The F-Box Protein OsFBK12 Targets OsSAMS1 for Degradation and Affects Pleiotropic Phenotypes, Including Leaf Senescence, in Rice

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Leaf senescence is related to the grain-filling rate and grain weight in cereals. Many components involved in senescence regulation at either the genetic or physiological level are known. However, less is known about molecular regulation mechanisms. Here, we report that OsFBK12 (an F-box protein containing a Kelch repeat motif) interacts with S-ADENOSYL-L-METHIONINE SYNTHETASE1 (SAMS1) to regulate leaf senescence and seed size as well as grain number in rice (Oryza sativa). Yeast two-hybrid, pull-down, and bimolecular fluorescence complementation assays indicate that OsFBK12 interacts with Oryza sativa S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN and with OsSAMS1. Biochemical and physiological data showed that OsFBK12 targets OsSAMS1 for degradation. OsFBK12-RNA interference lines and OsSAMS1 overexpression lines showed increased ethylene levels, while OsFBK12-OX lines and OsSAMS1-RNA interference plants exhibited decreased ethylene. Phenotypically, overexpression of OsFBK12 led to a delay in leaf senescence and germination and increased seed size, whereas knockdown lines of either OsFBK12 or OsSAMS1 promoted the senescence program. Our results suggest that OsFBK12 is involved in the 26S proteasome pathway by interacting with Oryza sativa S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN and that it targets the substrate OsSAMS1 for degradation, triggering changes in ethylene levels for the regulation of leaf senescence and grain size. These data have potential applications in the molecular breeding of rice.

F-box proteins are components of E3 ubiquitin ligase S-PHASE KINASE-ASSOCIATED PROTEIN, CULLIN, F-BOX CONTAINING COMPLEXES (SCFs), which mediate a wide variety of biological processes (Schulman et al., 2000). The N terminus of F-box proteins, which interacts with S-Phase Kinase-Associated Protein1 (Skp1), is conserved. The C terminus generally contains one or several highly variable protein-protein interaction domains, such as Leu-rich repeat, Kelch repeat, tetra-tricopeptide repeat, or WD40 repeat domains (Jain et al., 2007). Kelch motifs consist of 44 to 56 amino acid residues, with four highly conserved residues, two adjacent Gly residues, and a Tyr and Trp pair separated by about six residues. The presence of Kelch repeats is a unique characteristic of a subset of F-box proteins in plants (Prag and Adams, 2003).

F-box proteins target their substrates for specific functions. Several key hormone signaling components, including receptors, have been identified as F-box proteins. The TRANSPORT INHIBITOR RESPONSE1 (TIR1) F-box protein acts as an auxin receptor regulating the stability of auxin/indole-3-acetic acid proteins in Arabidopsis (Arabidopsis thaliana; Gray et al., 2001; Zhang et al., 2011). CORONATINE-INSENSITIVE1 is an F-box protein that is a coreceptor with JASMONATE ZIM-DOMAIN PROTEIN1 as a central regulator of jasmonate signaling (Sheard et al., 2010). SNEEZY and SLEEPY1 regulate DELLA through interaction with the DELLA-GIBBERELLIN-INSENSITIVE DwarF1 complex in GA signaling (Dill et al., 2004; Strader et al., 2004). In addition, ETHYLENE-INSENSITIVE2 and ETHYLENE-INSENSITIVE3 are quickly degraded by the F-box proteins ETHANOL TOLERANCE PROTEIN1/ETHANOL TOLERANCE PROTEIN2 and EARLY B-CELL FACTOR1/EARLY B-CELL FACTOR2 during ethylene signaling (Guo and Ecker, 2003; Potuschak et al., 2003; Qiao et al., 2009; Wang et al., 2009a). Only a few F-box proteins containing Kelch motifs (FBKs), however, have been characterized. The FBK proteins ZEITLUPE, FLAVIN-BINDING, KELCH REPEAT, F-BOX1, and LOV KELCH PROTEIN2 are involved in light signaling, flowering, and circadian control via a proteasome-dependent pathway in Arabidopsis (Imaizumi et al., 2005). The rice (Oryza sativa) FBK gene LARGER PANICLE/ERECT PANICLE3 was reported to regulate panicle architecture (Fiao et al., 2009) and modulate cytokinin levels through Oryza sativa CYTOKININ OXIDASE2 expression (Li et al., 2011a). However, only a
few substances for F-box proteins with specific functions are known in plants. In other words, it is still not clear how F-box proteins mediate plant developmental processes such as leaf senescence and seed size.

Leaf senescence and the related ethylene regulation impact grain filling, which is an important determinant of yield, especially in the last stage of maturation in rice. Delayed leaf senescence was reported to be mediated by a nucleus-localized zinc finger protein, *Oryza sativa* DELAY OF THE ONSET OF SENESCENCE, in rice (Kong et al., 2006). The *STAY GREEN RICE* gene is involved in regulating pheophorbide a oxygenase that causes alterations in chlorophyll breakdown during senescence (Jiang et al., 2007). Physiologically, the progression of leaf senescence is dependent on ethylene levels, which also regulate grain filling (Wuriyanghan et al., 2009; Agarwal et al., 2012). In plants, it is well established that ethylene is biosynthesized from S-adenosyl-l-methionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC). ACC synthase catalyzes the first step of the biosynthesis by converting S-adenosylmethionine into ACC, and ACC oxidase catalyzes the second step by metabolizing ACC and dioxygen into ethylene. S-Adenosyl-l-methionine synthase (SAMs) is involved in developmental regulation mediated by methylation alterations of DNA and histones in rice (Li et al., 2011b). The physiological function of ethylene in rice is dependent not only on its biosynthesis but also on signal transduction components such as the ETHYLENE-RESPONSE2 receptor (Zhu et al., 2011). However, less is known about how F-box protein regulation is involved in the coordination of senescence progression.

Here, we show that a rice F-box gene, *OsFBK12*, that contains a Kelch repeat domain is involved in the regulation of ethylene-mediated senescence and seed size. Transgenic lines with reduced or increased expression of *OsFBK12* showed phenotypes in germination, panicle architecture, and leaf senescence as well as in seed size. Our data suggest that *OsFBK12* directly interacts with OsSAM51 to induce its degradation, which affects ethylene synthesis and histone methylation, leading to pleiotropic phenotypes.

**RESULTS**

**Overexpression and Knockdown of *OsFBK12* Causes Pleiotropic Phenotypes, Including Leaf Senescence**

To explore the network of F-box proteins, an approach using transgenic rice plants as well as molecular interaction was used. The LOC_Os03g07530 gene, located on chromosome 3 (DNA sequence 3832950–3835744), is predicted to encode an F-box protein (http://rice.plantbiology.msu.edu/cgi-bin/ORF_infoPage.cgi?orf=LOC_Os03g07530.1), and based on its homologs (http://rice.plantbiology.msu.edu/ca/gene_fams/1194.shtml), it is termed *OsFBK12*. An unrooted phylogenetic tree shows the relationship of the FBK proteins in rice and Arabidopsis (Supplemental Fig. S1).

*OsFBK12* is predicted to be a protein of 431 amino acids containing an F-box domain (amino acid residues 94–141) at the N terminus and a Kelch repeat motif protein-protein interaction domain (amino acid residues 159–365) at the C terminus.

To investigate the biological function of *OsFBK12*, transgenic lines with reduced or increased expression were generated. We obtained 12 transformed RNA interference (RNAi) lines and 15 overexpression transgenic lines. Real-time PCR analysis confirmed that the transcription of *OsFBK12* was reduced in the RNAi transgenic lines (FR) and increased in the transgenic overexpression lines (FO; Fig. 1D). The FO transgenic lines showed delayed germination and bottle-green leaves under normal growth conditions, whereas the FR lines developed precociously and had light green leaves (Fig. 1, A and C). At the tillering stage, overexpression of *OsFBK12* caused a decrease in tiller number and an increase in plant height compared with the wild type. By contrast, the RNAi (FR) lines displayed increased tiller numbers and decreased plant height (Fig. 1, B, E, and F). At 45 d after heading, leaves of the FR lines became withered and the chlorophyll a/b content showed a faster reduction than in the wild type, which is a signature of earlier senescence. By contrast, the FO lines stayed green, with suppressed chlorophyll reduction and decelerated senescence (Fig. 1, C and G). Overall, alterations in germination and leaf senescence were the most dramatic developmental phenotypes of the transgenic plants.

**OsFBK12 Expression Levels Impact Panicle Architecture and Grain Size**

Transcription pattern analysis showed that *OsFBK12* was expressed in all organs and tissues but predominantly in panicle and seed (Supplemental Fig. S2). Transgenic *OsFBK12p::GUS* rice plants showed strong GUS staining signals in panicle, root tip, young leaf, and leaf sheath but little in mature leaf and stem at the heading stage (Supplemental Fig. S3). Examination of panicle architecture in the FR transgenic lines revealed a significant increase in branch numbers (both primary and secondary) and in the total numbers of spikelets per panicle as well as a slight decrease in panicle axis length (Fig. 2, A, B, and E). Conversely, branching and spikelet number per panicle were reduced in FO lines compared with those in the wild type, while the panicle length was increased (Fig. 2, A, B, and E). In addition, the number of grains and the grain-filling rate (the ratio of seed-filled over total florets) were altered in the transgenic lines (Fig. 2E). The grains of FR lines were a little thinner and shorter than those of the wild type (Fig. 2, C and D), causing a slight decrease in the 100-grain weight (1.71 g) compared with that of the wild type (2.25 g; Fig. 2E). The grains in FO lines were significantly wider and longer than those in the wild type (Fig. 2, C and D), leading the 100-grain weight to increase by 0.7 g (Fig. 2E).

Histological analysis on the spikelet hull showed that the outer parenchyma cell layer in the FO line was...
increased compared with the wild type, while the FR line was reduced (Fig. 3, A–C). Scanning electron microscopy analysis showed that some of the starch granules packed together and appeared "football like"; those football-like starch granules in the middle zone were larger in the FO lines than in the wild type, whereas the starch granules were smaller in the FR lines (Fig. 3D). The cell numbers in the lemma were also different between the wild type and the transgenic lines (Fig. 3E). The FR-4 line showed more cells than in the wild type, whereas the FO-9 line had fewer. There was no difference in cell size in the other zones of the endosperm. Therefore, OsFBK12 may be involved in the regulation of cell division in the hull.

OsFBK12 Interacts with a SKP1-Like Protein in the Nucleus

Yeast two-hybrid assays were performed to screen for proteins that interact with OsFBK12. The entire coding region of OsFBK12 was inserted into the pGBKT7 vector as bait. Positive clones were identified based on both survival on restrictive medium (synthetic dextrose/His/Ade/Trp/Leu) and expression levels of β-galactosidase (lacZ) reporter gene. There were 216 colonies that survived on restrictive medium of His/Ade/Trp/Leu, and 102 of them expressed the β-galactosidase (lacZ) reporter gene. Among the 96 positive clones that were sequenced, 16 corresponded to Oryza sativa S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN (OSK1). To confirm the interaction further, a full-length complementary DNA (cDNA) of OSK1 was used as prey. Fragments of OsFBK12 encoding the F-box domain (OsFBK12Dkelch), the Kelch repeat domain (OsFBK12DF-box), and the full-length cDNA were used as baits. Colonies expressing lacZ were obtained with the F-box domain and full-length OsFBK12, but not with the Kelch repeat domain (Fig. 4A). These results suggest that OsFBK12 interacts with OSK1 through the F-box domain in yeast (Saccharomyces cerevisiae) cells.
In bimolecular fluorescence complementation (BiFC) assays, strong GFP fluorescence was observed when PSPYNE(R)173-OsFBK12 and SPYCE>(MR)-OSK1 were coexpressed in the nuclei of tobacco (Nicotiana benthamiana) leaf epidermal cells (Fig. 4B). Cells transformed with single constructs alone did not show...
Figure 3. Cell number and size of hulls and endosperm in the transgenic lines. A, Grain shape. WT, The wild type; FR-4, OsFBK12 RNAi line 4; FO-9, OsFBK12 overexpression line 9. White lines indicate the positions of the cross sections in B. Bar = 1 mm. B, Cross sections of the hulls. The boxes indicate the regions enlarged in C. Bars = 500 μm. C, Magnified view of the boxes in B. Bars = 50 μm. D, Scanning electron microscopy images of transections of endosperm. I, II, and III represent the outer, middle, and inner parts of the endosperm, respectively. Enlarged regions corresponding to I, II, and III are shown below. The wild type is shown in a, d, g, and j; FR-4 is shown in b, e, h, and k, and FO-9 is shown in c, f, i, and l. White arrows denote football-like starch granules. Bars = 1 mm (a–c) and 20 μm (d–l). E, Statistical analysis of the total length, cell number, and cell length in the outer parenchymal cell layers of the hulls. The data of FR and FO indicate averages of three individual FR lines and two FO lines. Data are means ± se. Student’s t test was performed. Asterisks represent $P < 0.05$ compared with wild-type values.
any fluorescence. Protein localization assays showed that the signal from an OsFBK12-GFP fusion protein overlapped with nuclear H33342 staining in protoplasts (Fig. 4C; Supplemental Fig. S4A) but was not coexpressed with the endoplasmic reticulum marker mCherry-HDEL in rice protoplasts (Supplemental Fig. S5). By contrast, the OSK1-GFP fusion protein was localized in both the nucleus and the cytoplasm (Supplemental Fig. S4B).

OsFBK12 Interacts with OsSAMS1 for Degradation in Plant Cells

Kelch repeat domains in FBKs function in protein-protein interactions, which specify the protein substrates for degradation via the ubiquitin pathway (Sun et al., 2007). We used the Kelch repeat domain in the pGBK7 vector as bait to screen a rice cDNA library in yeast. Of the 167 positive colonies that survived on restrictive medium of −Trp−His−Ade, β-Galactosidase activity of positive clones was analyzed using 5-Bromo-4-chloro-3-indolyl β-o-galactopyranoside. The proved interaction between OsGSR1 (a GA-stimulated transcript family gene in rice) and DWF1 was used as a positive control. B, BiFC assay for interaction between OsFBK12 and OSK1 in tobacco. Shown is the coexpression of Yellow fluorescence protein N-terminal (YN)-OsFBK12 and Yellow fluorescence protein C-terminal (YC)-OSK1 (top row), YN-OsFBK12 and YC vector (middle row), and YC-OSK and YN vector as a control (bottom row). Bars = 100 μm. C, Subcellular localization of OsFBK12-GFP and OSK1-GFP fusion proteins in rice protoplasts. GFP protein alone shows fluorescent signals in nucleus, membrane, and cytoplasm. H33342 is a staining dye for the nucleus. Bars = 10 μm.

Among the 78 positive clones that were sequenced, one encoded SAMS1. Assays in yeast cells using truncation constructs showed that OsSAMS1_222-316 (OsSAMS1 amino acids 222–316) interacted with both full-length OsFBK12 and the Kelch repeat domain truncation (Fig. 5A). In a pull-down assay, purified glutathione S-transferase (GST)-OsSAMS1 was immobilized to glutathione-Sepharose beads, and OsFBK12 tagged with maltose-binding protein (MBP) was incubated with the beads. GST and MBP alone were used as negative controls. A band at 92 kD was recognized by an antibody against MBP in immunoblot assays. By contrast, the negative controls did not show the corresponding signal. This suggests that OsFBK12 can interact with OsSAMS1 in vitro (Fig. 5B). To explore the possibility that OsSAMS1 is the substrate of OsFBK12, OsSAMS1 levels were monitored in the transgenic lines. Immunoblot assays showed that OsSAMS1 was increased in the knockdown transgenic lines of OsFBK12, FR-4, FR-10, and FR-12. By contrast, OsSAMS1 levels were decreased in the OsFBK12 overexpression lines FO-5 and FO-9 (Fig. 5C). Tobacco leaves expressing OsSAMS1-GFP were then treated with...
the 26S proteasome inhibitor MG132, showing that OsSAMS1-GFP was stable in the presence of MG132 for up to 8 h (Fig. 5D), whereas in the control (without MG132 treatment), OsSAMS1 showed a gradual decrease. When purified OsFBK12-myc was added into the extracts, OsSAMS1-GFP degradation was accelerated. Furthermore, we tested the polyubiquitination in the transgenic leaves of OsSAMS1-GFP. The western-blot assay indicated that a series of bands with higher molecular masses were recognized by the ubiquitin antibody in the transgenic leaves of OsSAMS1-GFP (Fig. 5F). Together, these data suggest that OsFBK12 targeted OsSAMS1 for ubiquitination and subsequent degradation by the 26S proteasome.

Similarities between OsFBK12 and OsSAMS1 Transgenic Plants in Germination and Senescence

Germination is suppressed in knockdown transgenic OsSAMS1 rice plants (Li et al., 2011b). This suppression of germination was rescued by supplementation...
Figure 6. Germination and senescence phenotypes of the OsFBK12 and OsSAMS1 transgenic lines. A, Effect of SAM on seed germination in the OsFBK12 transgenic plants. The morphology of seedlings was observed after 40 h for germination. SAM concentration was 1 mM. Bar = 1 cm. Data are means ± SD of triplicate experiments with 30 seeds per sample. B, The time course of germination and the effect of SAM (1 mM). Data are means ± SD of triplicate experiments with 30 seeds per sample. C, Effect of ethephon on seed germination in the OsFBK12 and SAMS1 transgenic plants. The morphology of seedlings was observed after 40 h for germination. Ethephon concentration was 50 μL L⁻¹. Bar = 1 cm. Data are means ± SD of triplicate experiments with 30 seeds per sample. D, The effect of ethephon on ACC and ethylene production in the OsFBK12 and SAMS1 transgenic plants. ACC content was determined by gas chromatography, and ethylene production was measured using a gas chromatograph. Data are means ± SD of triplicate experiments with 30 seeds per sample.
with SAM (1 mM; Fig. 6A). Similarly, in OsFBK12-OX (FO) transgenic plants, there was a block of germination, although the germination patterns were not different between OsFBK12-RNAi (FR) lines and the wild type. The germination suppression in OsFBK12-OX (FO) was also alleviated with SAM treatment (Fig. 6, A and B). The germination suppression of OsFBK12-OX (FO) lines and OsFBK12-RNAi (FR) lines can also be alleviated with 50 μL L⁻¹ ethephon (2-chloroethylsulfonic acid; Fig. 6, C and D). These data are consistent with the idea that altered expression of SAMS caused a corresponding change in the production of SAM and ethylene as well as the resulting alteration of physiological function (He et al., 2006).

SAM is a precursor for ethylene biosynthesis, raising the question of whether ethylene levels were affected by changes in SAM. We found that the ACC and ethylene contents were increased in OsFBK12-RNAi (FR) lines and in the OsSAMS1 overexpression transgenic lines (SO) relative to the wild type. Correspondingly, decreased ACC and ethylene levels appeared in the OsFBK12-OX (FO) plants and OsSAMS1-RNAi transgenic lines (SR) plants (Fig. 6, E and F).

We further monitored the phenotypes of the OsSAMS1 transgenic plants. In the OsSAMS1-OX (SO) transgenic plants, most leaves turned yellow and grain filling was completed by 100 d after germination, when only a few leaves of wild-type plants were yellow and wild-type plants remained in the grain-filling stage. Under the same conditions, the OsSAMS1-RNAi (SR) transgenic plants were still green and not heading yet (Fig. 6G).

A leaf senescence assay showed that the OsFBK12-RNAi (FR) and OsSAMS1-OX (SO) plants displayed faster senescence than wild-type plants, whereas OsFBK12-OX (FO) and OsSAMS1-RNAi (SR) plants maintained green leaves much longer than wild-type plants (Fig. 6H). To determine whether this phenotype is caused by ethylene content in the transgenic plants, we treated the wild-type and transgenic plants with 50 μL L⁻¹ ethephon and 200 mM aminoethoxyvinylglycine (an ethylene biosynthesis inhibitor). Leaf senescence in FR and SO lines was prevented by the biosynthesis blocker, and ethylene treatment promoted the leaf senescence in the FO and SR lines (Fig. 6, H and I). These results suggest that OsSAMS1 promotes plant developmental processes and leaf senescence and that OsFBK12 suppresses them.

DISCUSSION

OsFBK12 Targets OsSAMS1 for 26S Proteasome-Mediated Degradation

F-box proteins are subunits of E3 ubiquitin ligase complexes called SCFs. The F-box motif can bind to SKP1 via the N terminus to form a complex and recognize the target proteins via a protein-protein interaction domain at the C terminus (Sonnberg et al., 2009). Although F-box proteins have been identified to mediate multiple biological processes, less is known about their specific degraded substrates, especially for F-box proteins containing Kelch motifs (Han et al., 2004; Imaizumi et al., 2005; Piao et al., 2009). Our data suggest that OsFKB12, as a Kelch-type F-box protein, might form an SCF complex with SKP1 and bind to OsSAMS1, targeting it for 26S proteasome-mediated degradation.

The well-conserved N-terminal domain of OsFBK12 interacted with OSK1, and its C-terminal Kelch repeat domain interacted with OsSAMS1 (Figs. 4 and 5). The interaction of the full-length proteins (Fig. 5B), along with three lines of biochemical and physiological evidence, support that OsSAMS1 is targeted by OsFBK12 for degradation in the 26S proteasome. First, the degradation of OsSAMS1 was inhibited by treatment with MG132 and promoted by treatment with purified OsFBK12 (Fig. 5, D and E). Second, OsSAMS1 was increased in the OsFBK12 knockdown lines. Conversely, a decrease of OsSAMS1 appeared in the OsFBK12 overexpression transgenic line (Fig. 5C). Third, the germination block in the OsSAMS1-RNAi (SR) transgenic line could be rescued by supplementation with SAM, indicating that SAM production was likely reduced in the OsSAMS1 knockdown lines (Li et al., 2011b). The OsFBK12-OX (FO) transgenic plants showed phenotypes identical to those of OsSAMS1 knockdown lines, supporting that the OsFBK12 promotes the degradation of OsSAMS1 and causes the reduction in SAM (Fig. 6A). Moreover OsSAMS1-GFP was detected in the nucleus and the cytoplasm (Supplemental Figs. S5 and S6), while OsFBK12 was...

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**Figure 6.** (Continued.)

Experiments with 30 seeds per sample. D, The time course of germination and the effect of ethephon (50 μL L⁻¹). Data are means ± SD of triplicate experiments with 15 seeds per sample. E, ACC content in different transgenic plants (using 30-d-old seedlings). Multiple comparisons were performed by Tukey’s honestly significant difference post hoc test. Letters indicate significantly different results. Tests were carried out by the R library multcomp. F, Ethylene content in different transgenic plants (using 30-d-old seedlings). G, Phenotypic comparison of the wild type (WT), the OsSAMS1-RNAi (SR) line, and the OsSAMS1 overexpression (SO) line at 120 d after germination. Bar = 20 cm. H, Detached leaf senescence for 3 d in darkness (using 30-d-old seedlings) with and without treatment of aminoethoxyvinylglycine (AVG) and ethephon. Bar = 20 cm. I, Chlorophyll content of the detached leaves in H. Data are means ± SD of triplicate experiments. FW, Fresh weight. The data of FR and FO indicate averages of three individual FR lines and two FO lines; the data of SR and SO indicate averages of two individual SR lines and two SO lines.
localized in the nucleus; this result hinted that OsSAMS1 may be degraded in the nucleus.

**OsFBK12 Is Involved in Ethylene-Mediated Leaf Senescence**

Ethylene is synthesized from Met via the intermediates SAM and ACC (Bouvier et al., 2006). The conversion of Met to S-adenosylmethionine is catalyzed by SAMS, and the conversion from SAM to ACC is catalyzed by ACC synthase, which are the rate-limiting enzymes in ethylene biosynthesis. Regulation of ethylene biosynthesis occurs at both the gene expression level and the protein activity level (Bouvier et al., 2006). Thus, OsFBK12 can be considered a negative regulator of ethylene biosynthesis by virtue of its function in promoting the degradation of OsSAMS1. Overexpression of OsFBK12 and knockdown of OsSAMS1 caused a decrease in the ethylene-responsive genes OsEATB and OsCTR1 and ethylene production, delaying leaf senescence, whereas overexpression of OsSAMS1 and knockdown of OsFBK12 resulted in increased ethylene levels, promoting early leaf senescence (Fig. 6; Supplemental Fig. S7). Therefore, OsFBK12 is a negative regulator of ethylene-mediated senescence through the degradation of OsSAMS1.

**OsFBK12 Might Regulate Seed Size**

Altered OsFBK12 gene expression led to changes in seed size resulting from changes in the cell number and the size of the football-like granules. Football-like granules is an important part of the completion of starch granule packaging, which is similar to the types of granules that associate with the grain size in wheat endosperm (Xu et al., 2010).

The cell numbers in the lemma of the OsFBK12 over-expression lines were reduced, while the cell length and the football-like granule size were increased (Fig. 3E). This can be explained by the compensatory mechanisms in monocot species (Barrôco et al., 2006). That is, the reduced cell production can be partly compensated by an increased cell size. The cell size in the OsFBK12-OX (FO) lines was compensated through cell expansion.

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**Figure 7.** Proposed working model for the OsFBK12 regulation of leaf senescence and seed size in rice. This model proposes that OsFBK12 was involved in 26S proteasome-mediated degradation by interacting with OSK and targeted the substrate OsSAMS1. When OsSAMS1 degraded, it caused a corresponding change in ethylene level and regulated leaf senescence. Meanwhile, OsFBK12 might target another substrate or might regulate the transcription of some genes to affect seed size. PPi, pyrophosphate; Pi, phosphate; MAT, 5'-methylthioadenosine.
The knockdown of OsFBK12 reduced the cell size during spikelet hull development. Cell production in the OsFBK12-RNAi (FR) lines increased while the spikelet hull was smaller, and the cells size in spikelet hull and the football-like starch granules in the endosperm were restricted and partly compensated by decreased cell size.

OsFBK12 interacts with SAMS1 to regulate leaf senescence. The mRNA levels of OsSAMS1 as well as other OsSAMSs in OsSAMS1-RNAi (SR) have been reported (Li et al., 2011b). The mRNA levels of either OsSAMS1 or OsSAMS2 were decreased in the OsFBK12-OX (FO) transgenic plants, whereas the mRNA level of OsSAMS3 was increased in OsFBK12 RNAi (FR) transgenic plants (Supplemental Fig. S8). This indicated that the transgene of OsFBK12 impacts OsSAMS1 on both the protein interaction and RNA transcription levels, which are both upstream of ethylene biosynthesis. It is notable that SAM is not only a precursor for ethylene biosynthesis but also a universal methyl group donor and involved in numerous transmethylation reactions (Frostesjö et al., 1997; Rocha et al., 2005; Zhang et al., 2011). That SAM also functions as a precursor of polyamines might explain the diverse phenotypes between the lines of OsFBK12-OX (FO) and OsSAMS1-RNAi (SR) except for the consensus ones (Tomosugi et al., 2006; Kusano et al., 2007; Yang et al., 2008).

Based on our data, we propose a model (Fig. 7) for how OsFBK12 regulates senescence. OsFBK12 interacts with OsSK1 to form an SCF complex and degrade its substrate, such as OsSAMS1. The degradation of OsSAMS1 results in a decrease in SAM content as well as in ethylene levels, which affects germination and leaf senescence. Additionally, it is possible that OsFBK12 might affect cell division, resulting in changes in cell numbers in the spikelet hull. These findings may lead to a better understanding of senescence control in rice.

MATERIALS AND METHODS

Plant Germination and Transformation

For seed germination, dehulled rice (Oryza sativa) seeds of different transgenic lines and the wild type were surface sterilized and immersed in water. Seeds were placed in a growth chamber under dark conditions for the first 72 h, and then with a 12/12-h light/dark cycle at 25°C. Germination was determined by the number of plants that had radicle emergence. The SAM treatment experiment was performed according to Li et al. (2011a). That SAM also functions as a precursor of polyamines might explain the diverse phenotypes between the lines of OsFBK12-OX (FO) and OsSAMS1-RNAi (SR) except for the consensus ones (Tomosugi et al., 2006; Kusano et al., 2007; Yang et al., 2008).

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Total RNA Extraction and Real-Time PCR

Total RNA was extracted by use of the TRIzol RNA extraction kit (Invitrogen) and treated with RNase-free DNase I (MBI Fermentas). Total RNA (2 μg) was reverse transcribed into cDNA by AMV Reverse Transcriptase (Promega). Real-time PCR amplification was performed in 20-μL reactions containing 5 μL of 50-fold diluted cDNA, 0.2 μM of each primer, and 10 μL of SYBR Green PCR Master Mix (Toyobo). Quantitative real-time PCR was performed on Mx3000p (Stratagene) using SYBR Green reagent (Toyobo). Expression was normalized to that of ACTIN. Primer sequences used for amplification are listed in Supplemental Table S1.

Vector Construction and Rice Transformation

The entire open reading frame (ORF) of OsFBK12 was amplified by RT-PCR and then inserted upstream of GUS in the binary plasmid pUn1301 (Chen et al., 2011). The pTCK303-OsFBK12 construct was used to create an RNAi knockdown transgenic line. The detailed protocols for construct generation were described previously (Wang et al., 2004). The resulting constructs were used for transformation using Agrobacterium tumefaciens strain EHA105 as described previously (Ge et al., 2004). All primers used in this study are listed in Supplemental Table S1.

Scanning Electron Microscopy

Rice seeds were fixed in 50% (v/v) ethanol, 5% (v/v) acetic acid, and 37% (v/v) formaldehyde for more than 24 h and then dehydrated through a graded series of alcohol-isooamyl acetate. After being critical-point dried in carbon dioxide for 1 h with a Hitachi HCP-2, the plant material was coated with gold and observed with a Hitachi S-4800 scanning electron microscope at 10.0 kV.

Histological Analysis

Rice spikelet hulls were fixed in 50% (v/v) ethanol, 5% (v/v) acetic acid, and 37% (v/v) formaldehyde at room temperature overnight and then dehydrated in an ethanol series, cleared with xylene, and embedded in Paraplast (Sigma). Tissue sections (10 μm thick) were cut and stained with 0.02% (w/v) toluidine blue for 5 min at room temperature after dewaxing. Photographs were taken on an Olympus VANOX microscope.

Yeast Two-Hybrid Assay

The BD Matchmaker library construction and screening kit (Clontech Laboratories) was used for yeast two-hybrid assays. All protocols were carried out according to the manufacturer's user manual. The cDNA encoding the full-length OsFBK12 protein was inserted into the GAL4 DNA-binding domain vector pGBKT7. The pGADT7 clones were selected on synthetic dextrose/-Leu/−Trp/−His/−Ade/5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside plates and sequenced at the Invitrogen Sequencing Facility to ensure that the prey proteins were in-frame fusions with the GAL4 pGADT7 Vector domain using the system described previously (Wang et al., 2009a).

Pull-Down Assay

To test the interaction between OsFBK12 and OsSAMS1, the ORF of OsSAMS1 was cloned into the pGEM4T-1 vector as a GST fusion protein, and OsFBK12 was subcloned into the plasmid pMAL-c2 to allow the expression of OsFBK12 as a fusion with MBP in Escherichia coli. Expression of GST fusion proteins and in vitro binding experiments were performed as described previously (Wang et al., 2009a).

BiFC and Protein Degradation Assays in Tobacco

BiFC assays followed the described protocol (Waadt et al., 2008). For BiFC assays, the ORF of OsFBK12 was cloned into the PSPYNE(R173 vector and the ORF of OsSK1 was cloned into the PSPYCE(MR) vector. The plasmids were electroporated into A. tumefaciens strain GV3101 and coinfected into tobacco (Nicotiana benthamiana) leaves (Liu et al., 2010). GFP fluorescence was visualized with a confocal scanning microscope after infiltration for 48 to 72 h.

The coinfected tobacco leaves were used in the in vivo assays of OsSAMS1 protein stability. For protein degradation assays, full-length OsSAMS1 (OsS5g0135700) fused with GFP was transformed into tobacco leaves. Three days after infiltration, the OsSAMS1-GFP sample, the wild type, and the coinfected PSPYNE(R173-OsFBK12 and PSPYCE(MR)-OSK1 were separately extracted as described in NaCl-free native extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 mM Suc, 1 mM MgCl2, 10 mM EDTA, 5 mM dithiothreitol, and protease inhibitor cocktail tablets). A final concentration of 10 μM ATP was added to preserve the function of the 26S proteasome. The...
OsSAMSI-GFP extract was divided into three parts: the first part was mixed with PSYNE(R)1173-OsFBK12 and SPYCE(MR)-OSK1, the second part was mixed with the wild-type extract, and the third part was mixed with MG132 to a final concentration of 50 μM. The mixtures were incubated at 4°C with gentle shaking. Samples were removed at different time points, and quantification and normalization were carried out according to the previously described protocol (Zhang et al., 2011).

ACC and Ethylene Measurements
ACC was extracted from 1.5 g of leaves in 80% (v/v) ethanol and centrifuged, and the supernatant was evaporated to dryness. The residue was resuspended in water, and the ACC content was determined following conversion to ethylene as described previously (Compeçon et al., 1979).

Leaves were cut into 10-cm pieces, and 10 pieces were placed in a 50-mL flask containing distilled water. After incubation at 28°C for 60 h, a 1-mL gas sample was withdrawn by syringe from the head space of each bottle and the ethylene concentration was measured by gas chromatography (Shimadzu GC-2014C) on a device equipped with an activated alumina column and flame ionization detectors. Separations were carried out at 50°C, using N2 as the carrier gas, and the ethylene peak was detected with a flame ionization detector. The peak area was integrated and compared with an eight-point standard ethylene curve. Ethylene standards from 0.01 to 5 μL L−1 were used for the calibration. The quantified data, divided by fresh weight and time, were converted to specific activities.

Chlorophyll Content
Chlorophyll was extracted from 50-mg leaf samples in 10 mL of 95% (v/v) ethanol for 16 h in the dark and measured spectrophotometrically at 660 nm using the previously described protocol (Inskoop and Bloom, 1985).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AC144991 (OsFBK12), AP008217 (OSK1), and AP008211 (OsSAMSI).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Unrooted phylogenetic tree of FBK protein in rice and Arabidopsis.

Supplemental Figure S2. OsFBK12 expression from the eFP browser and Gene Investigator.

Supplemental Figure S3. Analysis of the expression pattern of OsFBK12 in rice.

Supplemental Figure S4. Subcellular localization of OsFBK12-GFP and OSK1-GFP fusion proteins in onion epidermal cells.

Supplemental Figure S5. OsSAMSI-GFP and OSK-GFP are co-expressed with the endoplasmic reticulum marker mCherry-HDEL in rice protoplasts.

Supplemental Figure S6. Subcellular localization of OsSAMSI in onion epidermal cells.

Supplemental Figure S7. RT-PCR analysis of the expression of ethylene-induced genes in OsFBK12 and OsSAMSI transgenic seedlings.

Supplemental Figure S8. RT-PCR analysis of the expression of OsSAM genes in OsFBK12 transgenic rice.

Supplemental Table S1. Sequences of primers used in this study.

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LITERATURE CITED

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