F-Box Proteins in Rice. Genome-Wide Analysis, Classification, Temporal and Spatial Gene Expression during Panicle and Seed Development, and Regulation by Light and Abiotic Stress

Mukesh Jain, Aashima Nijhawan, Rita Arora, Pinky Agarwal, Swatismita Ray, Pooja Sharma, Sanjay Kapoor, Akhilesh K. Tyagi, and Jitendra P. Khurana*

Interdisciplinary Centre for Plant Genomics and Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi 110 021, India

F-box proteins constitute a large family in eukaryotes and are characterized by a conserved F-box motif (approximately 40 amino acids). As components of the Skp1p-cullin-F-box complex, F-box proteins are critical for the controlled degradation of cellular proteins. We have identified 687 potential F-box proteins in rice (Oryza sativa), the model monocotyledonous plant, by a reiterative database search. Computational analysis revealed the presence of several other functional domains, including leucine-rich repeats, kelch repeats, F-box associated domain, domain of unknown function, and tubby domain in F-box proteins. Based upon their domain composition, they have been classified into 10 subfamilies. Several putative novel conserved motifs have been identified in F-box proteins, which do not contain any other known functional domain. An analysis of a complete set of F-box proteins in rice is presented, including classification, chromosomal location, conserved motifs, and phylogenetic relationship. It appears that the expansion of F-box family in rice, in large part, might have occurred due to localized gene duplications. Furthermore, comprehensive digital expression analysis of F-box protein-encoding genes has been complemented with microarray analysis. The results reveal specific and/or overlapping expression of rice F-box protein-encoding genes during floral transition as well as panicle and seed development. At least 43 F-box protein-encoding genes have been found to be differentially expressed in rice seedlings subjected to different abiotic stress conditions. The expression of several F-box protein-encoding genes is also influenced by light. The structure and function of F-box proteins in plants is discussed in light of these results and the published information. These data will be useful for prioritization of F-box proteins for functional validation in rice.

In plants, like other living organisms, protein turnover is a key regulatory mechanism in many cellular processes, including cell cycle, circadian rhythms, cell lineage specification, metabolic control, flower development, embryogenesis, stress responses, and various signal transduction pathways. The ubiquitin (Ub)/26S proteasome pathway is responsible for selective degradation of most intracellular proteins in eukaryotes (Smalle and Vierstra, 2004). Proteins targeted by this pathway are first modified by the attachment of Ub polymers. These polyubiquitinated substrates are recognized by the 26S proteasome and degraded. Ubiquitination of target protein occurs in three steps (Deshaies, 1999; Smalle and Vierstra, 2004). In first step, Ub moiety is activated by the Ub-activating enzyme (E1) and then transferred to the Ub-conjugating enzyme (E2). Finally, substrate recognition and ubiquitination occurs through Ub-protein ligases (E3), which catalyze the transfer of activated Ub to appropriate targets. Skp1p-cullin-F-box (SCF) protein is a major class of plant E3 ligases, which is composed of four subunits: cullin1/Cdc53, Rbx1/Roc1/Hrt1, Skp1, and an F-box protein. The F-box protein performs the crucial role of conferring specificity to the complex for appropriate targets.

F-box proteins contain a conserved F-box domain (40–50 amino acid) at their N terminal, which interacts with Skp1. The name F box was given because it was identified first at the N-terminal region of cyclin F (Bai et al., 1994). The C terminus of F-box proteins generally contains one or several highly variable protein-protein interaction domains, for example, Leu-rich repeat (LRR), kelch repeat, tetratricopeptide repeat (TPR), and WD40 repeat that interact with specific targets. Many reported F-box proteins have been identified as SCF components. However, F-box proteins function in non-SCF complexes as well (Clifford et al., 2000; Galan et al., 2001; Kim et al., 2002). Over the past few years, several F-box proteins have been identified...
in eukaryotes such as yeast (Saccharomyces cerevisiae), nematodes, flies, humans, and plants (Kipreos and Pagano, 2000; Gagne et al., 2002; Kuroda et al., 2002; Jin et al., 2004).

Regulation of various developmental processes in plants, for example morphogenesis, circadian clock regulation, self incompatibility, and floral meristem and floral organ identity determination, involves F-box proteins (Sullivan et al., 2003; Moon et al., 2004; Smalle and Vierstra, 2004). So far, functions of only 23 F-box proteins (18 from Arabidopsis [Arabidopsis thaliana]) have been reported from plants (Supplemental Table S1). UNUSUAL FLORAL ORGANS (UFO), the first F-box protein identified in plants, has a role in floral meristem identity and floral organ development (Levin and Meyerowitz, 1995; Samach et al., 1999; Laufs et al., 2003; Hepworth et al., 2006). LKP1/ZTL, LKP2/KLF, and FKFI F-box proteins are involved in photomorphogenesis, circadian clock regulation, and control of flowering time (Nelson et al., 2000; Somers et al., 2000, 2004; Schultz et al., 2001; Imaizumi et al., 2003, 2005). Recently, it has been demonstrated that many F-box proteins, including TIR1, which has been known for a long time to regulate the stability of Aux/IAA proteins, act as auxin receptors in Arabidopsis (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). The role of F-box proteins in ethylene (EBF1 and EBF2) and gibberellic acid (SLY1 and SNE) signaling has also been established (Guo and Ecker, 2003; McGinnis et al., 2003; Potuschak et al., 2003; Dill et al., 2004; Gagne et al., 2004; Strader et al., 2004). The F-box protein COI1 has been implicated in jasmonate-regulated defense responses (Xie et al., 1998; Xu et al., 2002; Devoto et al., 2005). Another F-box protein, SON1, negatively regulates systemic acquired resistance and NIM1-independent defense response (Kim and Delaney, 2002). S-locus F-box genes have been identified in several plants that control pollen function of self incompatibility (Lai et al., 2002; Qiao et al., 2004a, 2004b; Ushijima et al., 2004; Wang et al., 2004; Sonneveld et al., 2005).

The Arabidopsis genome encodes a superfamily of F-box proteins (approximately 600–700 members) with diverse C-terminal domains (Gagne et al., 2002; Kuroda et al., 2002). The presence of a strikingly large number of F-box proteins implies that a plethora of SCF complexes, which recognize a wide array of substrates, are possible. Yeast two-hybrid analysis showed that several putative F-box proteins interact with one or more members of the Arabidopsis Skp1-like (ASK) family (Gagne et al., 2002; Kuroda et al., 2002), indicating that many of these proteins are components of SCF complexes. Different groups have classified Arabidopsis F-box proteins in several subfamilies or subgroups on the basis of phylogenetic relationship or unique functional domains (Gagne et al., 2002; Kuroda et al., 2002). The functions of most of the F-box proteins are yet to be studied, despite the fact that they play crucial roles in many aspects of plant growth and development. A great deal of experimental work will be required to determine the specific biological function of each of these genes comprising the F-box family. The current availability of rice (Oryza sativa) genome sequence (International Rice Genome Sequencing Project, 2005) allows the identification of F-box protein-encoding genes in this monocotyledonous species and their comparative analysis with Arabidopsis, which will be useful in terms of studying the functional and evolutionary diversity of this superfamily in plants.

To our knowledge, the function of only two F-box proteins has been reported in rice (Supplemental Table S1). Gibberellin-insensitive dwarf2 acts as a positive regulator of gibberellic acid signaling (Itoh et al., 2003; Sasaki et al., 2003; Gomi et al., 2004) and dwarf3 (D3) controls tiller bud activity (Ishikawa et al., 2005). In this study, 687 potential F-box proteins have been identified in rice genome and classified into 10 sub-families on the basis of type of domain(s) present at their C terminus. Several putative novel conserved motifs have also been identified in proteins that do not harbor any known functional domain other than the F-box. The chromosomal locations and phylogenetic relationship of F-box proteins have been reported. A comprehensive expression analysis of F-box protein-encoding genes at several stages of development of rice, including vegetative growth, floral transition, and panicle and seed development has been performed. In addition, evidence has been provided for the regulation of expression of several F-box protein-encoding genes by light and abiotic stress.

RESULTS AND DISCUSSION

Identification and Classification of F-Box Proteins

F-box proteins are represented by a large family in various organisms. There are 11 F-box protein-encoding genes predicted in yeast, 22 in Drosophila, 68 in human, 74 in mouse, and 326 in Caenorhabditis elegans (Kipreos and Pagano, 2000; Jin et al., 2004). In Arabidopsis, however, a strikingly large number of F-box protein-encoding genes (600–700) have been predicted (Gagne et al., 2002; Kuroda et al., 2002). The recent completion of genome sequencing of rice (International Rice Genome Sequencing Project, 2005) provides a powerful tool to identify genes encoding F-box proteins in this most important cereal crop. BLAST searches of the rice (subsp. japonica cv Nipponbare) genome performed using Hidden Markov Model (HMM) profile of F-box domain as query and subsequent analysis identified 687 nonredundant potential F-box proteins. The Institute of Genomic Research (TIGR) locus, open reading frame length, protein length, and chromosomal location of each of these genes are listed in Supplemental Table S2. The BLASTP search of these 687 F-box proteins in the annotated proteins of indica rice (cv 93–11) genome available at BGI-RiSe Rice Genome Database (http://rise.genomics.org.cn; Yu et al.,
Many F-box proteins are predicted to contain various protein-protein interaction domains at their C terminus (Bai et al., 1996; Patton et al., 1998; Gagne et al., 2002; Kuroda et al., 2002) in diverse organisms. In mammals, F-box proteins have been classified into three groups, FBXW containing WD40 repeat domains, FBXL containing LRR domains, and FBXO with other domains (Jin et al., 2004). Genome-wide analyses of Arabidopsis F-box proteins revealed the presence of several domains such as LRR, kelch repeats, FBD, WD40, PAS/PAC, ring finger, tubby (TUB), and PPR (Gagne et al., 2002; Kuroda et al., 2002), allowing their classification into 19 groups (Kuroda et al., 2002). The domain search of rice F-box proteins in the SMART and Pfam databases identified several other known functional domains similar to Arabidopsis. This search led us to classify the rice F-box proteins in 10 subfamilies (Table I). A large number (465) of rice F-box proteins did not show the presence of any known functional domain other than the F box and were classified as FBX subfamily members. The other 222 proteins exhibited the presence of one or more known functional domains and were classified in FBDDUF (66) containing domain of unknown function (DUF), FBL (61) containing LRR domains, FBK (25) containing kelch repeats, FBD (17) containing FBD domain, FBT (14) containing TUB domain, FBLD (9) containing both LRR and FBD domains, FBA (4) containing FBA domain, FBO (24) containing other domains, including PAS, PAC, PPR, TPR, ring finger, zinc finger, MYND, helicase, and SEL1 repeats (Table I; Fig. 1; Supplemental Fig. S1; Supplemental Table S2). The most abundant domains containing F-box proteins are those containing DUF domain. Most of the members of FBDDUF subfamily contain DUF295 domain except for Os05g18660, Os07g33400, and Os09g37590, which contain DUF1618, DUF635, and DUF246 domains, respectively. LRRs are 20 to 29 amino acid motifs with positionally conserved Leu or other aliphatic residues. Only 25 F-box proteins containing kelch repeats were identified, a number much less than that of Arabidopsis. Kelch repeats are present in a large fraction of rice and Arabidopsis F-box proteins (Gagne et al., 2002; Kuroda et al., 2002; this study) and appear to be the unique feature of plant F-box proteins, as currently no yeast or mammalian F-box protein displays the presence of kelch repeats. WD40 repeats and LRRs are the predominant domains present at the C terminus of yeast and mammalian F-box proteins (Bai et al., 1996; Cenciarelli et al., 1999; Winston et al., 1999; Jin et al., 2004). Although kelch and WD40 repeats have similar tertiary structure like a β-propeller (Smith et al., 1999; Adams et al., 2000), they have no sequence similarity. This suggests the convergent evolution of a subset of F-box proteins originating a common tertiary structure specialized in protein-protein interaction. Seventeen rice F-box proteins were predicted to contain FBD domain, a plant-specific domain of approximately 80 amino acids, which is also present in BRCT domain containing proteins. Fourteen F-box proteins exhibited the presence of C-terminal TUB domain. This 120 amino acid domain was first identified in mouse TUB1, which is involved in controlling obesity (Kleyn et al., 1996). The other nine F-box proteins harbor both the LRR repeats and FBD domains. FHA domain was predicted in four F-box proteins.

Three of the F-box proteins (Os06g47890, Os11g34460, and Os02g05700) have PAS/PAC domain in addition to kelch repeats and may function as flavin-binding photoreceptors. Similar proteins in Arabidopsis, ZTL, LKP2, and FKF1 have been implicated in control of flowering time and circadian rhythms (Nelson et al., 2000; Somers et al., 2000, 2004; Schultz et al., 2001; Imaizumi et al., 2003, 2005). JmjC domain, which is supposed to be a metal-binding site (Clissold and Ponting, 2001), was found in two F-box proteins (Os03g27250 and Os11g36450). DNA-binding domains such as zinc finger, MYND, C2H2 zinc finger, helix loop helix, and ring finger were also found in rice F-box proteins, which may be directly or indirectly involved in transcriptional regulation. In addition, PPR and TPR domains were predicted in rice F-box proteins that are responsible for processing and/or translation of organellar mRNAs (Small and Peeters, 2000).

A large fraction (465) of predicted rice F-box proteins do not harbor any known functional domain other than the F box. Therefore, the unknown putative conserved motifs in F-box proteins of FBX subfamily were investigated using the Multiple Em (Expectation Maximization) for Motif Elicitation (MEME) program (Bailey and Elkan, 1995). The statistical significance of a motif predicted by MEME is based on its log likelihood ratio, its width, and number of occurrences. The e value is an estimate of the expected number of motifs with the given log likelihood ratio (or higher) and with the same width and number of occurrences that one would find in a similarly sized set of random sequences. More than 30 putative statistically significant (e value less than e-100) motifs could be identified (Supplemental Table S3). Each of these motifs was more than 10 amino acids in length and was conserved in at

| Subfamily | Domain Content | No. in Category |
|-----------|----------------|-----------------|
| FBX       | Unknown/none   | 465             |
| FBDDUF    | DUF            | 66              |
| FBL       | LRR            | 61              |
| FBK       | Kelch          | 25              |
| FBD       | FBD            | 17              |
| FBT       | TUB            | 14              |
| FBLD      | LRR and FBD    | 9               |
| FBA       | FBA            | 4               |
| FBW       | WD40           | 2               |
| FBO       | Others         | 24              |

Table I. Classification of 687 F-box proteins based on their domain architecture.
least five of the predicted F-box proteins. Some of these motifs (motifs 1, 5, and 19) were conserved in as large as 138 rice F-box proteins. BLAST searches showed that most of these motifs were conserved in other plants also; however, some of them were present only in rice sequences (data not shown). These putative novel motifs may be critical for interaction of F-box proteins with other proteins, like other known domains, and may provide specific function(s) to these proteins. However, the biological significance of these motifs remains to be elucidated. Based on the BLAST searches, large-scale multiple alignments, and analysis using PFam-B databases, some new motifs have been identified in Arabidopsis F-box proteins (Gagne et al., 2002; Kuroda et al., 2002). Although the consensus sequences of these motifs have not been described, some of these motifs were found to be conserved in other plant species also (Kuroda et al., 2002). The BLAST search of some representative Arabidopsis F-box proteins harboring these motifs showed very weak similarity with rice F-box proteins (data not shown). These results suggest that rice and Arabidopsis F-box proteins contain some specific motifs, which may perform species-specific functions.

### Gene Structure and Chromosomal Distribution

To study the gene structure, the number of introns present within the open reading frame of each F-box protein gene was determined by analysis of their exon-intron organization. Interestingly, 40.76% (280 of 687) of F-box protein-encoding genes are predicted to be intronless (Supplemental Table S2), which is a much higher percentage than predicted for rice genes overall (19.9%; M. Jain, P. Khurana, A.K. Tyagi, J.P. Khurana, unpublished data). Forty five of 66 members of FBDFU subfamily, 18 of 25 members of FBK subfamily, and all the four members of FBA subfamily are intronless. Such intronless gene families can evolve rapidly either by gene duplication or reverse transcription/integration (Lecharny et al., 2003; Lurin et al., 2004; Jain et al., 2006c).

The 687 F-box protein-encoding genes were found to be distributed randomly on all the 12 rice chromosomes. The huge size of F-box family indicates that it has evolved through a large number of duplication events in rice. The rice genome has undergone genome-wide duplication events, including polyploidy, which has great impact on the amplification of members of a gene family in the genome. For fine mapping, the position (in bp) of each F-box protein gene was determined on rice chromosome pseudomolecules available at TIGR (release 4) and has been represented diagrammatically in Figure 2 (the exact position in bp and orientation of each F-box protein gene on rice chromosome is given in Supplemental Table S2). Substantial clustering of F-box protein-encoding genes was evident on different chromosomes. Several F-box protein-encoding genes were arranged in tandem repeats of two to nine genes.

#### Family TIGR ID Protein structure

| Family | TIGR ID | Structure |
|--------|---------|-----------|
| FB2    | Os01g052980 |                       |
| FBDFU  | Os01g22430 |                       |
| FBL    | Os02g10700 |                       |
| FBK    | Os02g36210 |                       |
| FBD    | Os01g41280 |                       |
| FBT    | Os01g64700 |                       |
| FBD    | Os02g06520 |                       |
| FBA    | Os02g10600 |                       |
| FBW    | Os11g07970 |                       |
| FBO    | Os09g27660 |                       |
| FBO    | Os01g72310 |                       |
| FBO    | Os01g09790 |                       |
| FBO    | Os11g36790 |                       |
| FBO    | Os03g12940 |                       |
| FBO    | Os07g36360 |                       |
| FBO    | Os03g22750 |                       |
| FBO    | Os02g41910 |                       |
| FBO    | Os03g69250 |                       |
| FBO    | Os01g09270 |                       |
| FBO    | Os02g05700 |                       |
| FBO    | Os11g34460 |                       |
| FBO    | Os08g20480 |                       |
| FBO    | Os06g02460 |                       |
| FBO    | Os12g44130 |                       |
| FBO    | Os09g39190 |                       |
| FBO    | Os04g40030 |                       |
| FBO    | Os02g29150 |                       |
| FBO    | Os15g04850 |                       |

**Figure 1.** Domain organization of representative F-box proteins from each family. Members of FBO family with different domain(s) are also shown. On the left, family name to which corresponding F-box protein belongs and TIGR locus ID are given. Domain abbreviations are: ANTH, ANTH domain; DEXD, DEAD-like helicase domain; FBA_1, F-box associated domain; FBD, domain present in F-box and BRCT domain containing proteins; H2B, histone H2B; HATC, hAT family dimerization domain; HELICC, helicase superfamily C-terminal domain; HLH, helix-loop-helix domain; HGWP, HGWP motif; JmjC, Jumonji; Kelch, kelch repeats; KNOT1, knottins; LysM, Lys motif; MYND, MYND finger; PAC, motif C terminal to PAS motifs; PAS, PAS domain; Peptidase C48, Ulp1 protease family C-terminal catalytic domain; PPR, pentatricopeptide repeats; RING, ring finger; RRM, RNA recognition motif; SEL1, sel1-like repeats; UBPQ, Ub domain; VWA, von Willebrand factor type A domain; WD40, WD40 repeats; ZnF, zinc fingers.
either in the same or inverse orientation, representing localized gene duplications. Interestingly, at several positions, the F-box protein-encoding genes present in tandem belonged to different families, suggesting the diversification by domain shuffling after tandem duplications. Recently, Yu et al. (2005) presented evidence for the ongoing individual gene duplications in rice, which provide a never ending raw material for studying gene genesis and their functions. The duplication of the F-box protein-encoding genes is also associated with chromosomal block duplications in rice. Fifty F-box protein-encoding genes were found to be located on the duplicated segmental regions of rice chromosomes mapped by TIGR (Fig. 2, Supplemental Table S4). All but four of the F-box protein-encoding genes located on duplicated segments belong to same subfamily. However, four F-box protein-encoding genes present on duplicated chromosomal segments between chromosome 8 and 9 (Os08g35930 and Os09g27090) and chromosome 11 and 12 (Os11g04330 and Os12g04130) belong to different families, further supporting the idea of diversification of F-box protein-encoding genes by domain shuffling. Interestingly, eight of the 14 FBT subfamily proteins were present on duplicated chromosomal segments. Since the number of F-box protein-encoding genes present on duplicated chromosomal segments is much smaller than those present in tandem, the tandem duplications of chromosomal regions appear to have played a major role in expansion of this gene family. These results suggest that the expansion of F-box protein-encoding genes in rice is probably, in large part, due to localized gene duplications. In Arabidopsis also, tandem duplications have been implicated in the expansion of F-box protein gene family (Gagne et al., 2002).

Phylogenetic Analysis

To examine the phylogenetic relationship among rice F-box proteins, an unrooted tree was constructed from alignments of their full-length protein sequences along with F-box proteins with known function in Arabidopsis and other plants (Fig. 3; Supplemental Fig. S2). Manual analysis of the phylogenetic tree revealed 11 distinct clades (A–K) of rice F-box proteins. The clades A to D, F, and H were further divided into 18 subclades, giving 23 distinct groups of proteins in total. Two proteins, Os06g49530 and Os01g14270, did not seem to belong to any of these groups. The investigation of C-terminal domains present in F-box proteins clustered together within a group revealed a striking clustering. Most members of the same subfamily were clustered together. For example, all the F-box proteins in group A2, including Arabidopsis AFR protein, belong to FBK subfamily. Similarly, clade B (groups B1–B4) mostly contains the members of FBOX subfamily. All but one of the 14 members of FBT subfamily grouped together in group C2. Likewise, S6 (of 66) members of FBDUF subfamily were included in group D2. All the F-box proteins in group F1 and most of proteins in group F2 belong to FBL subfamily. Similarly, the F-box proteins with identical C-terminal domain(s) clustered together in the phylogenetic tree constructed from the alignments of their F-box domain.
Figure 3. Phylogenetic relationship among the rice F-box proteins. The unrooted tree was generated using ClustalX program by neighbor-joining method. F-box proteins grouped into 11 distinct clades (A–K). The clades A to D, F, and H were further divided into 18 subclades (A1–A3, B1–B4, C1–C2, D1–D3, F1–F2, and H1–H4). The position of F-box proteins with known function from Arabidopsis and other plants used for construction of phylogenetic tree is indicated. A larger version of the tree is available as Supplemental Figure S2.
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vis-à-vis Arabidopsis, harboring similar domains, protein diversity (Morgenstern and Atchley, 1999). Although F-box proteins belonging to other subfamilies. Such clustering with similar F-box domain but dissimilar C-terminal protein-protein interaction domain, corroborating the results from phylogenetic analysis of Arabidopsis F-box proteins (Gagne et al., 2002). However, some groups of F-box proteins had widely different C-terminal domains. The members of FBA, FBW, and FBO subfamilies were grouped with F-box proteins belonging to other subfamilies. This analysis revealed that most rice and Arabidopsis F-box proteins cluster in species-specific distinct clades. This result indicates that F-box proteins harboring similar domains expanded in a species-specific manner; probably only a few members originated from the common ancestral genes that existed before divergence of monocots and dicots. This type of divergence between a monocotyledonous (rice) and a dicotyledonous (Arabidopsis) species has been observed for other large gene families as well (Bai et al., 2002; Zhang et al., 2005; Jain et al., 2006a, 2006c). Furthermore, significant differences were found in the degree of expansion of F-box proteins. For example, Arabidopsis contains 100 kelch repeat proteins (Gagne et al., 2002), however, only 25 such proteins are predicted in rice. Whether the degree of expansion is species-specific and represents unique functional requirement, will be unraveled when more and more genome sequences become available and gene function analyzed. Further analysis of phylogenetic tree constructed from rice and Arabidopsis F-box proteins containing kelch repeats revealed that one major group of proteins present in Arabidopsis was completely absent in rice except for one protein (Supplemental Fig. S3B). It can be speculated that these proteins were lost in rice or evolved in Arabidopsis after divergence of monocots and dicots and may perform dicot-specific functions.

Digital Expression Analysis

Three different approaches were undertaken for expression analysis of rice F-box protein-encoding genes, which involved the use of already available information. The first approach was the survey for availability of any full-length cDNA (FL-cDNA), expressed sequence tag (EST), or peptide sequence(s) in databases corresponding to rice F-box protein-encoding genes. For this search, the gene expression evidence search page available at TIGR was used. This analysis revealed that one or more FL-cDNA, EST, and/or peptide sequence(s) were available for 368 (53.6%) of 687 F-box protein-encoding genes (Supplemental Table S5), indicating that a large percentage of these genes are expressed. However, the frequency of ESTs for F-box protein-encoding genes varied greatly from one to 62. Also, the EST sequences were derived from various rice tissue/organ genes, including seedlings, shoots, roots, heading-stage panicles, and filling-stage panicles. Expression for a total of 361 F-box protein-encoding genes (each of them represented by a unique 70-mer oligo on the microarray) could be retrieved and among them 287 genes were expressed in at least one of the above-mentioned rice organs/tissues (Supplemental Fig. S4). Several F-box protein-encoding genes exhibited tissue/organ-specific expression. There were 10, 10, 11, 21, and 13 F-box protein-encoding genes specifically expressed in seedlings, shoots, roots, heading-stage panicles, and filling-stage panicles, respectively (Supplemental Fig. S4). The specific expression of highest number of F-box protein-encoding genes in heading panicle demonstrates their predominant role in panicle development.

Massively parallel signature sequencing (MPSS) provides a sensitive quantitative measure of gene expression for nearly all genes in the genome (Brenner et al., 2000). The third approach was to extract information about MPSS tags available from the Rice MPSS Project (http://mpss.udel.edu/rice/) for each F-box protein gene. Data from 16 MPSS libraries representing 12 different tissues/organs of rice was extracted for both 17 base and 20 base signature libraries. MPSS tags were available for 456 and 350 F-box protein-encoding genes in 17 base and 20 base signature libraries, respectively. This further strengthens the idea that most of F-box protein-encoding genes are expressed. However, significant signatures (that uniquely identify individual genes) were found for only 290 and 260 F-box protein-encoding genes in 17 base and 20 base signature libraries, respectively. Substantial differences were found in number of tags (in tpm) representing F-box protein-encoding genes, indicating from marginal (1–3 tpm) to strong (>250 tpm) expression (Supplemental Table S6). A large percentage (40–50%) of F-box protein-encoding genes exhibited moderate expression (26–250 tpm). Only a few genes were expressed at a high level (>250 tpm). Further, we identified F-box protein-encoding genes with signatures present in only one library by comparisons between different MPSS libraries for both 17 base and 20 base signature datasets. Subsequently, the genes common in both 17 base and 20 base signature libraries were identified with tissue-specific expression (Supplemental Table S7). These F-box protein-encoding genes may play important and specific roles in the development.
biology of these diverse tissues and their upstream regions may have experimental utility as tissue-specific promoters. Highest number of genes (7) showed specific expression in mature pollen followed by root (5). This observation is consistent with the role of F-box protein-encoding genes in controlling pollen function of self-incompatibility (Lai et al., 2002; Qiao et al., 2004a, 2004b; Ushijima et al., 2004; Wang et al., 2004; Sonneveld et al., 2005).

Microarray Analysis of Expression Profiles of F-Box Protein-Encoding Genes during Panicle and Seed Development

DNA microarrays can measure the individual transcript level of tens of thousands of genes simultaneously, thus providing a high-throughput means to analyze gene expression at the whole genome level. The whole genome microarray data can also be used to analyze the expression of a subset of genes of interest. To achieve gene expression profiling of F-box protein-encoding genes, microarray analysis was performed using Affymetrix rice whole genome arrays. The rice tissues/organisms and developmental stages selected for microarray analysis include seedling, seedling root, mature leaf, Y leaf (leaf subtending the shoot apical meristem [SAM]), SAM, and various stages of panicle (P1–P6) and seed (S1–S5) development. Different developmental stages of panicle and seed development have been categorized according to panicle length and days after pollination (dap), respectively, as follows: up to 0.5 mm, SAM; 0 to 3 cm, floral transition and floral organ development; 3 to 10 cm, meiotic stage; 10 to 15 cm, young microspore stage; 15 to 22 cm, vacuolated pollen stage; 22 to 30 cm, mature pollen stage; 0 to 2 dap, early globular embryo (approximately 25 cell stage); 3 to 4 dap, middle and late globular embryo (150–800 cell stage, onset of coleoptile, SAM and radicle differentiation; early milky endosperm); 5 to 10 dap, embryo morphogenesis (protrusion of leaf primordia and organ enlargement; late milky endosperm); 11 to 20 dap, embryo maturation (soft dough endosperm); 21 to 29 dap, dormancy and desiccation tolerance (hard dough endosperm). These stage specifications are approximations based on information from Itoh et al. (2005).

Following whole-chip data processing (quality controls, normalization, probe summarization, variance stabilization, and log transformation), the log signal values for 617 F-box protein-encoding genes represented on the array were extracted. Average log signal values for all the 617 F-box protein-encoding genes from three biological replicates of each sample are given in Supplemental Table S8. A hierarchical cluster display of average log signal values of these genes is also presented in Supplemental Figure S5. The signal values indicate that most of these F-box protein-encoding genes are expressed in at least one of the rice vegetative organs and/or stages of development analyzed. Subsequently, differential expression analysis was performed to identify F-box protein-encoding genes with most abundant expression both during panicle and seed development stage(s). We defined a gene as differentially expressed at a given stage only if the expression level of the gene at that stage was significantly higher (more than 2-fold) than the levels at all the other stages. For this, differentially expressed genes were identified for two datasets of panicle and seed development with respect to vegetative organs, including seedling, root, mature leaf, and Y leaf. This analysis revealed that a total of 125 and 81 F-box protein-encoding genes were differentially expressed in at least one of the stages of panicle and seed development, respectively, as compared to vegetative organs. Further analysis revealed that 33 genes were common among the differentially expressed genes during panicle and seed development stages as compared to vegetative organs. In the next step, the differentially expressed F-box protein-encoding genes at any stage of the panicle development as compared to seed development stages were identified. Similarly, the differentially expressed F-box protein-encoding genes at any stage of the seed development as compared to panicle development stages were identified. This analysis revealed that 64 and 31 F-box protein-encoding genes were preferentially expressed in at least one of the stages of panicle and seed development, respectively (Figs. 4 and 5). The differential expression of some representative genes in panicle and seed developmental stage(s) identified from microarray data analysis has also been confirmed by realtime PCR analysis (Supplemental Fig. S6). It is thus conceivable that these F-box protein-encoding genes may perform specific roles during different stages of development.

SAM comprises a small group of dividing cells that give rise to aerial parts of the plant. After producing stem and leaves during vegetative growth SAM undergoes transition in its fate to reproductive development marked by formation of floral meristem followed by flowers. Molecular studies have identified several genes whose activities are induced during transition to flowering and that play a primary role in the determination of both floral meristem and floral organ identity in rice (Tang et al., 2005; Furutani et al., 2006). The role of F-box protein, UFO1, the first F-box protein identified in plants, in determination of floral meristem and floral organ identity and flower development in Arabidopsis is well established (Patton et al., 1998; Samach et al., 1999; Wang et al., 2003; Hepworth et al., 2006). Our analysis revealed a strikingly large number (36 of 64) of F-box protein-encoding genes are differentially expressed in SAM and/or early panicle development (P1) stage (Fig. 4), suggesting that F-box protein-encoding genes play crucial roles in floral transition, determination of floral meristem and floral organ identity, and early flower development in rice. As many as 28 F-box protein-encoding genes were also differentially expressed in other stages of panicle...
Figure 4. Expression profiles of rice F-box protein-encoding genes differentially expressed during panicle development. Expression profiles of 60 up-regulated (A) and four down-regulated (B) F-box protein-encoding genes in SAM or any stage of panicle development (P1–P6) as compared to vegetative reference tissues/organs (seedling, root, mature leaf, and Y leaf) and seed developmental stages (S1–S5) are presented. The average log signal values of F-box protein-encoding genes in various tissues/organs and developmental stages (mentioned at the top of each lane) are presented by cluster display. The color scale (representing log signal values) is shown at the bottom.
development (P2–P6), indicating their role in gameto-
 PHYTE development (Fig. 4). The role of several plant 
F-box proteins in controlling pollen function of self 
incompatibility has been reported in other plant spe-
cies (Lai et al., 2002; Qiao et al., 2004a, 2004b; Ushijima et al., 2004; Wang et al., 2004; Sonneveld et al., 2005).

Several genes play a critical role in seed develop-
ment and grain filling in crop plants. A close coordi-
nation of gene expression among many important 
pathways is required during this biological process of 
paramount importance. In rice, the expression of genes 
involved in different pathways has been demonstrated 
to be coordinately controlled in a synchronized fash-
ion during grain filling (Anderson et al., 2003). Re-
cently, the differential expression of a significantly 
large number of genes involved in various cellular 
processes during seed maturation in hexaploid winter 
wheat (\textit{Triticum aestivum} cv Mercia) has been reported 
(Wilson et al., 2005). Because the cellular gene ma-
achinery is affected greatly during seed development 
and grain filling, it is expected that the expression of 
F-box protein-encoding genes involved in protein 
turnover is affected as well. In our study, 31 F-box 
protein-encoding genes were differentially expressed 
in at least one or more of the developmental stages of 
seed development in rice (Fig. 5), as described earlier. 
The transcript abundance of 29 F-box protein-encoding 
genes was more than 2-fold higher during various 
stages of seed development (Fig. 5A). Conversely, two 
(Os03g02550 and Os12g06630) F-box protein-encoding 
genes showed decreased transcript levels during later 
stages (S4 and/or S5) of seed development (Fig. 5B). 
These results suggest the involvement of F-box pro-
teins in rice seed development.

F-Box Protein-Encoding Genes in Anther Development

Recently, a rice basic helix-loop-helix transcription 
factor, UNDEVELOPED TAPETUM 1 (UDT1), has 
been identified as a major regulator of early tapetum 
development and pollen mother cell meiosis (Jung et al., 2005). Tapetum is the innermost sporophytic 
layer in the anther wall, which is thought to play a 
crucial role in the development and maturation of 
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several genes were down-regulated in \textit{udt1}-1 mutant 
anthers (Jung et al., 2005). During the survey of these 
genes, we found that at least eight F-box protein-encoding 
genes (Os07g04790, Os05g40610, Os02g28600, 
Os11g09670, Os08g09460, Os06g04690, Os04g56250, 
and Os10g10390) reported here were significantly 
down-regulated (more than 2-fold) in \textit{udt1}-1 anthers 
as compared to wild-type anthers (Fig. 6). These genes 
were differentially expressed at different stages of
anther development, including meiosis, young microspore, vacuolated pollen, and pollen mitosis also, as compared to paleas/lemmas (Fig. 6). One of these genes, Os05g40610, was found to be up-regulated during early panicle development (P1–P4 stages) in our analysis also. The difference between our study and the Jung et al. (2005) study for other genes are most likely due to the different tissues (panicle in our study and anthers in study by Jung et al., 2005) used for the microarray analysis. Taken together, these results suggest that these F-box protein-encoding genes might be involved in rice anther development and represent the targets of UDT1 transcription factor.

The F-box proteins are known to interact with Skp1 protein, another component of the SCF complex. The Arabidopsis genome contains 20 Skp1-like genes called ASK, which exhibit clear tissue-specific expression during floral organ development (Marrocco et al., 2003). Several of the ASK promoters directed the postmeiotic expression of GUS in the male gametophyte (Marrocco et al., 2003). A Pfam profile search for Skp1 family dimerization domain (PF01466) in the TIGR rice genome annotation database revealed the presence of at least 23 putative skp1-like genes in the rice genome, some of which are preferentially expressed in panicle development stages (data not shown). The coregulation of gene expression of F-box and Skp1-like gene family members, which interact with each other, may be crucial for the regulation of floral organ development, particularly male gametophyte by Ub degradation pathway.

**Light Regulation**

In addition to the direct effects, light also exerts its influence by modifying the rhythms generated by circadian clock (Somers et al., 1998; Chen et al., 2004). Although the molecular components of circadian clock in plants are not fully understood, the major control is likely to be the protein turnover mediated by Ub-proteasome pathway. The F-box proteins, LKP1/ZTL, LKP2/FKL, and FKF1, represent the important components of circadian clock. ZTL acts as a light-dependent regulator of proteolytic degradation of clock-associated proteins (Somers et al., 2000). FKF1 regulates the expression of the flowering time gene CONSTANS (CO) by degrading CYCLING DOF FACTOR 1, a repressor of CO transcription (Nelson et al., 2000; Imaizumi et al., 2003, 2005). In addition, COP9 signalosome has been known to interact with SCF complexes and act as a negative regulator of photomorphogenesis (Chamovitz et al., 1996). Also, the involvement of two F-box proteins, AFR and EID1, in phytochrome A-mediated light signaling in Arabidopsis has been demonstrated (Dieterle et al., 2001; Harmon and Kay, 2003; Marrocco et al., 2006).

Earlier, a genomic study of rice gene expression in response to light has been performed by microarray analysis, which demonstrated that the expression of a
significant component of rice genome is regulated by different light qualities (Jiao et al., 2005). To investigate the light regulation of rice F-box protein-encoding genes, microarray data obtained in the study by Jiao et al. (2005) was analyzed. The expression of only 327 F-box protein-encoding genes could be retrieved under different light conditions. Examination of expression ratios between light- and dark-grown rice seedlings indicates that a significant number (54) of F-box protein-encoding genes were regulated by white light (Supplemental Table S9). We found that 28 genes were up-regulated and 26 genes were down-regulated by at least 2-fold, with a \( p \) value less than 0.05. However, under different light quality conditions, less number of genes was regulated as compared to white light (Supplemental Table S9) and most of them were regulated by specific monochromatic lights. Only three (two up-regulated and one down-regulated) genes were differentially expressed in far-red light. In red light, three F-box protein-encoding genes were up-regulated. However, four genes were up-regulated and two down-regulated in blue light. Quantitative difference was found between white light and monochromatic light regulated gene expression in rice at the whole genome level also (Jiao et al., 2005). Putative orthologs of Arabidopsis F-box proteins known to be involved in light signaling could be demarcated in rice on the basis of their phylogenetic relationship, Os06g44500 for AFR1, Os11g34460 for FKF1, Os03g27250 for EID1, and Os06g47890 and/or Os02g05700 for LKP2 and/or ZTL. Although these genes did not show any differential light regulation in the data analyzed, the possibility of their involvement in light signal transduction pathway cannot be completely ruled out. These F-box proteins containing similar functional domains may perform similar functions in rice and Arabidopsis. Taken together, these results suggest that expression of a number of F-box protein-encoding genes is regulated by light, indicating light control of protein turnover in rice, which may be critical for several light-dependent cellular processes.

Expression of F-Box Protein-Encoding Genes under Abiotic Stress Conditions

Plants respond to adverse environmental conditions by eliciting various physiological, biochemical, and molecular responses, leading to changes in gene expression. Because abiotic stresses affect the cellular gene machinery, it is quite likely that the components of protein degradation machinery, such as F-box proteins, are affected as well. To address this question, expression of rice F-box protein-encoding genes was analyzed under abiotic stress conditions by microarray analysis performed with total RNA isolated from rice seedlings subjected to salinity, desiccation, and conditions are shown. The color scale (representing log signal values) is shown at the bottom.

![Figure 7](image-url)
cold treatment. We were able to identify 43 F-box protein-encoding genes that are differentially expressed (36 and 7 genes were up- and down-regulated, respectively) equal to or more than 2-fold with 95% confidence (p value less than 0.05), under one or more of the above-mentioned stress conditions (Fig. 7). Seven of these genes were up-regulated under all the abiotic stress conditions analyzed (Fig. 7A). The other 12 F-box protein-encoding genes were differentially expressed under any two stress conditions (Fig. 7B). Nine F-box protein-encoding genes were up-regulated and two were down-regulated under salt and desiccation stresses but not under cold stress. One F-box protein gene showed up-regulation under salt and cold stress. However, 24 other F-box protein-encoding genes were differentially expressed under only one stress condition; 16 under desiccation stress (14 up-regulated and 2 down-regulated), five under salt stress (3 up-regulated and 2 down-regulated), and three under cold stress (2 up-regulated and 1 down-regulated; Fig. 7C). The real-time PCR results of differential expression of some representative genes under abiotic stress condition(s) are consistent with the microarray data (Supplemental Fig. S6).

The role of COI1, an Arabidopsis F-box protein, is well established in jasmonic acid (JA)-regulated defense responses. COI1 forms an integral part of an SCF E3 Ub ligase that is predicted to target repressors of JA signaling to the proteasome for degradation (Devoto et al., 2002; Xu et al., 2002). COI1 binds a histone deacetylase, RPD3b, and may regulate the expression of genes involved in JA response through modulating the activity of RPD3b and possibly other proteins (Devoto et al., 2002). Recently, requirement of COI1 for a significantly large number of JA- and wound-induced gene transcription has been demonstrated (Devoto et al., 2005). Another F-box protein, SON1, has also been reported to negatively regulate defense response independent of salicylic acid and systemic acquired resistance, possibly via Ub-proteosome pathway (Kim and Delaney, 2002). The F-box proteins, Os05g39370 and Os01g63420, represent the closely related orthologs of Arabidopsis COI1 and may perform similar function in rice. These genes did not show significant differential expression under abiotic stress conditions analyzed in this study. However, no closely related ortholog of SON1 could be predicted in rice. The direct role of F-box proteins in abiotic stress responses has not been demonstrated so far. Our study demonstrates that F-box proteins play a significant role in abiotic stress pathway and the corresponding genes may be a valuable resource for producing stress-tolerant transgenic crop plants.

Plant stress responses often mimic certain normal developmental processes (Cooper et al., 2003). Interaction between plant development and environmental conditions implies that some genes must be coregulated by both environmental factors and developmental cues. We found nine (Os01g47050, Os01g59690, Os02g15950, Os02g51350, Os04g33820, Os05g43490, Os06g39370, Os07g09710, and Os10g30280) and three (Os02g54240, Os07g13830, and Os08g41750) F-box protein-encoding genes that were differentially regulated under stress conditions and were specifically expressed at one or more panicle and seed developmental stage, respectively. Several examples of such interaction of developmental regulation and stress responses exist. Phospholipase D and its product phosphatidic acid that play a role in pollen germination and pollen tube growth in tobacco (Nicotiana tabacum; Potocky et al., 2003; Zonia and Munnik, 2004) are also involved in various stress responses like water deficit, salts, wounding, and elicitation (Wang, 2002). A systematic regulation of gene expression has been found to drive the developmental processes and stress responses in Arabidopsis (Chen et al., 2002), indicating that an overlap of genes occurs between the developmental processes and stress responses. Moreover, a network of rice genes that are associated with stress response and seed development has been reported (Cooper et al., 2003). Recently, microarray analysis demonstrated that a significant number of pollination/fertilization-related genes are indeed regulated by dehydration and wounding in rice (Lan et al., 2005). This suggests that the genetic programs regulating them are likely to be related. This study also provides evidence that a number of candidate F-box protein-encoding genes are likely to be involved in critical developmental processes and stress responses, but their direct relationship needs experimental validation.

CONCLUSION

The F-box protein-mediated targeted protein degradation is critical for several key cellular processes. A very large number of F-box proteins have been predicted in rice and Arabidopsis. However, the function of only a few plant F-box proteins has been established as of now. The field of F-box proteins is in fact currently emerging. This study provides insights into the functions of F-box proteins in rice. For example, several F-box protein-encoding genes displayed specific expression during various stages of panicle and seed development. In addition, F-box proteins appear to serve as the key components of the machinery involved in regulating plant growth and development throughout its life cycle and their expression is influenced by light and abiotic stresses. The leads provided here would pave the way for elucidating the precise role of individual F-box protein in rice by adopting RNAi strategy or insertion mutagenesis.

MATERIALS AND METHODS

Database Search

The BLAST search of all the annotated proteins in whole rice (Oryza sativa) genome at TIGR (release 4) was performed using the HMM profile (build 2.3.2) of F-box domain (PF00646) downloaded from Pfam, as query. The
HMM profile for 44 amino acid-long F-box domain has been generated by alignments of 545 seed sequences. This search resulted in the identification of 901 proteins with an e-value cutoff of 1.0. The unknown conserved motifs were investigated by MEME (http://meme.sdsc.edu) version 3.5.2 (Bailey and Elkan, 1995). A limit of 50 motifs with other options set to default values was specified. MEME is a tool for discovering motifs in a group of related DNA or protein sequences. MEME uses statistical modeling techniques to automatically choose the best width, number of occurrences, and description for each motif. Multiple alignment analyses were performed using ClustalX (version 1.83) program (Thompson et al., 1997). The unrooted phylogenetic trees were constructed by neighbor-joining method (Saitou and Nei, 1987) and displayed using NJPLOT program (Perriere and Gouy, 1996). Exon-intron organization was determined using genome browser tool at TIGR (http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/). Manual curation and assessment of the gene structure of each predicted F-box protein remains to be done.

Localization of F-Box Protein-Encoding Genes on Rice Chromosomes

All the sequenced contigs of japonica cv Nipponbare have been physically constructed as pseudomolecules at TIGR (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml), representing the 12 rice chromosomes. Each of the F-box protein-encoding genes was positioned on these rice chromosome pseudomolecules by the BLASTN search. The presence of F-box protein-encoding genes on duplicated chromosomal segments was investigated by segmental genome duplication of rice available at TIGR (http://www.tigr.org/tdb/e2k1/osa1/segmental_dup/index.shtml) with the maximum length distance permitted between collinear gene pairs of 100 kb.

Plant Material and Growth Conditions

Rice (subsp. indica var IR64) seeds were obtained from the Indian Agricultural Research Institute. After disinfection with 0.1% HgCl₂ for 10 min and thorough washing with reverse-osmosis (RO) water, seeds were soaked overnight in RO water. Seedlings were grown in RO water in a culture room maintained at 28°C ± 1°C with a daily photoperiodic cycle of 14-h light and 10-h dark. For salt treatment, 7-d-old light-grown rice seedlings were transferred to a beaker containing 200 mls NaCl solution for 3 h. For desiccation, 7-d-old light-grown rice seedlings were dried for 3 h between folds of tissue paper at 28°C ± 1°C in a culture room. For cold treatment, the seedlings were kept at 4°C ± 1°C for 3 h. The seedlings kept in water for 3 h, at 28°C ± 1°C, served as control. Mature leaf, Y leaf, and different stages of panicle (up to 10-h dark. For salt treatment, 7-d-old light-grown rice seedlings were trans-
Analysis and Expression of Rice F-Box Protein-Encoding Genes

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