Theor Appl Genet (2012) 125:381–390
DOI 10.1007/s00122-012-1840-8

ORIGINAL PAPER

A novel gene *IBF1* is required for the inhibition of brown pigment deposition in rice hull furrows

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Received: 27 December 2011 / Accepted: 29 February 2012 / Published online: 15 March 2012

Abstract The role of flavonoids as the major red, blue, purple and brown pigments in plants has gained these secondary products a great deal of attention over the years. In this study, we characterized a rice *inhibitor for brown furrows1* (*ibf1*) mutant. In the *ibf1* mutant, brown pigments specifically accumulate in hull furrows during seed maturation and reach a maximum level in dry seeds. Higher amounts of total flavonoids and anthocyanin in hull may be responsible for the brown pigmentation of *ibf1*. The *IBF1* gene, which encodes a similar kelch repeat-containing F-box protein, was isolated by map-based cloning approach. Real-time RT-PCR and GUS activity assays revealed that *IBF1* specifically expressed in reproductive tissues. GFP–IBF1 fusion protein mainly localized in cytoplasm. The expression of some major structural enzymatic genes involved in flavonoids biosynthesis could be up- or down-regulated to some different extent in *ibf1* mutant. Our data suggested that *IBF1* as a suppressor could inhibit the brown pigmentation of rice hull furrows.

Communicated by M. Xu.

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Introduction

The pigments that color most flowers, fruits and seeds are flavonoid secondary metabolites. Flavonoids are synthesized by a branched pathway that yields both colorless compound (e.g., flavonols) and colored pigments (e.g., anthocyanins, the polymeric phlobaphenes and proanthocyanidins) (Koes et al. 2005). Although the most visible function of the flavonoids is the formation of the red and purple anthocyanin pigments, non-pigmented flavonoid compounds also play central roles in the biology of plants, serving as signals for pollinators and other beneficial organisms, participating in plant hormone signaling, facilitating pollen-tube germination, protecting plants from UV-B, and functioning as phytoalexins and allelopathic compounds (Mol et al. 1998). Thus, they have long been used as tractable markers for genetic analysis of a wide variety of biological processes and their evolution, including biochemical pathways, metabolic channeling, intracellular transport, cell differentiation, regulation of gene expression, and the activity of transposable elements (Lepiniec et al. 2006). Flavonoids also have significant activities when ingested by animals, and there is great interest in their potential health benefits, particularly for compounds such as isoflavonoids, which have been linked to the anticancer benefits of soy-based foods, and the stilbenes in red wine that are believed to contribute to reduced heart disease (Winkel-Shirley 2001a). The pathway of flavonoid biosynthesis has been well reviewed by Zhao et al. (2010).

Zea mays, *Antirrhinum majus* and *Petunia hybrida Vilm* were established as the major experimental models to

elucidate the flavonoid biosynthetic pathway. Moreover, the regulation and subcellular organization of flavonoid pathway have also been developed in *Arabidopsis thaliana*, showing the central flavonoid metabolism encoded by a single-copy gene (Winkel-Shirley 2001b). The flavonoid pathway in *Arabidopsis* has been characterized mainly using mutants called *transparent testa* (*tt*), and tannin-deficient seeds are affected in seed coat pigmentation (Kitamura et al. 2010). At least 20 loci have been identified; they correspond to enzymes, regulatory factors, and transports (Baxter et al. 2005; Debeaujon et al. 2003). *Oryza sativa* has become a model plant for monocots, but the molecular features of its flavonoid biosynthesis have not been extensively characterized. Nevertheless, flavonoids with significant biological activities have been described in rice, such as isovitexin in hull, tricin in bran, proanthocyanidins in pericarp of pigmented grains, and anthocyanins in purple-leaf cultivars. In addition, some structural genes and regulators were identified in rice based on sequence homology and reverse genetics (Shih et al. 2008). Recently, the *Rc* (brown pericarp and seed coat) gene has been reported as the first well-characterized gene specially involved in the grain color of rice encoding a bHLH protein, which is a positive regulator of proanthocyanidin biosynthesis (Furukawa et al. 2007; Sweeney et al. 2006). An amino acid transporter protein Bh4 was also identified controlling the black straw of seed hull in wild rice, such as *Oryza rufipogon* and *Oryza nivara* (Zhu et al. 2011).

Rice is widely consumed around the world, and the most common type (>85 %) is white hulled. Rice with colored pericarps has long been consumed worldwide and is considered to be a healthy food (Oki et al. 2002). The rice *inhibitor for brown furrows1* (*ibf1*) mutant which exhibits brown furrows of hull was first described in 1917 and has long been used as a marker gene in rice breeding and genetic study. Previously, the rice *IBF1*, the gene responsible for the *ibf1* mutant phenotype, was fine mapped within a 90-kb region on chromosome 9 (Cui et al. 2007). Here, we report the successful isolation of *IBF1* from another allelic mutant *ibf1*-2 and molecular characterization of IBF1, which is a similar kelch repeat-containing F-box family protein that perturbs the accumulation of total flavonoids and anthocyanin in rice hull through regulating some structural gene expression of flavonoid pathway, thereby affecting the color of rice furrows.

Materials and methods

Plant materials

An *ibf1* mutant was obtained from the *indica* rice, Zhefu 802, and named as *ibf1-1*. The other *ibf* mutant was obtained from a *japonica* rice variety, A7053, which was verified as an allelic mutant of the *ibf1-1* and named as *ibf1-2*.

Complementation test

A 10.3-kb genomic DNA fragment containing the entire *IBF1* coding region, the 5,538-bp upstream sequence and the 3,578-bp downstream sequence was inserted into the binary vector pCAMBIA1300 to generate the transformation plasmid pCIBF1 for complementation test. Empty pCAMBIA1300 was used as a control plasmid. The two binary plasmids were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation and transformed to rice for complementation testing according to a published method (Hiei et al. 1994).

Gene expression analysis

Total RNA was extracted from various tissues using RNeasy Mini Plant Kit (Qiagen). After RNase free DNase (Invitrogen) treatment, 3.5 μg of RNA was reverse transcribed using oligo (dT) primer and M-MLV (Invitrogen). For semiquantitative RT-PCR analysis, 70 ng of cDNA was applied for a 20-μL PCR amplification using the following gene-specific primer pairs: 5′-GACTGGAACTC CATCTTCTG-3′ and 5′-ATCTCGTCGAGGATGAAGA G-3′ for *OsCHS1*, 5′-CAGTACTCGGACAAGGTGAC-3′ and 5′-GGAGTGGGTGAAGAGGAT-3′ for *OsCHI*, 5′-CA CAGAAGCCCAAGTCTCT-3′ and 5′-GATTTTCGACGG AAGATACA-3′ for *OsF3H*, 5′-GATTCATCAACGA AAGGAAG-3′ and 5′-AGTGAATAGGTTCAGGAGCA-3′ for *OsF3′H*, 5′-GATGGATGTACTTCGTGTCC-3′ and 5′-CTGCTTCAGGATCGAGTAGT-3′ for *OsDFR*, 5′-TCT CCTGGGTCGTCTTCT-3′ and 5′-ATCCTTGAGCTTCT TGAACA-3′ for *OsANS1*, 5′-GCCGAGAAGATCATCCT GTT-3′ and 5′-GAACAGCCTCTCGGACATCT-3′ for *IBF1*, and 5′-CAAGATGATCTGCCGCAAATGC-3′ and 5′-TTTAACCAGTCCATGAACCCG-3′ for *OsUBQ*. The PCR samples were collected after 31 cycles for *IBF1*, *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3′H*, *OsDFR*, *OsANS1*, and 25 cycles for *OsUBQ*. The primers of six structural genes in rice flavonoid biosynthesis were designed according to (Shih et al. 2008).

For quantitative real-time RT-PCR, first strand cDNAs were synthesized by reverse transcription from 3 μg of total RNA. The cDNAs were then used as templates in real-time PCRs using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The amplification of the target genes were analyzed using the ABI Prism 7000 Sequence Detection System and Software (PE Applied Biosystems). The relative expression levels of each transcript were obtained by

normalization of the *OsUBQ* gene. The following gene-specific primers were used for real-time PCR analysis: 5′-GCCGAGAAGATCATCCTGTT-3′ and 5′-GAACAGCCTCTCGGACATCT-3′ for *IBF1*, and 5′-CAAGATGATCTGCCGCAAATGC-3′ and 5′-TTTAACCAGTCCATGAACCCG-3′ for *OsUBQ*. Three independent RNA isolations were used for cDNA synthesis, and each cDNA sample was subjected to real-time PCR analysis in triplicate.

For promoter β-glucuronidase (GUS) assay, a 2.6-kb genomic fragment upstream of the *IBF1* gene translation start codon was PCR amplified with the primer 5′-CCTAGGTTCACCTCCTTTTCAATATA-3′ and 5′-ATACCATGGGTCCGTCGTGGCGAGCG-3′, and cloned into the vector pCAMBIA1301. The resulting plasmid was transformed into rice and the resulting transgenic plants were analyzed by GUS staining assay as described (Scarpella et al. 2003).

Subcellular localization of the GFP–IBF1 fusion protein

The *GFP–IBF1* fusion gene was constructed by fusion of *GFP* ORF to the N-terminal end of the *IBF1* ORF (amplified from genomic DNA with the following primers: 5′- AAGCTTATGCAGCACCTGCACGGCGA-3′ and 5′-GGATCCGTGTGCAAGCCGTTCGTCC-3′) and cloned into the *Hin*dIII/*Bam*HI sites of the vector pJIT163. Particle bombardment was performed with a PDS-1000/He particle gun (Bio-Rad) according to the manufacturer's instructions. Five micrograms of plastid precipitated onto 1.0-μm gold beads was delivered into onion (*Allium cepa*) epidermal cells put on ½-MS plates. Twenty-four hours after bombardment, protein expression was observed and images were captured with a Zeiss LSM 510 Meta confocal laser scanning microscope (Jena Germany). GFP fluorescence was excited with a 488-nm argon laser, and images were collected in the 500- to 530-nm range. The expression construct was transfected into the *Arabidopsis* protoplasts mediated by PEG4000 also (Zhou et al. 2009). The transformed protoplasts were examined using a confocal microscope (LSM 510 Meta, Zeiss).

Phylogenetic analysis

Homologous sequences of IBF1 were obtained through NCBI BLAST search. The collected protein sequences were then aligned by ClustalX1.8. The alignment was revised critically and used as input for MEGA3.1 to construct a phylogenetic tree. A neighbor-joining tree was built by MEGA3.1 using Poisson correction model with gaps complete deletion. Topological robustness was assessed by bootstrap analysis with 1,000 replicates (Varbanova et al. 2007).

Analyses of anthocyanin and total flavonoids

Anthocyanin analysis was performed according to Bariola et al. (1999). In brief, mature rice hulls were ground in liquid nitrogen and anthocyanin was extracted with HCl/methanol (1:99, v/v) at 4 °C for 24 h. The extracts were cleared by centrifugation at 12,000×*g* for 30 min. For the supernatant, A_{530} minus A_{657} (both read from a Beckman DU-800 spectrophotometer) was used as a measure of the anthocyanin content. Absorbance values were normalized to the fresh weight of the hull samples.

Total flavonoids were determined as follows. In principle, $AlCl_3$ reacts specially with flavonoids, which have 3-OH or 5-OH in the basic frame and 4′,5′-dihydroxy in the B ring, to a flavonoid–Al complex that has strong absorption at 420 nm. Mature rice hulls were ground in liquid nitrogen and incubated in 80 % methanol for 24 h. The extract was cleared by centrifugation at 12,000×*g* for 30 min. The supernatant was recovered, to which 10 % $AlCl_3$ was added to a final concentration of 1 %. Absorbance values were obtained at 420 nm, which were normalized as above. All sampling and measurement were repeated three times to eliminate systematic error.

Results

Isolation of the *IBF1* gene

In a previous study, we mapped the *IBF1* gene in a 90-kb region of rice chromosome 9 and selected a gene encoding F-box protein (Os09g12150), which is deleted in the original *ibf1-1* mutant, as the candidate gene for the *IBF1* locus (Cui et al. 2007). Another mutant named *ibf1-2* from a *japonica* rice variety, A7053, was verified as an allelic mutant of the *ibf1-1* by cross test. We sequenced the candidate gene in *ibf1-2* and found that three nucleotides (109G, 110C, 409G) deletion compared to the wild type led to a frame shift mutation (39–137aa) (Fig. 1a). Then, we selected this gene to perform a complementation test.

Genetic complementation of *IBF1*

To further clarify whether the above candidate gene AK070984 was indeed responsible for the brown furrows phenotype, we carried out a genetic complementation test by transforming *ibf1-2* with a wild-type genomic clone (pCIBF1) that encompasses the upstream sequences and coding region, followed by downstream sequences (Fig. 1b). Among the 15 independent transgenic lines of pCIBF1 screened through hygromycin resistance, all

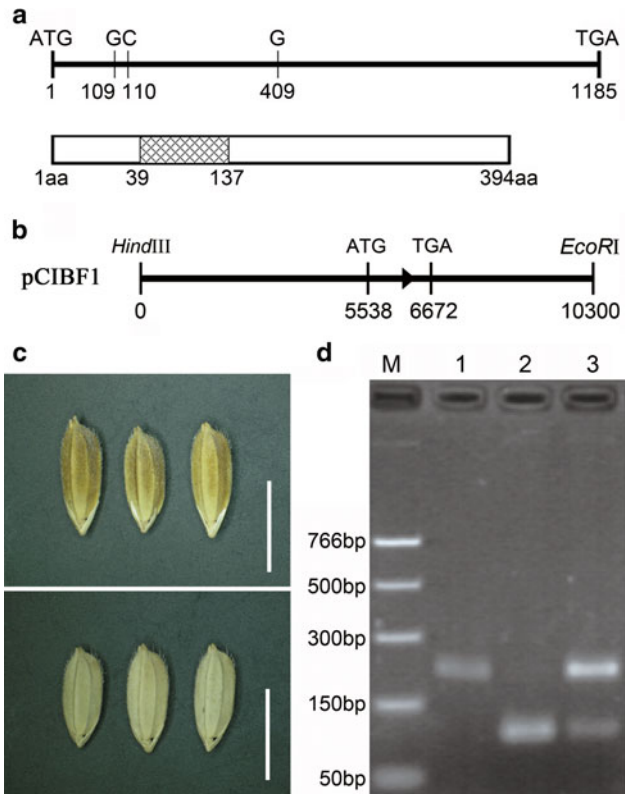

Fig. 1 Identification of the *IBF1* gene. **a** Schematic representation of *IBF1* structure, showing the mutation sites of *ibf1-2*. The start codon (ATG) and the stop codon (TGA) are indicated. *White box* indicates the only exon. Three nucleotides (GCG) deletion in *ibf1-2* led to a frame shift mutation (grid part). **b** Complementation construct. The construct pCIBF1 contains the entire ORF of *IBF1*, plus a 5,538-bp upstream region and 3,578-bp downstream region. **c** The seeds of transgenic lines. The furrows of transgenic plants by empty control vector remained brown (*upper*), whereas the color of rescued furrows by complementation construct was slight *yellow*. **d** Identification of transgenic plants. The GCG deletion lost a *Bss*HII site that is used for identifying the mutant line, the wild-type line and the complementation line. *Lane M* NEB PCR marker, *lane 1 ibf1-2*, *lane 2* wild type, *lane 3* the pCIBF1 transformed rice line. *Bars* 5 mm

furrows completely recovered to a normal color. On the other hand, all five control lines of the empty pCAMBIA1300 failed to rescue the brown furrows mutant phenotype (Fig. 1c). Additionally, the mutation in *ibf1-2* lost a *Bss*HIIsite in the genomic DNA, which can be used as a CAPS marker to determine the *ibf1-2* mutant background in the complementation test (Fig. 1d). Therefore, we conclude that the candidate gene AK070984 controls the brown furrows phenotype in rice.

Morphological characterization of *ibf1*

We evaluated the morphology of *ibf1-1* and compared it with the wild-type plant, Zhefu 802. *ibf1-1* exhibits an obvious slight-brown pigment accumulation in the furrows of glumes after pollination (Fig. 2a). During the desiccation of seeds, furrow color becomes darker and finally turns into typical brown. In contrast, the color of furrows in wild-type rice remains yellow (Fig. 2b).

Analyses of total flavonoids and anthocyanin

To identify whether abnormity of the flavonoid was responsible for the deposition of brown pigments in *ibf1* furrows, we investigated total flavonoids and anthocyanins contents in wild-type and *ibf1-2* seeds, individually. Altogether, 15 independent complementary transformants from *ibf1-2* were obtained and they all showed no difference in morphology, including their furrow color, growth characteristics, and fertility compared to wild-type rice (data not shown). We used these transgenic lines (*ibf1-2*-C) as wild type to perform analyses compared to the *ibf1-2* mutant. The seeds of transgenic plants accumulated lower levels of both total flavonoids and anthocyanin than the mutants and the differences were significant ($P < 0.01$) (Fig. 3).

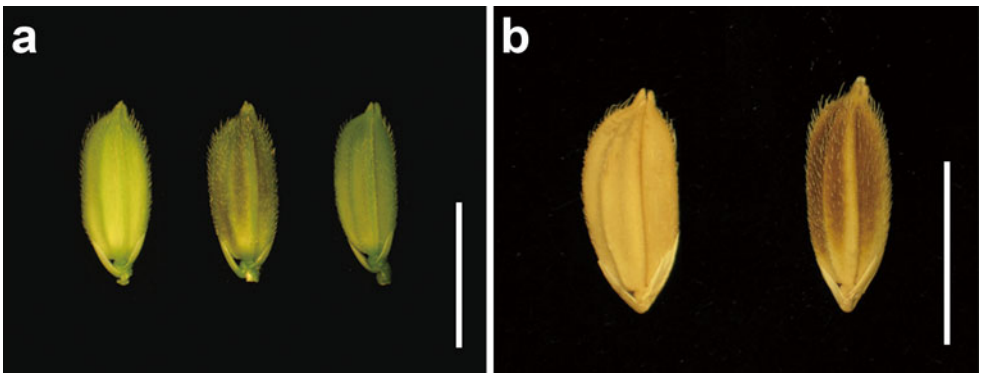

Fig. 2 Phenotype of wild type and the *ibf1* mutant. **a** The spikelet of wild type after pollination (*left*), mutant after pollination (*middle*), and mutant not pollinated (*right*). Only the mutant exhibits pigmentation in the furrows after pollination. **b** The seeds of wild type and *ibf1* at maturation. The furrows in wild type are *yellow* (*left*), while the *ibf1* are *brown*. *Bars* 5 mm

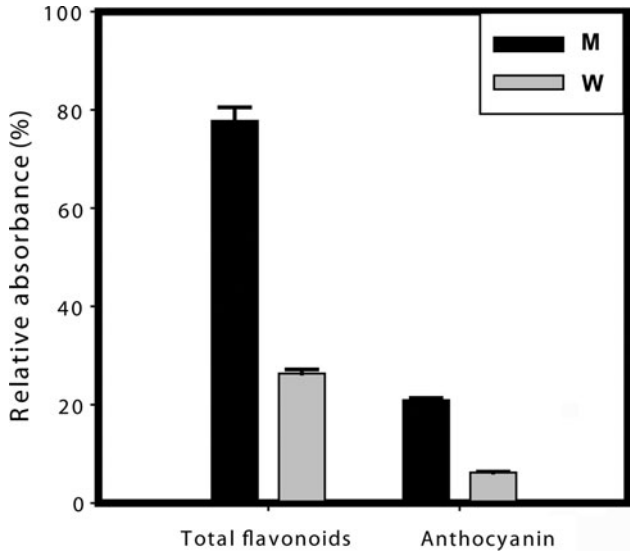

Fig. 3 Total flavonoid and anthocyanin measurement in rice hulls. *M* stands for the *ibf1-2* mutant and *W* stands for control wild type (*ibf1-2*-Complementation). Each value is the mean ± SE ($N = 3$). *Bars* 5 mm

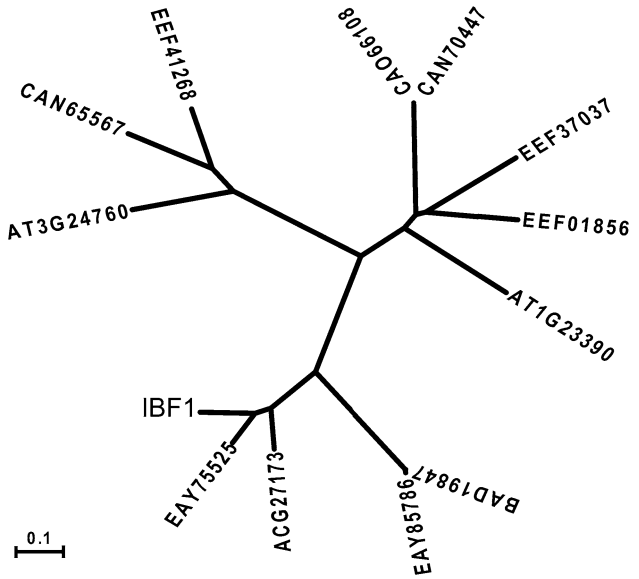

Fig. 4 Sequence characteristics of IBF1 and other homologs. The unrooted phylogenic tree for the deduced amino acid sequence of IBF1-related homologs. The *scale bar* is an indicator of genetic distance based on branch length. EAY75525, hypothetic protein OsI_03429 (*Oryza sativa indica* Group); ACG27173, hypothetic protein (*Zea mays*); EAY85786, hypothetic protein (*Oryza sativa indica* Group); BAD19847, kelch repeat-containing F-box-like protein (*Oryza sativa japonica* Group); AT1G23390, kelch repeat-containing F-box family protein (*Arabidopsis thaliana*); EEF01856, F-box family protein (*Populus trichocarpa*); EEF37037 & EEF41268, conserved hypothetic protein (*Ricinus communis*); CAN70447, hypothetic protein (*Vitis vinifera*); CAO66108, unnamed protein product (*Vitis vinifera*); CAN65567, hypothetic protein (*Vitis vinifera*); AT3G24760, F-box family protein (*Arabidopsis thaliana*)

Phylogenetic analysis

The *IBF1* cDNA was sequenced and aligned to the genomic fragments. The *IBF1* gene has only one exon and no intron (Fig. 1a). The 1,379-bp *IBF1* cDNA (AK070984) contained a longest open reading frame coding a 394-amino acid protein with a predicted molecular mass of 41.5 kDa and a calculated pl of 6.61. Sequence similarity searches using the BLAST algorithm on the National Center for Biotechnology Information Web site showed that IBF1 belonged to F-box family proteins. The best global similarities were from 12 proteins of unknown functions, and EAY75525 gave the highest score (72 % identity and 77 % similarity on a 385-amino acid stretch covering the IBF1 sequence). ACG27173 exhibits 65 % identity and 73 % similarity on a 387-amino acid stretch with IBF1. All of the proteins similar to IBF1 ranged in length from 381 to 450 residues, with the exception of the grape protein CAN70447, which had 541 amino acids. These IBF1-related sequences were all expressed tags from plant species including *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana*, *Vitis vinifera*, *Ricinus communis,* and *Populus trichocarpa*. Phylogenetic analysis indicated that the similarity between IBF1 and its grass family homologs was higher than that of the other family orthologs. An unrooted tree was built using the neighbor-joining method based on full-length protein sequences (Fig. 4). The result indicated that a low level of amino acid similarity spanning the whole protein sequence existed among the IBF1-like homologs, suggesting that these proteins were evolutionarily specified.

An alignment of the sequence of IBF1 with four closely related protein sequences is shown in Fig. 5. All five proteins contain two motifs that suggest modes of function. An about 40-amino acid F-box domain is located near the N-terminus of each protein and the central region of these proteins consist of three kelch repeats, which were originally identified as tandem repeats of ~50 amino acids in the *Drosophila* kelch protein (Xue and Cooley 1993).

Expression pattern of *IBF1*

The expression was investigated in various vegetative and reproductive tissues by quantitative RT-PCR. As shown in Fig. 6, the *IBF1* mRNA was detected only in reproductive tissues, from rapid elongation inflorescences to glumes of mature seeds throughout spikelet development and seed desiccation, with a peak at 1–3 DAF. These results showing that the expression of *IBF1* was restricted to reproductive organs were consistent with those reported by Cui et al. (2007).

Gene expression pattern of *IBF1* was also characterized by histochemical GUS assays of *IBF1*::*GUS* transgenic

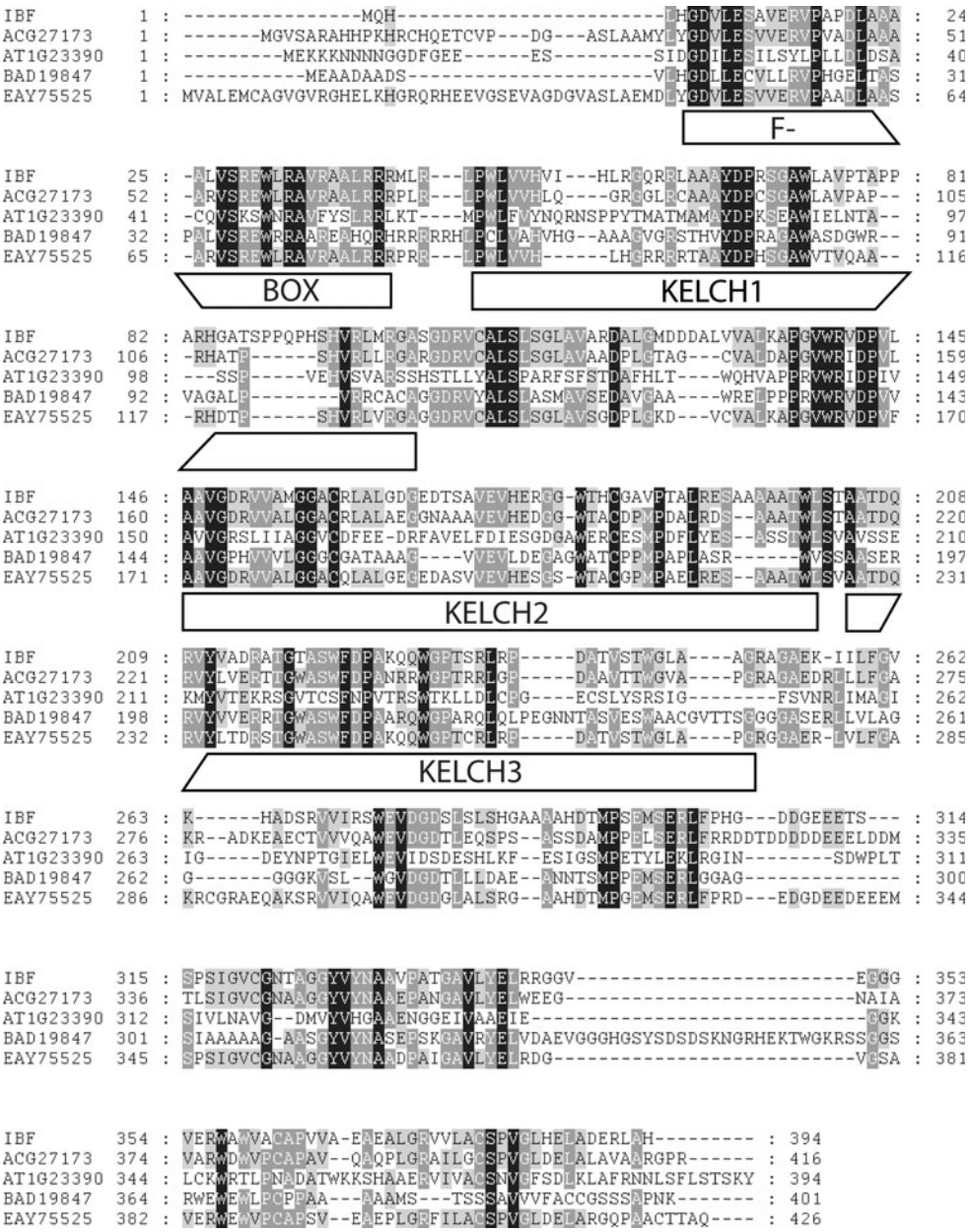

Fig. 5 The amino acid sequence of IBF1 is aligned with ACG27173, AT1G23390, BAD19847 and EAY75525. The *shaded areas* represent identical residues in at least three of the five proteins and completely conserved residues are highlighted with a *black* background. Predicted domains are indicated at the *bottom* of the alignment: F-box motif, predicted with SMART (Schultz et al. 2000) and/or PFAM (Bateman et al. 2002); kelch repeats, predicted with REP (Andrade et al. 2000)

plants. Staining observation reconfirmed that the expression of *IBF1* was restricted to panicles. During the development of glumous flowers, GUS activity was detected in pedicels, florets, and glumes (Fig. 7). In contrast, the GUS activity was not detected in aerial parts and roots of seedlings.

Subcellular localization of IBF1

Subcellular localization of a protein makes important contribution to its function frequently. To examine the subcellular localization of the IBF1, we transiently transformed IBF1–GFP construct in the epidermal cells of onion. Protein expression was observed under a fluorescence microscope. The results showed that the IBF1–GFP fusion protein was mostly separated in the cytoplasm of cells (Fig. 8a, b). The construct was also introduced into the *Arabidopsis* protoplasts and we found that GFP signals preferred to be detected in the cytoplasm, whereas scarce signals could be observed in the cell membrane (Fig. 8c–e).

Expression analysis of flavonoid biosynthetic genes in rice florets

The expression of major structural enzymatic genes after pollination was investigated in Zhefu802 and *ibf1-1*. Gene expression experiments were performed by semiquantitative RT-PCR using RNA samples prepared from the rice

florets pollinated after 1 day. As shown in Fig. 9, *OsCHS1*, *OsF3H*, *OsDFR*, and *OsANS1* were all differentially expressed between two rice lines. It should be noted that the transcript of *OsDFR* in the mutant was significantly increased, whereas the expression of *OsANS1* in *ibf1-1* was repressed dramatically compared to that in wild-type plants.

Discussion

In rice, the brown color in furrows of hull is controlled by two independently segregating dominated genes with opposite effects. *BF* functions in forming a brown color in furrows of hull, whereas *IBF* inhibits this reaction. Co-existence of *BF* and its inhibitor or deficiency of *BF* gives normal color of furrows. Since *BF* is available in common cultivated rice, the brown color in the furrows of hull may be caused by a defective *IBF*. Fine mapping was used to identify the location of *IBF1*, a locus for brown furrows of rice, in an about 90-kb region on the long arm of chromosome 9. A combination of mutant analysis, complementation analysis, and sequence comparisons demonstrated that a kelch repeat-containing F-box like protein corresponding to the gene Os09g12150 was responsible for *IBF1*.

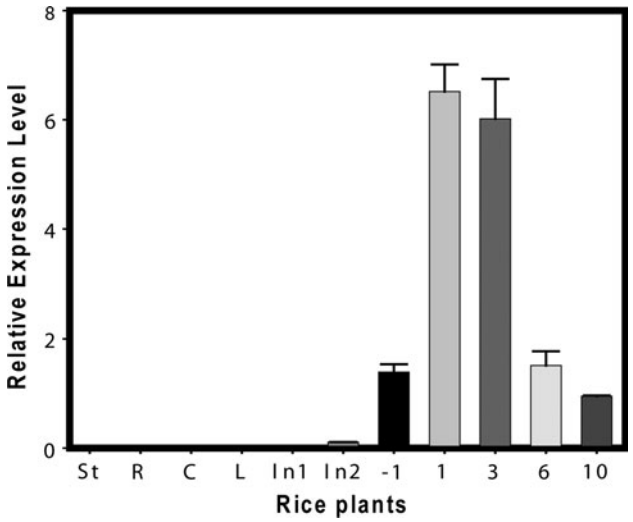

Fig. 6 *IBF1* gene expression pattern. Real-time RT-PCR analysis of *IBF1* from cDNA of seedlings (*St*), roots (*R*), culms (*C*), leaves (*L*), 1–2 cm inflorescences (*In1*), 8–10 cm inflorescences (*In2*), glumes of 1 day before pollination (*−1*), glumes of 1 DAF (*1*), glumes of 3 DAF (*3*), glumes of 6 DAF (*6*), and glumes of 10 DAF (*10*) of wild-type plants. Amplification of *OsUBQ* was used to ensure the quality of cDNA

In diverse plant systems, color changes of flower or seed coat were induced by gene mutations, which participated in flavonoid pathway frequently. Flavonoids are secondary metabolites that accumulate in most plant seeds and are involved in many important physiological functions such as dormancy or viability (Lepiniec et al. 2006). Thus, flavonoid biosynthesis becomes one of the most extensively studied areas of plant secondary metabolism. In *Arabidopsis*, PAs (proanthocyanidins) accumulate in seed coat and protect the embryo and endosperm. Their oxidation during the course of seed desiccation leads to the formation of brown pigments that confer color to the mature seed (Debeaujon et al. 2003). The recessive mutation *intensifier1* of maize apparently causes an overall increase in flavonoid production in the aleurone and *IN1* that encodes a known transcription factor in the anthocyanin pathway belonging to the *r1/b1* multigene family in maize (Burr et al. 1996). In soybeans, seed coat color is determined by the classically defined *I* (*Inhibitor*) locus. The dominant *I* allele inhibits seed coat pigmentation, and it has been suggested that there is a correlation between the inhibition of pigmentation by the *I* allele and chalcone synthase (*CHS*) gene silencing in the seed coat (Senda et al. 2002). Here, we report a specific recessive mutant *ibf1* of rice in which brown pigments accumulate significantly in the hull. Overt increases of total flavonoids and anthocyanin in this mutant furrows suggest that brown pigmentation is due to the disruption of *IBF1*, which could control the flavonoid metabolism in rice hull. Moreover, *IBF1* of rice acts a negative role in flavonoid biosynthesis similar to *IN1* of maize and *I* locus in soybean.

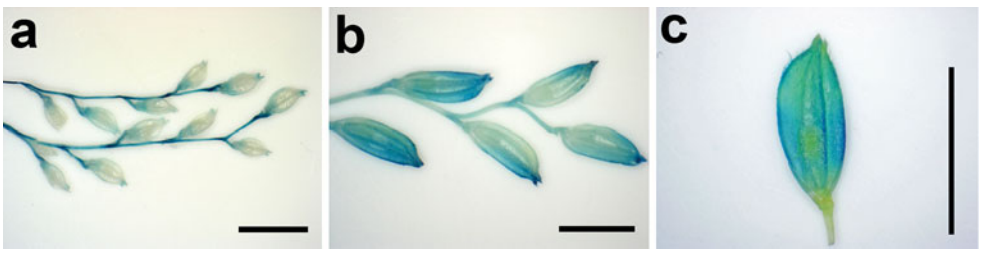

Fig. 7 Histochemical staining of GUS activity in *IBF1*::*GUS* transgenic rice plants. GUS activity was detected only in rice panicles. **a** GUS mainly expressed in pedicel of 5-cm panicles. **b** GUS expressed in florets of 10-cm panicles. **c** GUS activity was detected in glumes of 16-cm panicles. *Bars* 5 mm

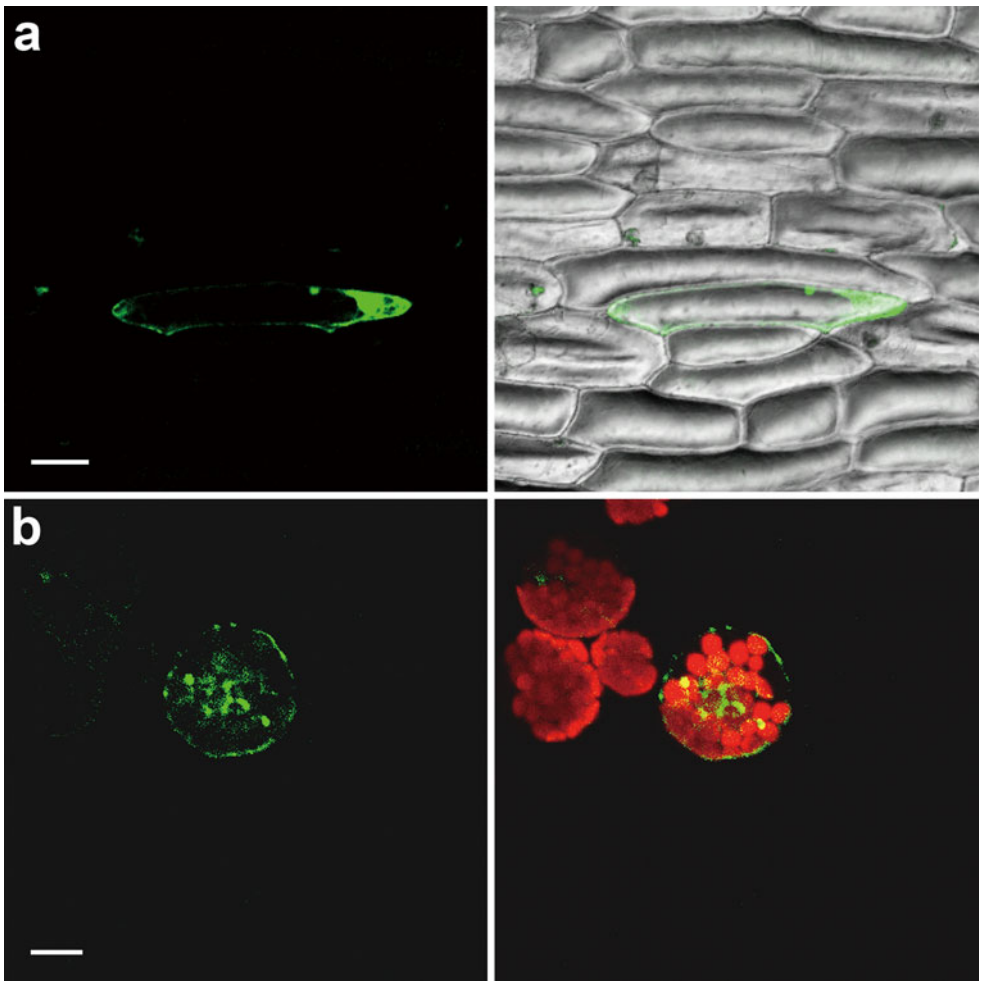

Fig. 8 Subcellular localization of IBF1–GFP fusion protein in onion epidermal cells and in *Arabidopsis* protoplast cells. **a** An onion skin epidermal cell expressing IBF1–GFP alone (*left*) and its merge (*right*), showing fluorescent signals mainly in the cytoplasm. **b** An *Arabidopsis* protoplast cell expressing IBF1–GFP alone (*left*) and its merge (*right*), showing a similar signal pattern. *Green* for GFP signals, *red* for chloroplast signals. *Bars* 10 μm (color figure online)

In recent years, through the study of flavonoid metabolism in *Arabidopsis* using *transparent testa* (*tt*) mutants, a great deal is known about the genes involved in this pathway. A total of 17 genes have already been identified at the molecular level, among which 8 are structure genes (*CHS*, *CHI*, *F3H*, *F3′H*, *DFR*, *LDOX*, *FLS* and *ANR*), 6 encode regulatory proteins (*TT1*, *TT2*, *TT8*, *TT16*, *TTG1*, *TTG2*) and 3 are involved in flavonoid compartmentation (*TT12*, *TT19*, and *AHA10*) (Baxter et al. 2005). Interestingly, to date, most of regulatory proteins always belong to the family of transcription factors in *Arabidopsis* or other studied model plants, including MYB, bHLH, MADS, WD40 or WRKY families (Kranz et al. 1998; Stracke et al. 2001; Heim et al. 2003). However, in this study, IBF1 takes part in regulating the flavonoid biosynthesis in another novel way. Firstly, IBF1 protein localized in cytoplasm mostly demonstrates that it does not belong to the transcription factor family. Secondly, based upon the initial BLAST run and a later PSI-BLAST iteration, we conclude that *IBF1* encodes a similar kelch motif-containing F-box family protein, where the amino (N)-terminal F-box motif is followed by a defined kelch motif and a characteristic carboxy-terminal domain. Such a novel family protein, which combines the F-box motif with a number of predicted kelch repeats, was discovered in *Arabidopsis* and this combination is unique to plants (Andrade et al. 2001; Bork and Doolittle 1994). Thereinto, F-box proteins were originally identified in yeast and human as components of a particular E3 ubiquitin ligase complex, named Skp1p-Cdc53p-F-box protein complex (SCF complex) (Bai et al. 1996; Skowyra et al. 1997). They have been shown in some cases to be critical for the controlled degradation of cellular regulatory proteins via the ubiquitin pathway (Hershko and Ciechanover 1998). On the other hand, according to the only crystal structure solved of a protein containing kelch repeats (Ito et al. 1994), these modules form a β-propeller, which could provide multiple potential protein–protein contact sites. So we propose that IBF1, a flavonoid biosynthesis-related repressor, could participate in the proteolysis as a part of the SCF complex during seed maturation in rice hull. Moreover, it is possible that pollination could stimulate these protein–protein interaction activities, since the expression level of *IBF1* was highest at 1–3 days after pollination. We need more in vivo and in vitro assays to

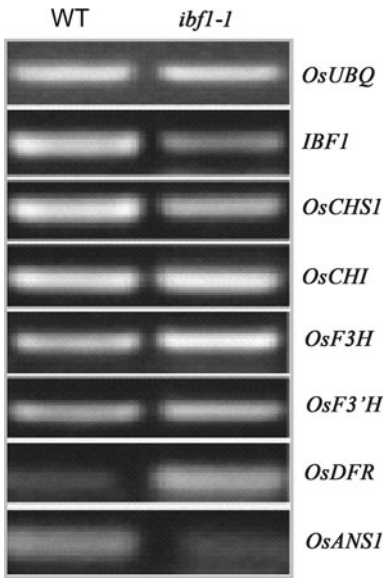

Fig. 9 Semiquantitative RT-PCR expression analyses of flavonoid structural genes in wild-type and the *ibf1-1* mutant glumes from panicles 1 day after pollination. *OsUBQ* expression was used as the internal control. Note the differential expression of the structural genes *OsCHS1*, *OsF3H*, *OsDFR* and *OsANS1* between two rice lines. Intriguingly, *OsDFR* was dramatically up-regulated in the mutant and *OsANS1* was on the contrary

investigate whether IBF1 is involved in the course of ubiquitination or other biochemical function as an inhibitor of BF, and what is the direct substrate of IBF1. Although IBF1 may not regulate the expression of flavonoid enzymatic genes during their transcription, the expression of several major structural genes in the flavonoid pathway changed in the *ibf1* mutant (Fig. 9). We noted that the expression of *OsDFR* was dramatically up-regulated in mutant florets 1 day after pollination. This may contribute to the significant increase of total flavonoids in *ibf1* hull, because OsDFR is the key enzyme for producing the precursors of colored anthocyanin or proanthocyanidins. Additionally, most of the flavonoid enzymes were recovered in the "soluble" cell fractions and loosely bound to the endoplasmic reticulum, possibly in a multi-enzyme complex (Saslowsky and Winkel-Shirley 2001; Winkel-Shirley 2001a), whereas the pigments themselves accumulated in the vacuole (anthocyanins and proanthocyanidins) or the cell wall (phlobaphenes). Thus, there is a possibility that regulation of some specific proteins by IBF1 in the cytoplasm could give feedback in regulating the expression of flavonoid enzymes.

In this study, we cloned a novel rice gene *IBF1*, which encodes a similar kelch repeat-containing F-box protein, and our results demonstrate that IBF1 participates in flavonoid biosynthetic pathway as a negative regulator, inhibiting the accumulation of brown pigments in rice hull. This new regulator may uncover a type of novel control in the future, as well as be very useful in further studies of the flavonoid metabolism.

Acknowledgments This work was supported by grants from the Ministry of Sciences and Technology of China (2011ZX08009-006 and 2009CB118500) and the National Natural Science Foundation of China (30760100 and 31160223).

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