BMC Plant Biology

Research article

Ds tagging of *BRANCHED FLORETLESS 1* (*BFL1*) that mediates the transition from spikelet to floret meristem in rice (*Oryza sativa* L)

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Published: 23 September 2003

BMC Plant Biology 2003, **3**:6

Received: 23 May 2003

Accepted: 23 September 2003

This article is available from: http://www.biomedcentral.com/1471-2229/3/6

Abstract

Background: The genetics of spikelet formation, a feature unique to grasses such as rice and maize, is yet to be fully understood, although a number of meristem and organ identity mutants have been isolated and investigated in *Arabidopsis* and maize. Using a two-element *Ac*/*Ds* transposon tagging system we have isolated a rice mutant, designated *branched floretless 1* (*bfl1*) which is defective in the transition from spikelet meristem to floret meristem.

Results: The *bfl1* mutant shows normal differentiation of the primary rachis-branches leading to initial spikelet meristem (bract-like structure equivalent to rudimentary glumes) formation but fails to develop empty glumes and florets. Instead, axillary meristems in the bract-like structure produce sequential alternate branching, thus resulting in a coral shaped morphology of the branches in the developing panicle. The *bfl1* mutant harbours a single *Ds* insertion in the upstream region of the *BFL1* gene on chromosome 7 corresponding to PAC clone P0625E02 (GenBank Acc No. AP004570). RT-PCR analyses revealed a drastic reduction of BFL1 transcript levels in the *bfl1* mutant compared to that in the wild-type. In each of the normal panicle-bearing progeny plants, from occasional revertant seeds of the vegetatively-propagated mutant plant, *Ds* was shown to be excised from the *bfl1* locus. *BFL1* contains an EREBP/AP2 domain and is most likely an ortholog of the maize transcription factor gene *BRANCHED SILKLESS1* (*BD1*).

Conclusions: *bfl1* is a *Ds*-tagged rice mutant defective in the transition from spikelet meristem (SM) to floret meristem (FM). *BFL1* is most probably a rice ortholog of the maize ERF (EREBP/AP2) transcription factor gene *BD1*. Based on the similarities in mutant phenotypes *bfl1* is likely to be an allele of the previously reported *frizzy panicle* locus.

Background

The orderly production of meristems with specific fates is crucial for the proper development of plant architecture. The shoot apical meristem (SAM) is the ultimate source of all aerial structures of the plant, including inflorescences and flowers. The identity of a given meristem is defined by the types of structures it produces [21]. The SAM produces leaves in its vegetative developmental stage, but begins to generate an inflorescence meristem after switching to the reproductive developmental stage [23].

The large number of genes involved in this complex process can be classified into three categories, namely flowering time genes, meristem identity genes and organ identity genes [18]. The flowering time genes are those involved in the transition from a vegetative meristem to a reproductive meristem. Many mutations that affect flowering time have been described in *Arabidopsis* [3,6,18,27] and some in rice [12,38]. Once this transition is achieved, the inflorescence meristem (IM) produces intermediate meristems that give rise to floret meristems (FMs) to produce floral organs [21].

A number of meristem and organ identity genes have been investigated in *Arabidopsis* and *Antirrhinum* [28]. Although monocots are expected to have molecular and genetic mechanisms of flowering similar to those of dicots, little is known about the genetics of spikelet formation – a feature unique to grasses such as rice and maize. Many developmental mutants that affect the elaboration of the inflorescence have been isolated in maize [21] but, to date, only a few of the corresponding genes have been cloned. The maize *KNOTTED1* (*KN1*) is one such spikelet meristem (SM) identity gene and encodes a homeodomain-containing protein which acts as a transcription factor [33]. The *kn1* mutant develops fewer branches and spikelet pairs because of defects in inflorescence meristem maintenance [14]. Another maize gene *LIGULELESS2* (*LG2*) has been shown to encode a basic-leucine zipper (bZIP) protein, and the *lg2* mutant plants have reduced long tassel branches [34]. One of the well characterized maize SM identity genes is *INDETERMINATE SPIKELET1* (*IDS1*) that encodes an AP2-like transcription factor, and the mutant gene *ids1* specifies determinate fate by suppressing indeterminate growth within the spikelet meristem [8]. Recently, yet another maize SM identity gene *BRANCHED SILKLESS1* (*BD1*), encoding a transcription factor containing an ERF (EREBP/AP2) domain has been cloned and characterized [9]. BD1 specifies SM identity by repressing indeterminate branch fate within the lateral domain of the SM [9] and plays a crucial role in mediating the transition from spikelet to floret meristem during maize ear development [10]. The *bd1* mutant shows highly branched ears [9,10].

Although more than 20 genes that control panicle morphology have been reported in rice [17], few have been analyzed in detail [15,22] and none has been cloned to date. However, some rice orthologs of floret meristem identity genes of *Arabidopsis* and *Antirrhinum* have been cloned and their expression patterns investigated. For instance, in *Arabidopsis* and *Antirrhinum*, the indeterminate state of the IM is maintained by *TERMINAL FLOWER1* (*TFL1*) and *CENTRORADIALIS* (*CEN*), respectively. Mutations in these genes result in the IM being converted into a terminal flower [7,25]. Over-expression of *RCN1* and *RCN2*, two rice *TFL1/CEN* orthologs, resulted in a delayed transition to the reproductive phase and more branched panicles due to a delayed switch from the branch shoot to FM [23]. The expression pattern of *RFL*, the rice ortholog of the *Antirrhinum FLORICAULA (FLO)* and *Arabidopsis LEAFY* (*LFY*) genes, indicates that it plays a distinctly-different role from that of *LFY* in *Arabidopsis* and *FLO* in *Antirrhinum* [16]. More recently, a *BD1*-like gene has also been cloned from rice but has yet to be functionally characterized [9].

Here we report the isolation of a *Ds*-tagged rice mutant designated *branched floretless 1* (*bfl1*) and identification of the *BRANCHED FLORETLESS 1* (*BFL1)* gene. The *bfl1* mutant showed normal differentiation of the primary rachis-branches leading to initial SM (bract-like structure equivalent to rudimentary glumes) formation but failed to develop empty glumes and florets. Instead, axillary meristems in the rudimentary glumes produced sequential alternate branching resulting in a highly-branched panicle. *BFL1* encodes a transcription factor containing an EREBP/AP2 domain, identical to the rice *BD1*-like gene cloned recently [9]. Genotypic and phenotypic analyses of the *bfl1* mutant and *BFL1* revertants suggest that the BFL1 is essential for the transition from SM to FM in rice.

Results

bfl1 *is a mutant defective in the transition from SM to FM*

Out of 20 progeny of a mutagenic plant B2-8A-2-18 (F_2 generation) derived from a cross between *iAc* and *Ds*G transgenic lines [31], we found a mutant plant (B2-8A-2-18-16) with panicle characteristics similar to a previously reported EMS-induced *frizzy panicle* mutant *fzp* [20] and a γ-ray induced *frizzy panicle* mutant *fzp2* [15]. We have designated this mutant *branched floretless 1* (*bfl1*).

The panicle of a *bfl1* mutant looks like a stick and its rachis-branches never stretch because of tangling (Fig. 1B). Development of the primary rachis-branches appeared to be normal (Fig. 1C). The panicle length of wild-type and the *bfl1* mutant were 15.5 cm and 14.9 cm, respectively and the number of primary rachis-branches of wild-type and the mutant were 6.1 and 6.4, respectively. However, lateral and terminal spikelets of primary and secondary order rachis-branches failed to produce florets. Instead, they continued to produce next-order rachis branches in alternate axis (Fig. 1G, 2E) most likely after initiation of bract-like structures (Fig. 1F, 1G, 2E, 2F). As observed in the scanning electron mirgrographs (SEMs), the bract-like structure in the mutant (Fig. 2F,2G,2H) corresponds to rudimentary glumes of wild-type (Fig. 2B,2C,2D) because they have comparable positions and surface appearances. The mutant showed no difference from the wild-type plant with respect to vegetative growth,

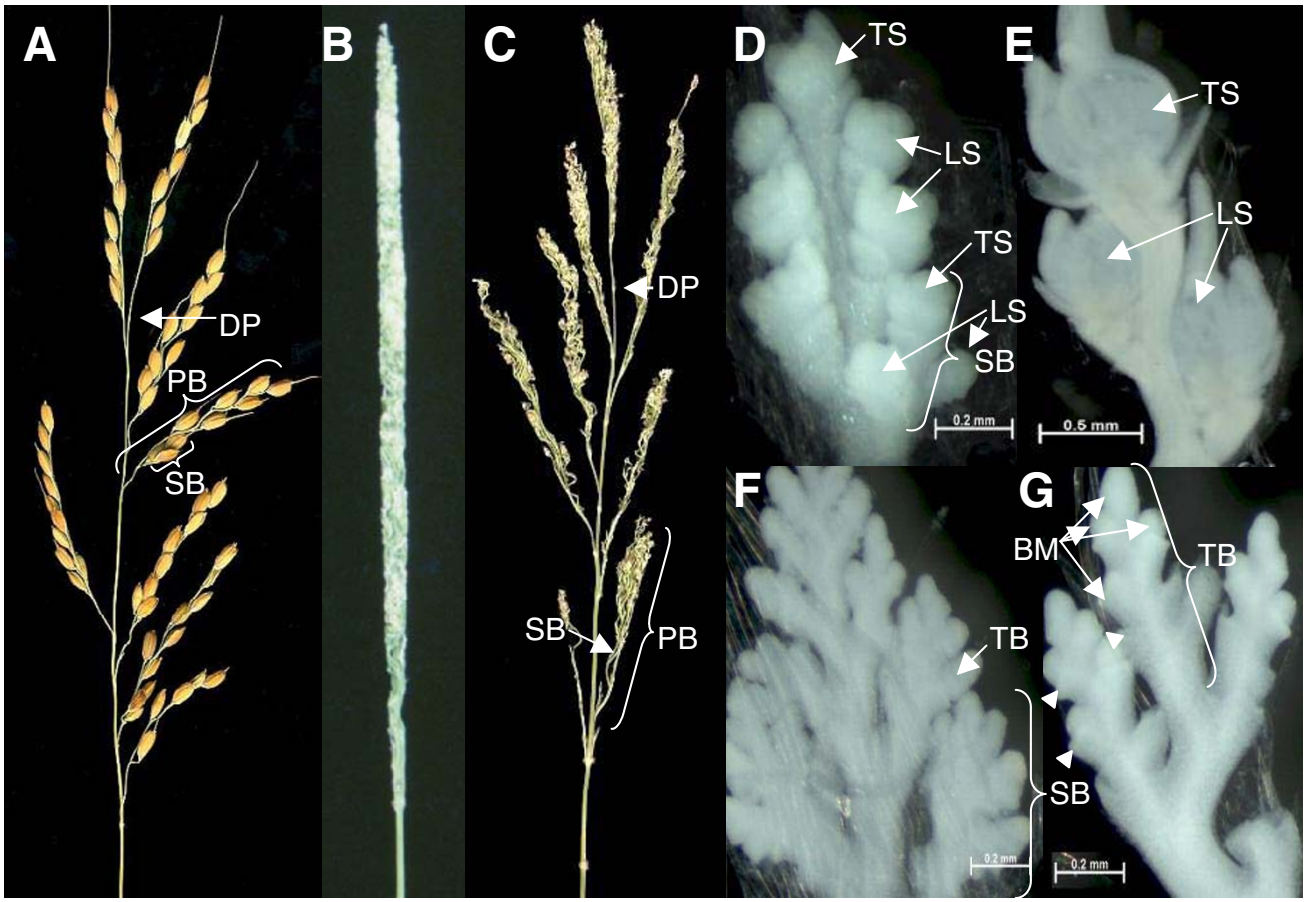

Figure 1
Inflorescence morphology of wild-type and *bfl1* mutant. A, Mature panicle of wild-type. B, Mature panicle of *bfl1* mutant. C, Manually stretched mature panicle of *bfl1* mutant. D, Primary rachis-branch (PB) of wild-type at the stage between spikelet primordium and spikelet organ differentiation, which is composed of secondary rachis-branch (SB), lateral spikelets (LS) and terminal spikelet (TS). E, Secondary rachis-branch of wild-type at microspore developmental stage, which is composed of lateral spikelets and terminal spikelet. F, Primary rachis-branch of *bfl1* mutant at the developmental stage equivalent to D, alternating higher level rachis-branches are continuously developed instead of spikelets. G, Secondary rachis-branch of *bfl1* mutant at the developmental stage equivalent to E. Abbreviations: DP: degenerate point; PB: primary rachis-branch; SB: secondary rachis-branch; LS: lateral spikelet; TS: terminal spikelet; TB: tertiary rachis-branch; BM: branch meristem; RG: rudimentary glume (indicated by triangles).

or to the time of transition from vegetative phase to reproductive phase.

bfl1 *is a* Ds*-tagged mutant*

PCR analyses showed that the original *bfl1*mutant contained both *iAc* and *Ds* (Table 2) and was negative for GFP (the T-DNA or *Ds* launching pad selection marker), indicating that the transposed *Ds* and *iAc* have not segregated but the original *Ds* launching pad has segregated away. As *iAc* was present, excision of *Ds* and possible reversion to wild-type phenotype could be expected. The mutant plant was therefore propagated vegetatively to recover seeds produced after reversion events.

In such vegetatively-propagated plants, several revertant sectors were found within a tiller, or in different tillers (Fig. 3A, 3B). In total, 15 normal mature seeds (Fig. 3E), 11 sterile and abnormal seeds (Fig. 3F, 3G) and 6 incomplete spikelets (Fig. 3H) were obtained. Out of these 15 putative revertant seeds, 10 germinated (PR1 to PR10) and were grown to maturity. PR2, PR5 and PR7 showed normal panicle development and partial sterility (Fig. 3C;

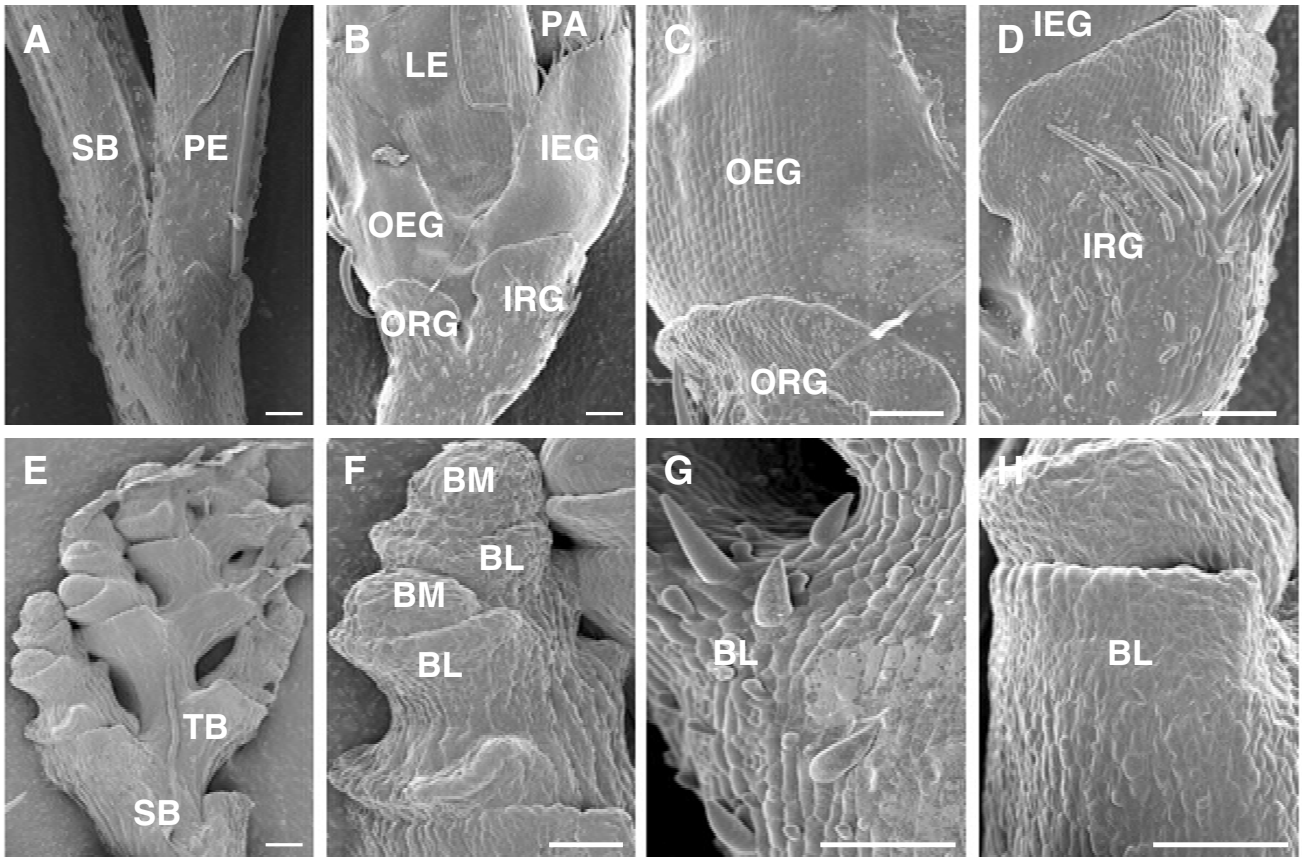

Figure 2

Scanning electron mircrographs (SEMs) of spikelet structures of wild-type and *bfl1* mutant plants. A, Junction of secondary rachis-branch (SB) and pedicel (PE) of spikelet in wild-type. B, A close-up of the basal part of a spikelet showing all the spikelet organs of wild-type. C, A close-up of the surface of outer rudimentary glume (ORG) and outer empty glume (OEG) of a wild-type spikelet. The cells of empty glume are orderly arranged and have a smooth flat surface, whereas the cells of rudimentary glume have irregular shapes and a rugose surface bearing hairy structures. D, Surface of inner rudimentary glume (IRG) of wild-type, having short or long hairs. E, Secondary rachis-branches of *bfl1* and subsequently formed tertiary and higher order rachis-branches which develop continuously in alternating axes. F, Bract-like structure (BL) and rachis-branch meristem (BM) of *bfl1*. Following differentiation BL, BM either arrests its growth or continues to produce new BL. Most likely BL is equivalent to rudimentary glume because of the resemblance of the surface structures. G and H, Close-up view of the surface of BL of *bfl1*. Bars in all diagrams represent 100 μm. Abbreviations: SB: secondary rachis-branch; TB: tertiary rachis-branch; PE: pedicel of spikelet; ORG: outer rudimentary glume; IRG: inner rudimentary glume; OEG: outer empty glume; IEG: inner empty glume; PA: palea; LE: lemma; BL: bract-like structure; BM: branch meristem.

Table 2), whereas the other 7 plants (PR1, PR3, PR4, PR6, PR8, PR9 and PR10) still displayed the *bfl1* phenotype (Fig. 3D; Table 2). *Ds*-specific PCR analysis showed positive results for PR2 to PR10 and a negative result for PR1 (Table 2). The latter was attributed to a possible *Ds3'* end truncation as indicated in the subsequent Southern blot hybridization analyses (see below).

Ds3' (LW1125; GenBank Acc. No. AY343496) and *Ds5'* (LW1455; GenBank Acc. No. AY343495) flanking sequences were rescued from the *bfl1* mutant and PR1 using TAIL-PCR [19] and plasmid rescue system, respectively. Initial searches (February 2002) failed to identify any homologous sequences from GenBank (NCBI) database but the China Rice Genome database http://btn.genomics.org.cn/rice/ contained one entry (contig

Table 1: Primers used in this study.

Primer	Sequence	Target
Ds5_112-	ATCGGTTATACGATAACGGTC	*Ds5'*
Ds3_1	ACCCGACCGGATCGTATCGGT	*Ds3'*
Ds3_3	GTATTTATCCCGTTCGTTTTCGT	*Ds3'*
Ds3_6587+	CCGTCCCGCAAGTTAAATATG	*Ds3'*
AD2	NGTCGA(G/C)(A/T)GANA(A/T)GAA	
GPAInt	TCCAAGTCCACAAGGAAAATTG	*Ds*
GUS_313-	TCACTTCCTGATTATTGACCCAC	*Ds*
Ac_1931+	CAGCTCCAAAGACAAAGACAAC	*Ac*
Ac_2382-	TGCAGCAGCAATAACAGAGTC	*Ac*
LW1125_For	TGTGGAGGAGAAATTAGACAGG	*Ds* insertion flank (P0625E02)
LW1125_Rev	CAGTGTGAAATGTGTAGAAGGG	*Ds* insertion flank (P0625E02)
OsBfl1_For	GCACCAACTTCGTCTACACCCA	*BFL1* (P0625E02)
OsBfl1_Rev2	TGAATGGAGAGTAGGAGTCGGAGC	*BFL1* (P0625E02)
RSs1_F	TGCCTTGATCGAAGCTGAC	*RSs1*
RSs1_R	AGCAAGGGGTAGAGGCTCTC	*RSs1*

239) showing homology to *bfl1* flanking sequences. PCR characterization of the *bfl1* locus and gene prediction were therefore based on contig 239. Subsequently, the *Ds* insertion in the *bfl1* mutant was mapped to rice chromosome 7 corresponding to PAC clone P0625E02 (GenBank Acc No. AP004570).

Southern blot hybridization analyses with the *gus* probe (which hybridizes with the 3' end of the *Ds* insertion) showed that the *bfl1* mutant had a single-copy of *Ds* showing one 7.2 kb positively-hybridizing band (Fig. 4). PR3, PR4, PR6, PR8, PR9 and PR10 also had the same 7.2 kb band and showed mutant panicles, indicating that they were homozygous *Ds* insertion plants resulting from self-pollination of gametes with *Ds* in the *bfl1* locus. PR1, however, produced mutant panicles but did not show any positively-hybridizing band, suggesting a deletion of the 3' region of *Ds* comprising the *gus* gene sequences. PR2 and PR5 did not have the same 7.2 kb band, but had a band of a different size indicating that they were homozygous revertants with *Ds* excised from the *bfl1* locus and re-inserted into other locations in the rice genome. PR7 had one extra positively-hybridizing band besides the 7.2 kb band, indicating that it was a heterozygous revertant resulting from fertilization of a gamete with *Ds* in the *bfl1* locus by a gamete with a re-transposed *Ds*. Thus, normal panicle-development appeared to result from excision of *Ds* from the *bfl1* locus. Based on the sequences of contig 239 and the PAC clone P0625E02, the expected size of the positively-hybridizing band in the Southern blot analyses of *Hin*dIII-digested *bfl1* mutant plant DNA with the*gus* probe is 9.3 kb. However, the observed band size was ~7.2 kb. A close observation of the published sequence of this fragment revealed that a single nucleotide substitution could result in an additional *Hin*dIII restriction site, and a shorter positively-hybridizing band. We tried locating the possible nucleotide substitution in various sources of genomic DNA including a freshly amplified DNA but failed to see any nt transition. Most likely this particular transition varies between different samples of Nipponbare. The position of the *Hin*dIII RE recognition site formed due to possible transition is indicated in Fig. 6. Nevertheless, our genomic sequences flanking both *Ds3* (GenBank Acc. No. AY343496) and *Ds5* (GeneBank Acc. No. AY343495) ends unambigously maps the location of the *Ds* insertion.

Three sets of PCRs were performed to confirm the presence or absence of a *Ds* insertion in the *bfl1* locus: PCR1 to amplify the region flanking the *Ds* insertion; PCR2 and PCR3 to amplify part of the 5' and 3' ends of the *Ds* element and their flanking genomic regions, respectively (Table 1; Fig. 6). PR2 and PR5 were positive for PCR1 and negative for PCR2 and PCR3 confirming that they were homozygous revertants. PR7 was positive for PCR1, PCR2 and PCR3 confirming that it was a heterozygous revertant (Table 2). The genotypes of PR2, PR5 and PR7 were confirmed by subsequent analyses of progeny plants. The PCR results of PR1 confirmed that it had a *Ds3*' truncation (Table 2). All other plants were negative for PCR1 and positive for PCR2 and PCR3, suggesting that there had been no excision of the *Ds* element from the *bfl1* locus.

Taken together, the above analyses strongly suggest that the insertion of the *Ds* is the cause of the *bfl1* mutation and the excision of the *Ds* from the *bfl1* locus is associated with the reversion of the mutation.

It is a well known fact that during *Ds* insertion it creates 8 bp host sequence duplication. However, when the *Ds* is

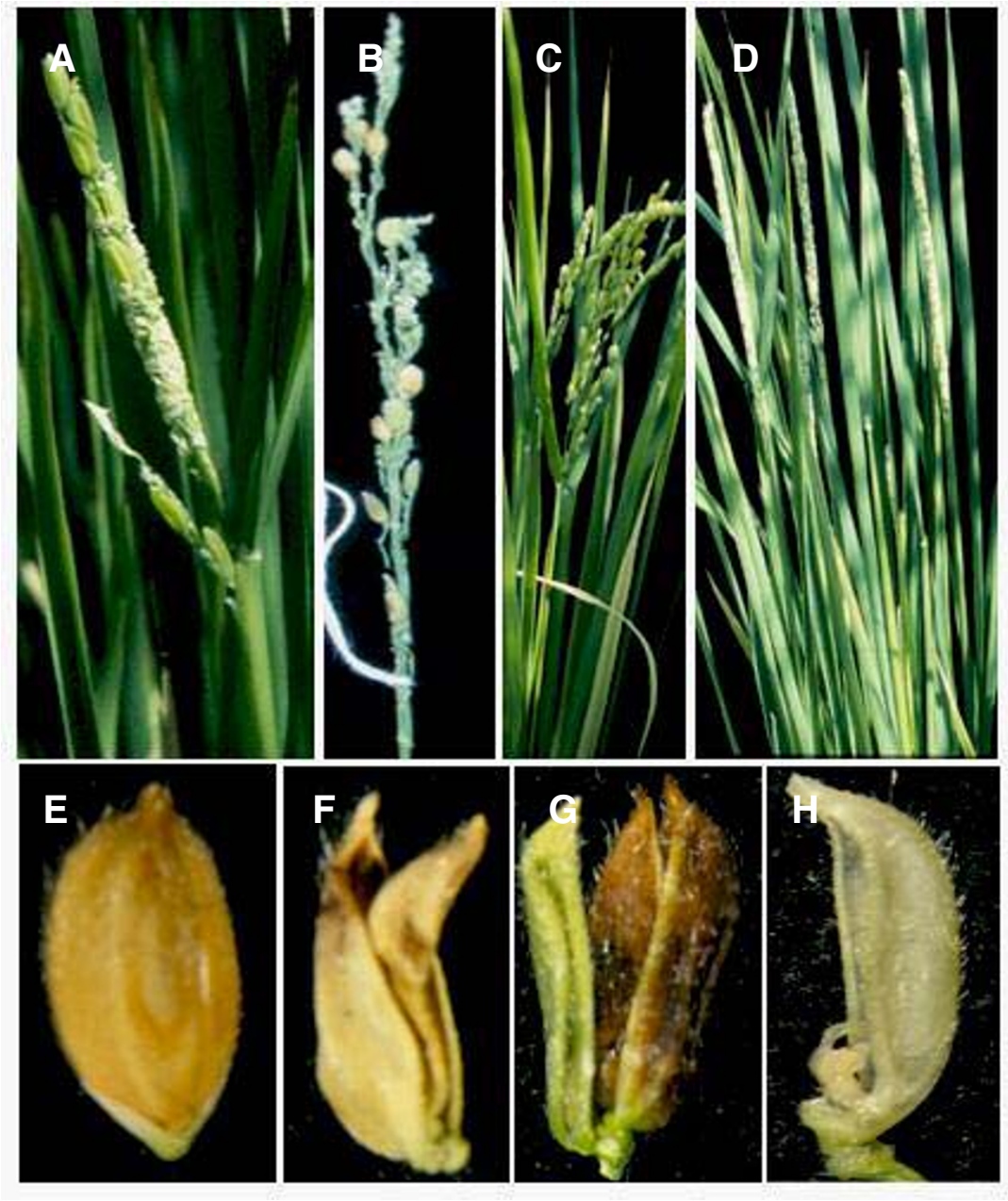

Figure 3
Revertants of *bfl1* mutant. A, Heading panicle with revertant spikelets. B, Mature panicle with revertant seeds. C, Panicles of PR2 showing normal developed spikelets. D, Panicles of PR3 showing *bfl1* mutant phenotype. E, Normal looking revertant seed. F, Abnormal sterile revertant seed. G, Sterile revertant spikelet with an extra sterile spikelet inside. H, Sterile revertant spikelet with just lemma.

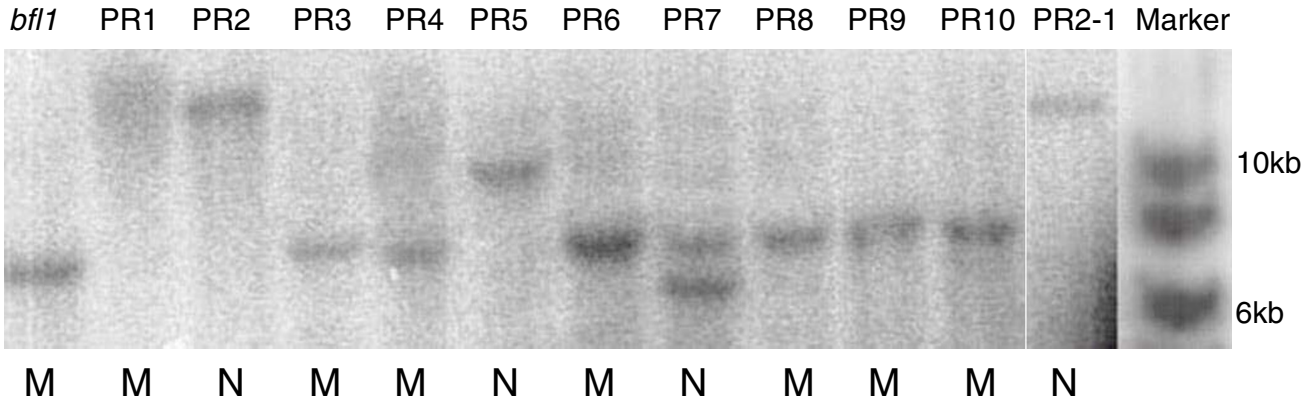

Figure 4
Southern blot hybridization analyses of *Ds* insertion in the *bfl1* mutant and *Ds* excision in revertants. Genomic DNA (~10 μg) was extracted from putative revertants, digested with *Hind*III, fractionated on a 0.7% agarose gel, and blotted to a Hybond-N$^+$ membrane. The membrane was hybridised with a *gus* probe (see Figure 6). PR1 to PR10 are revertants of the original *bfl1* mutant and PR2-1 is one of the progenies of PR2. Abbreviations: N, wild-type phenotype; M, mutant phenotype.

Table 2: PCR characterization of *bfl1* mutant and its putative revertants (PR)

Plant	*Ac* PCR	*Ds* PCR	PCR1[a]	PCR2[a]	PCR3[a]	Genotype of *Ds* insertion	Phenotype
bfl1	+	+	-	+	+	*Ds*/*Ds*	Mutant
PR1	+	-	-	+	-	*Ds*/*Ds*	Mutant
PR2	+	+	+	-	-	+/+	Normal
PR3	+	+	-	+	+	*Ds*/*Ds*	Mutant
PR4	+	+	-	+	+	*Ds*/*Ds*	Mutant
PR5	-	+	+	-	-	+/+	Normal
PR6	+	+	-	+	+	*Ds*/*Ds*	Mutant
PR7	+	+	+	+	+	*Ds*/+	Normal
PR8	+	+	-	+	+	*Ds*/*Ds*	Mutant
PR9	+	+	-	+	+	*Ds*/*Ds*	Mutant
PR10	-	+	-	+	+	*Ds*/*Ds*	Mutant
PR12	ND	+	+	-	-	+/+	Normal
WT	-	-	+	-	-	+/+	Normal

a): PCR1, PCR2 and PCR3 were done with primers LW1125_For and LW1125_Rev, LW1125_Rev and Ds5_112$^-$, and LW1125_For and Ds3_6587$^+$, respectively.

re-excised it often leaves footprints of this direct repeat sequences. *Ds* excision footprints of PR2, PR5, PR7 and PR12 showed retention of 4, 7, 0 and 7 bp of the duplicated host sequence, respectively (Fig. 5). Although the proper reading frame could be restored with the excision of *Ds* in PR7, the reading frame would not be restored for PR2, PR5 and PR12, suggesting that the *Ds* insertion was not in the coding region of the *BFL1* gene, but in its non-coding or regulatory region.

Subsequently, we obtained additional revertants, including a whole normal panicle with 34 seeds. When 26 seeds of this panicle were germinated and grown to maturity, they showed segregation of homozygous un-excised *Ds* (*Ds*/*Ds*), heterozygous *Ds* excision (*Ds*/+) and

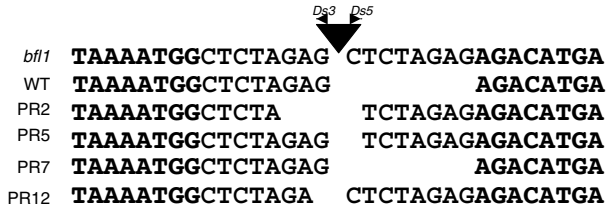

Figure 5
Identification of *Ds* excision footprints. *Ds* excision footprints of PR2, PR5, PR7 and PR12 that showed normal looking panicle. The footprints were amplified using primers LW1125_For and LW1125_Rev, and sequenced with primer LW1125_For (see Table 2 and Fig. 6 for details)

homozygous *Ds* excision (+/+) in the ratio of 1:2:1 (6:13:7). All *Ds* excision plants had the same excision footprint as PR12 (Table 2 and Fig. 5), indicating that there had been germinal excision of *Ds* during the transition from vegetative to reproductive stage.

BFL1 is a transcription factor gene with an EREBP/AP2 domain

Analyses of 10-kb sequence of the ~30-kb contig 239 from the China Rice Genome database harboring the *BFL1* locus by gene prediction programs FGENESH http://www.softberry.com/berry.phtml/ and GENSCAN http://genes.mit.edu/GENSCAN.html identified a single-exon gene capable of encoding a protein with the DNA binding domain of the EREBP/AP2 family of plant transcription factors [26,36], 1515 bp downstream from the *Ds* insertion. Subsequently, the same gene was predicted in the rice PAC clone P0625E02 sequences (Fig. 6). The predicted P0625E02.25 protein corresponds to our predicted protein of 318 amino acids. In view of the function of EREBP/AP2 domain genes, and the excision of *Ds* from the *bfl1* locus resulting in revertants with normally-developed panicles, we concluded that this EREBP/AP2-domain gene is likely to be the *BFL1* gene. Furthermore, the loss or reduction of the BFL1 expression in mutant plants was confirmed by gene-specific RT-PCR using total RNA isolated from leaf and developing panicles (Fig. 7). BFL1 was expressed in wild-type leaves and developing panicles but was greatly reduced in the *bfl1* mutant. The expression of BFL1 in revertant panicles was evident in the RT-PCR analysis (Fig. 7). However, we could not backup the RT-PCR result with Northern blot analysis possibly due to very low expression level.

The EREBP/AP2 domain of *BFL1* was found to be identical to the ERF domain of a recently-isolated maize *BD1* gene and that of a rice *BD1*-like gene [9]. The EREBP/AP2 domain of BFL1 also shared a high level of similarity with those of class II ERFs, such as LEAFY PETIOLE and TINY of *Arabidopsis* [32,37] and, as in LEAFY PETIOLE, the EREBP/AP2 domain of BFL1 was located close to the N-terminus of the protein (Fig. 8).

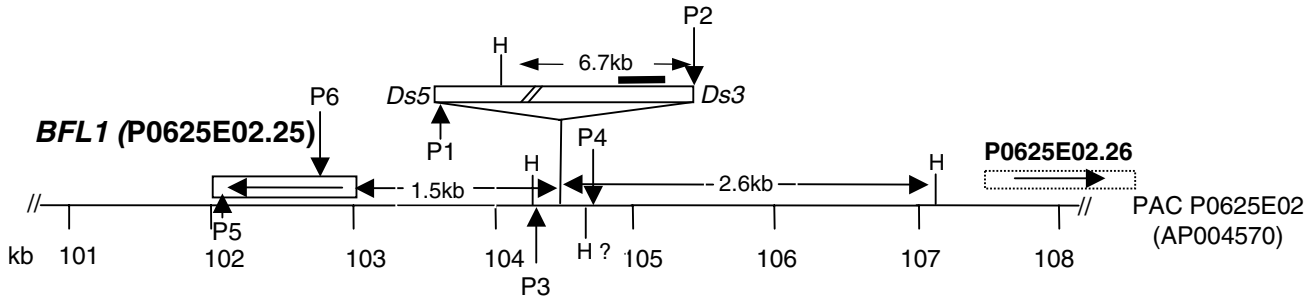

Figure 6
Genomic location of *BFL1* and *Ds* insertion in *bfl1* mutant. Schematic representation of the positions of the *BFL1* gene and the *Ds* insertion in *bfl1* mutant in the Rice genomic PAC clone P0625E02 (GenBank Acc. No.AP004570). The orientation of the *BFL1* gene (P0625E02.25) is indicated by arrow. Binding sites of primers Ds5-112$^-$ (P1), Ds3-6587$^+$ (P2), LW1125_Rev (P3), LW1125_For (P4) used in PCR1, PCR2 and PCR3, and OsBfl1_Rev2 (P5) and OsBfl1_For (P6) used in RT-PCR (see Table 2 for details) are indicated. Solid line represents *gus* probe used for Southern blot hybridization analysis. *Hin*dIII restriction sites are indicated (H).

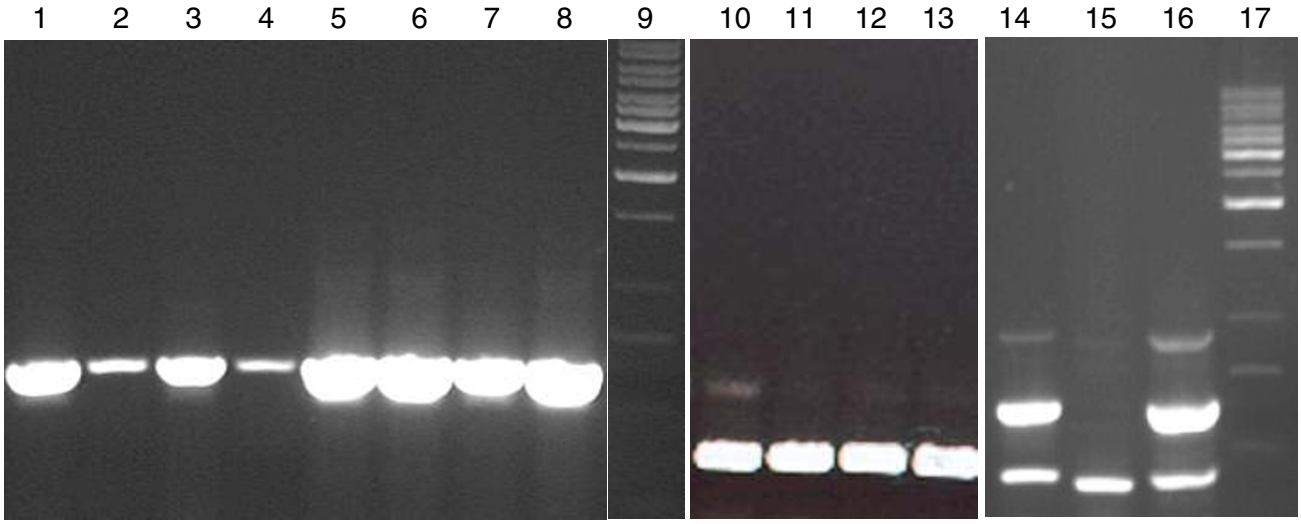

Figure 7
BFL1 transcript analysis by RT-PCR. Semi-quantitative detection of BFL1 transcripts in total RNA samples isolated from wild-type leaf (lane 1) and developing panicle (lane 3) and mutant leaf (lane 2) and developing panicle (lane 4), and same samples without DNase treatment (lanes 5–8) amplified using *BFL1* gene-specific primers. Lanes 10–13 are same samples amplified using rice *RSs1* gene specific primers (binding to two exons flanking an intron) to serve as internal controls for RNA quantity and DNA contamination. Semi-quantitative detection of BFL1 transcripts along with internal control (*RSs1*) in total RNA samples isolated from wild-type (lane 14), *bfl1* mutant (lane 15) and revertant (lane 16) panicles. Lanes 9 and 17 are the molecular wt. markers.

Discussion

BFL1 *mediates the transition from SM to FM during the development of the rice panicle*

In rice, immediately after the transition to the reproductive stage, the SAM switches into an IM, from which primary rachis-branch meristems (PBMs) initiate in a spiral phyllotaxy. The IM degenerates after a given number of PBMs have been initiated. Concurrently, primary rachis-branches differentiate into secondary rachis-branch meristems (SBMs) or lateral spikelet meristems (LSMs) alternatively, and are terminated by terminal spikelet meristems (TSMs). SMs produce two pairs of glumes, rudimentary and empty glumes, as well as a single floret composed of lemma, palea, lodicules, stamens and carpel [29]. Therefore, the rice inflorescence or panicle represents a determinate branched structure composed of a main spike bearing primary and secondary rachis-branches, lateral and terminal spikelets (Fig. 1A). Each rice spikelet contains a single floret, a pair of empty glumes and a pair of rudimentary glumes in contrast to the pair of glumes and two florets present in maize but it is still to be resolved whether empty glumes or rudimentary glumes are analogous to maize glumes [5,13,29].

In the *bfl1* mutant, although the development of the SAM is normal until primary rachis-branches (PBs) are produced, lateral and terminal spikelets are replaced by secondary rachis-branches (SBs) which then continually initiate higher order branches in a distichous phyllotaxy resulting in an indeterminate branched panicle. Based on our observation, the rudimentary glumes of spikelets seemed to be initiated normally as bract-like structures in the *bfl1* mutant (Fig. 1G, 2F), but subsequent SMs ceased development and failed to produce empty glumes and FM, instead, their identity was converted to that of a branch meristem (BM), indicating that SM identity is acquired but fails to transit from SM to FM. Therefore, we propose that the function of BFL1 is to repress BM identity within the spikelet, or to positively regulate the initiation of FM. Similarly, in the maize *bd1* mutant, ear SMs could not transit to FMs and continue to produce glumes and spikelet-like structure [10]. As we believe that the spikelet initiation has occurred in *bfl1* it is logical to assume that the bract-like structure (rudimentary glume) are in fact actual glumes as discussed previously [29].

```
           1                                                    50
     BFL1  MNTRGSGSSS SSSSSQASLM AFSEPPKPA. ...SQPSPPS SPMSERPPSG
      BD1  MNTRACGSSG SGGRNDQTMM GFSEHPKPAA SGQAQPSPPS SP.SERPPAG
    LEAFY  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~MNTTSSKS
   EREBP3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MAVKN KVSNGNLKGG
     ERF4  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~MAKMGLKP DPATTNQTHN
     TINY  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MIASE STKSWEASAV RQENEEEKKK
Consensus  MNTR--GSS- S--------M -FSE-PKPA- S---Q--P-S SPMNE-P-KG
           51                                                   100
     BFL1  RSRRRAQEPG RFLGVRRRPW GRYAAEIRDP TTKERHWLGT FDTAQEAALA
      BD1  RGRRRAQEPG RFLGVRRRPW GRYAAEIRDP TTKERHWLGT FDTAQEAALA
    LEAFY  KKKQDDQVGT RFLGVRRRPW GRYAAEIRDP TTKERHWLGT FDTAEEAALA
   EREBP3  NVKTDGVKEV HYRGVRKRPW GRYAAEIRDP GKKSRVWLGT FDTAEEAAKA
     ERF4  N.....AKEI RYRGVRKRPW GRYAAEIRDP GKKTRVWLGT FDTAEEAARA
     TINY  PVKDSGKHPV .YRGVRKRNW GKWVSEIREP RKKSRIWLGT FPSPEMAARA
Consensus  -VKR--Q-P- R--GVR-RPW GRYAAEIRDP T-KERHWLGT FDTAEEAALA
           101                                                  150
     BFL1  YDRAALSMKG AQARTNFVYT H..AAYNYPP FLAPFHAPQY AAAAAA..PS
      BD1  YDRAALSMKG AQARTNFVYT H..AAYNYPP FLAPFH.... .....H..PS
    LEAFY  YDRAARSMRG TRARTNFVYS DMPPSSSVTS IVSPDDPPPP PPPPAP..PS
   EREBP3  YDTAAREFRG PKAKTNFPS. ........PT E.NQSPSHSS TVESSS..GE
     ERF4  YDTAARDFRG AKAKTNFPTF LELSDQKVPT GFARSPSQSS TLDCAS..PP
     TINY  HDVAALSIKG ASAILNFP.. DLAGSFPRPS SLSPRDIQVA ALKAAHMETS
Consensus  YDRAA-SM-G A-ARTNF-YT ---A-YN-P- FLAP--S-SS -L-AA---PS
           151                                                  200
     BFL1  S..VQYGGGV GAAPHIGSYG HHHHHHHHHG HGAASGASSV GEC.ST...M
      BD1  S..VQQ.QHY GAAPHVGSYG ..HHHYHHQG SAAAVGASS. GECSST...M
    LEAFY  NDPVDYMMMF NQYSSTDSPM LQPHCDQVDS YMFGGSQSSN SYCYSND..S
   EREBP3  NGV....... HAPPHAPLEL DLTRRLGSVA ADGGDNCRRS GEVGYPI..F
     ERF4  TLVVPS.... ATAGNVPPQL EL.....SLG GGGGGSC... ....YQI...
     TINY  QSFSSSSSLT FSSSQSSSSL ESLVSSSATG SEELGEIVEL PSLGSSYDGL
Consensus  --VVQ----- GAAPHV-SYL ELHHH-H--G SG-GG--SS- GECGSTI--M
           201                                                  250
     BFL1  PVMVPVDPHR SSMSS.SLLD MDRNGH.... DFLFSGADDN SGYLSSVVPE
      BD1  ATAVP..PVE RADGT.LLLD RGGGGHHHHP EFLFASADDN SGYLSSVVPE
    LEAFY  SNELPPLPSD LSNSCYSQPQ WTWTGDDYSS EYVHSPMFSR MPPVSDSFPQ
   EREBP3  HQQPTVAVLP NGQPVLLFDS LWRAGVVNRP QPYHVT.... .PMGFNGVNA
     ERF4  .........P MSRPVYFLD. LMGIGNVGRG QPPPVTSAFR SPVVHVATKM
     TINY  TQLGNEFIFS DSADLWPYPP QWSEGDYQMI PASLSQDWDL QGLYNY~~~~
Consensus  -Q-VPV-P-P -S--VY-L-D LW--G-V-RP --L-STADÐ S-Y- SSVVPE
           251                                                  300
     BFL1  SCLRPR.GGG AAADHQDMRR YSDADAYGM. MGLREDVDDL AQMVAGFWGG
      BD1  SCLRPR.SSA AAV..EDLRR YSDADAYGMG VGLREDVDDL AQMVAGFWGG
    LEAFY  G.FNYF.GS~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   EREBP3  GVG.PTVSDS SSAVEENQYD GKRG..IDLD LNLAPPMEF~ ~~~~~~~~~~
     ERF4  ACGAQSDSDS SSVVDFEGGM EKRSQLLDLD LNLPPPSEQA ~~~~~~~~~~
     TINY  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Consensus  -C-RPR-S-S ---V-ED-RR Y--ADAY--D L-LR--V-DL AQMVAGFWGG
           301                                                  343
     BFL1  GDA.ADQLGA CGFPASGGAA D..MVASSQG .SDSYSPFSF LSH
      BD1  GAGDADQLCG GGFP.SGGAG DSMAVASSQG SSDGYSPFSF LSH
    LEAFY  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~
   EREBP3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~
     ERF4  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~
     TINY  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~
Consensus  G---ADQL-- -GFP-SGGA- D---VASSQG -SD-YSPFSF LSH
```

Figure 8

Comparison of EREBP/AP2 domain of BFL1 with that of other ERF transcription factors. Multiple sequence alignment of BFL1, the maize BD1, the tobacco EREBP3, and the *Arabidopsis* LEAFY PETIOLE, TINY and ERF4 proteins using PILEUP program of GCG [11] with gap weight of 8 and gap length weight of 2. Consensus sequence was derived with PRETTY program of GCG with the minimum plurality of 2. EREBP/AP2 (ERF) domain is boxed. Note that BFL1 and BD1 have 80% identity at the predicted amino acid sequence and identical EREBP/AP2 domains.

***BFL1** is a rice ortholog of the maize **BD1** gene*

Public availability of the near-complete rice genome sequences (http://www.ncbi.nlm.nih.gov/;http://rgp.dna.affrc.go.jp/; http://btn.genomics.org.cn/rice; http://portal.tmri.org/rice/) allowed us to locate the single *Ds* insertion in the *bfl1* mutant on rice chromosome 7 corresponding to the PAC clone P0625E02 and indicated that the gene most likely to have been affected by this *Ds* insertion is one which encodes an EREBP/AP2 domain-like protein (P0625E02.25). Since this domain is identical to the ERF domains of the recently reported maize *BD1* gene and the rice *BD1* – like gene [9] we conclude that *BFL1* is the rice ortholog of maize *BD1*.

Although ERF proteins are known as plant-specific transcription factors involved in the ethylene response [24], they are also implicated in plant development [4,32,37]. The direct evidence for the role of ERF proteins in organogenesis comes from the characterization of the maize *BD1*. The *BD1* gene mediates the transition from a spikelet to a floret meristem during maize ear development [9,10] and its expression is temporally and spatially regulated during spikelet and floret initiation [9]. Strong phenotypic resemblance between the maize *bd1* mutant and our rice *bfl1* mutant further supports our claim that *BFL1* is the rice ortholog of *BD1*.

Failure to detect BFL1 transcript by Northern blot analysis was most probably due to its low expression. It is not uncommon to have such a low expression levels of transcription factor genes such as *BFL1*. However, expression studies using RT-PCR under optimal conditions indicated substantial reduction in the BFL1 transcript levels in the *bfl1* mutant compared to wild-type and was restored in the revertant (Fig. 7). Further studies on spatial and developmental expression patterns of BFL1 in wild-type, mutant, and revertant plants are needed to define the regulation of BFL1 expression.

***The* Ds *insertion in* bfl1 *blocks the formation of the transcription initiation complex of the* BFL1**

Based on our prediction, the transcription of *BFL1* starts at 121 nt upstream of the translation start site (TSS). A TATA box and the *Ds* insertion are located 157 and 1515 nt upstream of the TSS, respectively, suggesting that the *Ds* insertion is not in the core promoter region of *BFL1* gene. The observed mutant phenotype and the RT-PCR data, however, suggest that the *Ds* insertion severely affects the expression of the *BFL1* gene, thus suggesting that the region upstream of the core promoter is required for efficient transcription initiation of the *BFL1* gene. The *Ds* insertion in *bfl1* is most likely to block assembly of the transcription initiation complex, which includes not only the core promoter but also proximal and distal enhancer elements. Further investigation into such regulatory elements is required to unravel the transcriptional control of *BFL1*. It is obvious that there are several other genes involved in this complex regulatory pathway and identification of these genes is crucial to understand the three-step transition unique to grasses i.e. branch to spikelet to florets.

Conclusion

Phenotypic, genotypic and expression analyses of a *Ds* tagged mutant and its *Ds*-excised revertants and/or wild-type plants in conjunction with publicly-available rice genome sequences have allowed us to identify a rice gene, *BRANCHED FLORETLESS 1* (*BFL1*), involved in the transition from SM to FM. *BFL1* is most likely an ortholog of the maize transcription factor gene *BD1* and encodes a transcription factor protein containing an EREBP/AP2 domain identical to that of BD1 and its orthologs in other cereals [9]. Because of the phenotypic similarities *bfl1* is most likely to be an allele of the previously reported *fzp* [20] and *fzp2* [15] mutants.

Methods

Plant materials and growth conditions

All rice lines used in this study were derived from the japonica cultivar Nipponbare. The *bfl1* mutant described in this paper was recognised initially among F_3 generation plants derived from a cross between the *iAc* (pSK300, TT3-24-1-1) and *Ds* gene trap (*DsG*, pSK200, TT2-10-1-1) transgenic lines described previously by Upadhyaya et al. (2002). The *iAc* and *DsG* binary vector constructs, tissue culture and *Agrobacterium* transformation procedures used have been described previously [30,31]. Plants were grown under controlled glasshouse conditions with 25 ± 3°C day and 21°C night temperatures with 16 h day length.

DNA extraction and* Ds *flanking sequence rescue

Genomic DNA was extracted from plants using the PureGene nucleic acid isolation kit (Gentra Systems Inc. Minneapolis, MN, USA) according to the manufacturer's instructions. *Ds5'* and *Ds3'* flanking sequences were rescued using the built-in plasmid rescue system [31] and TAIL-PCR [19], respectively. For plasmid rescue, ~2 μg of genomic DNA was digested with *Sac*I, then extracted with phenol/chloroform, precipitated and self-ligated in 500 μl of ligation mix containing 5 Weiss units of T4 DNA ligase at 16°C overnight. The ligated DNA was used for electroporation after ethanol precipitation. Plasmid clones were analysed by appropriate restriction enzyme analyses before being selected for sequencing. TAIL-PCR was performed using three nested primers Ds3_1, Ds3_3, Ds3_6587+ and an arbitrary degenerate primer AD2 (Table 1) according to Liu and others [19] with minor modifications. The purified PCR product was re-amplified using Ds3_6587+ and AD2 to ensure success of the

sequencing reaction. Sequencing was performed with the reagents of the ABI Prism BigDye termination cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Sequence analysis

DNA sequence comparisons were performed using the BLAST program [1,2] searching against the NCBI GenBank http://www.ncbi.nlm.nih.gov/ and China's indica rice sequence database http://btn.genomics.org.cn/rice. The ORF was identified by using FGENESH http://www.softberry.com/berry.phtml/ and GENSCAN http://genes.mit.edu/GENSCAN.html. Alignment of EREBP/AP2 domains was performed using programs of Genetics Computer Group Wisconsin software suit [11].

Southern blot hybridization

For Southern blot hybridization analysis, genomic DNA (~10 μg) was digested with *Hin*dIII, fractionated on a 0.7% agarose gel, and blotted onto a Hybond-N^+ membrane (Amersham Life Science, England) according to the manufacture's instructions. The *gus* gene of the *DsG* construct was used to prepare radioactively-labelled probes with the Megaprime™ DNA labelling system (Amersham Life Science, England) according to the manufacturer's instructions. The membrane was hybridized at 42°C for 6 h, washed at 60°C with 0.1 × SSC and 0.1% SDS and visualized by autoradiography using a phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA).

PCR conditions

PCR primers (Table 1) were designed using the 'prime' program of the Genetics Computer Group (GCG) Wisconsin software suit [11]. *Ds* insertion plants were analysed by *Ac*- (primers Ac_1931$^+$ and Ac_2382$^-$) and *Ds*- (primers GUS_313$^-$ and GPAInt) specific PCR amplification. The following PCR program was used: 30 cycles of 94°C for 2 minutes, 58°C for 30 seconds, and 72°C for 1 minute, followed by a final 72°C for 5 minutes. To identify the presence or absence of *Ds* insertion in the *bfl1* locus, a set of primers annealing to the flanking region of the *Ds* insertion were designed based on the published rice genomic sequences (contig 239 of China Genome database at http://btn.genomics.org.cn/rice. Three sets of PCRs were then performed: PCR1 with primers LW1125_For and LW1125_Rev amplified the region flanking the *Ds* insertion. PCR2 with primers LW1125_Rev and Ds5_112$^-$ and PCR3 with LW1125_For and Ds3_6587 $^+$amplified part of the 5' and 3' ends of the *Ds* element and their flanking genomic regions, respectively (Table 1; Fig. 6). The PCR conditions were 30 cycles of 94°C for 2 minutes, 60°C for 30 seconds, and 70°C for 1 minute, followed by a final 70°C for 5 minutes.

BFL1 transcript levels in RNA samples of mutant and wild-type were visualized by RT-PCR with *BFL1* specific primers (Table 1) using OneStep RT-PCR kit (QIAGEN Inc, California, USA) reagents according to manufacture's instructions. Primers specific to the rice sucrose synthase gene *RSs1* [35] was used as an internal control for RNA integrity and DNA contamination.

Authors' contributions

The mutant was isolated among the screening population which was a combined effort of Plant Industry Rice Functional Genomics group http://www.pi.csiro.au/fgrttpub/members.htm. Most of the plant analyses were performed by QHZ and some by MSH. Sequence analyses were performed by MNU and QHZ. QHZ, MNU and ESD participated in the design, conception and coordination of the study. All authors read and approved the final manuscript.

Note added while this manuscript was under review

The *FRIZZY PANICLE* gene has also been shown to be an ortholog of maize *BD1* in the recently published article "Komatsu M, Chujo A, Nagato Y, Shimamoto K, Kyozuka J (2003) *FRIZZY PANICLE* is required to prevent the formation of axillary meristems and to establish floral meristem identity in rice spikelets. Development, 130(16):3841-50" and hence *bfl1* is an allele of *frizzy panicle*.

Acknowledgements

The authors thank Kerrie Ramm, Ramani Shivakkumar, Celia Miller, Shu-Ting Pan, and Kathryn Smith for their invaluable technical assistance. We also thank Drs John Watson, Ming-Bo Wang and Andrew Eamens for their critical reading and discussions. This work was supported by GrainGene, Rural Industries Research and Development Corporation (RIRDC) and NSW Agricultural Genomics Centre. We also wish to acknowledge Dr Junko Kyozuka for information on *frizzy panicle* mutants.

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