The eukaryotic translation initiation factor 5A (eIF-5A) is a highly conserved protein found in all eukaryotic organisms. Although originally identified as a translation initiation factor, recent studies in mammalian and yeast (Saccharomyces cerevisiae) cells suggest that eIF-5A is mainly involved in RNA metabolism and trafficking, thereby regulating cell proliferation, cell growth, and programmed cell death. In higher plants, the physiological function of eIF-5A remains largely unknown. Here, we report the identification and characterization of an Arabidopsis (Arabidopsis thaliana) mutant fumonisin B1-resistant12 (fbr12). The fbr12 mutant shows an antiapoptotic phenotype and has reduced dark-induced leaf senescence. Moreover, fbr12 displays severe defects in plant growth and development. The fbr12 mutant plant is extreme dwarf with substantially reduced size and number of all adult organs. During reproductive development, fbr12 causes abnormal development of floral organs and defective sporogenesis, leading to the abortion of both female and male germline cells. Microscopic studies revealed that these developmental defects are associated with abnormal cell division and cell growth. Genetic and molecular analyses indicated that FBR12 encodes a putative eIF-5A-2 protein. When expressed in a yeast mutant strain carrying a mutation in the eIF-5A gene, FBR12 cDNA is able to rescue the lethal phenotype of the yeast mutant, indicating that FBR12 is a functional eIF-5A. We propose that FBR12/eIF-5A-2 is fundamental for plant growth and development by regulating cell division, cell growth, and cell death.

The eIF-5A protein was initially identified as a translation initiation factor from rabbit reticulocytes in an in vitro assay (Kemper et al., 1976). The eIF-5A function in protein translation is partly supported by the observation that the putative translation initiation factor interacts with the ribosomal protein L5 (Schatz et al., 1998) and the translating 80S ribosomal complex (Jao and Chen, 2006). However, accumulating evidence suggests that eIF-5A is not required for protein synthesis in vivo because of the lack of correlation between eIF5A and the general protein synthesis (Park et al., 1997; Thompson et al., 2004). More recently, eIF-5A was found to function as a regulator of p53 and p53-dependent apoptosis (Li et al., 2004). At the cellular level, Hpu-containing eIF-5A has been shown to be involved in the control of cell proliferation and apoptosis (Park et al., 1997; Thompson et al., 2004). 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et al., 1993, 1997; Kang and Hershey, 1994). Instead, Hpu-containing eIF-5A has been shown to stabilize mRNA and transport of specific subsets of mRNA from the nucleus to the cytoplasm (Bevec and Hauber, 1997; Zuk and Jacobson, 1998). In yeast, a temperature-sensitive mutant ts1159, which carries a mutation in the eIF-5A/TIF51A gene, causes the accumulation of uncapped mRNAs at the nonpermissive temperature, suggesting that eIF-5A is critical for mRNA turnover, possibly acting downstream of decapping (Zuk and Jacobson, 1998). The involvement of eIF-5A in the translocation of RNA has also been documented (Bevec and Hauber, 1997; Rosorius et al., 1999; Caraglia et al., 2001). In agreement with these observations, the solved crystal structure of eIF5A is characteristic of some RNA-binding proteins (Murzin, 1993), such as cold shock protein CspA (Schindelin et al., 1994) and the Escherichia coli translation initiation factor IF1 (Sette et al., 1997). Moreover, binding of eIF-5A to RNA requires Hpu on the protein and appears to be sequence specific to two core motifs of the targets (Xu and Chen, 2001). On the other hand, eIF5A was also found to interact with distinctive cellular proteins, including the exportin protein CHROMOSOME REGION MAINTENANCE1 (Rosorius et al., 1999), exportin 4 (Lipowsky et al., 2000), nucleoporins (Hofmann et al., 2001), tissue transglutaminase II (Singh et al., 1998), syntenin (Li et al., 2004), DHS, and Lia1 (for ligand of eIF5A; Thompson et al., 2003). On the basis of these observations, eIF5A was proposed to function as a bimodular protein capable of binding to both RNA and proteins (Liu et al., 1997; Jao and Chen, 2006), thus involved in multiple aspects of cellular signaling activities.

In plants, genes encoding eIF-5A and DHS have been cloned from several species. Similar to its mammalian and yeast counterparts, a tomato (Lycopersicon esculentum) eIF-5A recombinant protein can be deoxyhypusine modified by a DHS recombinant protein (Wang et al., 2001). Whereas direct functional analysis of eIF-5A in planta has not been reported, suppression of DHS expression, which presumably causes partial inactivation of eIF-5A, has gained important information on the eIF-5A function. In Arabidopsis (Arabidopsis thaliana), constitutive overexpression of an antisense DHS cDNA causes delayed senescence and resistance to drought stress (Wang et al., 2003). Similarly, in tomato, overexpression of an antisense DHS construct results in delayed fruit softening and leaf senescence.

**Figure 1.** The fbr12 mutant shows an FB1-resistant phenotype and reduced PR gene expression. A, Two-week-old seedlings of wild type (Ws) and fbr12 germinated and grown in Murashige and Skoog medium in the absence or presence of 0.8 μM FB1. Bar = 5 mm. B, Cell death induced by FB1 in wild-type and fbr12 plants. Three-week-old seedlings were treated with dimethyl sulfoxide (DMSO; 0.05%; control) or 2 μM FB1 for 24 h. Leaves were collected and stained with Trypan blue. Black staining represents dead or dying cells. C, Nuclear DNA fragmentation induced by FB1 in wild type and fbr12. Protoplasts prepared from wild-type and fbr12 leaves were treated with DMSO (0.002%; control) or 50 nM FB1 for 12 h and then analyzed by TUNEL staining as described in "Materials and Methods." TUNEL-positive cells were counted under a fluorescent microscope (n > 1,000 in each experiment). Data presented were mean values of three independent experiments. Bars represent S.E. D, FB1-induced expression of PR1 and PR5 genes. Total RNA was prepared from 3-week-old seedlings treated with 3 μM FB1 for various times as indicated and used for northern-blot analysis. Each lane contains 15 μg of RNA. The blot was hybridized with a full-length PR1 or PR5 cDNA probe, respectively.
(Wang et al., 2005). In addition, because expression of both DHS and eIF-5A appears to be correlated with senescence or stress (Wang et al., 2001, 2003, 2005), it has been proposed that different isoforms of eIF-5A may facilitate the translation of mRNAs required for cell division and cell death, thereby regulating plant growth and development (Thompson et al., 2004). In this study, we report functional characterization of an Arabidopsis eIF-5A gene. A loss-of-function mutation in the eIF-5A-2 gene causes severe developmental defects throughout the life cycle, characteristics of abnormal cell division, cell growth, and cell death. These results demonstrate the biological importance of eIF-5A in plant growth and development.

RESULTS

Identification and Genetic Analysis of the fumonisin B₁-resistant12 Mutant

The fumonisin B₁-resistant12 (fbr12) mutant was identified from a genetic screen of a T-DNA-mutagenized population of approximately 5,000 independent lines in the pga22 mutant background (Sun et al., 2003,

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**Figure 2.** Defective growth and development in fbr12. A, Four-day-old seedlings of wild type (Ws; left) and fbr12 (right) germinated and grown in Murashige and Skoog medium under continuous white light. No substantial difference between wild type and fbr12 is observed at this stage. Bar = 1 mm. B, Ten-day-old seedlings of wild type (Ws; left) and fbr12 (right) germinated and grown in Murashige and Skoog medium under continuous white light. Bar = 5 mm. C, Four-week-old plants of wild type (Ws; left) and fbr12 (right) germinated and grown in soil under continuous white light. Bar = 5 mm. D, Rosette leaves collected from 4-week-old plants of wild type (Ws; top) and fbr12 (bottom) germinated and grown in soil under continuous white light. Bar = 5 mm. E, Seven-week-old plants of wild type (Ws; left) and fbr12 (right) germinated and grown in soil under continuous white light. Bar = 2 cm. F, Enlarged view of an fbr12 plant shown in E. The plant was germinated and grown in soil under continuous white light for approximately 7 weeks. Bar = 2 cm. G, Comparison of wild-type (left) and fbr12 (right) floral inflorescences derived from 6-week-old plants germinated and grown in soil under continuous white light. Bar = 2 mm. H, Analysis of growth rates of adult organs of wild-type and fbr12 plants germinated and grown in soil under continuous white light. Leaf initiation rate was calculated as the number of new leaves produced per day between the second and the seventh true leaves. Total number of leaves refers to rosette leaves 40 d after germination. Total number of flowers refers to flowers at stage 12 and above (flower development stages are defined according to Sanders et al. [1999]). At least 30 plants were analyzed in each experiment and average values were shown. Bars = ses.
The pga22 mutant (Wassilewskija [Ws] accession) carries an estradiol-inducible T-DNA transcription activation tag (Zuo et al., 2000) upstream from the ISOPENTENYL TRANSFERASE8 (AtIPT8/PGA22) gene. Expression of the AtIPT8/PGA22 gene is highly inducible by the chemical inducer estradiol, which causes overproduction of cytokinin in planta. However, the pga22 mutant shows a phenotype indistinguishable from wild-type plants in the absence of estradiol (Sun et al., 2003). We have generated a T-DNA insertion population by transforming pga22 mutant plants with a second binary vector pTA231 (Sun et al., 2005). To screen for mutants, pooled T2 seeds (20 lines/pool) were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.8 μM fumonisin B1 (FB1). The fbr12 mutant was identified from this screen and showed strong resistance to FB1 (Fig. 1A). The original mutant (T3) was backcrossed with the wild type (Ws) twice and the resulting F2 or F3 progeny that did not contain the pga22 mutation were used for all experiments described below except for initial genetic analysis.

Because fbr12 was infertile (see below), we crossed putative fbr12/+ plants (in the pga22 background) with the wild type (Ws). In F2 progeny derived from self-pollinated F1 plants, the FB1-resistant phenotype segregated in a 1:3 ratio (FB1 resistant:sensitive = 72:22; x^2 = 3.58; P < 0.05). These results indicate that the fbr12 mutation is recessive in a single nuclear locus. Because of lethality of the mutation, fbr12 was maintained as heterozygous.

The fbr12 Mutation Alters FB1-Mediated Programmed Cell Death

To investigate the cellular and molecular alterations induced by FB1 in fbr12, we first compared toxin-induced cell death by Trypan blue staining. In wild-type leaves, FB1 induced massive cell death. However, substantially reduced cell death was observed in fbr12 leaves (Fig. 1B). In some cases, no cell death was found in the mutant leaves treated by FB1, (data not shown). We next examined nuclear DNA fragmentation, a molecular hallmark of apoptotic cells, in protoplasts treated by FB1, using the TUNEL method. In untreated protoplasts derived from both the wild type and fbr12, TUNEL-positive signal was rarely detected. However, upon FB1 treatment, whereas more than 90% of wild-type protoplasts showed TUNEL-positive signals, <25% of fbr12 protoplasts displayed distinctive positive signals under the identical assay conditions (Fig. 1C). At the molecular level, FB1 is known to induce the expression of PATHOGEN-RELATED (PR) genes, and the induction is compromised in fbr1 and fbr2 mutants (Stone et al., 2000). Similar to that of fbr1 and fbr2, FB1-induced expression of PR1 and PR5 was reduced in fbr12 (Fig. 1D). Collectively, data presented in Figure 1

**Figure 3.** Developmental defects in the fbr12 floral organs. A, Comparison of wild-type and fbr12 flowers at different development stages. Bar = 5 mm. B, Comparison of wild-type and fbr12 siliques. Bar = 5 mm. C, Reduced numbers of floral organs in the fbr12 mutant. The x axis indicates distribution of floral organ numbers in the examined fbr12 flowers (n = 105). Floral organ numbers of wild-type flowers are marked by asterisks above corresponding bars.
suggest that FB₁-elicited PCD and/or defense response is altered by the fbr₁₂ mutation.

**The fbr₁₂ Mutation Causes Pleiotropic Phenotype during Plant Growth and Development**

The fbr₁₂ mutant phenotype became apparent shortly after germination. Compared to the wild type, fbr₁₂ was substantially smaller and more slender, with significantly shorter roots. However, all embryonic organs, including roots, hypocotyls, and cotyledons, appeared to be well defined (Fig. 2, A and B). The initiation of true leaves was substantially delayed in fbr₁₂ (Fig. 2B). During later growth stages, fbr₁₂ displayed a stunted phenotype (Fig. 2, C, E, and F), producing less rosette and cauline leaves that were smaller than those of the wild type (Fig. 2, D and E). In addition, fbr₁₂ plants also had few flowers that were abnormally developed (Fig. 2, F and G). Overall, fbr₁₂ affected the growth rate and organogenesis characteristics of dwarfism, a reduced leaf initiation rate and reduced numbers of adult organs (Fig. 2H).

In fbr₁₂, development of the floral organs was severely affected by the mutation. Compared to the wild type, fbr₁₂ plants produced fewer flowers (Fig. 2, G and H) in which all floral organs displayed various developmental defects. In general, all fbr₁₂ floral organs were smaller than those of the wild type (Fig. 3A). Compared to that of the wild type, sepals in fbr₁₂ flowers were often misshapen and occasionally fused together. Petals in fbr₁₂ flowers were smaller than that of the wild type (Fig. 3A). Stamens and stigmas appeared to be morphologically normal. However, two lateral stamens were usually absent in the mutant and the gynoecium stigmatic papillae was shorter than that of the wild type (see also below for more details on germline cell development). In wild-type Arabidopsis, a flower consists of four sepals, four petals, six stamens, and a carpel. Whereas a carpel was often found in fbr₁₂ flowers,

**Figure 4.** Delayed development of the SAM in fbr₁₂. A to E, Light microscopy of longitudinal sections of wild-type and fbr₁₂ seedlings. A, Four-day-old wild-type seedling. B, Six-day-old wild-type seedling. C, Four-day-old fbr₁₂ seedling. The dome-structured SAM is smaller than that of wild type in the same stage shown in A, although apparent morphological alterations are not observed at this stage (see also Fig. 2A). D, Six-day-old fbr₁₂ seedling. Compared to wild type at the same developmental stage shown in B, delayed initiation of true leaf projections is observed. E, Nine-day-old fbr₁₂ seedling showing a development stage equivalent to that of 6-d-old wild-type seedlings shown in B. F to J, Scanning electron microscopy of the SAMs of wild type and fbr₁₂. F, Four-day-old wild-type seedling. G, Six-day-old wild-type seedling. H, Four-day-old fbr₁₂ seedling. I, Six-day-old fbr₁₂ seedling showing a phenotype similar to that of 4-d-old wild type in F. J, Nine-day-old fbr₁₂ seedling that appears to be in a developmental stage equivalent to that of 6-d-old wild-type seedlings shown in G. Bar = 20 μm (A–E) and 50 μm (F–J).
other floral organ components had significantly reduced numbers in the mutant compared to the wild type (Fig. 3C). In particular, the number of petals and stamens was altered more dramatically by the fbr12 mutation, with <30% and 10% of flowers producing correct numbers of these organs, respectively (Fig. 3C). Approximately 50% of flowers formed the correct number (four) of sepals. As a result of these defects, fbr12 siliques were markedly shorter and did not contain any seeds (Fig. 3B).

The fbr12 Mutation Affects Cell Proliferation and Cell Growth

To investigate the cellular basis of the fbr12 mutant phenotype, we analyzed the mutant by microscopy. Light microscopy revealed that the structure of the shoot apical meristem (SAM) was unaffected in fbr12, but SAM development was delayed compared to that of the wild type (Fig. 4, A–E). Notably, 9-d-old fbr12 SAM appeared to be equivalent to that of 6-d-old wild type. A similar observation was made by scanning electron microscopy (Fig. 4, F–J). These results suggest that the fbr12 mutation may cause a slow division or growth rate of the meristem cells.

Transverse sections of stems revealed that fbr12, similar to that of the wild type, contained three distinctive layers of cell files that include, from outside to inside, the epidermis, cortex, and central cylinder. Development of epidermal cells appeared to be unaffected in the fbr12 mutant. This result suggests that radial patterning remains relatively normal in fbr12. In
the cortex, however, cells were substantially enlarged in fbr12 compared to the wild type (Fig. 5, A–D). By contrast, the mutant had smaller cells in the central cylinder than those of the wild type (Fig. 5, A–D). The central cylinder contained the vascular bundles consisting of phloem and xylem. In xylem, fbr12 had an increased number of cells that were smaller, presumably representing a group of incompletely differentiated cells. Compared to that of the wild type, no distinctive cell layers were observed in the fbr12 phloem. Moreover, both the number and the size of the phloem cells were greatly reduced (Fig. 5, C and D). Quantitative analysis also indicated that fbr12 had increased cell numbers in xylem and parenchyma, but reduced cell numbers in phloem and cortex (Fig. 5E). Scanning electron microscopy indicated that, in fbr12 petals, whereas the cell number remains unaltered, the cell size is smaller than that of the wild type (Fig. 6, A and B). Quantitative analysis of cell numbers in petals revealed that the total cell numbers remained nearly unaltered, but the cell size was reduced in fbr12 (Fig. 6C), thus causing smaller petals. Collectively, these observations indicate that the fbr12 mutation affects cell proliferation, cell growth, and cell differentiation in both vegetative and reproductive organs/tissues.

**FBR12 Is Essential for Male and Female Sporogenesis**

The fbr12 mutant could grow and develop into mature plants with significantly smaller size, which could not set any seeds. Reciprocal crosses between the wild type and fbr12 did not yield any F1 seeds, suggesting that the mutant was both male and female sterile. To reveal the cellular basis of the sterile phenotype, we followed the entire reproductive developmental stages by light microscopy and scanning electron microscopy. During male germline cell development, no apparent defects were found in fbr12 before stage 7 (another development stages were defined according to Sanders et al. [1999]). In the wild type, at this stage, microspores are generated from pollen mother cells by meiosis and are confined as tetrads in a wall rich in callose, which, in turn, are contained in tapetum (Fig. 7A). In fbr12, tetrads appeared to be correctly generated (Fig. 7D). At stage 8, a wild-type pollen sac contains three layers of well-defined cell files, from outside to inside, epidermis, middle layer, and the tapetum. Inside the wild-type pollen sac, microspores are released from tetrads (Fig. 7B). At the same developmental stage, the middle layer and tapetum of the fbr12 pollen sac became disorganized with excessive numbers of cells that are irregularly shaped and stained darker compared to those in the wild type (Fig. 7, B and E). More severe defects were observed in later stages. In contrast to that of the wild type, no vacuole formation was found in fbr12 microspores; instead, microspores appeared to collapse and eventually formed dark-stained debris inside the disorganized tapetum (Fig. 7, C and F). Consequently, in mature anthers, no viable pollen grains were found in fbr12 by either toluidine blue O staining (Fig. 7, G and H) or scanning electron microscopy (Fig. 7, I–K).
Figure 7. Male-sterile phenotype of the fbr12 mutant. A to F, Light microscopy of cross-sections of pollen sacs derived from wild-type (A–C) and fbr12 (D–F) anthers. The images show anthers at different developmental stages (anther developmental stages are defined according to Sanders et al. [1999]): stage 7 (A and D), stage 8 (B and E), and stage 9 (C and F). At stage 7, no apparent developmental abnormality was observed in pollen sacs of fbr12 (D) compared to that of wild type (A). After entering stage 8, the tapetum of fbr12 had an excess of cell layers that encompassed degenerated pollen grains (E and F). E, Epidermis; En, endothecium; ML, middle layer; MSp, microspores; T, tapetum; Tds, tetrads. Bar = 10 μm. G and H, Anthers of wild-type (G) and fbr12 (H) plants stained by toluidine blue O. Mature pollen grains are stained as dark blue. Bar = 2 mm. I to K, Scanning electron microscopy of anthers of wild type (I) and fbr12 (J) and K). Note the empty pollen sacs in fbr12 anthers. Bar = 40 μm.
During female gametophyte development, the *fbr12* mutation also appears to express after meiosis, approximately at stage 2-III (ovule development stages are defined according to Schnetzi et al. [1995]). In the wild type at this stage, meiosis is completed to give rise to four megaspores of which only one survives and undergoes three rounds of nuclear divisions to eventually form a mature embryo sac. Meanwhile, the developing ovule grows toward the septum and the base of the carpel, whereas both the inner and outer integuments are initiated (Fig. 8, A and G). In *fbr12*, no apparent defects were observed at this stage and a megaspore appeared to be normally formed in the ovule (Fig. 8, D and H). In the wild type, whereas the inner integument continues to grow symmetrically, the outer integument starts asymmetric growth throughout development (Fig. 8B), eventually leading to the formation of a hook-shaped, mature embryo sac (Fig. 8, C and G). By contrast, both inner and outer integuments stopped further growth in *fbr12* ovules (Fig. 8, E and H). Therefore, ovule development was completely arrested at this stage.

We have also followed the entire reproductive development in *fbr12/+* heterozygous plants. An *fbr12/+* plant should give rise to haploid germline cells carrying a mutant or a wild-type allele, with approximately 50% of each genotype, respectively. On the other hand, the diploid sporophytic tissues (e.g. tapetum and ovules) should normally be developed because of the recessive nature of the mutation. No abnormally developed germ line cells were observed in *fbr12/+* plants, which showed no difference in fertility compared to wild type (data not shown). Moreover, in progeny derived from self-pollinated *fbr12/+* plants, the mutation was segregated in a 1:3 ratio (mutant: wild-type; see above) characteristic of sporophytic mutations (Yang et al., 1999; McCormick, 2004). These results suggest that the *fbr12* mutation only affects sporogenesis, but not gametogenesis. Similarly, no abnormality was observed in developing embryos of *fbr12/+* plants. Taken together, these results suggest that *FBR12* is essential for sporophytic development, but dispensable for gametogenesis and embryogenesis, and that defective gametogenesis is likely caused by abnormal development of sporophytic tissues in the *fbr12* mutant.

Molecular Cloning of the *FBR12* Gene

The *fbr12* mutant was identified from a T-DNA-mutagenized population and the mutant genome appears to contain a single T-DNA insertion. We identified the genomic sequences flanking the left border by thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995). DNA sequencing analysis indicated that the left border was inserted in the coding sequence region of At1g26630, 9 bp upstream from the stop codon, whereas the right border faced the 5'-end of the gene (Fig. 9A). Northern-blot analysis did not reveal any expression of At1g26630 in the mutant plants (Fig. 9B), suggesting that *fbr12* is likely a null mutation.

To verify the identity of *FBR12*, we carried out a molecular complementation experiment. A 2.5-kb wild-type genomic DNA fragment, which encompassed the promoter region, 5'-untranslated region (UTR), the coding sequence, and part of the 3'-UTR of At1g26630, was cloned into binary vector pE8 (Zuo et al., 2000). The resulting construct was then transformed into *fbr12/+* heterozygous plants via *Agrobacterium tumefaciens*-mediated transformation (Bechtold et al., 1993). Multiple independent transgenic lines were obtained by double selection with BASTA (carried by the *fbr12* mutant) and hygromycin (carried by pE8). In the 20 tested T2 lines, all hygromycin-resistant plants displayed normal phenotype. In T3 lines derived from BASTA- and hygromycin-resistant T2 plants, we analyzed four families that did not show segregation of either the selective markers (BASTA and hygromycin) or the *fbr12* phenotype. PCR analysis revealed that all tested plants (24) were homozygous for the T-DNA insertion at the At1g26630 locus. In these transgenic plants, the transgene fully rescued the developmental defects (Fig. 9, D and F) and restored the sensitivity of the mutant to FB1 (Fig. 9E). These data suggest that the At1g26630 transgene was able to fully complement the *fbr12* mutant phenotype, thus representing *FBR12*.

**FBR12 Encodes a Functional eIF-5A-2**

Database search identified a full-length *FBR12* cDNA clone (accession no. NM_102425). Comparison of the cDNA and genomic sequences revealed an open reading frame (ORF) interrupted by four introns (Fig. 9A). The ORF encodes a polypeptide of 159 amino acid residues, with a predicted molecular mass of 17.1 kD and a pi of 5.8. Sequence comparison revealed that *FBR12* encodes a putative eIF-5A-1 (accession no. NP_173985; also annotated by The Arabidopsis Information Resource [TAIR]) or eIF-5A-2 (accession nos. Q93VP3 and BE039424; Thompson et al., 2004). Hereafter, we refer to this protein as FBR12 or eIF-5A-2 according to Thompson et al. (2004).

Consistent with the pleiotropic phenotype of *fbr12*, *FBR12* appears to be ubiquitously expressed in all examined tissues and organs (Fig. 9C). Members of the eIF-5A family are highly conserved across different kingdoms of eukaryotic organism cells (Jenkins et al., 2001). The Arabidopsis genome contains two additional *FBR12/eIF-5A-like* genes, At1g69410 and At1g13950, which encode proteins sharing 86% and 82% identity with *FBR12*, respectively. In addition, *FBR12* shares significant homology with representative eIF-5A proteins from other species, including rice (*Oryza sativa*; 79%; accession no. AAK1617679), mammals (human and mouse; 51%–54%), *Drosophila* (54%; accession no. AAG17032), *Caenorhabditis elegans* (55%; accession no. NP_499152), yeast (57%; accession no. NP_012581), and fission yeast (*Schizosaccharomyces pombe*; 52%; accession no. CA16195).

To functionally characterize *FBR12*, we carried out a genetic complementation experiment in a yeast mutant.
strain PSY1249. This strain carries a temperature-sensitive mutation in an eIF-5A gene TIF51A that allows the mutant to grow only under the permissive temperature (25°C; Valentini et al., 2002). An FBR12 cDNA containing the entire ORF was cloned into a yeast expression vector under the control of the GAL1 promoter that can be induced by Gal, but repressed by Glc. The resulting construct pYES2-FBR12 was transformed into PSY1249. The transformed PSY1249 cells were able to grow at the nonpermissive temperature (36°C) in the presence of the Gal inducer, but not the Glc repressor (Fig. 10). Therefore, inducible expression of FBR12 cDNA is able to rescue the growth defects of PSY1249 caused by a mutation in the yeast eIF-5A gene. This result demonstrates that FBR12 is a functional eIF-5A.

The fbr12 Mutation Delays Dark-Induced Leaf Senescence

Previous studies showed that overexpression of antisense DHS in Arabidopsis and tomato resulted in delayed leaf senescence. Because DHS is essential for hypusination of eIF5A proteins, this transgenic phenotype was attributed to the inactivation of eIF5A proteins by knocking down DHS expression (Wang et al., 2003, 2005). To investigate the possible role of FBR12/eIF5A-2 in regulating senescence, we compared dark-induced leaf senescence in mutant and wild-type plants.

Fully expanded leaves detached from fbr12 and wild-type plants were placed in the dark and their senescence rates were analyzed. Under assay conditions, leaves derived from wild-type plants showed an
apparent dark-induced senescence syndrome at day 7 and became completely bleached at day 10. Under the identical assay conditions, leaves derived from fbr12 mutant plants displayed a slower senescence rate (Fig. 11A). To quantitatively analyze the senescence rate, we measured the chlorophyll levels in fully expanded leaves of wild type and fbr12. Under normal growth conditions, the chlorophyll level was slightly lower in fbr12 leaves compared to that of the wild type. However, dark treatment caused a greater loss of chlorophyll in wild-type leaves (37% at day 4, 47% at day 7, and 80% at day 10) compared to that of fbr12 mutant leaves at the same stages (17% at day 4, 33% at day 7, and 48% at day 10; Fig. 11B). Taken together, these results suggest that FBR12/eIF5A-2 is involved in the regulation of senescence-type PCD in Arabidopsis.

**DISCUSSION**

In this study, we present evidence showing that the Arabidopsis FBR12 gene encodes a functional eIF5A that is involved in the regulation of cell proliferation, cell growth, and cell death. FBR12 shares significant homology with eIF-5A proteins characterized across different kingdoms. Moreover, an FBR12 cDNA clone was able to complement the temperature-sensitive lethal phenotype of yeast PSY1249 cells carrying a mutation in the TIF51A/eIF-5A gene. These results demonstrate that FBR12 is a functional eIF-5A.

The eIF-5A protein was originally characterized as a component of the translation initiation complex. However, recent studies suggest that this class of highly conserved proteins is involved in RNA metabolism and RNA trafficking (Bevec and Hauber, 1997; Zuk and Jacobson, 1998; Thompson et al., 2004), although very limited evidence is available for the proposed biochemical function of eIF-5A proteins. Nevertheless, because of its fundamental cellular function, eIF-5A has been shown to be required for growth and development in several organisms. In both yeast and mammalian cells, eIF-5A is essential for cell proliferation (Park et al., 1993, 1997; Kang and Hershey, 1994). In C. elegans, two copies of eIF-5A homologs, IFF-1 and IFF-2, were identified. Knockout of iff-2 results in slow growth.
larval growth and disorganized somatic gonadal structures in hermaphrodites, whereas lack of IFF-1 activity causes sterility with underproliferated germline cells. Double mutants of iff-1/iff-2 displayed a slightly stronger phenotype than single mutants, but did not affect the viability of the animal (Hanazawa et al., 2004). In Arabidopsis, we found that a loss-of-function mutation in the FBR12/eIF-5A-2 gene causes more severe defects, including an underdeveloped SAM, reduced sizes and numbers of all adult organs, defective development of floral organs, and abnormal sporogenesis. Nevertheless, the loss of eIF-5A activity in both organisms shows some phenotypical similarities characteristic of slow growth and defects in reproductive development. On the other hand, we notice that gametogenesis and embryogenesis appear to be unaffected by the fbr12 mutation, suggestive of the presence of germline- and/or embryo-specific eIF-5A activity. A similar view is also shared by Thompson et al. (2004), who proposed that different isoforms of eIF-5A function distinctively in the regulation of cell divisions and cell death. This notion is reinforced by the observation that a null mutation in an Arabidopsis homologous gene eIF-5A-3 (SALK_022515) does not have substantial effects on plant growth and development (H. Feng, J. Feng, and J. Zuo, unpublished data). These results suggest that FBR12 represents major eIF-5A-2 activity during plant growth and development.

The extreme dwarf phenotype of the fbr12 mutant may be caused by reduced cell division and/or cell growth. Microscopic studies suggest that both cell division and cell growth are affected by the fbr12 mutation. However, the regulatory roles of FBR12 in cellular activity appear to be cell type- or tissue-specific with distinctive mechanisms. In stem development, for example, no apparent abnormality was observed in the epidermal cell layer, but various defects were found in the cortex and the central cylinder. More strikingly, the FBR12 gene appears to function differently, characteristically by inhibiting cell growth in the cortex, but promoting cell growth in the central cylinder. In a similar mode, the fbr12 mutation causes increased cell numbers in xylem and parenchyma, but reduced cell numbers in phloem and cortex. These observations suggest that FBR12 regulates cell division and cell growth in a tissue- and development-specific manner. We notice that fbr12 shows some phenotypic similarity with fbr6, whose wild-type allele encodes a transcription activator AT5SPL14 (Stone et al., 2005). In particular, both fbr6 and fbr12 affect vascular tissue development, suggesting that they may act in a linear pathway. It will be interesting to analyze the possible interaction of these two loci.

In addition to its role in cell division and cell growth, FBR12 also appears to play a role in regulating cell death. Similar to other fbr mutants, fbr12 shows resistance to FB1 with substantially reduced cell death induced by the toxin. Compared to the wild type, fbr12 protoplasts showed substantially less DNA fragmentation induced by FB1, suggesting that FBR12/eIF-5A-2, similar to its mammalian homologs, is likely involved in the regulation of apoptotic cell death. Because FB1 is known to inhibit ceramide synthase, thereby perturbing sphingolipid metabolism (Wang et al., 1991; Abbas et al., 1994; Gilchrist et al., 1994), FBR12 may be involved in the regulation of a subset of RNA responsible for sphingolipid metabolism or signaling. In addition to the antiapoptotic phenotype induced by FB1, fbr12 also shows delayed leaf senescence induced by dark, another form of PCD in plant cells. This phenotype is consistent with two earlier observations made in Arabidopsis (Wang et al., 2003) and tomato (Wang et al., 2005) in which antisense suppression of DHS, which presumably inactivates eIF-5A by blocking hypusination, leads to delayed senescence and tolerance to stresses. However, because fbr12 grows slower than wild-type plants, we could not exclude the possibility that delayed senescence in the mutant is partly attributed to slower initiation of the developmental program induced by dark. Nevertheless, in parallel to these findings in plants, the hypusination activity on eIF-5A was significantly reduced during senescence of IMR-90 human diploid fibroblasts (Chen and Chen, 1997). Taken together, these observations suggest that FBR12 and/or other eIF-5A genes may be directly or indirectly involved in the regulation of distinctive forms of cell death in plants.

In conclusion, FBR12/eIF-5A-2 plays a critical role in plant growth and development by regulating cell division, cell growth, and cell death. Identification of the
fbr12 mutant provides unique materials to functionally characterize this class of highly conserved proteins in eukaryotic organisms. Clearly, identification and characterization of direct targets of FBR12/eIF-5A-2 will be critical to better understand its function.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Genetic Screen for fbr Mutants

TheWs etecotype of Arabidopsis (Arabidopsis thaliana) was used in this study unless otherwise indicated. Plants were grown under a 16-h-light/8-h-dark cycle (white light; 120 μmol m⁻² s⁻¹) at 22°C in soil or on Murashige and Skoog medium (1× Murashige and Skoog salts, 3% Suc, 0.8% agar) as described previously (Sun et al., 2003). A T-DNA-mutagenized population of approximately 5,000 independent lines was generated in the pga22 (Ws) background (Sun et al., 2003, 2005). These T-DNA insertion lines were screened for FBR-resistant mutants as described (Stone et al., 2000). T2 seeds were germinated and grown on Murashige and Skoog medium containing 0.8 μM Fbr, (purchased from Sigma) under continuous white light for 1 to 2 weeks. Putative fbr mutants were identified and then transferred onto fresh Murashige and Skoog medium without Fbr. From this screen, two fbr mutants were identified. The fbrF mutant (originally named p250) was outcrossed with wild-type plants (Ws) twice to segregate out the pga22 mutation, and F2 or F3 progeny in the Ws background lacking the pga22 mutation were used in all experiments.

Protoplast Preparation, Detection of Nuclear DNA Fragmentation, and Detection of Cell Death

Leaves were collected from 4- to 5-week-old seedlings and protoplasts were prepared according to Danon and Gallis (1998). In situ detection of nuclear DNA fragmentation was performed as described (Danon and Gallis, 1998) with minor modifications. The TUNEL reaction was carried out in an Eppendorf tube by using an in situ cell death detection kit (Roche Diagnostics) according to the manufacturer’s instructions. Nuclear DNA was stained with Hoechst 33342 (5 μg/ml; Sigma). After staining, the samples were mounted on a polylysine slide and visualized using a fluorescent microscope.

Cell death was analyzed by Trypan blue staining as described (Mou et al., 2000).

Light and Electron Microscopic Analyses

Semithin sections were prepared and analyzed as described with minor modifications (Yang et al., 1999). Briefly, samples were fixed in 2.5% glutaraldehyde, 0.2 M sodium phosphate buffer, pH 7.2, at 4°C overnight and then postfixed in 1% osmium tetroxide for 2 h at 4°C. After dehydration in an acetone and graduated ethanol series, samples were embedded in Spurr’s resin (Sigma). Semithin sections (2 μm) were stained with 0.1% (w/v) toluidine blue O and observed under a light microscope. For differential interference contrast observations, young flower buds were cleared with Herr’s solution (Herr, 1971), and then analyzed under an inverted light microscope. Pollen grains were stained by 0.1% (w/v) toluidine blue O and observed under a light microscope. For scanning electron microscopic analysis, samples were fixed, postfixed, and dehydrated as described above. After being critical point dried in liquid CO₂ and mounted, samples were sputter-coated with gold in an E-100 ion sputter and then observed under a scanning electron microscope (model S-570, Hitachi).

For comparison of cell size and numbers, the distal portion of the petal epidermis or stem sections was analyzed as described (Mizukami and Ma, 1992; Mizukami and Fischer, 2000). For statistical analysis, images were photographed with an Olympus digital camera and then analyzed using Image-Pro Plus 5.1. Statistical calculations were performed with Microsoft Excel.

Analysis of Leaf Senescence and Measurement of Chlorophyll

Fully expanded leaves collected from 4-week-old wild-type or fbr12 plants were used for the analysis of dark-induced leaf senescence essentially as described (Guo and Crawford, 2005). Total chlorophyll was extracted from dark-treated or not-treated leaves and analyzed as described (Lichtenhalber, 1987).

Molecular Complementation of the fbr12 Mutant Phenotype

The T-DNA-tagged genomic sequence in the fbr12 genome was identified by TAIL-PCR as previously described (Liu et al., 1995; Sun et al., 2003). For molecular complementation, a 2.5-kb FBR12 genomic clone was obtained by PCR using primer pairs (FBR12F1, 5’-CCCGAGTGCTGAGCAAGACCATGTA-3’; and FBR12R1, 5’-CAGTGAATCTTTGAAATGGA-3’), and then cloned into a pGEM-T Easy vector (Promega). This cDNA fragment, containing the entire ORF of Fbr12, was inserted into the pER8 vector (Zuo et al., 2000). The resulting construct was transformed into Agrobacterium tumefaciens strain GV3101, which was used for transformation of fbr12/+ heterozygous plants by vacuum infiltration (Bechtold et al., 1993).

All other molecular manipulations were carried out according to standard methods (Sambrook and Russell, 2001). RNA northern-blot and real-time PCR analyses were carried out as described previously (Sun et al., 2003; Feng et al., 2006). Semi-quantitative reverse transcription (RT)-PCR was performed as previously described (Sun et al., 2003), Actin8 (At1g49240) was used as an internal control in all assays. Primer pairs used in the RT-PCR analyses were (all sequences are from the 5’-end to the 3’-end): ATIG19890F, GAGTT-CTTCTTCCTCTCCCA and ATIG11980R, GAAACCGTGCCAGCCGTA; AT35G9845F, GTGTTGAAGTGATCTCTCGA; AT35G9845R, GTACG-TGAGGACATCCGA; AT35G03840F, CATGATGACTTCTCCCT and AT35G03840R, GTGTGAAAGTATCTCCGA; FBR12F, TCAAAACAGCT- CACTTGAAAAAGT; and FBR12R, CATTTGGCGTGACCGTGCT; and Actin8F, TGTCGACATGCAAGCAGACCATGTA; and Actin8R, TGGTCGACACTGTAACCCTTTCCCA.

Genetic Complementation in Yeast Cells

An FBR12 cDNA fragment was PCR amplified using primer pairs (ATIG263602P, 5’-CCGGAGATGTGCGAGCAGCAGACCATGTA-3’ and ATIG263602S, 5’-CAGTGAATCTTTGAAATGGA-3’), and then cloned into a pGEM-T Easy vector (Promega). This cDNA fragment, containing the entire ORF of FBR12 and 32 bp of the 3’-UTR, was verified by DNA sequencing. The insert was released by Smal and PstI digestion and cloned into the same sites of a pQE-82 L vector (Qagen). This cDNA fragment released by pQE-FBR12 was SacI and PstI was cloned into the SacI and XbaI of a pYES2 vector (Invitrogen) under the control of a GAL1 promoter. Therefore, FBR12 expression in yeast (Saccharomyces cerevisiae) is inducible by Gal and repressible by Glc (West et al., 1987; Giniger et al., 1985). Culture and transformation of yeast cells were carried out according to standard protocols (Aubusel et al., 1994).

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LITERATURE CITED


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