RM3774 and RM6307 (Figure 7A). In order to further access the locus, 52 pairs of markers were developed and five pairs of the markers exhibited the polymorphism. Using all 869 F2 recessive individuals, AFD1 was finally narrowed down within a 65 kb region between two insertion or deletion (ID) markers ID21 and ID42 in the BAC clone AP004081 (Figure 7A). Fortunately, a gene that encoded a DUF640 domain transcription factor with a single nucleotide T deletion in this region resulted in coding frame shift (Figure 7A, B). This gene has been reported by Li et al. (2012). To test whether the mutation was causally linked to the mutant phenotypes, the LOC_Os02g56610 wild type genomic fragment that contained the coding sequence, 2,272 base pair (bp) of sequence upstream of the start codon, and 1,478 bp of sequence downstream of the stop codon was transformed into the afd1 mutant. As a result of this, all mutant phenotypes were rescued in the afd1 mutant (Figure 8). Taken together, these results confirmed that LOC_Os02g56610 is the AFD1 gene.

Figure 7. Isolation of the AFD1 gene

(A) Map position of the AFD1 locus. The relative position of the BAC clone is showed. Genomic structure of AFD1. The site of the mutation in the afd1 mutant is showed. (B) AFD1 gene encodes a 248 amino acid expression protein, while afd1 encodes a 320 amino acid expression protein due to frame shift.
AFD1 regulates vegetative and reproductive development

AFD1 is responsible for plant height and grain size in rice

Dwarfism can be attributed to reduced cell size and cell elongation in rice. For instance, cell numbers and/or cell sizes were changed dramatically in dwarf mutant88 (d88), dwarf mutant50 (d50), high tillering, reduced height, and infertile spikelets (this) and ddf1 (Gao et al. 2009; Sato-Izawa et al. 2012; Duan et al. 2012; Liu et al. 2013). In the afd1 mutant, the internode was shorter and the cell size was reduced. The qPCR analysis showed that the expression levels of cell proliferation and expansion related genes were upregulated. These results indicated that AFD1 was involved in regulation of cell numbers and sizes. Currently, a large body of evidence exhibited that lots of genes underlying dwarf phenotype were related with GA and BR pathways in rice (Ueguchi-Tanaka et al. 2000; Yamamuro et al. 2000; Hong et al. 2003; Itoh et al. 2004; Lin et al. 2009). d1 and dwarf mutant35 (d35) were involved in GA pathway, and dwarf mutant2 (d2) and d3t were related with BR pathway (Ueguchi-Tanaka et al. 2000; Hong et al. 2003; Itoh et al. 2004; Tong et al. 2009). In this study, no differences were observed in the wild type and afd1 plant by GA and BR treatment, suggesting that the afd1 mutant did not depend on GA and BR biosynthetic pathway and some other mechanisms existed for explanation of dwarfism phenotype in the afd1 mutant.

Grain size is an important determinant of yield in rice. Although several genes that influence rice grain size have been described such as GW5, qGL3, XIAO and SMALL GRAIN1 (SMG1), the molecular mechanisms remain unclear in rice (Qi et al. 2012; Wang et al. 2012; Zhang et al. 2012; Duan et al. 2014). In the study, the afd1 mutant displayed the slender and thicker hulls. Further paraffin sections and SEM analysis revealed that the abnormal hulls were resulted from smaller cells, and more cell layers and numbers. The qPCR tests showed that AFD1 regulated the expression of cell proliferation and expansion related genes. Meantime, the afd1 brown grain size and weight were closed to those of the wild type when parts of hulls were removed in the afd1 mutant, so we determined that the hull size and thickness controlled the brown grain size and weight by regulations of cell sizes and numbers in afd1 hulls. In addition, our results also showed that the thick hulls and defective pollen grains

Figure 8. Complementation test

(A–F) Complementation of the afd1 mutation by a genomic fragment containing the AFD1 locus. (A–F) all the mutant phenotypes (including dwarf stature, panicle, and deformed floral organs) were recovered. (A) Dwarfism phenotype was recovered in the rescued lines. (B) Longitudinal sections of the 1st internode in the rescued lines. (C–E) Deformed floral organs (lemma, palea and lodicule) were recovered in the rescued lines. (F) Lodicule with smooth epidermal surface in the rescued lines. Bars = 30 cm in (A), 100 μm in (B) and (F), 2 cm in (C), 2,000 μm in (D), and 500 μm in (E).

Expression pattern of AFD1 and protein localization

To determine the expression pattern, we used qPCR to investigate the AFD1 expression in the wild type plants. The qPCR analysis showed that AFD1 transcripts appeared in all examined tissues and organs, including roots, internodes, leaves, panicles at different developmental stages, main rachillae of panicles, lemmas, paleae, lodicules, stamens and pistils (Figure 9A). However, the results revealed that AFD1 exhibited more abundant expressions in internodes, young panicles, lemmas, paleae, and lodicules than those in other tissues or organs examined (Figure 9A), consistent with the phenotypes defects in the afd1 mutant. Next, we examined the subcellular localization of AFD1. Vectors harboring the AFD1-GFP fusion and the single GFP fusion were transiently expressed in rice protoplasts. The fluorescent signals of AFD1-GFP fusion protein were detected exclusively in the nucleus of rice protoplasts (Figure 9B–G). These results suggested that AFD1 encoded a nuclear protein and may act as a transcription factor.

AFD1 affects the expression of cell proliferation and expansion related genes

Histological analysis and SEM tests revealed that AFD1 probably played important roles in plant height, hull thickness and grain size by affecting both cell numbers and cell sizes in rice. Now, several genes have been reported to be involved in regulation of cell proliferation or cell expansion such as xyloglucan endotransglycosylase related genes (XTR), ß-Expansin (EXPA), Ras-like nuclear GTPase (RAN) and Histone (Duan et al. 2012). In the study, we investigated two genes that were mainly related to cell proliferation (Histone H4 and OsRAN2) and related to cell elongation (OsXTR2 and OsEXPA2) in the afd1 mutant by qPCR. In afd1 internodes, main rachillae of panicles and hulls (lemmas and paleae), the expression levels of all four tested genes were upregulated (Figure 10). Meantime, we also detected the expression levels of 31 genes that were involved in regulation of cell cycle and cell expansion in 1st internodes. Among them, the transcript levels of five genes were upregulated including one cell cycle related gene and four cell expansion genes (Figure S6). The results supported the phenotype observations and the notion that AFD1 may influence cell sizes and cell numbers by regulating the expression of cell proliferation and expansion related genes in rice.

DISCUSSION

AFD1 is responsible for plant height and grain size in rice

Dwarfism can be attributed to reduced cell size and cell elongation in rice. For instance, cell numbers and/or cell sizes were changed dramatically in dwarf mutant88 (d88), dwarf mutant50 (d50), high tillering, reduced height, and infertile spikelets (this) and ddf1 (Gao et al. 2009; Sato-Izawa et al. 2012; Duan et al. 2012; Liu et al. 2013). In the afd1 mutant, the internode was shorter and the cell size was reduced. The qPCR analysis showed that the expression levels of cell proliferation and expansion related genes were upregulated. These results indicated that AFD1 was involved in regulation of cell numbers and sizes. Currently, a large body of evidence exhibited that lots of genes underlying dwarf phenotype were related with GA and BR pathways in rice (Ueguchi-Tanaka et al. 2000; Yamamuro et al. 2000; Hong et al. 2003; Itoh et al. 2004; Lin et al. 2009). d1 and dwarf mutant35 (d35) were involved in GA pathway, and dwarf mutant2 (d2) and d3t were related with BR pathway (Ueguchi-Tanaka et al. 2000; Hong et al. 2003; Itoh et al. 2004; Tong et al. 2009). In this study, no differences were observed in the wild type and afd1 plant by GA and BR treatment, suggesting that the afd1 mutant did not depend on GA and BR biosynthetic pathway and some other mechanisms existed for explanation of dwarfism phenotype in the afd1 mutant.

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affected the setting percentage and resulted in reduction of grain yield in the \textit{afd1} mutant.

\textbf{AFD1 controls the lemma and palea development in rice}

In grasses flowers, the identities of the highly specific organs, namely the lemma and palea have been widely debated and have still been controversial for a long time. Generally, the palea is considered homologous to the prophyll (the first leaf produced by the axillary meristem) that is formed on a floret axis, whereas the lemma corresponds to the bract (the leaf subtending the axillary meristem) that is formed on a spikelet axis, suggesting the lemma and palea have a different identity and origin. (Ohmori et al. 2009; Sang et al. 2012; Ren et al. 2013). Recently, some evidence has revealed that the rice palea is a congenital fusion organ of the bop and the mrp,
which potentially have distinct origins (Verbeke 1992; Zanis 2007; Ren et al. 2013). Firstly, the lemma and bop showed highly similar cellular structure, which was obviously different from that of the mrp (Prasad et al. 2005; Ohmori et al. 2009; Sang et al. 2012). Secondly, the rice mutant osmads16 and MAD52þ-MADS4 double RNAi plants exhibited transformation of the lodicule into the mrp-like organ but not the bop. Moreover, loss of function of B class genes underwent homeotic transformation of petals (equivalent to lodicules) into sepals (equivalent to lemma or paleae) in Arabidopsis (Nagasawa et al. 2003; Yadav et al. 2007; Yao et al. 2008; Sang et al. 2012; Ren et al. 2013). These results suggested that the mrp, but not the bop, was homologous to the lodicule. Now, a series of defective lemma and palea mutants have been identified in rice. In df1 and multi-floret spikelet1 (mfst) mutants, the bop was lost and retarded, and two mrp-like organs appeared in the original place (Luo et al. 2005; Jin et al. 2011; Ren et al. 2013). Whereas, the mrp was enlarged and acquired the lemma or the bop-like organ identity in cfo1/osmads32 and mfo1/osmads6 mutants (Ohmori et al. 2009; Li et al. 2011b; Sang et al. 2012). In curved chimeric palea/deformed floral organ1 (ccp1/dfoil), the bop was incurved and shrunken and the mrp was converted to an ovary-like structure, but the lemma retained its identity (Zheng et al. 1997; Nardmann et al. 2004; Jin et al. 2011). Therefore, we speculated that an organ consisted of two parts (margin region and central region) originated from different meristematic domains established early during organ initiation in grass.

**AFD1 is required for the normal lodicule in rice**

Generally, the lodicules in grass flowers are regarded as homologous to the eudicot petals, and play very important roles in opening the florets for better pollination. Here, our results showed that the afd1 lodicules had rough epidermis cell and acquired the hull identity in part, suggesting the homeotic transformation of the lodicule to the hull-like organ in the afd1 mutant. Recent studies revealed that MFO1 and CFO1 were expressed in the lodicule and the mutations of these genes resulted in transformation of the lodicule into the hull-like structure (Ohmori et al. 2009; Li et al. 2011b; Sang et al. 2012). However, the lodicule displayed complete hull-like histological identity in mfo1, but the afd1 and cfo1 lodicules only possessed a rough epidermis, and inner cells were different from those of the mfo1. The information indicated that the rice lodicule was controlled by two classes of genes at least. One class genes maintain proper lodicule identity by prevention of the establishment of hull-like identity in the lodicule including AFD1 and CFO1, whereas the others prevented the formation of hull-like lodicules such as MFO1.

**MATERIALS AND METHODS**

**Plant materials**

The rice (*Oryza sativa*) afd1 mutant was identified from ethylmethane sulfonate-treated cultivar ZH11. ZH11 plants were used as the wild type strain for phenotypic observation. All plants were transplanted in the experimental farms at China National Rice Research Institute, Hangzhou and in Lingshui, Hainan Province in the rice growing season.

**Isolation of AFD1**

The afd1 mutant was crossed with NJ6, and 869 F2 mutant plants that showed the mutational phenotype were used as a mapping population in the study. SSR markers from publicly available rice databases were used for the initial gene mapping in the Gramene and the Rice Genomic Research Program websites. Next, fine mapping was conducted using IRID primers developed from comparisons of genomic sequences from Nipponbare and 9311 (Guo et al. 2014). The primers used are in Table S1.

**Microscopy observation**

First, a series of panicles were fixed in 50% FAA (including ethanol, formaldehyde, and glacial acetic acid and sterile water) overnight at 4 °C. Second, panicles were dehydrated with ethanol series, infiltrated with xylene series, and embedded in paraffin. The paraffin sections (about 10 µm thickness) were fixed on glass slides, deparaffinized with xylene series and dehydrated with ethanol series. Finally, paraffin sections were stained with 1% safranine and 1% Fast Green, then dehydrated using a series of ethanol, infiltrated using a series of xylene and covered with neutral resins. Light microscopy analysis was conducted using a NIKON ECLIPSE 90i microscope. Fresh materials were observed using a HITACHI S-3500 scanning electron microscope with a ~45 °C cool stage for electron microscopy analysis (Ren et al. 2013).

**RNA extraction and expression analysis**

RNA from roots, internodes, leaves, inflorescences at different developmental stages, main rachillae of panicles, and floral organs (lemmas, paleae, lodicules, stamens and pistils) was isolated using trizol reagent (Invitrogen). The first strand of cDNA was synthesized from 2 mg of total RNA using oligo (dT) 18 primers in a 20 µL reaction volume using the ReverTra Ace quantitative PCR RT Master Mix Kit with gDNA remover (Toyobo). The qPCR test was performed with an ABI Prism 7000 Sequence Detection System and the SYBR Green PCR Master Mix kit (Applied Biosystems). At least three biological repeats were carried out, and mean values were used for the expression of each gene.

**Vector construction**

In the study, a 4,497 bp genomic fragment from the wild type which encompassed the AFD1 coding sequence, 2,272 bp upstream and 1,478 bp downstream sequences, was amplified using the primers AFD1com-F and AFD1com-R for the complementation test. PCR products were digested with HindIII and EcoRI and inserted into the vector pCAMBIA1301 (pcAI1301). The recombinant pcAI1301 plasmid was introduced into the afd1 mutant using the Agrobacterium tumefaciens-mediated transformation method. The primers used are in Table S1.
Subcellular localization

The CDS (Coding Sequence) of AFD1 except the stop codon was amplified using the primers AFD1OE-F and AFD1OE-R, which contains HindIII and EcoRI sites, respectively. The target fragment was inserted into the modified expression vector 35S-GFP (S65T)-NOS (pCA1301) to generate the AFD1- GFP fusion vector (Ren et al. 2015). At last, the plasmids of GFP and AFD1-GFP were transformed into rice protoplasts and transiently expressed in rice protoplasts. After 10 h of incubation at 28 °C, GFP signals were detected using OLYMPUS IX71 confocal microscope. The pr (Table S1).

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AUTHOR CONTRIBUTIONS

D.R. and Y.R. performed most of the research. Q.Q. and D.Z. drafted the manuscript. L.W., J.H., L.Z., Z.G., G.D., G.Z. and L.G. performed gene expression analysis. D.R., Z.L., H.Y., Y.Z. and Y.L. performed phenotype investigations. D.R., Y.R., L.W., H.Y., Q.X. and Z.L. carried out vector construction and subcellular localization. D.R., Q.X. and Z.L. carried out microscopy observation. D.R. and Q.Q. revised the manuscript. Q.Q. and D.Z. designed the experiment, supervised the study, and revised the manuscript.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of this article at the publisher’s web-site.
Figure S1. Investigation of afd1 plant growth
Figure S2. Investigation of organs size in the wild type and afd1 mutant
Figure S3. The related agronomic characteristics of wild type and afd1 mutant
Figure S4. Epidermal surface of awn and hull
Figure S5. Investigation of related grain characteristics in the wild type and afd1 mutant
Figure S6. Expression levels of cell cycle and cell expansion related genes in 1st internodes
Table S1. Primers used in the study