RESULTS AND DISCUSSION
PART C
Molecular Characterization and Functional Analysis of an F-box coding gene OsFBLD10

Introduction to F-box proteins

It is very important to regulate the protein turnover which helps in removal of unnecessary and abnormal proteins and therefore maintain cellular homeostasis required for growth. The ubiquitin/26S proteasome pathway (UPS) plays a key role in degradation of proteins, required in all biological processes like cell division, signal transduction and apoptosis (Smalle and Vierstra, 2004, 2009). The pathway involves action of three enzymes, which includes E1 (Ub activating), E2 (Ub conjugating) and E3 (Ub ligase), respectively. They all act in a sequential manner to tag the target protein with a poly-ubiquitin chain in an ATP dependent reaction, which then is degraded by the multi-subunit 26S proteasome complex (Hellman and Estelle, 2002). Substrate specificity is largely determined by E3 ubiquitin ligases which have been studied well and divided into various families. SCF (SKP1-CUL1-F-box) is the largest and well-characterised among them, which consists of four subunits, SKP1, cullin1/Cdc 53, F-box protein and RBX1/Roc1/Hrt1 (Skowyra et al., 1997; Zheng et al., 2002). Among them, cullin1 acts as a scaffold and organizes the other subunits to form a functional SCF complex. The N-terminal of Cullin1 binds both SKP1 and the F-box protein (FBP) that recognizes and binds targets (Bai et al., 1996; Skowyra, 1997; Deshaies, 1999; Zheng et al., 2002; Lechner et al., 2006) and its C-terminal binds to RBX1.

F-box proteins constitute a large family of proteins which were discovered in humans (Bai et al., 1994). F-box represents a conserved domain of ca 40 aa residues unravelled first in cyclin F. The F-box proteins have been found to be present in all organisms, for example, Drosophila has 22 F-box proteins, yeast has 11 F-box members and there are 68 members in humans and 74 in mouse (Jain et al., 2007). In plants, the F-box proteins represent a rather large family with a number of 897 F-box genes in Arabidopsis thaliana, 971 in rice, 417 in maize, 817 in sorghum, 702 in soybean, 88 in Chlamydomonas, 425 in Populus, 315 in grapes and 1350 in Arabidopsis lyrata (Hua et al., 2011). The N-terminal region of F-box proteins contains conserved 40-60 amino acid long F-box domain, which binds to
SKP1, and the C-terminal region binds to the substrates (Schulman et al., 2000; Zheng et al., 2002). The C-terminal confers diversity to these proteins, it may have DUF295 domain or LRRs or kelch repeats or WD-40 repeats or FBD, which all are important in protein-protein interaction and thus recruit substrate to the SCF-complex (Kipreos et al., 2000; Hermand et al., 2006; Jain et al., 2007; Xu et al., 2009).

The F-box proteins have been found to play a role in a variety of processes in plants. F-box protein SKP2 regulates the degradation of E2Fc, which plays a role in cell division and during transition from skoto-morphogenesis to photomorphogenesis in *Arabidopsis* (Pozo et al., 2002). Another FBP, FBL17 has been shown to regulate cell cycle during pollen development (Kim et al., 2008; Gusti et al., 2009). These proteins have been found to mediate hormone signalling in plants. A very well documented example is of TIR1 that acts as auxin receptor and stimulates binding of SCF<sup>TIR1</sup> to the substrate, AUX/IAA repressor proteins (Tan et al., 2007). It regulates auxin dependent gene expression by ubiquitinating the AUX/IAA repressors for their degradation which releases active ARFs to transcribe auxin responsive genes (Dharmasiri et al., 2005; Villalobos et al., 2010). Other F-box proteins like AFB1-3 and AFB5 have been shown to play a role in auxin response pathway (Walsh et al., 2006). LRR containing F-box protein, COI1, has been shown to regulate JA responses by acting as jasmonic acid receptor (Mosblech et al., 2011). It activates the transcription of JA responsive genes by causing degradation of JAZ (JA-ZIM domain containing) proteins, which actually are repressors of the JA response pathway (Chua et al., 2010). SLEEPY1 (SLY1) and SNEEZY in *Arabidopsis*, and GID2 in rice are F-box containing proteins that act as regulators of GA signalling (McGinnis et al., 2003; Sasaki et al., 2003; Ariizumi et al., 2011). Similarly, other FBPs like EBF1, EBF2 and DOR, and TLP9 have been reported to play a role in ethylene and ABA signalling, respectively (Lai et al., 2004; Zhang et al., 2008; Vierstra, 2009). Numerous reports have been published till date demonstrating the role of these F-box proteins in plant development. UFO (Unusual Floral Organ) activates the transcription of APETALA3 and thus influences floral meristem identity. It acts antagonistic to AGAMOUS and affects petal and stamen development (Durfee et al., 2003). HAWAIIAN SKIRT, another FBP in *Arabidopsis*, was found to regulate organ fusion and organ growth and development.
RMF protein plays a role in anther development by causing tapetum degeneration (Kim et al., 2010). APO1, the UFO ortholog in rice, causes suppression of the rachis branch meristem, into spikelet meristem, thus controlling the number of spikelets and also regulating floral organ identity and vegetative growth (Ikeda et al., 2005; Ikeda-Kawakatsu et al., 2009). MAX2 protein controls inflorescence architecture and senescence (Stirnberg et al., 2002; Shen et al., 2007). ARABIDILLO-1/2, VIER1 and CEGENDUO are other group of F-box proteins that regulate root growth (Coates et al. 2006; Dong et al. 2006; Schwager et al., 2007). The F-box proteins have been shown to play role in light signalling. For example, EID1 and AFR1 are involved in PHY-A mediated light signaling (Dieterle et al., 2001; Harmon and Kay, 2003). ZTL, FKF1 and LKP2 are involved in blue light signalling. They also regulate levels of TOC1 and PRR5, which are components of the circadian oscillator which further regulate the levels of CCA1 and LHY (Schultz et al., 2001; Baudry et al., 2010). ZTL has also been shown to interact with GIGANTEA (which controls the circadian rhythm). It has also been demonstrated to have role in flowering and photomorphogenesis (Somers et al., 2000; Mas et al., 2003; Kim et al., 2007). F-box containing proteins have been shown to be present in pathogenic organisms. The necrotic yellow virus harbours CLINK protein and P0 proteins of polerovirus are such examples. VirF of \textit{Agrobacterium tumifaciens} also is an FBP which enters the nucleus and assembles with the host SCF complexes to target components of host DNA replication (Lechner et al., 2006; Vierstra, 2009). OsDRF1 from rice controls defence response in rice (Cao et al., 2008; Paquis et al., 2010) and CPR30 is another FBP in \textit{Arabidopsis} which regulates SA-dependent and SA-independent defence signalling (Gou et al., 2009). Apart from the above cited examples, numerous other reports implicate these FBPs in mediating stress tolerance in plants. ORE9, TLP9, MAX2 and DOR are involved in stress response in \textit{Arabidopsis} (Woo et al., 2001; Lai et al., 2004; Shen et al., 2007; Zhang et al., 2008). Similarly, in rice, OsDRF1 and MAF1 affect ABA sensitivity (Cao et al., 2008; Yan et al., 2010). BIG-24.1, a FBP from grapevine, was induced by ABA, ethylene and wounding. Genome wide analysis of rice F-box proteins was done and a total of 43 proteins were found to be induced by abiotic stress (Jain et al., 2007). However, their functional validation still remains to be done.
As mentioned above, F-box proteins constitute a large family of proteins which are characterised by the presence of 40 amino acid long F-box domain. They are a component of the SCF (SKP1-CUL1-F-box) complexes, which are E3 ubiquitin ligases, and thus play an important part in protein degradation. Jain et al. (2007) did a genome wide analysis in rice and identified 687 F-box genes and classified them into various subfamilies based on their domain composition, as an update, rice genome now harbours 971 F-box genes (Hua et al., 2011). Jain et al. (2007) studied the expression of all the family members during different stages of rice development. Various family members were found to have a differential expression during various stages of reproductive development and under abiotic stress conditions. One of the F-box family members had not been annotated previously and thus was not included in above analysis. For reasons given below, this gene was designated as OsFBLD10.

Expression analysis of the F-box gene, OsFBLD10

Development dependent expression

The microarray based expression profile for rice F-box family members reported by Jain et al. (2007) showed that a large number of genes had high expression in shoot apical meristem (SAM) and/or early inflorescence stages. The expression profile for OsFBLD10 was also checked in the available microarray data and it was found to have a strikingly higher expression in SAM, followed by P1 and P2 stages, and then declined in later stages of panicle and seed development. The expression of OsFBLD10 was relatively low in tissues like root, leaf and even young seedling (Figure 59A). Real time PCR analysis was done to confirm this expression pattern and essentially similar results were obtained. The transcript levels of OsFBLD were highest in SAM tissue and it declined gradually in developing panicle and were virtually negligible during seed development (Figure 59B).

Changes in expression under abiotic stress

The microarray data available on abiotic stress treatments showed that there was no significant change in transcript levels of OsFBLD10 by any kind of abiotic stress.
treatment. Although a slight induction was seen by cold stress (Figure 59C). This was confirmed by real time PCR, using RNA isolated from stress treated tissues.

Figure 59: The expression profile of OsFBLD10 gene. (A) Microarray based expression profile in various stages of rice development. (B) Real time PCR analysis of OsFBLD10 gene for various rice tissues. (C) Microarray based profile in various abiotic stress treatment conditions. (D) Real-time PCR to check transcript levels under light and dark conditions.
In comparison to OsFBLD10, a few other F-box proteins have been found to be up- or down-regulated in one or more stress conditions (Jain et al., 2008). In fact, there are many reports where functional validation of these genes has proved their role in imparting stress tolerance (Vierstra et al., 2003; Cao et al., 2008; Yan et al., 2010). However, since OsFBLD10 did not show any clear response to abiotic stresses, this aspect was not pursued further and this study was focused more on its role in regulating development.

**Light responsiveness**

F-box proteins like ZTL and FKF1 have been found to be important members of circadian clock (Somers et al., 2000) and Arabidopsis proteins AFR and EID1 also been shown to regulate light signalling (Dieterle et al., 2001; Harmon and Kay, 2003). They have been shown to be differentially regulated under dark and light conditions. The monochromatic lights like red, far-red light and blue light also affect the expression of F-box genes (Jiao et al., 2005; Jain et al., 2007). The expression of OsFBLD10 was checked in dark-grown and light-grown seedlings. White light did cause an up-regulation in transcript accumulation (Figure 59D). The effect of red, far-red and blue light remains to be examined.

**Structure of OsFBLD10 protein**

The FBLD subfamily of F-box proteins is rather small and comprised of only 9 members. It is characterized by the presence of LRR (important for protein-protein interactions) and FBD domain in addition to the F-box domain. When the domain composition of our protein (AK103754) was checked, it was found to contain all the three domains. Thus, it was given name as OsFBLD10, a new member of this family. The position of domains in the protein is diagrammatically represented in Figure 60A. Further, this new OsFBLD10 protein was compared with other OsFBLD subfamily members in rice and multiple sequence alignment was done using Clustal X program. As can be seen below, there was considerable conservation of residues within the F-box domain between different OsFBLD members (Figure 60B). Similarly, the F-box domain of OsFBLD10/AK103754 was aligned with domain from other characterized F-box proteins from different organisms like humans, yeast and
Arabidopsis. There was less degree of similarity between the amino acid residues of the F-box domain from other organisms (Figure 60C).

Figure 60: Sequence analysis of OsFBLD10 protein. (A) Domain composition of protein showing the presence of F-box domain, LRR domain and FBD domain. (B) Multiple sequence alignment of F-box domain of OsFBLD10 protein with other FBLD family members (OsFBLD101 to OsFBLD109 from rice. (C) Sequence alignment of F-box domain with other characterised F-box proteins from different organisms- OsMAF1 (NP_001047698.1), OsGID2 (BAC81428), OsDRF1 (DQ237916) from Oryza sativa, UFO (NP_564368.1), FKF1 (NP_564919), ZTL (NP_061867), EBF1 (CAE75865) from Arabidopsis thaliana, SKP2 (AAK31593.1), FBX42 (NP_061867), Cyclin F (AAB60342) from Homo sapiens and Cdc4 (NP_116585) from Saccharomyces cerevisiae.
Subcellular localization of OsFBLD10 protein

Since F-box proteins play a role in various cellular compartments, it was decided to check its intracellular localization. Onion peel bombardment was done to check the subcellular localization of OsFBLD10 protein. For the generation of the construct, full-length open reading frame was cloned into GFP containing pCAMBIA 1302 vector. It was amplified using gene specific primers (having Ncol and BglII and restriction sites) from KOME clone as template (Figure 61).

![PCR amplification of complete open reading frame of OsFBLD10.](image1)

![Cloning of of OsFBLD10 ORF into pCAMBIA1302 vector for checking its localization.](image2)

**Figure 61:** PCR amplification of complete open reading frame of OsFBLD10.

**Figure 62:** Cloning of of OsFBLD10 ORF into pCAMBIA1302 vector for checking its localization. (A) Agarose gel electrophoresis for examining mobility shift of recombinant clones. (B) Confirmation of cloning into pCAMBIA localization vector by restriction digestion with Ncol and BglII (C) Schematic representation of OsFBLD10::pCAMBIA construct generated for particle bombardment studies.
The amplicon was ligated into Ncol and BglII digested pCAMBIA 1302 vector and then transformed into E. coli competent cells. The transformants obtained were screened on kanamycin and digested with Ncol and BglII to check for the presence of insert. The positive clone released a band of 1.5 Kb which corresponded to the ORF and vector backbone after restriction digestion (Figure 62A, 62B).

![35S::OsFBLD-GFP](image1)

![35S::GFP](image2)

**Figure 63: Subcellular localization of OsFBLD10 in onion peel cells.** (A) OsBLH::GFP fusion construct cloned in pCAMBIA vector seen to be localized in both nucleus and plasma membrane. (B) Empty vector pCAMBIA harbouring GFP reporter gene used as control.

Plasmid was isolated in bulk from this positive clone and onion peel bombardment was performed. As apparent from the confocal images, OsFBLD10 protein is localized in both nucleus and the plasma membrane (Figure 63). This implies that this F-box protein is membrane localized but after receiving a signal or interacting with other protein it can enter into the nucleus to execute its function. However, this conclusion is purely speculative and is based on some earlier data available on other F-box proteins (Santagata et al., 2001; Sakaguchi et al., 2008).
Functional analysis of OsFBLD10 in Arabidopsis

PCR amplification and cloning of full-length cDNA

Full-length cDNA of OsFBLD10 was obtained in the form of the KOME clone (Figure 64A) where it had been cloned into Lambda-FLC vector between SfiI enzyme sites. The plasmid was isolated and full-length cDNA amplified using gene-specific primers (Figure 64B).

![Figure 64A](https://example.com/image1)

![Figure 64B](https://example.com/image2)

Figure 64: Plasmid isolation and amplification of FBLD10 cDNA from KOME clone. (A) Plasmid isolated from KOME clone quantified on gel. (B) PCR amplification of cDNA of OsFBLD10 from KOME clone using gene specific primers.

For generation of the over-expression construct in pMDC32 vector, gateway cloning technique was employed. Gene-specific primers were designed with forward primer having four extra bases CACC at its 5’ end. The cDNA of 1.8 kb was amplified from the KOME clone and cloned into pENTR-dTOPO entry vector. Transformants were screened on kanamycin and the cloning was confirmed by digestion with NotI and Xhol having one site in vector and insert, respectively, and thus a band of 744 bp and vector backbone were released (Figure 64A, 64B). The TOPO clone thus obtained was mobilised into destination vector pMDC32 by a ligation-recombination (LR) reaction. The ligation mix was transformed into E.coli and the transformants screened on kanamycin and hygromycin. Next, restriction digestion with BamHI released a band of 1.2 Kb and the vector backbone and thus confirmed the cloning of cDNA into pMDC32 vector (Figure 64C, 64D).
Figure 64: Cloning of OsFBLD10 cDNA for generating its over-expression construct
(A) Plasmid isolated from pENTR/d-TOPO clones checked on gel to check mobility shift.
(B) Restriction digestion of clones with NotI and XhoI to confirm cloning in TOPO vector.
(C) Agarose gel electrophoresis of pMDC32 plasmids on gel. (D) Restriction digestion of
clones with BamHI to confirm cloning.

Plasmid was isolated from the positive pMDC32 clone and mobilised into Agrobacterium tumifaciens which was then used for Arabidopsis transformation by floral dip method as described in Materials and Methods.

Characterization of Arabidopsis transgenics expressing OsFBLD10

The seeds obtained after floral dip were screened on MS agar medium containing hygromycin selection and a total of fourteen T1 transgenic lines were obtained. Approximately 100 seeds from each T1-line were screened on hygromycin containing medium to check for their segregation ratio. Ten plants each from a total of three lines showing 3:1 ratio were transferred to soilrite-containing pots to obtain their T2 generation. Seeds form all the lines were again screened to obtain
hygromycin resistant T3-homozygous lines. T3 lines were further selfed to get T4 generation, which were then used for subsequent analysis. PCR was done from the genomic DNA of the homozygous lines obtained, using \( HPTII \) primers, which confirmed the presence of transgene in them. Real time PCR was also done to check the expression levels of \( OsFBLD10 \) in these homozygous lines (Figure 66A, 66B).

![Figure 66A](image)

**Figure 66:** Analysis of \( OsFBLD10 \) over-expression transgenics. (A) PCR from genomic DNA of wild type and transgenics with \( HPTII \) primers to confirming the presence of transgene. (B) Real time PCR analysis to check the expression levels of \( OsFBLD10 \) in \( Arabidopsis \) transgenics obtained.

On phenotypic analysis, it was seen these over-expression transgenics were found to be similar to the wild-type plants at the seedling stage. During initial vegetative phase, the rosette size was similar and there was no difference in number of leaves. However, later, when adult plants were compared, the \( OsFBLD10 \) over-expression
lines had a larger sized rosette. The plants were slightly shorter in height than the wild-type plants (Figure 67A, 67B). There was no difference in flowering time or flower morphology per se.

Figure 67: Phenotypic comparison of the wild-type and transgenic plants over-expressing OsFBLD. (A) Wild-type and OsFBLD10 transgenic at younger stage of development (20-day-old). (B) 35-day-old adult plants of wild-type and OsFBLD10 transgenic plants.
Functional analysis of *OsFBLD10* by raising transgenics in rice

As described previously, *OsFBLD10* has an interesting expression profile with highest expression in SAM and early panicle stages, indicating that it may regulate floral transition or early stages of panicle development. However, there was no change in flowering time or morphology of inflorescence when it was over-expressed in a heterologous system, i.e. *Arabidopsis*. To know about its role in floral transition or rice development, attempts were made to functionally characterize it by generating transgenics in rice, using both over-expression strategy and RNAi strategy.

**Generation of over-expression construct**

Full-length cDNA was cloned into rice over-expression vector pB4NU. For its cloning, the *OsFBLD10* cDNA was first amplified from KOME clone, using gene specific primers having SmaI restriction sites at their ends (Figure 68A). The amplicon obtained was ligated into SmaI digested vector and transformed into *E. coli*. The transformants were selected on kanamycin and then confirmed for cloning by digestion with BamHI, which had a site at 1211 bp in *OsFBLD10* cDNA. Therefore, in the positive clone, a fragment of 1.2 Kb was obtained alongwith the vector backbone (Figure 68B, 68C).

The over-expression construct was mobilised into *Agrobacterium tumifaciens* and then transformed in rice by tissue culture following the protocol by Mohanty et al. (1999) as mentioned in Materials and Methods. The steps involved in rice tissue culture, including seed inoculation, subculturing after callus formation, co-cultivation with the *Agrobacterium* strain harbouring construct, followed by washing of callus are diagrammatically represented below. The calli were kept on selection and then after shoot initiation, they were transferred successively to regeneration medium, rooting medium and root growth medium, before transferring them to the greenhouse (Figure 69).
Figure 68: Amplification of OsFBLD10 cDNA and cloning in pB4NU vector. (A) PCR amplification of OsFBLD10 cDNA with gene specific primers from its KOME clone. (B) Plasmid isolation from clones to check their mobility shift on gel. (C) Restriction digestion with BamHI to confirm the cloning in pB4NU vector. (D) Schematic representation of OsFBLD10::pB4NU construct generated.
Figure 69: Diagrammatic representation of steps involved in rice tissue culture for transformation. 21-day-old calli were subcultured and co-cultivated with *Agrobacterium* strain harbouring over-expression or silencing construct. The calli were kept in succession on selection medium, regeneration medium and rooting medium. Finally, the plantlets obtained were kept in root growth medium (RGM) for hardening them before transferring to soil in a clay pot in the greenhouse.
Characterization of OsFBLD10 over-expression lines in rice

The Agrobacterium clone harbouring the ubq::OsFBLD10 construct was used to transform rice. To begin with rice seeds were kept in dark on 2 MS medium and 21-day-old calli thus generated were transformed with Agrobacterium strain. These transformed calli were selected on hygromycin for 45-50 days with intermittent transfer to fresh medium. Later, white coloured, solid proliferating calli were kept on regeneration medium supplemented with BAP. The shooting started on this medium and once the shoots grew up to 2-3 cm and started touching the Magenta box lid, these plantlets were transferred to rooting medium. Each plantlet was transferred to a single culture tube containing rooting medium. Finally, they were shifted from rooting medium to rice growth medium for hardening. A total of sixteen lines were obtained, which were transferred to soil in a pot and kept in a greenhouse. These plants matured and set seed between 100-120 days. Seeds were harvested individually and 25-30 seeds of each line were inoculated on hygromycin selection to check for 3:1 segregation. Around five lines showing near about 3:1 ratio were selected for further characterisation. PCR was done from genomic DNA of these over-expression lines using HPTII primers to confirm the presence of the transgene (Figure 70A).

As mentioned before, OsFBLD10 had maximum level of expression in SAM and P1 and P2 stages of panicle development. Therefore, to examine the level of the transgene expression in transgenic lines, real time PCR was done. P1 stage (upto 3 cm panicle) was harvested from individual transgenic lines and the wild-type plants grown in the greenhouse and their RNA isolated for real time PCR analysis for studying the expression of OsFBLD10 gene (Figure 70B).

The transgenics over-expressing OsFBLD10 were checked for any phenotypic alteration vis-a-vis the wild-type PB1 plants. As such, there was no apparent difference in their morphology except that the over-expression lines were shorter in height than the wild-type PB-1 plants. Preliminary analysis (of first generation transgenics) also revealed that the lines over-expressing OsFBLD10 are somewhat late flowering. This observation needs to be confirmed in the subsequent generations and work is in progress in this direction.
Figure 70: Expression analysis of transgenics over-expressing *OsFBLD10*. (A) PCR from genomic DNA of transgenics using HPTII primers to confirm the presence of transgene. (B) Real-time PCR analysis of transgenics to check the expression levels of the transgene.

Figure 71: Adult rice wild-type PB1 plants and the transgenics over-expressing *OsFBLD10*.

**Generation of *OsFBLD10* RNAi construct**

In order to study the effect of loss of function of the gene, RNA silencing of *OsFBLD10* was also attempted. For generating the RNAi construct, a short stretch of 252 bp, unique to this gene, was amplified from the available KOME clone by
using gene specific primers. Four extra bases (CACC) were added at the 5’ end of the forward primer to facilitate cloning in entry vector pENTR/d-TOPO (Figure 72).

![Figure 72: PCR amplification of OsFBLD10 RNAi fragment. A short stretch specific to the F-box gene was amplified from KOME clone using gene specific primers.](image)

The blunt ended PCR product was used to put TOPO reaction (as described in Materials and Methods) and then this ligation mix was transformed into *E. coli* competent cells and the transformants screened on kanamycin selection. The isolated TOPO plasmids were then confirmed for the presence of insert by PCR using vector specific M13 primers, which gave a band of around 600 bp (Figure 73A, 73B). The RNAi fragment cloned in TOPO vector was ligated into destination pANDA vector (for silencing of genes) by ligation-recombination reaction. After transformation into *E. coli*, the transformants obtained were screened on hygromycin (Figure 74A). To confirm the cloning, PCR with a combination of gene specific primer and GUS-linker primer was carried out where the positive clones gave an amplicon of 1.2 Kb (Figure 74B).
Figure 73: Cloning of RNAi fragment in pENTR/d-TOPO vector. (A) Plasmid isolation from TOPO clones to check for the mobility shift. (B) PCR using vector specific M13 primers to confirm cloning of RNAi fragment in TOPO vector. (C) Schematic representation of OsFBLD10::TOPO construct generated.

Figure 74: Cloning of RNAi fragment in rice silencing vector pANDA. (A) Plasmid isolation of pANDA clones. (B) PCR with vector specific and gene specific primers to confirm cloning in the vector. (C) Schematic representation of construct generated for studying the effect of silencing.
This OsFBLD10-RNAi construct was then transformed into Agrobacterium tumifaciens and used finally for rice transformation through rice tissue culture, in a similar way as done for the over-expression transgenics.

**Characterization of RNAi lines**

Twenty-five PB-1 RNAi transgenic lines were obtained by tissue culture. They were grown in greenhouse and seeds were harvested individually as described earlier. For checking 3:1 segregation, 25-30 seeds of individual lines were inoculated on hygromycin selection and their germination percentage calculated. Six lines showing ratio close to 3:1 were chosen for further analysis. These RNAi lines were checked for the presence of transgene by PCR with HPTII primers (Figure 75A). Real-time PCR was also conducted, using RNA from panicle tissue and leaf tissue, to confirm silencing of OsFBLD10 (Figure 75B, 75C).

**Figure 75:** Analysis of rice RNAi transgenic lines. (A) PCR from genomic DNA of wild type and OsFBLD10 RNAi lines with HPTII primers. (B, C) Real time PCR to check expression levels of RNAi transgenic lines in panicle tissue and leaf tissue.
When checked for their phenotype, no apparent difference was observed in RNAi lines as compared to wild type with respect to their morphology both at the vegetative stage and reproductive stage. However, flowering in these OsFBLD10-RNAi lines occurred much earlier than PB-1 parent line (Figure 76). Both wild-type and transgenic plants were transferred at the same time to greenhouse and OsFBLD10 silenced plants flowered ca 12-15 days before the wild-type PB-1 plants.

![Wild-type PB1 and OsFBLD::RNAi plants](image)

**Figure 76: Phenotype of adult RNAi plants.** The adult transgenic rice plants were healthier than the wild-type plants. These RNAi lines showed early flowering as compared to the wild-type plants. Inset shows a close-up of the panicle.

Based on the data presented above, it can be concluded that OsFBLD10 is localized both in the nucleus and the plasma membrane. Whether it interacts with another co-factor on the plasma membrane and then moves inside the nucleus to perform its function, in a way analogous to some other F-box proteins, remains to be validated experimentally. The maximum transcript of OsFBLD was observed in shoot apical meristem and early stages of inflorescence development. Realising that this gene may code for a protein involved in SAM maintainence or floral transition, over-expression and RNAi transgenics of rice were generated, which did show early flowering in RNAi lines and delayed flowering in over-expression lines of OsFBLD10. Two of the FBP’s, ZTL and FKF, are already known to be involved in photoperiodic pathway of floral induction (Somers et al., 2000; Song et al., 2012). But, OsFBLD10 belongs to a separate class altogether. It will be our endeavour now
to decipher the target of OsFBLD10 in the floral pathway by identifying the proteins which interact with it and elucidating their role in transition to flowering. One can at least conclude from the functional analysis done in this study that OsFBLD is a negative regulator of transition to flowering, that is why down-regulation of OsFBLD in RNAi lines caused precocious flowering in rice.