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Arabidopsis *AtSPL14*, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1

Julie M. Stone
University of Nebraska - Lincoln, jstone2@unl.edu

Xinwen Liang
University of Nebraska - Lincoln, xliang2@unl.edu

Emily R. Nekl
University of Nebraska - Lincoln

Justin J. Stiers
University of Nebraska - Lincoln

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Arabidopsis *AtSPL14*, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1

Julie M. Stone, Xinwen Liang, Emily R. Nekl, and Justin J. Stiers

Department of Biochemistry and Plant Science Initiative, University of Nebraska-Lincoln, 1901 Vine Street, Lincoln, NE 68588, USA

Corresponding author — Julie M. Stone, fax 402 472-3139, e-mail jstone2@unl.edu

Present address for Emily R. Nekl — Department of Entomology, University of Nebraska–Lincoln, Lincoln, NE, USA

Abstract

The recessive *Arabidopsis thaliana* fumonisin B1-resistant (*fbr6*) mutant was identified by its ability to survive in the presence of a programmed cell death (PCD)-inducing fungal toxin FB1. The *fbr6* mutant also displays altered plant architecture in the absence of FB1, most notably elongated petioles and enhanced leaf margin serration. These phenotypes are a result of a T-DNA insertion in the *SQUAMOSA* promoter binding protein (SBP) domain gene, *AtSPL14*. *AtSPL14* encodes a plant-specific protein with features characteristic of a transcriptional regulator, including a nuclear localization signal sequence, a plant-specific DNA binding domain (the SBP box), and a protein interaction motif (ankyrin repeats). A transiently expressed fusion of the AtSPL14 protein to green fluorescent protein is directed to the plant nucleus. DNA sequences immediately upstream of the translation start site direct expression of the *β*-glucuronidase reporter gene primarily in the vascular tissues, consistent with the phenotypes of the *fbr6* mutant. AtSPL14 activates transcription in yeast, with a transactivation domain residing within the N-terminal region of the protein. Recombinant AtSPL14 protein binds *A. thaliana* genomic DNA *in vitro* in the absence of other proteins. These results indicate that FBR6/SPL14 functions as a transcriptional regulator that plays a role not only in sensitivity to FB1, but also in the development of normal plant architecture.

Keywords: *Arabidopsis thaliana,* transcription, SBP domain, fumonisin B1, programmed cell death

Introduction

Cell fate decisions are essential for the growth, development, and survival of multicellular organisms. Programmed cell death (PCD), the intentional elimination of specific cells, is critical for proper development and defense against pathogen infection in both plants and animals. The mechanistic details and molecular components controlling PCD in eukaryotes are not fully understood. However, the pathways appear to be evolutionarily and functionally conserved, given that plant components can function in animals and vice versa (Chae *et al.*, 2003; Dickman *et al.*, 2001; Kawai-Yamada *et al.*, 2001; Lacomme and Santa Cruz, 1999; Lincoln *et al.*, 2002; Richael *et al.*, 2001). In vascular plants, PCD is a prominent feature of xylem tissue development (Demura *et al.*, 2002; Fukuda, 2000; Groover and Jones, 1999) as well as defense responses to pathogen attack (Beers and McDowell, 2001; Gilchrist, 1998).

Genetic approaches have been used to identify genes involved in plant PCD pathways. For example, mutations that cause plant 'lesion mimic mutants', which spontaneously undergo PCD in the absence of pathogen infection, have revealed roles for lipid metabolism, light perception, and hormone signaling in plant PCD (Brodersen *et al.*, 2002; Gray *et al.*, 2002; Liang *et al.*, 2003; Lu *et al.*, 2003; Mach *et al.*, 2001; Pruzinska *et al.*, 2003; Rate *et al.*, 1999; Vanacker *et al.*, 2001; Yang *et al.*, 2004). We reasoned that identifying plant mutants defective in undergoing PCD in response to pathogens and/or compounds that mimic pathogen infection would provide a complementary genetic approach to investigate the molecular mechanisms regulating PCD.

Fumonisin B1 (FB1) is a fungal toxin that disrupts sphingolipid metabolism in eukaryotes by acting as a competitive inhibitor of ceramide synthase (Desai *et al.*, 2002). FB1 induces PCD (or apoptosis) in both plants and animals (Asai *et al.*, 2000; Tolleson *et al.*,

1999; Wang *et al.*, 1996) and inhibits growth in yeast (Mao *et al.*, 2000). In *Arabidopsis thaliana*, FB1 treatment initiates the formation of 'apoptotic bodies' that closely resemble those typically associated with PCD in animal cells, and this FB1-induced cell death is dependent on active transcription and translation, as well as reversible protein phosphorylation (Asai *et al.*, 2000). Moreover, sensitivity to FB1 is dependent on light and the hormone signaling pathways mediated by salicylic acid, jasmonic acid, and ethylene (Asai *et al.*, 2000; Stone *et al.*, 2000).

We exploited the fact that micromolar levels of FB1 inhibit growth of *A. thaliana* seedlings to identify FB1-resistant (*fbr*) mutants. These mutants were selected on FB1-containing agar media at FB1 levels that prevented wild-type plants from developing (Stone *et al.*, 2000). Because FB1 induces PCD and PCD functions in responses to pathogen infection, we predicted that at least some of the *A. thaliana fbr* mutants would also exhibit defense-related phenotypes. Indeed, *fbr1* and *fbr2* mutants showed enhanced resistance to virulent bacterial pathogen growth and changes in defense gene induction (Stone *et al.*, 2000). Because FB1 sensitivity is also impacted by light perception and hormone signaling, which are important factors in development, we expected that some *A. thaliana* FB1-resistant mutants might also display altered morphology. A subset of the identified *fbr* mutants exhibit a characteristic alteration in plant architecture, including elongated petioles and enhanced leaf margin serration.

In this paper, we describe the identification of the gene corresponding to the recessive *fbr6* mutant, which displays altered plant architecture in addition to resistance to FB1. The *fbr6* mutant phenotypes are the result of a T-DNA insertion in *AtSPL14*, a member of the SQUAMOSA PROMOTER BINDING PROTEIN-box (SBP-box) gene family. Functions of SBP-box genes are largely unknown, but they are predicted to act as transcriptional regulators based on the presence of a plant-specific putative DNA binding domain. We further delineate additional functional domains of AtSPL14, and demonstrate that the protein localizes to the nucleus, possesses a transcriptional activation domain and binds Arabidopsis DNA. These data support a role for *AtSPL14* as a transcriptional regulator of genes that function in plant development and sensitivity to FB1.

Results

Isolation, genetic and phenotypic characterization of the fbr6 mutant

The *fbr6* mutant was identified in a high-throughput selection for *A. thaliana* mutants resistant to FB1-mediated growth inhibition. Seeds from enhancer trap T-DNA insertion lines (Campisi *et al.*, 1999) were plated on MS-agar supplemented with 0.5 μm FB1, and surviving plants were transferred to soil (Stone *et al.*, 2000).

The *fbr6* mutant was backcrossed to the parental genotype (Col6 *gl1-1*), four F_1 progeny were self-fertilized, and the resulting F_2 progeny were tested for their ability to survive selection on agar media containing 0.5 μm FB1. A chi-square goodness-of-fit test confirmed that the *fbr* phenotype of the F_2 progeny segregated at the expected 3:1 (sensitive:resistant) ratio for a single recessive mutation ($\chi^2 = 2.466$, $n = 286$). The FB1-resistant plants were transferred to MS-agar plates lacking FB1 for recovery then transplanted into soil to collect seed. The FB1-resistant F_3 progeny derived from the backcross exhibited the aberrant plant architecture observed in the original *fbr6* mutant (see below).

In addition to resistance to FB1, the *fbr6* mutant (grown in the absence of FB1) displays elongated petioles and enhanced leaf margin serration compared with wild-type plants (Figure 1a,b). Transition to flowering occurs a few days later in the *fbr6* mutant than in wild-type plants, but no significant alterations in inflorescence branching pattern or floral morphology were observed (Figure 1c). A serrated leaf margin phenotype is associated with altered phase transition during rosette leaf development (Clarke *et al.*, 1999; Prigge and Wagner, 2001). Because developmental phases can be distinguished by venation pattern and the number of water pores or hydathodes (Candela *et al.*, 1999; Poethig, 2003; Tsukaya *et al.*, 2000), we compared wild type and *fbr6* mutant leaves that had been cleared with ethanol to reveal venation patterns. The venation patterns of *fbr6* and wild-type cotyledons were similar (Figure 1d). However, whereas the venation pattern of the fourth true wild-type leaves generally had five hydathodes as expected for a juvenile leaf (Candela *et al.*, 1999; Clarke *et al.*, 1999), the fourth *fbr6* mutant leaves typically resembled mature wild-type leaves with seven hydathodes (Figure 1e). In some cases, the fourth leaf of *fbr6* mutants was asymmetrical with six hydathodes (data not shown). These observations suggest that there is a slight acceleration of the juvenile to adult vegetative phase transition in the *fbr6* mutant. In contrast to the *fbr1* and *fbr2* mutants characterized previously (Stone *et al.*, 2000), growth of bacterial pathogens in *fbr6* was not significantly different from wild-type plants.

The fbr6 phenotypes are due to T-DNA insertion in the AtSPL14 gene

Genomic DNA flanking the T-DNA insertion in the *fbr6* mutant was recovered using TAIL-PCR (Liu *et al.*, 1995). The DNA sequence of the cloned PCR product indicated that the T-DNA sequences in *fbr6* are inserted on chromosome I in the 3′ UTR region (Figure 1f) of a gene formerly designated as *SPL1R2*, *SQUAMOSA*promoter binding protein-like related 2 (Cardon

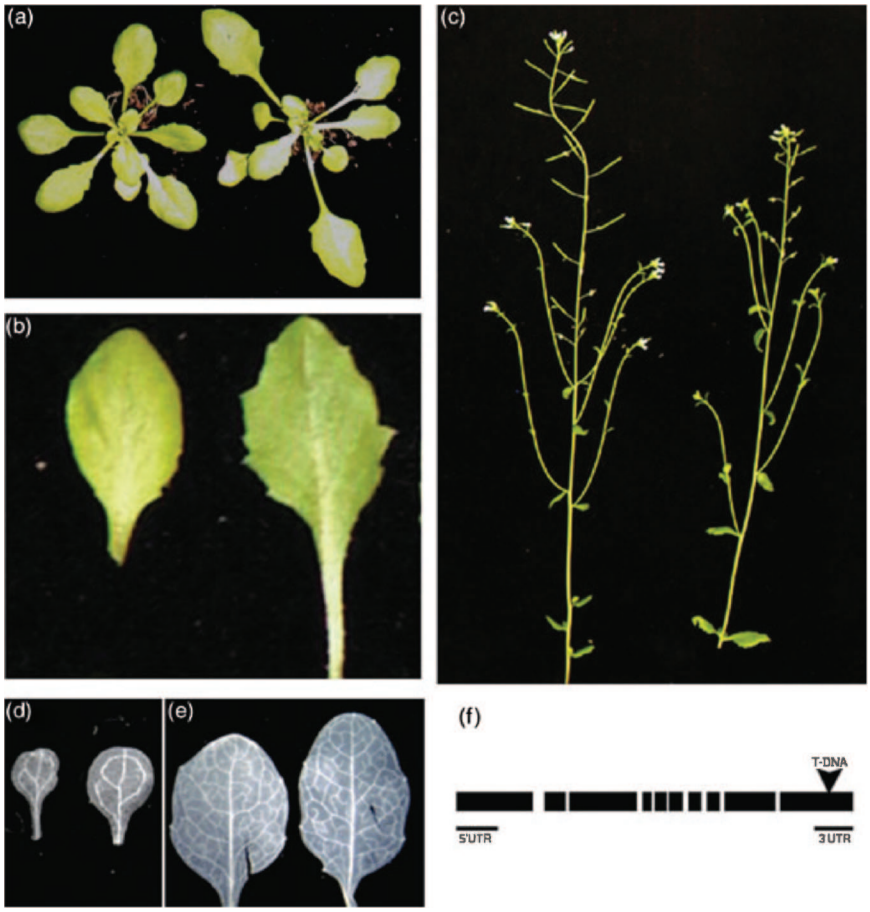

Figure 1. The *Arabidopsis thaliana fumonisin B1-resistant6* (*fbr6*) mutant has altered plant architecture and a T-DNA insertion in the 3′UTR of the *AtSPL14* gene.

(a–e) Comparison of the 'wild-type' Col6 *gl1-1* (left) with the Col6 *gl1-1 fbr6* mutant (right).

(a) Rosette morphology of plants grown in soil with an 8-h photoperiod. The *fbr6* mutant displays enhanced leaf margin serration and elongated petioles.

(b) A close up view of the fifth true leaf shows the serrated leaf morphology of *fbr6*.

(c) Inflorescences show no significant difference in branching pattern or floral morphology, however, the *fbr6* mutant transitions to flowering somewhat later than wild type.

(d) The cotyledon venation pattern observed in a dark-field image of cleared tissues displayed no significant differences.

(e) The venation pattern of the fourth true leaf shows five hydathodes for the wild-type leaf, whereas the corresponding *fbr6* mutant leaf has seven hydathodes.

(f) Organization of the AtSPL14 (At1g20980) gene based on genomic DNA sequence, EST sequences, and RT-PCR. Exons are represented as black boxes, and the UTRs are underlined. The *fbr6* mutant was found to have T-DNA inserted in the 3′UTR as indicated.

et al., 1999). This gene (At1g20980) corresponds to *AtSPL14*, according to more recent nomenclature for the 16-member squamosa promoter binding protein-like (SPL) gene family (http://www.bio.uni-frankfurt.de/botanik/mcb/AFGN/Huijser.htm).

To verify that the phenotypes observed in *fbr6* were due to the disruption of the *SPL14* gene, *fbr6* transgenic plants harboring a wild-type genomic copy of *AtSPL14* driven by its native promoter were generated. Several independent transgenic lines show that both the sensitivity to FB1 (Figure 2a) and normal plant architecture (Figure 2b) were restored by molecular complementation, indicating that both *fbr6* phenotypes are a result of *AtSPL14* disruption.

The T-DNA insertion in *fbr6* occurs upstream of the predicted polyadenylation signal suggesting that maturation of *AtSPL14* mRNA might be defective in the *fbr6* mutant. As the *AtSPL14* transcript was undetectable by total RNA Northern blot analyses in both wild-type and *fbr6* mutant plants, semiquantitative RT-PCR was performed to determine whether the *AtSPL14* mRNA was expressed in the *fbr6* mutant. RNA was isolated from wild type, *fbr6* mutant and complemented *fbr6* mutant plants. *SPL14* transcripts were detected in wild-type plants, at diminished levels in the *fbr6* mutant plants, and at wild-type (or greater) levels in the complemented *fbr6* mutant plants (Figure 2c). These data, together with the recessive nature of the *fbr6* mutant, verify that the *fbr6* phenotypes are due to a reduction-of-function of *AtSPL14*.

AtSPL14 encodes a putative transcriptional regulator

AtSPL14 (At1g20980) encodes a 1035 aa protein predicted to function as a plant-specific transcriptional regulator. Analyses of the predicted AtSPL14 protein sequence using the InterPro database (http://www.ebi.ac.uk.interproscan) revealed that it has a highly conserved SBP DNA binding domain (IPR004333), a Cys- and His-rich region (consensus – $CX_4CX_{13}HX_5HX_{15}C$-$QQCX_3HX_{11}C$) found only in plant proteins (Cardon *et al.*, 1999). The founding members of the SBP domain-containing superfamily were originally identified in *Antirrhinum majus*, where they were identified by their ability to bind to the upstream regulatory region of the *SQUAMOSA* gene involved in floral meristem identity (Klein *et al.*, 1996). This suggests that the SBP domain of AtSPL14 may also function in DNA binding.

Consistent with the presence of a DNA binding domain, AtSPL14 is predicted to be localized to the plant nucleus. An amino acid sequence (**KR**SCRRR-LAGHN**RRRRK**) fitting the consensus for a bipartite

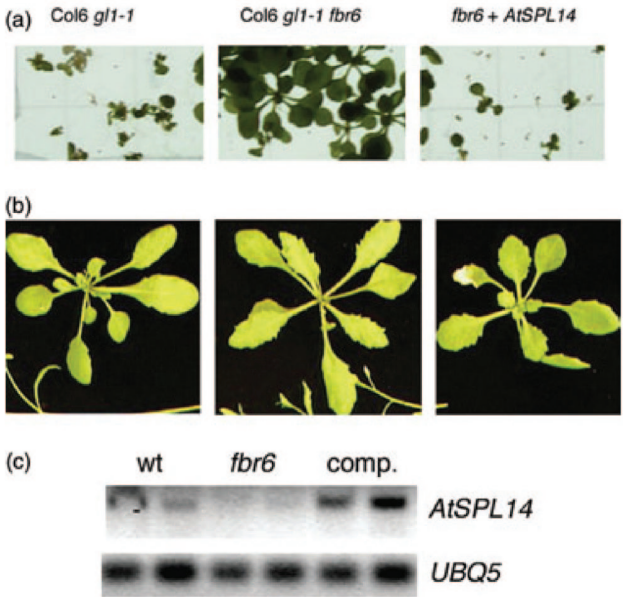

Figure 2. Molecular complementation of the *fbr6* mutant with a wild-type genomic DNA copy of the *AtSPL14* gene restores fumonisin B1 sensitivity, normal plant morphology, and expression of *AtSPL14* mRNA.

(a) Growth of the wild-type parent Col6 *gl1-1*, Col6 *gl1-1 fbr6* mutant, and a homozygous transgenic Col6 *gl1-1 fbr6* line transformed with a wild-type copy of the *AtSPL14* gene (*fbr6* + *AtSPL14*) on MS plates supplemented with 0.5 μm fumonisin B1.

(b) Comparison of 3-week-old soil-grown Col6 *gl1-1*, Col6 *gl1-1 fbr6* mutant, and a molecularly complemented plant (*fbr6* + *AtSPL14*).

(c) Semiquantitative RT-PCR was used to detect *AtSPL14* transcript accumulation in wild-type, *fbr6* mutant, and a molecularly complemented transgenic line. *AtSPL14* mRNA accumulation is reduced in the *fbr6* mutant and is restored by molecular complementation of the *fbr6* mutant with the wild-type *AtSPL14* gene. Accumulation of transcripts corresponding to *UBQ5* was used as a control.

nuclear localization signal (NLS) was found within the highly conserved SBP DNA binding domain (aa 117–193), using PSORT (http://psort.nibb.ac.jp) for prediction of protein localization (Robbins *et al.*, 1991).

The InterPro analysis also revealed that the AtSPL14 protein possesses ankyrin repeats (IPR002110) in the C-terminal region of the protein (aa 821–941). Ankyrin repeats are a common protein–protein interaction motif consisting of approximately 33 amino acid modules found in transcription factors and other eukaryotic proteins (Dechend *et al.*, 1999; Ely and Kodandapani, 1998; Niggeweg *et al.*, 2000). These analyses of the predicted protein encoded by the *AtSPL14* gene indicate that it is likely to function as a transcriptional regulator.

AtSPL14 is expressed in vascular tissues and floral organs

To determine the spatial and developmental expression pattern of *AtSPL14*, we generated transgenic plants harboring the DNA sequence immediately upstream of the translation start site of *AtSPL14* fused to the *β*-glucuronidase (GUS) reporter gene. The *AtSPL14*'promoter'::GUS fusion was transformed into wild-type (Col-0) plants, and several independent transgenic plants homozygous for the transgene were analyzed by histochemical staining for GUS activity.

Under the influence of the *AtSPL14*'promoter' expression of the GUS gene was detected primarily in the vascular tissues of aerial portions of the plant. No GUS activity was detected in the hypocotyl (Figure 3a), while strong staining was observed predominantly in the leaf petioles and the primary vascular tissues of both leaves (Figure 3a,b) and cotyledons (Figure 3c). In leaves, there was intense staining in the hydathodes (Figure 3b), and somewhat lower levels in the secondary vascular of leaves (Figure 3b) and cauline leaves (data not shown). GUS activity was occasionally detected in root tissues of plants grown on agar, but not in plants grown in soil. GUS-dependent staining in the vascular tissues of inflorescences and at the base and tips of developing siliques post-pollination increased during seed formation and persisted through maturation (Figure 3d).

AtSPL14 localizes to the nucleus

The presence of a putative bipartite NLS in AtSPL14 suggested that it would be localized to the plant nucleus (Robbins *et al.*, 1991). The *AtSPL14* cDNA was cloned into the binary vector pEGAD to produce an in-frame fusion downstream of the green fluorescent protein (GFP) (Cutler *et al.*, 2000). Transient transformation of *Nicotiana tabacum* leaves was achieved by 'agroinfiltration' (Yang *et al.*, 2000), and subcellular localization of GFP was visualized by confocal microscopy (Figure 4). Controls showed GFP expressed throughout the cytoplasm (Figure 4a,b), whereas the GFP-AtSPL14 fusion was targeted to the nucleus (Figure 4c,d).

AtSPL14 was tested for its ability to activate transcription in yeast when fused to the GAL4 DNA binding domain. cDNAs encoding the entire AtSPL14 protein or various deletions were fused in-frame to sequences encoding the GAL4 DNA binding domain and transformed into yeast strain AH109 containing GAL4-responsive upstream activator sequence (UAS) binding sites upstream of different reporter genes. The ability of these GAL4BD/AtSPL14 fusion proteins to activate transcription was assessed by the ability to grow in the absence of histidine (conferred by the *HIS3* reporter gene) and to induce *α*-galactosidase activity (conferred by the *MEL1* reporter gene). A GAL4BD fusion to AtSPL14 activated transcription in yeast, while the control (GAL4BD alone) failed. Deletion analyses suggest that the capacity to activate transcription in yeast resides within the N-terminal 184 amino acid residues of AtSPL14 (Figure 5).

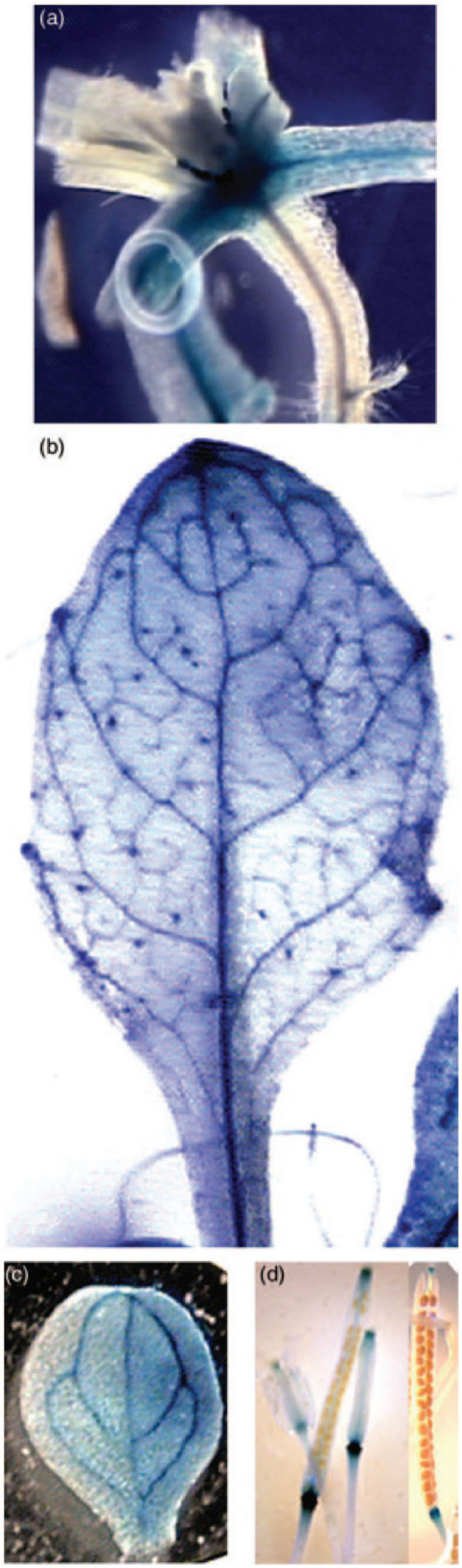

Figure 3. DNA sequences immediately upstream of the translation start site for the *AtSPL14* gene drives expression of the *β*-glucuronidase (GUS) reporter gene in several plant tissues. Histochemical staining for *β*-glucuronidase activity was performed on homozygous transgenic T_3 and T_4 plants harboring a *AtSPL14*::GUS fusion construct. The upstream region of *AtSPL14* drives expression of GUS in: (a) the vascular tissues of leaf petioles, but not the hypocotyl; (b) the vascular tissues of petioles and true leaves, the hydathodes, and the base of trichomes; (c) the vascular tissue of cotyledons; and (d) the stigma and base of inflorescences and developing siliques post-pollination.

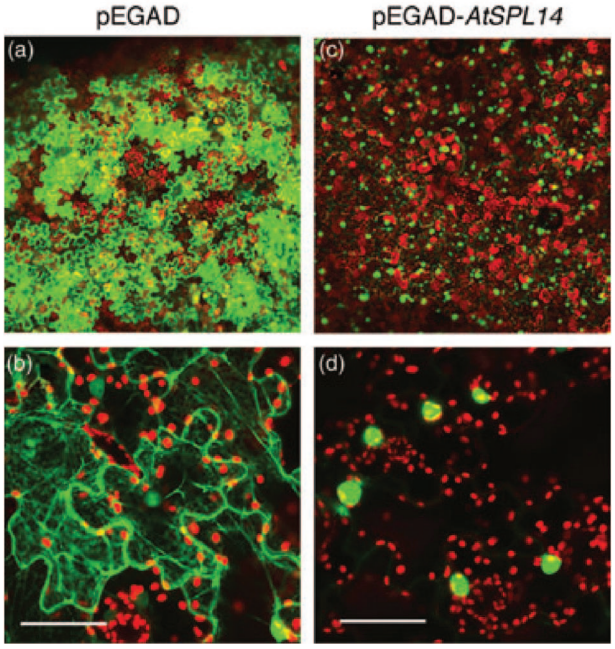

Figure 4. Nuclear localization of a GFP-AtSPL14 fusion protein. Tobacco leaves were transiently transformed with *Agrobacterium tumefaciens* carrying either a control vector pEGAD or pEGAD-*AtSPL14* to produce an in-frame GFP-AtSPL14 fusion protein. Images were obtained by confocal laser scanning microscopy and merged Z-series images are shown.

(a, b) Transformation with pEGAD shows expression of GFP throughout the cytoplasm.

(c, d) Transformation with pEGAD-AtSPL14 shows GFP localized primarily to the nucleus. (a) and (c) are low magnification images, while (b) and (d) are high magnification images (bar = 50 μm).

To assess whether AtSPL14 binds to *A. thaliana* genomic DNA sequences, recombinant fusion proteins were produced in *Escherichia coli*. Maltose binding protein (MBP) and a MBP fusion to the N-terminal 409 residues of AtSPL14 (encompassing the SBP domain) were immobilized on a PVDF membrane and incubated with ^{32}P-labeled *A. thaliana* genomic DNA. The *A. thaliana* genomic DNA bound to the MBP-FBR6 fusion protein, but did not bind to the MBP control protein (Figure 6). Therefore, AtSPL14 binds to target sequences in the *A. thaliana* genome in the absence of other proteins.

Discussion

The existence of small gene families encoding the putative DNA-binding SBP domain in plants has been known for over a decade. However, little is known of the physiological functions of these putative tran-

Figure 5. Transcriptional activation activity of AtSPL14 in a modified yeast two-hybrid assay. Schematic representation of the protein encoded by *AtSPL14* showing positions of the 'SBP' DNA binding domain and the ankyrin-repeat region (ANK). GAL4 DNA binding domain (BD) fusions to portions of AtSPL14 expressed in a yeast strain AH109 carrying the GAL4-responsive upstream activator sequences upstream of the *HIS3* and *MEL1* reporter genes. Ability to grow in the absence of exogenous histidine (His-) and α-galactosidase activity (α-GAL) of individual fusion proteins is shown. Serial dilutions of yeast harboring the indicated GAL4 BD fusions to AtSPL14 were spotted onto plates lacking histidine and supplemented with X-α-GAL. These data indicate that AtSPL14 can activate transcription in a heterologous eukaryotic system, and that a transcriptional activation domain resides in the N-terminal 184 amino acids of AtSPL14.

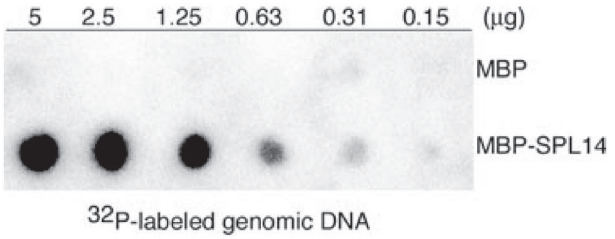

Figure 6. Recombinant AtSPL14 binds *Arabidopsis thaliana* genomic DNA in the absence of additional proteins. Purified recombinant fusion proteins (MBP and MBP-AtSPL14) were immobilized on a membrane (Hybond-N; Amersham Biosciences) and incubated with ^{32}P-labeled *A. thaliana* genomic DNA. Recombinant fusion protein containing the SBP domain of AtSPL14 binds to DNA, while the control protein MBP alone fails to bind to DNA.

scriptional regulators beyond their ability to bind DNA (Cardon *et al.*, 1999; Klein *et al.*, 1996; Riechmann *et al.*, 2000). Only two SBP domain-containing gene mutants with observable phenotypes have been previously described. The *A. thaliana spl8* mutant has reduced fertility due to the function of *AtSPL8* in pollen sac development (Unte *et al.*, 2003). The *Zea mays liguleless1* mutation affects plant development at the boundary between the leaf blade and sheath, and the LIGULELESS1 protein was also shown to be nuclear-localized, consistent with its presumed function as a transcription factor (Moreno *et al.*, 1997). Our T-DNA insertion in the 3′UTR of the *AtSPL14* gene provides the third example of an observable phenotype in an SBP domain-containing gene mutant. This insertion reduces the levels of *AtSPL14* mRNA and causes both the FB1-resistant and altered plant architecture phenotypes associated with the *fbr6* mutant.

The FB1-resistant (*fbr*) mutant screen was designed to identify components of plant PCD pathways, which have been linked to sphingolipid metabolism, light perception, and hormone signaling (Brodersen *et al.*, 2002; Gray *et al.*, 2002; Liang *et al.*, 2003; Lu *et al.*, 2003; Mach *et al.*, 2001; Pruzinska *et al.*, 2003; Rate *et al.*, 1999; Vanacker *et al.*, 2001; Yang *et al.*, 2004). Several of the identified *fbr* mutants (including *fbr6*) display abnormal plant architecture. However, because sensitivity to FB1 is influenced by hormone signaling and light (Asai *et al.*, 2000; Stone *et al.*, 2000), perturbation of these or other signal transduction pathways could be responsible for the altered plant architecture of *fbr6*.

Analysis of several *fbr* mutants has revealed differing phenotypes. For example, the previously characterized *fbr1* and *fbr2* mutants were less susceptible to the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (Stone *et al.*, 2000). In contrast, growth of virulent and avirulent strains of *P. syringae* pv. *maculicola* in the *fbr6* mutant was not significantly different from the wild type using the same assay conditions (data not shown).

The FB1 sensitivity of adult *fbr6* mutant leaves, assayed by leaf infiltration (Asai *et al.*, 2000) followed by quantitative electrolyte leakage measurements, was not significantly different from wild type, and a subset of the other *fbr* mutants fail to show resistance to FB1 in the leaf infiltration assays (J.M. Stone, unpublished data).

While the *fbr* mutants were all identified by the ability to germinate and develop in the presence of FB1, differences among the *fbr* mutants in sensitivity to FB1 in mature leaves and protoplasts might be due to cell type-specific expression. Only mutations in genes expressed in certain tissues during early development are likely to be identified in the *fbr* selection scheme. But if these genes are not highly expressed in mature leaf cells, the mutants will fail the FB1 resistance tests in the leaf infiltration and protoplast assays. Cell type-specific expression of AtSPL14 is a possible explanation for the observed sensitivity to FB1 and pathogen infection in mature leaves of the *fbr6* mutant.

Expression of AtSPL14 and the fbr6 phenotype

The expression patterns observed for the AtSPL14 'promoter'::GUS reporter construct are generally consistent with the leaf abnormalities associated with the *fbr6* mutant. The most obvious morphological defects in the *fbr6* mutant are elongated petioles, serrated leaf margins, and an accelerated vegetative phase change. The *AtSPL14*'promoter' drives GUS expression in the vascular tissues of petioles, so reduced levels of this transcription factor are consistent with altered petiole development. Other *A. thaliana* mutants with elongated petioles have been described, including light-sensing *phyB* mutant linked to hormone signaling pathways (Genoud *et al.*, 2002; Morelli and Ruberti, 2002; Tsukaya *et al.*, 2002).

Significant expression was also observed in the leaf vascular tissues. The two major tissue types of

the plant vasculature, xylem and phloem, are composed of multiple cell types. Phloem tissue consists of at least two differentiated cell types – sieve cells and companion cells, whereas xylem tissue contains tracheary elements that differentiate from provascular cells by a process of programmed cell death (PCD) and subsequent lignification (Demura *et al.*, 2002; Fukuda, 2000; Groover and Jones, 1999). Therefore, altered xylem differentiation might contribute to the altered plant architecture of the *fbr6* mutant.

The *fbr6* mutant appears to have a truncated juvenile phase, producing 'adult' leaves earlier than wild-type plants. Vegetative phases are distinguished by venation pattern, hydathode numbers, and the capacity to produce trichomes (Candela *et al.*, 1999; Poethig, 2003; Tsukaya *et al.*, 2000). *AtSPL14*-driven expression in these tissues – leaf vasculature, hydathodes, and the base of trichomes – resembles the patterns observed with the auxin-responsive marker, *DR5::GUS* (Aloni *et al.*, 2003). Hydathode numbers were used to assess leaf phases, as the *fbr6* mutant was isolated in a trichomeless (*gl1-1*) background. It will be interesting to know whether the early 'adult' leaves also have trichomes in a different background. The serrated leaf margin phenotype is shared with other mutants with accelerated phase changes (Berardini *et al.*, 2001; Bollman *et al.*, 2003; Candela *et al.*, 1999; Clarke *et al.*, 1999). Zinc finger transcription factors, such as *SERRATE* and *JAGGED* (Clarke *et al.*, 1999; Ohno *et al.*, 2004; Prigge and Wagner, 2001), might represent transcriptional regulators that act in concert with AtSPL14.

The expression at the base of siliques and in the stigma does not correlate with any gross morphological defects in the mutant, however, the base of siliques is the site of floral organ abscission requiring PCD (Jinn *et al.*, 2000).

Molecular characterization of AtSPL14

The N-terminal region of AtSPL14 (aa 1–184) exhibited transcriptional activation in yeast. The structural basis of transcriptional activation domains is essentially unknown, however, 'typical' transcriptional activation domains such as glutamine-rich or acidic regions were not detected in this region. The prevalent proline, serine, and threonine residues (approximately 20%) might comprise a transcriptional activation domain functionally similar to those described for some mammalian transcription factors (Liu *et al.*, 2003; Prado *et al.*, 2002). AtSPL14 is also rich in hydrophobic leucine residues. Three putative EAR motifs (LXLXL, aa 81–85, 388–392, 459–463), which function as a transcriptional repression domains in several plant transcription factors (Hiratsu *et al.*, 2002, 2003; Ohta *et al.*, 2001; Tiwari *et al.*, 2004), and five LLXXL motifs, which mediate interactions between transactivation domains and eukaryotic coactivators (Chen, 1999; Heery *et al.*, 1997) were detected. Further analyses are necessary to validate the importance of these motifs in AtSPL14.

Secondary structure predictions of AtSPL14 (aa 120–194) comprising the SBP domain suggested that this region may form a helix-loop-helix structure commonly found in many DNA binding proteins (Massari and Murre, 2000; Tan and Richmond, 1998). The highly conserved SBP DNA binding domain (consensus – $CX_4CX_{13}HX_5HX_{15}CQQCX_3HX_{11}C$) is particularly rich in Cys and His residues. Although Cys/His-rich DNA-binding regions of proteins often function to coordinate zinc, the SBP domain sequence does not correspond to any previously described zinc finger motifs (Dietrich *et al.*, 1997; Laity *et al.*, 2001). A recent report describing the solution structures of the SBP domains from SPL4 and SPL7 suggest that these do indeed coordinate two zinc ligands (Yamasaki *et al.*, 2004). We have verified that recombinant AtSPL14 is capable of binding to *A. thaliana* genomic DNA. However, incubation of recombinant AtSPL14 protein with metal-chelating compounds, EDTA or *o*-phenanthroline, had no significant effect on DNA binding activity (data not shown), similar to results with the *A. majus* SBP proteins (Klein *et al.*, 1996).

AtSPL14 contains a C-terminal extension lacking in AtSPL8 and LIGULELESS1. This C-terminal extension contains ankyrin repeats, which are found in a number of DNA-binding transcriptional regulators and partner proteins (Dechend *et al.*, 1999; Ely and Kodandapani, 1998; Niggeweg *et al.*, 2000). Because combinatorial control of gene expression plays a critical role in achieving functional diversity (Chen and Hampsey, 2002; Messenguy and Dubois, 2003), AtSPL14 is likely to function in concert with other proteins through its ankyrin repeats.

Identification of direct target genes of AtSPL14 and AtSPL14 regulatory partners should enhance our understanding of transcription regulation in vegetative development and/or FB1 resistance. Moreover, the finding that the *fbr6* mutant phenotypes are due to the T-DNA disruption in *AtSPL14* will facilitate genetic interaction analyses with other plant mutants to link the developmental alterations with FB1 resistance.

Experimental procedures

Arabidopsis thaliana growth and isolation of the fbr6 mutant

For isolation of the *fbr6* mutant, seed pools obtained from the ABRC (CS31087) were surface-sterilized with 50% bleach, 0.02% Tween-20 for 15 min, rinsed three times with sterile H_2O, and sowed on Murashige–Skoog media supplemented with 2% sucrose, 0.8% phytagar and 0.5 μm fumonisin B1 as described (Stone *et al.*, 2000). Plants were grown in soil (Metro-Mix 360; Scotts, Maryville, OH, USA) in a growth chamber (Percival AR36L; Percival, Perry, IA, USA) at 22°C, 70% RH and approximately 100 μE m^{-2} sec^{-1} under cool-white fluorescent lights supplemented with incandescent lamps, with either an 8 or 12 h photoperiod.

Identification of the T-DNA disruption in AtSPL14

The *fbr6* mutant was identified from a population of *Col-6 gl1-1* plants transformed with the enhancer trap vector pD991 (Campisi *et al.*, 1999). Thermal asymmetric interlaced PCR, TAIL-PCR, was used to rescue the DNA flanking the right border sequence using nested oligonucleotide primers corresponding to the right border of pD991 (oligo123, oligo124 and oligo86; http://www.dartmouth.edu/~tjack/) and primer TAIL-AD2 5′-ngtcgaswganawgaa-3′ as described (Campisi *et al.*, 1999; Liu *et al.*, 1995). The PCR product from the tertiary reaction was cloned into pGEM-TEasy (Promega, Madison, WI, USA) and subjected to DNA sequencing with T7 and SP6 oligonucleotide primers.

Semiquantitative RT-PCR

Total RNA was isolated from individual 3-week-old plants using the Qiagen RNeasy midi kit (Valencia, CA, USA) according to manufacturer's instructions. The RNA was treated with DNase I using a DNA-*free*™ kit (Ambion, Inc., Austin, TX, USA). RNA concentration was determined spectrophotometrically. Reverse transcription was performed in a 20 μl reaction with 1 μg total RNA, 0.5 μg oligo $(dT)_{18}$ primer, 40 U RNasin (Promega), 500 μm dNTPs, and 40 U M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA), then diluted to 50 μl. PCR was performed for 40 cycles 94°C 30 sec, 52°C 30 sec, 72°C 1.5 min in a reaction containing 100 μm dNTPs, 1 mm $MgCl_2$, 250 nm oligonucleotide primers. Oligonucleotide primers were cFBR6F7: ′5-CCGCTTCAAGTTTTTGCT-3′; cFBR6STOPSma: 5′-CCTCCCGGGTATAGTTCTCTAGATTGAGCCATAATCC-3′; UBQ5-F: 5′-GTGGTGCTAAGAAGAGGAAGA-3′; UBQ5-R: 5′-TCAAGCTTCAACTCCTTCTTT-3′. The *AtSPL14* primers were designed to span an intron to exclude DNA contamination. The primers correspond to exon 9 and the region encompassing the STOP codon in exon 10 (154 bp upstream of the T-DNA insertion site in *fbr6*).

Molecular complementation and promoter::GUS reporter gene fusions

The wild-type *AtSPL14* gene containing 1448 bp upstream of the ATG start codon and 443 bp downstream of the STOP codon was amplified from *A. thaliana* Col-0 genomic DNA by PCR using oligonucleotide primers 'prom *Sma*I F': 5′-CCTCCCGGGTTGAGGTTCGAAATAACGTGGTCAAG-3′ and 'polyA *Sma*I R': 5′-CCTCCCGGGTTATGCATTTTGACTTTCGAGAATAAG-3′. A fragment corresponding to only the 1448 bp upstream of the ATG start codon was amplified from *A. thaliana* Col-0 genomic DNA by PCR using oligonucleotide primers 'prom *Sma*I F' and 'prom *Sma*I R':5′-CCTCCCGGGATCTCTCGATCTGAGTCTGACCCTTTTTC-3′. The resulting fragments were subcloned in pGEM-TEasy (Promega), and fidelity of the PCR was confirmed by DNA sequencing.

The fragment containing the entire promoter and coding sequence (full-length) for molecular complementation was cloned into pCAMBIA 3300, and the FBR6 promoter only (pFBR6) fragment was cloned into pCAMBIA 3301, both vectors carry a gene that confers resistance to the herbicide, Basta™. These constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 MP90 by electroporation. Col-6 *gl1-1 fbr6* plants and Col-0 plants were transformed with 3300FBR6 'full-length' and 3301pFBR6, respectively, by the floral dip method (Clough and Bent, 1998). Primary transformants were selected on soil by spraying with a 1:100 dilution of Finale™ (AgrEvo Environmental Health, Montvale, NJ, USA). Transgenic lines homozygous for single insertions were selected by analyzing the segregation of herbicide resistance in T_2 and T_3 populations.

Molecular complementation of the *fbr6* mutant phenotypes was assessed by sowing wild-type, *fbr6* mutant and 'complemented' seeds on MS-agar media supplemented with 2% sucrose (w/v) and 0.5 μm FB1. Reversion of the morphological phenotype was assessed visually in soil-grown plants.

Tissue expression of the AtSPL14 'promoter'::GUS construct was assessed by histochemical staining of several independent transgenic lines at different stages of development using 5-bromo-4-chloro-3-indolyl-β-glucuronic acid as a substrate (Jefferson, 1987).

Nuclear localization of FBR6

The *AtSPL14* cDNA was amplified from reverse-transcribed RNA by PCR using oligonucleotide primers with engineered *Bam*HI restriction sites SBPF2: 5′-GGATCCAAATGGATGAGGTAGGAGCTCAAGTG-3′ and SBPR: 5′-ACTAGTCCGGATCCGATTGAGCCATAATCCAAACCTCC-3′. The resulting *AtSPL14* cDNA fragment was cloned into pGEM-TEasy (Promega) and subjected to DNA sequencing. A *Bam*HI fragment was cloned into the appropriately digested binary vector pEGAD to produce an in-frame fusion to GFP (Cutler *et al.*, 2000). pEGAD with no insert and pEGAD-*AtSPL14* were transformed into *A. tumefaciens* strain GV3101 MP90 by electroporation. The resulting strain was grown overnight at 30°C with shaking in LB supplemented with 25 mg l^{-1} rifampicin, 50 mg l^{-1} gentamycin and 50 mg l^{-1} kanamycin, pelleted at 3000 ***g*** 5 min and diluted to an OD_{600} of 1 in 10 mm MES pH 5.6, 10 mm $MgCl_2$, 100 μm acetosyringone (Sigma-Aldrich, St Louis, MO, USA). Transient transformation of *N. tabacum* leaves was achieved by 'agroinfiltration' by infiltrating the strains into leaves using a syringe without a needle (Goodin *et al.*, 2002; Schob *et al.*, 1997; Yang *et al.*, 2000). After 48 h, GFP was visualized with a laser scanning confocal microscope (BioRad MRC-1024ES) and analyzed using BioRad LaserSharp (v3.3) software (Bio-Rad, Hercules, CA, USA). Images shown were merged from Z-series scans.

Transcriptional activation assays in yeast

The *Saccharomyces cerevisiae* strain AH109 and the GAL4 binding domain vector pGBKT7 used to test transcriptional activation in yeast were obtained from BD Biosciences as part of the Matchmaker Two-Hybrid System 3 (Clontech, Palo Alto, CA, USA). The full-length cDNA (a *Bam*HI fragment using engineered restriction enzyme sites) and various truncated versions (*Nco*I/*Bam*HI, Δ1–304; *Bam*HI/*Pst*I, Δ473–1035; *Bam*HI/*Bal*I, Δ185–1035; *Bal*I/*Bam*HI, Δ1–185) were generated by restriction enzyme digestions and cloned into appropriately digested vector preparations of pGBKT7 to produce in-frame fusions to the GAL4 DNA binding domain. Constructs were transformed into yeast strain AH109 that has GAL4-recognized UAS driving expression of four different reporter genes and selected on media lacking tryptophan. Yeast were grown at 30°C with shaking. The ability of the AtSPL14 protein to activate transcription in yeast was assayed by the ability to grow in the absence of histidine (conferred by the *HIS3* reporter gene) and histochemical detection of α-galactosidase activity (conferred by the *MEL1* reporter gene). Cell counts of individual strains were determined by with a hemacytometer. Serial dilutions were plated on media lacking histidine and supplemented with 5-bromo-4-chloro-3-indolyl-α-galactopyranoside (X-α-Gal; 40 mg l^{-1}). The GAL4 binding domain fusion proteins also contained a c-myc epitope. Expression of the various GAL4BD/FBR6 fusion proteins was confirmed by Western

blot analysis using a monoclonal antibody against the c-myc epitope (data not shown).

Recombinant protein expression, purification, and DNA binding assay

To assess whether the SBP domain of AtSPL14 is capable of binding to *A. thaliana* genomic DNA sequences, recombinant fusions proteins were produced in *E. coli* strain Rosetta (EMD Biosciences, San Diego, CA, USA). A *Bam*HI/*Xba*I fragment of the *AtSPL14* cDNA was cloned into vector pMalK (derived from pMalcRI) and affinity-purified with amylose-agarose (New England Biolabs, Beverly, MA, USA). pMalK-FBR6s produces a recombinant protein of maltose binding protein in-frame with the N-terminal 409 amino acids of AtSPL14, encompassing the SBP domain. Purity was confirmed by SDS-PAGE analysis (Laemmli, 1970), and protein concentration was determined with the BCA protein assay according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as a standard. Dilutions of MBP alone and an MBP fusion to the N-terminal 409 amino acid residues of FBR6 were spotted onto a PVDF membrane (Hybond-P; Amersham Biosciences, Piscataway, NJ, USA) in 'binding buffer' (25 mm Hepes-KOH pH 7.4, 50 mm NaCl, 1 mm DTT). The membrane was blocked in 'binding buffer' supplemented with 5% dry milk and probed with 'binding buffer' supplemented with 1% dry milk and random-primed ^{32}P-labeled *A. thaliana* genomic DNA (Sambrook and Russell, 2001). The membrane was washed three times with 'binding buffer' plus 1% milk and analyzed with a Bio-Rad Molecular FX and QuantityOne software (Bio-Rad).

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