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“Role of a Transcriptional Regulator in Programmed Cell Death and Plant Development”

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Summary of outcomes:

Other manuscripts directly based on the funded project include:


Additional manuscripts closely related to the proposed work include:


Objectives, Experimental Plan, and Outcomes

Project DE-FG02-05ER15648, “Role of a Transcriptional Regulator in Programmed Cell Death and Plant Development”, was funded by the Department of Energy from June 2005 to June 2008 at a total cost of $354,000. The long-term goal of the Stone lab research is to understand the role(s) and molecular mechanisms of programmed cell death (PCD) in the controlling plant growth, development and responses to biotic and abiotic stress. We developed a genetic selection scheme to identify A. thaliana FB1-resistant (fbr) mutants as a way to find genes involved in PCD (Stone et al., 2000; Stone et al., 2005; Khan and Stone, 2008). The disrupted gene in fbr6 (AtSPL14) responsible for the FB1-insensitivity and plant architecture phenotypes encodes a plant-specific SBP DNA-binding domain transcriptional regulator (Stone et al., 2005; Liang et al., 2008).

This research plan is designed to fill gaps in the knowledge about the role of SPL14 in plant growth and development. The work is being guided by three objectives aimed at determining the pathways in which SPL14 functions to modulate PCD and/or plant development:
1) determine how SPL14 functions in plant development,
2) identify target genes that are directly regulated by SPL14, and
3) identify SPL14 modifications and interacting proteins.

We made significant progress during the funding period. Briefly, some major accomplishments are highlighted below:

• To identify potential AtSPL14 target genes, we identified a consensus DNA binding site for the AtSPL14 SBP DNA-binding domain using systematic evolution of ligands by exponential selection (SELEX) and site-directed mutagenesis (Liang et al., 2008). This consensus binding site was used to analyze Affymetrix microarray gene expression data obtained from wild-type and fbr6 mutant plants to find possible AtSPL14-regulated genes. These candidate AtSPL14-regulated genes are providing new information on the molecular mechanisms linking plant PCD and plant development through modulation of the 26S proteasome.
• Transgenic plants expressing epitope-tagged versions of AtSPL14 are being used to confirm the AtSPL14 targets (by ChIP-PCR) and further dissect the molecular interactions (Nazarenus, Liang and Stone, in preparation)
• Double mutants generated between fbr6 and various accelerated cell death (acd) mutants indicate that sphingolipid metabolism is influenced by AtSPL14 and sphingolipidomics profiling supports this conclusion (Lin, Markham and Stone, in preparation).
• A new set of phenotypes have been uncovered in the original fbr6-1 mutant, including a short-root phenotype related to auxin signaling and altered photosynthetic parameters related to stomatal density and conductance (Lin and Stone, in preparation; Lin, Madhavan and Stone, in preparation). Additional AtSPL14-related mutants and transgenic plants have been generated to effectively dissect the functions of AtSPL14, including a dominant negative fbr6-2 allele and transgenic plants overexpressing FBR6/AtSPL14 that display an accelerated cell death (acd) phenotype.

Objective #1) Determine how SPL14 functions in plant development and PCD
The working hypothesis for Objective #1 is that the function of SPL14 in plants is related to one or more of the pathways influenced by FB1: cell death, sphingolipid signaling, phase transitions, light perception/signaling, and hormone signaling.

Cell death-related double mutants and phenotypic analyses
Selected lesion mimic mutants that spontaneously form lesions in the absence of pathogen infection (accelerated cell death, acd, proposed to act in opposition to fbr6 in regulating PCD) were chosen with the hypothesis that the double mutants might regain sensitivity to FB1 and/or suppress spontaneous cell death. We have been unable to recover fbr6 acd11 mutants, but we successfully generated double mutants between fbr6 and acd1, acd2 and acd5 (single mutants obtained from Jean Greenberg, U. of Chicago). These double mutants, the single mutants, and wild-type controls were
tested for FB1 sensitivity using our \textit{fbr} phenotype assay (ability to develop when sown on FB1-containing agar plates) and systemic FB1-resistance assay, leaf infiltration followed by monitoring systemic cell death at distal leaves (systemic assay, sys-r). At the germination/development stage we found that \textit{fbr6} suppresses the enhanced sensitivity of \textit{acd5}, but not \textit{acd1} nor \textit{acd2} (data not shown). In the systemic assay, we found that the \textit{fbr6} mutant was clearly resistant to systemic cell death (sys-r). Similar to the results obtained in the \textit{fbr} plate assay, the \textit{fbr6 acd1} and \textit{fbr6 acd2} double mutants were supersensitive to systemic cell death (sys-s). However, the \textit{fbr6 acd5} mutant was as resistant to systemic cell death as the \textit{fbr6} mutant alone (sys-r). That is, the \textit{fbr6} mutation effectively and fully suppressed the \textit{acd5} cell death phenotype using this assay (Figure 1).

\textbf{Figure 1. FB1-induced systemic cell death (sys-r/sys-s) in \textit{fbr6 acdx} double mutants.} Relevant genotypes were subjected to the FB1-resistance systemic assay. Fifty plants of each genotype were assayed in four independent experiments, and the proportion of plants showing systemic cell death was determined. Different colors indicate significantly different values (P<0.05). Nearly 100% of \textit{acd1}, \textit{acd2}, \textit{acd1 fbr6} and \textit{acd2 fbr6} mutants were sys-s, while \textit{fbr6} was sys-r with less than 30% of plants displaying systemic lesions. \textit{acd5} was more sensitive than wild-type plants, and the percentage of \textit{acd5 fbr6} sys-s plants was significantly less than that of \textit{acd5} or wild type and not significantly different from \textit{fbr6} (sys-r).

We also noted genotype differences in production of reactive oxygen species (ROS) and expression of some FB1-induced genes. ROS accumulation was determined by histochemical staining with nitroblue tetrazolium (NBT) or 3,3′–diaminobenzidine (DAB) to detect O$_2^•$– or H$_2$O$_2$, respectively. Four-week-old plants of the relevant genotypes were mock-treated or FB1-treated by infiltrating a single lower leaf. Systemic leaves were stained after 5 days. In mock-treated plants, faint NBT or DAB precipitation was detected for WT, \textit{fbr6}, \textit{acd1 fbr6}, \textit{acd5} and \textit{acd5 fbr6}, whereas stronger NBT or DAB precipitation was seen in \textit{acd1}, \textit{acd2} and \textit{acd2 fbr6} leaves, indicating that introducing the \textit{fbr6} mutant to \textit{acd1} could reduce the O$_2^•$– and H$_2$O$_2$ levels in the double mutant, but the O$_2^•$– and H$_2$O$_2$ levels in \textit{acd2 fbr6} were no different from \textit{acd2}. NBT and DAB staining was stronger for all the genotypes after FB1 infiltration. O$_2^•$– and H$_2$O$_2$ levels in \textit{fbr6} were lower than those in wild type, and there was no difference in O$_2^•$– and H$_2$O$_2$ levels between \textit{acd1} and \textit{acd1 fbr6} or between \textit{acd2} and \textit{acd2 fbr6}. O$_2^•$– and H$_2$O$_2$ levels were significantly lower in \textit{acd5 fbr6} than in \textit{acd5}. Therefore, the \textit{fbr6} mutation could effectively suppress ROS accumulation in the \textit{acd5} mutant. For molecular markers, we examined expression of \textit{PATHOGENESIS-RELATED1} (\textit{PR1}), a vacuolar processing enzyme (\textit{gVPE}) and a gene encoding a sphingosine-1-phosphate lyase (\textit{DPL1}) for steady-state transcript levels with and without FB1 treatment (Stone et al., 2000; Shimada et al., 2003; Kuroyanagi et al., 2005; Niu et al., 2007; Tsegaye et al., 2007). \textit{PR1} gene expression levels didn’t correlate with FB1 sensitivity (data not shown), whereas the \textit{fbr6} mutation suppressed accumulation of the other two markers in \textit{acd5} (Figure 2).

\textbf{Figure 2. Semi-quantitative RT-PCR analyses of (A) Vacuolar processing enzyme (\textit{gVPE}) and (B) Sphingoid Long Chain Base-Phosphate Lyase (\textit{DPL1}) steady-state transcript levels.} Total RNA was isolated from rosette leaves 5 days after FB1 treatment, reverse transcribed with oligo-dT, and used as a template for PCR with \textit{ACTIN2}-, \textit{DPL1}-, and \textit{gVPE}-specific oligonucleotide primers to determine transcript levels. Relative expression was determined as the ratio of \textit{DPL1} or \textit{gVPE} to \textit{ACTIN2} (loading control). Different letters indicate significantly different values (P<0.05).
The different ability of \textit{fbr6} to suppress the cell death susceptibility (and other markers, such as ROS accumulation and FB1-induced gene expression) of these distinct lesion mimic mutants might be related to differences in tissue expression and/or the nature of the encoded proteins. Both \textit{ACD1} and \textit{ACD2} encode proteins implicated in controlling PCD-inducing chlorophyll breakdown products, whereas \textit{ACD5} and \textit{ACD11} encode proteins implicated in sphingolipid metabolism, a ceramide kinase and a sphingosine transfer protein, respectively (Greenberg et al., 2000; Mach et al., 2001; Brodersen et al., 2002; Liang et al., 2003; Pruzinska et al., 2003; Tanaka et al., 2003; Yang et al., 2004; Pruzinska et al., 2005; Yao and Greenberg, 2006). Thus, we conclude that the \textit{fbr6} mutation influences cell death pathways primarily through sphingolipid signaling, as opposed to inappropriate accumulation of chlorophyll breakdown products generated during photosynthesis. This conclusion is supported by our sphingolipidomics profiling data (Figure 3).

\textbf{Sphingolipidomics Profiling Reveals Dramatic Differences in the Response of \textit{fbr6-1} to FB1}

The ability of the \textit{fbr6} mutation to suppress the \textit{acd} phenotype in a mutant affected in a putative ceramide kinase, compelled us to determine sphingolipid profiles for wild-type and \textit{fbr6-1} mutant plants (+ and – FB1) in our systemic FB1-resistance (sys-r) assay by HPLC/MS at the Danforth Center, St. Louis, MO (Markham and Jaworski, 2007). With more than 200 different plant sphingolipid species differing in saturation, hydroxylation and head groups (Dunn et al., 2004; Markham et al., 2006), these analyses provide massive amounts of data representing a comprehensive snapshot of all major classes of sphingolipid species in the analyzed tissue, including ceramides, hydroxyceramides, glucosylceramides, glycosylinositolphosphoceramides, sphingoid long chain bases (LCBs) and their phosphorylated derivatives (LCB-Ps). Only the most relevant and interesting results are summarized briefly here. FB1 treatment clearly caused a shift in sphingolipids pools, as predicted given its function as a competitive inhibitor of ceramide synthase (sphinganine N-acyl transferase, Figure 4). In wild-type plants there was a shift from very long chain fatty acids (VLCFAs; C20 to C26) to shorter chain C16 FAs, but this shift was circumvented by the \textit{fbr6} mutation. FB1 treatment also caused significant accumulation of saturated LCBs and LCB-Ps, d18:0, t18:0, d18:0-P and t18:0-P, while there was little effect on d18:1, t18:1 and t18:1-P accumulation. The \textit{fbr6} mutant, however, accumulated very different levels and types of LCBs and LCB-Ps (which Jonathan Markham described as “really extraordinary!”), where both of the unsaturated versions (d18:1 and t18:1) are hyper-elevated at the expense of the saturated versions (d18:0 and t18:0) relative to wild-type plants (Figure 6).

Interestingly, the LCB-Ps were reduced relative to wild type, which is not completely consistent with our simplified models for why \textit{fbr6} might suppress the \textit{acd5} (deficient in a putative ceramide kinase, CERK) cell death phenotype. However the \textit{in vivo} substrate(s) for ACD5 are not yet known (Liang et al., 2003), and we should be able to reconcile this model with our pending sphingolipid profiling on both \textit{acd5} and the \textit{acd5 fbr6} double mutant. Sphingolipidomics profiling on wild-type plants and sphingolipid metabolism mutants have revealed an unsuspected complexity to plant sphingolipid metabolism/signaling (Liang et al., 2003; Dunn et al., 2004; Chen et al., 2006; Markham et al., 2006; Tsegaye et al., 2007; Dietrich et al., 2008).
Figure 4. Generalized scheme of sphingolipid metabolism as it relates to acd5 fbr6 double mutant analyses and LCB accumulation in response to FB1. A) FB1 inhibits ceramide synthase (aka sphinganine N-acyl transferase). Only a few of the well-studied key sphingolipid species in other eukaryotes (dihydro versions) most relevant to our acd5 fbr6 double mutant analyses are shown here. ACD5 encodes a putative ceramide kinase, DPL1 encodes a sphinganine-P lyase that catalyzes an irreversible step in LCB-P catabolism. B) LCBs (e.g., sphinganine d18:0 and sphinganine-1-P d18:0-P) differentially accumulate in fbr6 and wild-type plants treated with FB1. Error bars represent standard deviation (n=5).

Objective #2) Identify target genes that are directly regulated by SPL14
The working hypothesis for Objective #2 is that SPL14 binds to cis regulatory elements to regulate expression of genes that affect PCD and/or plant development.

SPL14 target gene identification – SELEX and gene expression microarrays

SELEX (systematic evolution of ligands by exponential enrichment)
Three complementary approaches were used to identify AtSPL14 target genes, WGPCR (whole genome PCR), SELEX (systematic evolution of ligands by exponential enrichment), and microarray gene expression analyses of wild-type and mutant plant tissues stimulated under specific conditions (Kehoe and Somerville, 1999; Schenk et al., 2000; Manuel et al., 2002; Wan et al., 2002; Kalifa et al., 2004; Ausubel et al., 2006). We used an affinity-based assay, referred to as SELEX or random binding site selection (RBSS), to screen a random pool of dsDNA fragments for sequences capable of binding to recombinant AtSPL14 protein to identify an AtSPL14-binding consensus DNA motif. Double-stranded DNA molecules containing a 26 nucleotide completely randomized central region were subjected to repetitive cycles of binding to recombinant AtSPL14 bound to a Ni²⁺-chelating affinity resin and PCR amplification. The individual binders were subsequently tested by electrophoretic mobility shift assays (EMSA) and competition with unlabeled probe, yielding twenty distinct dsDNA fragments. Alignment of the twenty individual binders using the web-based multiple expectation maximization for motif elicitation (MEME) analysis program (Grundy et al., 1997) yielded a consensus DNA binding motif (Figure 5). Mutational analyses indicated that predominantly the core motif, CGTAC, is essential for AtSPL14 protein binding to the DNA in vitro. In addition, we monitored the kinetic features of the AtSPL14 SBP domain binding to DNA by surface plasmon resonance (SPR) and compared and contrasted the target sequences we identified and the binding kinetics of AtSPL14 with those of other SBP domain proteins (Klein et al., 1996; Birkenbihl et al., 2005; Kropat et al., 2005). The representative Biochemistry Table of Contents Figure (Figure 5) represents the two Zn-finger structure proposed for AtSPL14 and our identified consensus DNA binding motif (Liang et al., 2008). Using the relaxed consensus DNA-binding motif we identified for AtSPL14 (CGTAC), more than 6000 genes with the motif within 500 basepairs of the translation start site were found as possible AtSPL14 targets, and one gene of unknown function has sixteen occurrences. However, after
analysis of microarray gene expression data comparing the transcriptomes of the fbr6 mutant to wild-type plants (see below), a subset of these candidate target genes for AtSPL14 were identified by their altered expression in fbr6 mutants.

**Figure 5.** Representation of the AtSPL14 SBP binding domain. The presumed Zn$^{2+}$ ion coordinating ligands and the consensus DNA binding motif identified by SELEX represented by WebLogo (Crooks et al., 2004) are shown (Liang et al., 2008).

**Microarray Gene Expression Analyses of the fbr6 Mutant Indicates AtSPL14 Might Function as a Repressor of Select Proteasome Subunit Genes**

To aid in determining FBR6/AtSPL14 target genes, the wild-type and fbr6 mutant transcriptomes were compared after mock treatment or imparting salicylic acid (SA)-induced oxidative stress using both slide-based 70-mer oligonucleotide arrays and Affymetrix ATH1 gene chips. Biological duplicates were performed with two genotypes (wild-type and fbr6 mutants) and two treatments (mock- and SA-treated). Specifically, 4 week-old plants were sprayed with either 0.1% Silwet (mock) or 1 mM SA in 0.1% Silwet (SA). Above ground tissues were harvested 8 hours after the treatment, flash frozen in liquid N$_2$, total RNA was isolated using TRIzol according to manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA) and further purified using Qiagen RNAeasy Min Elute Cleanup kit (catalog # 74204, Qiagen, Valencia, CA, USA). Fluorescent labeling with Cy5 and Cy3, hybridization to Affymetrix ATH1 24K arrays (which represent ~24,000 genes), scanning, and data analysis were provided by UNL’s Genomics Core Facility.

Using an average signal log ratio (SLR) cut-off of 0.5, we found that AtSPL14 expression was downregulated (an SLR of -0.85 in mock treated fbr6 mutant plants compared to wild type representing ~50% of wild-type levels), consistent with our observations that the fbr6-1 allele is not a null mutant, but rather the T-DNA insertion in the 3’UTR results in reduced expression (Stone et al., 2005). When a more stringent SLR cut-off value was used (representing > 2-fold difference), we found that the other genes downregulated in fbr6 do not tend to cluster into major classes based on Gene Ontology (GO) designations with the majority having unknown functions. However, greater than half of the genes with known or predicted functions upregulated in fbr6 are related to proteasome-mediated protein degradation (16 of 32 genes in mock-treated samples and 27 of 48 genes in SA-treated samples), primarily encoding subunits of the 26S proteasome. Therefore, the most obvious global difference between the wild-type and fbr6 transcriptomes (in leaf tissue) appears to be proteasome-related genes. Does AtSPL14 normally function to repress a subset of proteasome subunit genes?

Protein degradation plays an important role in plant PCD (Kim et al., 2003; Hatsugai et al., 2004; Schaller, 2004; Hara-Nishimura et al., 2005; Kuroyanagi et al., 2005; Kim et al., 2006; Vacca et al., 2007). Proteasome-dependent protein degradation has been intimately linked to plant hormone signaling pathways, and subsequently cell differentiation, plant development and stress responses, (Moon et al., 2004; Smalle and Vierstra, 2004; Brukhin et al., 2005; Abas et al., 2006; Arnaud et al., 2006; Huang et al., 2006; Jin et al., 2006; Gusmaroli et al., 2007; Vacca et al., 2007; Kurepa et al., 2008; Staswick, 2008). Effects of the fbr6 mutation on expression of proteasome subunit genes could be due to direct regulation by FBR6/AtSPL14 or indirect regulation due to an overall physiological alterations due to the fbr6 mutation. Indeed, many of the proteasome subunit genes appear to be co-regulated based on publicly available microarray gene expression experiments (e.g., CressExpress, Genevestigator) (Zimmermann et al., 2004; Srinivasasainagendra et al., 2008).

Several of the fbr6 upregulated genes, including the proteasome-related genes also possess the consensus DNA binding motif we identified by SELEX (CGTAC) within 1 kb of the annotated
translation start sites. Promoters for twenty of the altered genes predicted to have the binding motif were amplified by PCR and tested for in vitro binding using EMSA as described (Liang et al., 2008). We have also confirmed the microarray results for selected genes by semi-quantitative RT-PCR (Figure 6), and are attempting to use chromatin immunoprecipitation (ChIP) assays in transgenic lines expressing epitope-tagged versions of AtSPL14 to determine whether AtSPL14 binds directly to these promoters in vivo. This approach has been technically challenging, and due to some difficulty with reproducibility, results have been inconclusive.

**Figure 6. Proteasome subunits as potential AtSPL14 targets.** Semi-quantitative RT-PCR was performed with RNA isolated from fbr6 mutant and "wild-type" (WT) plants 8 hours after being subjected to either mock (-) or SA (+) treatments by spraying until run-off (1 mM SA, 0.01% Silwet). PCR was performed with oligonucleotide primers that anneal to PAE2 or ACTIN2 as an internal control. Steady-state PAE2 transcript levels are greater in the fbr6 mutant than in wild-type plants regardless of treatment, consistent with the Affymetrix microarray gene expression analyses, and four copies of the AtSPL14 consensus DNA binding site are present in the PAE2 promoter suggesting that AtSPL14 directly modulates PAE2 expression.

**Objective #3) Identify SPL14 modifications and interacting proteins**

The working hypothesis for Objective #3 is that SPL14 regulates gene expression within a signal complex involving multiple proteins and is redox sensitive.

Much of the progress on this objective is related to generating the appropriate genetic backgrounds and transgenic lines to confirm the in vivo relevance of our in vitro results. Stable transgenic plants that express epitope-tagged versions of SPL14 and the yeast two-hybrid interactors are at various stages of development. In many cases, we are first identifying homozygous knock-out insertion lines to uncover phenotypes and/or facilitate the in vivo protein-protein and protein-DNA interaction assays. We cannot express FBR6 under control of a constitutive promoter, but have observed some expression now under control of the native promoter. However, we have determined that we can effectively immunoprecipitate NTAPi-, HA- and GFP-tagged proteins (in complex) from plant extracts.

**References**


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

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Summary

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; Michael 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; Pruszinska 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., 2004).
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 morphogenesis. 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 F1 progeny were self-fertilized, and the resulting F2 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 F2 progeny segregated at the expected 3:1 (sensitive:resistant) ratio for a single recessive mutation (χ² = 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 F3 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 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_{4,13}H_{X,15}CQQCX_{4,11}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 (KRSCRRLAGHNRHRRR) fitting the
consensus for a bipartite 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 cauleine 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 activates transcription in yeast and binds A. thaliana DNA

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).
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 transcriptional regulators beyond 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.

**Figure 3.** DNA sequences immediately upstream of the translation start site for the *AtSPL14* gene drives expression of the $\beta$-glucuronidase (GUS) reporter gene in several plant tissues. Histochemical staining for $\beta$-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 $\mu$m).
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 ligueuleless1 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

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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 trichome-less (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 (LXXL, 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 – Cx4CxxHxx3Hx19QQCxx3Hx11C) 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; Leitly 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 H2O, 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 oligo68; http://www.dartmouth.edu/~tjack/) and primer TAIL-AD2 5'-ntgqasgwanawga-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)\textsubscript{18} primer, 40 U RNasin (Promega), 500 μM dNTPs, and 10 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\textsubscript{2}, 250 nm oligonucleotide primers. Oligonucleotide primers were cFB6R7: 5'-CGGCCCTCAATTTTTGCT-3'; cFB6R6STOP: 5'-CCTCCCGGTTAGTATGGTCTAGATTGAGCCATAATCC-3'; UBOS-F: 5'-GGTGTTCAAGAAGGAGAAGA-3'; UBOS-R: 5'-CAAGCTTCAACCTCTTTTT-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 Smal F’: 5'- CCTCCCGGTTAGTATGGTCTAGATTGAGCCATAATCC-3’ and ‘polyA Smal R’: 5’-CCTCCCGGTTAGTATGGTCTAGATTGAGCCATAATCC-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 Smal F’ and ‘prom Smal R’: 5’-CCTCCCGGTTAGTATGGTCTAGATTGAGCCATAATCC-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 of the ATG start codon and 443 bp downstream of the STOP codon were cloned into pGEM-TEasy (Promega) and subjected to DNA sequencing with T7 and SP6 oligonucleotide primers.

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::GUS construct was inserted into T-DNA of pCAMBIA 3300, both vectors carry a gene that confers resistance to gentamycin and 50 mg l\textsuperscript{-1} kanamycin, pelleted at 3000 g min and diluted to an OD\textsubscript{600} of 1 in 10 mM MES pH 5.6, 10 mM MgCl\textsubscript{2}, 100 μM acetylsyringone (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 pGBK7 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 BamH I fragment using engineered restriction enzyme sites) and various truncated versions (Ncol/BamH I, Δ1–304; BamH I/PstI, Δ473–1035; BamH I/Ball, Δ185–1035; Ball/BamH I, Δ1–185) were generated by restriction enzyme digestions and cloned into appropriately digested vector preparations of pGBK7 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 the hemacytometer. Serial dilutions were plated on media lacking histidine and supplemented with 5-bromo-4-chloro-3-indolyl-α-galactopyranoside (X-gal; 40 mg l\textsuperscript{-1}). The GAL4 binding domain fusion proteins also contained a c-myc epitope. Expression of the various GAL4DB/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 BamHI/XbaI 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 1% dry milk and probed with ‘binding buffer’ supplemented with 1% dry milk and random-primed 32P-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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Identification of a Consensus DNA-Binding Site for the Arabidopsis thaliana SBP Domain Transcription Factor, AtSPL14, and Binding Kinetics by Surface Plasmon Resonance†

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ABSTRACT: Proteins with a conserved Cys- and His-rich SQUAMOSA promoter binding protein (SBP) domain are transcription factors restricted to photosynthetic organisms that possess a novel two Zn-finger structure DNA-binding domain. Despite the fact that altered expression of some SBP-encoding genes has profound effects on organism growth and development, little is known about SBP domain protein target genes. Misexpression of the Arabidopsis thaliana AtSPL14 SBP domain gene confers resistance to programmed cell death and modifies plant architecture. A consensus DNA-binding motif for AtSPL14 was identified by systematic evolution of ligands by exponential enrichment (SELEX) or random binding site selection (RBSS). DNA recognized by AtSPL14 contained the core binding motif (GTAC) found for other SBP domain proteins, but mutational analyses indicated that at least one additional flanking nucleotide is necessary for effective AtSPL14−DNA interaction. Comparison of several SBP domain amino acid sequences allows us to hypothesize which specific amino acids might participate in this sequence-specific DNA recognition. Electrophoretic mobility shift assays (EMSA) with mutant AtSPL14 DNA-binding domain proteins indicated that not all of the Zn2+ ion coordinating ligands in the second Zn structure are strictly required for DNA binding. Surface plasmon resonance (SPR) was used to evaluate AtSPL14 in vitro binding kinetics for comparison of equilibrium binding constants with other SBP domain proteins. These data provide a strong basis for further experiments aimed at defining and distinguishing the sets of genes regulated by the closely related SBP domain family members.

Proper growth and development of multicellular organisms depend on a delicate balance between cell proliferation and programmed cell death (PCD). In plants, PCD is required for tracheary element differentiation to form the water-conducting xylem tissue and accurate formation of various reproductive organs (1−5). PCD is also an important aspect of plant defense against pathogen attack (6−8). Despite the essential nature of PCD, large gaps remain in our knowledge of the mechanistic details and molecular components controlling plant PCD. Therefore, to identify novel genes that might participate in plant PCD, we developed a mutant selection scheme for the model plant Arabidopsis thaliana using a PCD-inducing fungal toxin fumonisin B1 (FB1), to identify FB1-resistant (fbr) mutants (9−11). Misexpression of the AtSPL14 gene in the fbr6 mutant confers the ability to proliferate in the presence of FB1 and modifies normal plant architecture, linking the insensitivity to cell death to altered plant development (9). AtSPL14 has features of a DNA-binding transcription factor, including a Cys- and His-rich SQUAMOSA promotor binding protein (SBP) domain predicted to bind DNA, ankyrin repeats that mediate protein−protein interactions, nuclear localization, and ability to bind to A. thaliana genomic DNA (9).

SBP domain proteins are defined by a conserved approximately 80 amino acid−protein domain (the SBP domain or SBP box) found only in proteins from photosynthetic organisms, ranging from single-celled algae (e.g., Chlamydomonas reinhardtii) to higher plants (e.g., A. thaliana and Oryza sativa). The SBP gene families are comprised of 16 genes in A. thaliana and 19 genes in O. sativa that encode proteins that share the highly conserved SBP DNA-binding domain but are diverse in overall domain structure (12, 13). To date, only a few functions for SBP domain proteins have been reported, perhaps due to genetic redundancy of closely related family members. In all cases, SBP domain proteins have been implicated in various aspects of plant growth and development, including metal sensing in algae and directing development of leaves, embryos, and floral organs in higher

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1 Abbreviations: CuRE, copper-responsive element; dsDNA, double-stranded DNA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; FB1, fumonisin B1; fbr, FB1-resistant; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PCD, programmed cell death; PMSF, phenylmethanesulfonyl fluoride; RBSS, random binding site selection; RU, resonance (response) units; SA, streptavidin; SBP, SQUAMOSA promoter binding protein; SDS, sodium dodecyl sulfate; SELEX, systematic evolution of ligands by exponential enrichment; SPL, SQUAMOSA promoter binding protein-like; SPR, surface plasmon resonance; WT, wild type.

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plants (9, 13–22). Some of these SBP domain proteins may also be posttranscriptionally regulated by noncoding microRNAs to control their spatial and temporal expression (23–25). Despite the obvious importance of these proteins, the specific genes targeted by individual SBP domain family members and the molecular consequences of their actions are largely unknown.

To fully understand the physiological functions of AtSPL14 in regulating plant PCD and/or development, AtSPL14 target genes, their modes of regulation, and the consequences of expression repression need to be determined. As one step toward that goal, we used an affinity-based assay, referred to as systematic evolution of ligands by exponential enrichment (SELEX) or random binding site selection (RBSS) to screen a random pool of dsDNA fragments for sequences capable of binding to recombinant AtSPL14 protein. From this analysis, an AtSPL14-binding consensus DNA motif was derived. Mutational analyses indicated that predominantly the core motif, CGTAC, is essential for AtSPL14 protein binding to the DNA in vitro.

Recent structures of SBP domains determined by nuclear magnetic resonance (NMR) indicate that SBP domains form a unique two Zn-finger structure DNA-binding domain (26, 27). The SBP domain contains eight absolutely conserved Cys or His residues, some of which are critical for SBP domain DNA binding (28). We determined that all of the highly conserved cysteine in the two Zn+ ions binding structures of AtSPL14 SBP domain binding are important for DNA recognition by electrophoretic mobility shift assays (EMSA) and surface plasmon resonance (SPR). Yet, an AtSPL14 SBP domain with one of the Cys residues in the second Zn-finger structure mutated retained some DNA-binding ability. Moreover, we monitored the kinetic features of the AtSPL14 SBP domain—DNA binding by SPR. We further compare and contrast the target sequences and the equilibrium binding constants we determined for AtSPL14 with those of other SBP domain proteins (18, 28, 29).

**MATERIALS AND METHODS**

**Recombinant AtSPL14 Protein Expression and Purification.** Two different truncated and epitope-tagged versions of recombinant AtSPL14 proteins were used. The full-length AtSPL14 cDNA was generated by reverse transcription—polymerase chain reaction (RT-PCR) from RNA isolated from ecotype Col-0 (the reference genotype for the A. thaliana genome) using oligonucleotide primers SBPF (5′-GGATCCATGATTGAGAATTCAGCTTAAGTG-3′) and SBPR (5′-ACTAGTGCGATCCGATTTGACCATTAACTCAACCTC-3′) and verified by DNA sequencing (30). Engineered BamHI and existing internal SalI restriction enzyme recognition sites were used to subclone FBR6 short (FBR6s; aa 1–402) into BamHI/SalI-digested pET-28a(+) to produce a protein composed of an N-terminal His tag, thrombin cleavage site, and T7 tag fused to a region of AtSPL14 encompassing the DNA-binding domain (Novagen, EMD Chemicals, Inc., Darmstadt, Germany). The His-tagged FBR6s protein was 447 amino acids with a predicted molecular mass of 48.8 kDa.

For electrophoretic mobility shift assays (EMSA) and surface plasmon resonance (SPR) binding assays, recombinant epitope-tagged FBR6 supernshort (FBR6ss; aa 111–200) protein was used. The conserved SBP domain of AtSPL14 was amplified by PCR using oligonucleotide primers oJS86 (5′-CCGAAATTCCTCCGGAGGGAATTATCCC-3′) and oJS87 (5′-CCGATATCTCTATGGACCGGTTCGGCG-3′) and verified by DNA sequencing (30). The resulting PCR product was subcloned into pET-28a(+) to produce a protein composed of an N-terminal His tag, thrombin cleavage site, and T7 tag fused to a region of AtSPL14 encompassing the DNA-binding domain. The His-tagged FBR6ss protein was 126 amino acids with a predicted molecular mass of 14.3 kDa.

The recombinant proteins were expressed in *Escherichia coli* by inducing log phase cultures with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 2 h, and proteins were purified on Ni2+-conjugated affinity resin according to the manufacturer’s instructions (ProBond Purification System; Invitrogen, Carlsbad, CA). For SELEX experiments proteins were retained on the resin.

**Site-Directed Mutagenesis.** The site-directed mutagenesis was performed according to the Stratagene’s QuickChange site-directed mutagenesis kit instruction manual (Stratagene, Cedar Creek, TX). Oligonucleotide primers used to mutate the nucleotides in the AtSPL14-binding DNA and the cysteine residues of the AtSPL14 SBP domain are shown in Tables S1 and S2 of the Supporting Information, respectively.

**Systematic Evolution of Ligands by Exponential Enrichment (SELEX) or Random Binding Site Selection (RBSS).** A random pool of oligonucleotides (76 nucleotides) of sequence 5′-GCTGCAGAGGACACTCGGGTCTCN26CGACAGGATCGGCTGAAGTCTAGGACGCTGTG-3′, where N26 represents 26 randomized nucleotides, was synthesized by equimolar incorporation of A, G, C, and T at each “N” position (Integrated DNA Technologies, Coralville, IA). The two sets of 25 nucleotides flanking the 26-nucleotide random core were amplified by PCR to make double-stranded DNA (dsDNA), the random pool of oligonucleotides (100 ng) was subjected to PCR using the forward primer and Taq polymerase enzyme (94, 68, and 72 °C, 1 cycle), and the PCR products were purified by agarose gel electrophoresis (1.5% MetaPhor agarose; Cambrex Bio Science Rockland, Inc., Rockland, ME) to yield ds-R76, the substrate in the initial binding reaction.

The binding reactions were carried out on ice essentially as described with a few modifications (31). The recombinant protein T7-His6-FBR6s was purified using a His tag purification kit (ProBond; Invitrogen, Carlsbad, CA) and retained on the resin. The resin with immobilized protein (200 μL) was washed twice with binding buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM MgCl2, 0.2 mM EDTA, 5% glycerol, 0.5 mM DTT, 50 μM PMSF), mixed with 50 μg/mL poly(dIdC) (Amersham Biosciences, Cleveland, OH) to reduce nonspecific binding for 10 min, and incubated with ds-R76 DNA (200 ng) for 60 min, with gentle tapping every 10 min. The immobilized protein—DNA complexes were washed with TN buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) five times, and the DNA was eluted with 200 μL of dissociation buffer (0.5 M Tris-HCl, pH 7.4, 20 mM EDTA, 10 mM NaCl, 0.2% SDS). The bound DNA was amplified by PCR using 250 nM oligonucleotide primers that anneal to the defined terminal sequences of the 76-nucleotide oligonucleotide for 20 cycles. The resulting product was used as the substrate in the second round of SELEX. After five
serial selection rounds, the amplified DNA was cloned into vector pGEM-TEasy (Promega, Madison, WI) and subjected to DNA sequencing (University of Nebraska—Lincoln, Genomics Core Facility). Effective DNA binding was verified by electrophoretic mobility shift assay (EMSA) competition assays.

**Electrophoretic Mobility Shift Assay (EMSA).** DNA fragments were fluorescently labeled by PCR amplification in a reaction containing 100 μM dNTPs, 1× PCR buffer, 1.5 mM MgCl₂, 2 ng of DNA template (pGEM-TEasy subclones selected from SELEX), 300 nM 5′-IR-dye 700 GTACCTTCGTTGCGCTAG-3′ corresponding to the T7 promoter (Li-Cor, Lincoln, NE), 300 nM primer R or primer F, and Taq polymerase enzyme. The resulting PCR product was quantified using gel electrophoresis. Electrophoretic mobility shift assay binding reactions were performed in the same binding buffer used in the SELEX (exception: 1 mM DTT and no PMSF) in a total volume of 20 μL containing 60 nM protein and 4 nM labeled DNA and incubated for 30 min at 25 °C in darkness prior to electrophoresis. The protein–DNA binding mixture was electrophoretically resolved for 45 min at 4 °C in the dark on a prerun 8% nondenaturing polyacrylamide gel (polyacrylamide-bis ratio = 37.5:1) in Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA) at constant voltage (15 V/cm). Gels were analyzed using an Odyssey Infrared Imager (Li-Cor, Lincoln, NE).

**Immobilization of Biotinylated DNA on the SA Sensor Chip.** Cognate and noncognate DNA fragments (156 bp) containing dsR76 used in the SELEX experiments were biotinylated by PCR amplification with dsDNA cloned in pGEM-TEasy as templates, 5′-biotin-GTACCTTCGTTGCGCTAG-3′ oligonucleotide corresponding to the T7 promoter (IDTDNA, Cedar Rapids, IA), and either primer R or primer F. The PCR products were purified by the QiAquick PCR purification kit (Qiagen, Valencia, CA), and DNA was immobilized on streptavidin (SA) sensor chips (Biacore AB, Uppsala, Sweden).

5′-Biotin-labeled DNA (the random binding site selected DNA 14 containing the consensus FBR6ss binding site, CCGTAC, Figure 1) was immobilized onto the SA sensor chip surface [450 resonance (response) units, RU] to provide the maximal level of RU associated with protein binding in the range of 50–100 RU. SA chips were conditioned with three consecutive 1 min injections of 1 M NaCl and 50 mM NaOH prior to immobilization. Two reference cells were used as controls for background subtraction: reference 1 was the surface alone (no bound DNA), and reference 2 had a noncognate DNA (a random DNA that did not possess the consensus binding motif) immobilized to the reference cell at the same concentration as the cognate DNA.

**Surface Plasmon Resonance (SPR) Analysis.** The purified His-FBR6ss protein was desalted and equilibrated in HBS-EP buffer using Centricon-mediated centrifugal filtration (10 kDa cutoff; Millipore, Billerica, MA). The protein was diluted in HBS-EP buffer to yield several different concentrations ranging from 3.25 to 300 nM. Varying protein concentrations were injected at a 75 μL/min flow rate. The chip surface was regenerated by injection of 0.1% SDS and 3 mM EDTA buffer for 1 min after each protein injection. Responses from the reference cell(s) were subtracted to correct for refractive index changes and nonspecific binding.

**SPR Data Analysis.** Data were analyzed with BIAevalulation 3.0 software (Biacore, Piscataway, NJ), which automatically calculates binding parameters taking into account control and experimental results allowing for quantitative kinetic analyses. Association (kₐ) and dissociation (kᵯ) rates and overall affinity, the equilibrium dissociation constant (Kᵯ), were calculated using a simple bimolecular 1:1 Langmuir isotherm binding model (A + B ↔ A–B) and a mass transfer model that accounts for mass transfer limitations due to rapid association and/or dissociation rates (32). Nonspecific binding effects were subtracted using the sensogram generated from the control reference cell(s). Experiments were replicated in triplicate with similar results.
RESULTS

Identification of a Consensus Binding Site for the AtSPL14 SBP Domain by Systematic Evolution of Ligands by Exponential Enrichment (SELEX). SELEX or random binding site selection (RBSS), an in vitro oligonucleotide binding and PCR amplification method, was used to define consensus DNA-binding sequences for the AtSPL14 SBP DNA-binding domain (33–35). The AtSPL14-binding DNA was selected from a pool of 76 bp of double-stranded DNA (dsDNA) with a central core of 26 random nucleotides by repeated cycles of binding to the hexahistidine-tagged FBR6s protein (encompassing the SBP DNA-binding domain) immobilized on Ni²⁺-chelating affinity resin. Fifty-seven individual clones that were derived from five serial rounds of selection were subcloned and subjected to DNA sequencing, revealing that identical clones were identified multiple times. All of the unique clones were tested for binding to FBR6ss using EMSA assays and competition with unlabeled dsDNA probe.

FIGURE 2: Specific AtSPL14 DNA binding by electrophoretic mobility shift assays (EMSA) and competition assays. (A) An example of the initial competition assays by EMSA used to determine binding of the individual dsDNA fragments identified by SELEX. The double-stranded DNA probe was generated by PCR with IRDye 700 fluorescently labeled (probe) or unlabeled (competitor) oligonucleotide primers. The probe (4 nM) was incubated without (−) or with (+) recombinant His-tagged AtSPL14 SBP domain protein (FBR6ss, 60 nM). For testing specificity, increasing amounts of unlabeled competitor were included in the binding reaction; the ratio of competitor:probe is indicated. “Complexes” were separated on an 8% nondenaturing polyacrylamide gel and visualized by infrared imaging. Lanes: (1) free probe; (2) probe plus AtSPL14 SBP domain protein; (3–6) probe plus increasing amounts of unlabeled competitor. (B) For EMSA competition assays, single nucleotide substitutions in the core consensus binding site of a selected dsDNA-binding fragment were generated by site-directed mutagenesis. The core consensus motif is in color with flanking nucleotides in black for the wild-type (wt) dsDNA, and individual nucleotide changes for the mutated (M1–M13) dsDNA are indicated. (C) EMSA competition assays with the wild-type (WT) or mutated (M1–M13) dsDNA fragments. For the binding reactions, “FP” indicates free probe with no protein, “0” indicates no competitor, and the triangles represent increasing amount of competitor in the binding reaction (20× or 40× molar ratios). (D) Band intensities corresponding to bound complexes were determined by infrared imaging (Odyssey; Li-Cor, Lincoln, NE). Binding efficiencies were normalized to a control binding reaction with no competitor on each gel (“0” in panel C). Binding levels with a 20× molar ratio (black bars) and a 40× molar ratio (white bars) of unlabeled competitor are shown. Error bars represent 95% confidence levels from experiments performed in triplicate.
The final outcome of these analyses was 20 individual sequences. The consensus binding site was identified using the web-based multiple expectation maximization for motif elicitation (MEME) analysis program with manual manipulation and optimization to find the most representative binding motif; identified by SELEX (clone 14, Figure 1A) was immobilized to the SA sensor chip. For controls, reference cells were either surface (no immobilized DNA) or noncognate (with a 156 bp random DNA lacking the consensus motif immobilized). The AtSPL14 SBP domain–DNA interactions were then analyzed by SPR.

To determine ideal conditions for kinetic analyses, 15 nM recombinant protein was injected at two different flow rates, 15 and 75 µL/min. We found significant variation with flow rate, suggesting a mass transfer limitation (data not shown). Varying concentrations of recombinant protein (5, 10, 15, 20, 25, 30, and 50 nM) were injected at the higher flow rate (75 µL/min). The SPR response data were then fit to various models using BIAevaluation 3.0 software (Biacore). Non-specific binding was not observed when the protein concentration was less than 300 nM, and the noncognate DNA reference cell subtraction method was used (Figure S1 of the Supporting Information). The sensorgram data did not fit well to the simple bimolecular 1:1 Langmuir isotherm binding model (Figure 3A) but fit well using the binding mass transfer model (Figure 3B). According to these data, the AtSPL14 SBP domain has extremely rapid association and dissociation rates with cognate DNA possessing the consensus binding motif; indicated that these nucleotides are also important, as they markedly reduced competition for AtSPL14 SBP domain binding (Figure 3). These mutational analyses support that critical for DNA binding, and even conservative changes (i.e., pyrimidine-to-pyrimidine changes in the C and T) effectively abolished the ability of these mutated dsDNAs to compete in EMSA competition assays. These experiments revealed that the C in position 1 and the A or G in position 7 are dispensable for binding, as dsDNA fragments with mutations in these nucleotides could still effectively compete in EMSA competition assays (Figure 2). Positions 2, 3, and 4 (CGT) are critical for DNA binding, and even conservative changes (i.e., pyrimidine-to-pyrimidine changes in the C and T) effectively abolished the ability of these mutated dsDNAs to compete for binding. Mutation of the A and C in positions 5 and 6 indicated that these nucleotides are also important, as they markedly reduced competition for AtSPL14 SBP domain binding (Figure 2). These mutational analyses support that the AtSPL14 SBP domain recognizes a core consensus binding motif of CCGTACA.

AtSPL14 SBP Domain–DNA Interactions by Surface Plasmon Resonance (SPR). SPR analysis was performed using a BIAcore 2000 instrument to measure real-time interactions between DNA coupled to a sensor chip and an analyte (recombinant FBR6ss) in constant flow. A biotin-labeled 156 bp DNA containing the CCGTACA consensus binding motif identified by SELEX (clone 14, Figure 1A) was immobilized to the SA sensor chip. For controls, reference cells were either surface (no immobilized DNA) or noncognate (with a 156 bp random DNA lacking the consensus motif immobilized). The AtSPL14 SBP domain–DNA interactions were then analyzed by SPR.

Because of the mass transfer limitation, equilibrium dissociation constants were evaluated by the steady-state binding kinetics (R_kd). Nine different protein concentrations were injected at 15 µL/min for 10–15 min to ensure that the binding reaction reached equilibrium. The equilibrium binding constant (K_eq) was obtained by fitting the protein concentration corresponding to the steady-state binding level.
to a simple 1:1 binding model (Figure 4), resulting in an equilibrium binding constant $K_A = 3.8 \times 10^8 \text{ M}^{-1}$. Experimental error was determined by performing the experiment in triplicate; $K_A = 3.1 \times 10^8 \pm 1.0 \times 10^8 \text{ M}^{-1}$ (SEM). Therefore, the binding affinities obtained from determining both kinetic rate constants and steady-state equilibrium analyses were similar.

**Amino Acids Required for AtSPL14 SBP Domain–DNA Interactions.** Multiple sequence alignments of SBP domains from *A. thaliana*, *Antirrhinum majus*, and *C. reinhardtii* indicate that this is a highly conserved protein domain, with sequence identity ranging from 50% to 96% (Figure S2 of the Supporting Information). The consensus sequence is CX$_4$C-X$_2$YX$_2$HX$_2$CX$_4$RXCQCCX$_2$HX$_2$FDX$_2$SCRX$_2$LX$_2$HXXRR, where “X” is not absolutely conserved. The six absolutely conserved Cys residues and two of the absolutely conserved His residues have been shown to coordinate two Zn$^{2+}$ ions to form a novel two Zn-finger structure (26, 27). The presumed coordinating amino acids of the AtSPL14 SBP domain are shown in Figure S2 of the Supporting Information. To explore whether these six Cys amino acids are required for the AtSPL14 SBP domain to bind DNA, all six amino acids were individually mutated to Ala and Ser, and the mutant proteins were assayed for DNA-binding efficiency by EMSA. All 12 Cys substitutions almost completely abolished the ability of the AtSPL14 SBP domain to bind DNA, with the exception of C164-A and C164-S, which exhibited very weak binding at high protein concentrations (Figure 5). We also tested the ability of the mutant proteins to bind DNA by SPR. The binding of the mutant AtSPL14 SBP domain proteins was markedly reduced compared to the wild-type protein (Figure 5). Therefore, all six highly conserved Cys amino acids are important for DNA binding, but weak affinity is retained when one of the Cys amino acids is mutated to Ala or Ser.
acids predicted to coordinate a Zn$^{2+}$ ion in the second Zn-finger structure is mutated.

**DISCUSSION**

Our interest in determining the AtSPL14 SBP domain DNA-binding motif and binding kinetics derives from identification of an *A. thaliana* mutant with a disruption in the *AtSPL14* gene (At1g20980). The *A. thaliana* FB1-resistant (*fbr6*) mutant was originally identified by selecting for mutants capable of growth and development on media containing the fungal toxin fumonisin B1 (FB1). FB1 disrupts sphingolipid metabolism in eukaryotes (38) and induces PCD, dependent on transcription, translation, reversible protein phosphorylation, light, and hormone signaling pathways in *A. thaliana* (10, 11). In addition to resistance to FB1, the *fbr6* mutant also exhibits altered plant architecture, including elongated petals and enhanced leaf margin serration (9). Knowledge of the AtSPL14 DNA-binding site (and potentially regulated genes) is critical to fully understanding the physiological functions of this sequence-specific DNA-binding transcription factor.

The *AtSPL14* SBP Domain Binds to the Core GTAC Consensus Target Site but Requires an Additional Flanking Nucleotide for Effective Interaction. *AtSPL14* (At1g20980) encodes a 1035 amino acid–protein with an SBP DNA-binding domain (http://srs.ebi.ac.uk; IPR004333), a bipartite nuclear localization signal (aa 117–193), and ankyrin repeats that mediate protein–protein interactions (IPR002110; aa 821–941) in the C-terminal region of the protein (9, 13, 39, 40). The highly conserved SBP DNA-binding domain is a Cys-and His-rich region (consensus: CXC$_{12}$HX$_{1}$HX$_{15}$CQQCX$_{5}$HX$_{11}$C) found only in proteins from photosynthetic organisms (12, 13, 18, 28). The founding members of the SBP domain gene family (SBP1 and SBP2 from *A. majus*) were identified by their ability to bind to the upstream regulatory region of the *SQUAMOSA* floral meristem identity gene (29). Previous reports indicated that *A. majus* proteins and some *A. thaliana* SBP domain proteins bind DNA encompassing a ten nucleotide motif common to cis regulatory elements in the orthologous *A. majus* *SQUAMOSA* and *A. thaliana* *APETALA1* gene promoters and a similar motif in the *A. majus* *DEFH84* promoter in *vitro* (13, 17). Alignment of these sequences revealed a putative consensus DNA-binding motif of TNCGTACAA (13). It appears that GTAC is the core DNA-binding motif for all SBP domains described so far, but nucleotides flanking the core motif are preferred by different SBP domain proteins.

We used a completely random approach, referred to as systematic evolution of ligands by exponential enrichment (SELEX) or random binding site selection (RBSS), to identify the consensus DNA-binding site for AtSPL14. These analyses revealed CCGTAC(A/G) as the optimal binding site for the AtSPL14 SBP domain (Figure 1). We found no evidence for a palindromic binding motif, which would suggest that binding occurs as a dimer, consistent with a previous report that other SBP domains bind DNA with a 1:1 stoichiometry (26). To verify the importance of the consensus motif, we mutated the individual nucleotides of the predicted binding site CCGTAC(A/G) to obtain both conserved and nonconserved substitutions of each base except the 3′-end A/G (Figure 2B). Mutations in the CGTAC core markedly reduced competition for AtSPL14 binding, but the C at the 5′-end and the A/G at the 3′-end were dispensable (Figure 2). Therefore, each individual nucleotide of the core motif CGTAC is necessary for effective AtSPL14 SBP domain DNA binding (Figure 2).

Birkenbihl et al. (28) recently used a similar random binding site selection with G and T fixed at positions 7 and 8 (of 16 total “random” nucleotides) to identify binding sites for AtSPL3 and AtSPL8 SBP domains. A high preference for at least one more C at the 5′-end of the core consensus GTAC motif was found for AtSPL3 (83%), but not for AtSPL8. Therefore, the recognition site we determined for AtSPL14 is more similar to that of AtSPL3 than AtSPL8. The preference for a C flanking the GTAC core must be due to a specific amino acid–nucleotide interaction. The overall sequence identities between the AtSPL14 SBP domain and those of AtSPL3 (70%) and AtSPL8 (74%) are quite similar (Figure S2 of the Supporting Information), with only three amino acid differences in the Zn2 region proposed to interact directly with DNA (27). AtSPL14 and AtSPL3 have a Glu, Arg, and Gly whereas AtSPL8 has Asn, Lys, and Asp, respectively (Figure S2 of the Supporting Information). Whereas no simple universal code has been elucidated, specificity is imparted in most protein–DNA contacts by hydrogen bonding in the major groove (41). Statistical analysis of atomic interactions in 139 protein–DNA complexes analyzed from the Protein Data Bank (PDB) revealed favored amino acid–nucleotide pairs (42). The authors categorized direct amino acid–nucleotide contacts, including hydrogen bonds and electrostatic, hydrophobic, and other van der Waals interactions. Whereas contacts with the sugars or phosphates of DNA contribute to DNA–protein stability, only H-bonds to nucleotide bases can confer sequence specificity. In interactions with nucleotide bases, the most commonly observed interaction was Arg with G (42). Therefore, it seems most likely that the substitution of Lys in AtSPL8 for Arg in AtSPL14 and AtSPL3 renders the 5′ nucleotide flanking the core GTAC motif unimportant. This hypothesis will need to be tested experimentally.

Simple pattern matching searches for the consensus binding sequence reported for the *A. majus* SBP proteins “TNCGTACAA” (13, 29) upstream of annotated *A. thaliana* genes identified 331 and 640 putative SPL-regulated genes with the pattern within 500 and 1000 base pairs of the translation start site, respectively. Using the consensus DNA-binding motif, we identified for AtSPL14 (CGTAC) more than 6000 genes with the motif within 500 base pairs of the translation start site as possible AtSPL14 targets, and one gene of unknown function has 16 occurrences. Combined with microarray gene expression data comparing the transcriptomes of the *fbr6* mutant to wild-type plants, a subset of candidate target genes for AtSPL14 were identified (data not shown). Whereas some of these promoter sequences also bind to AtSPL14 in *vitro*, additional experiments, such as chromatin immunoprecipitation (ChIP), will be required to verify that these are true targets for AtSPL14 in *vivo*.

The Kinetic Binding Parameters of SBP Domains Differ. The SBP domain DNA binding is dependent on the presence of Zn$^{2+}$ ions (18, 28). The NMR-resolved structures of SBP domains revealed that the SBP domain forms a novel two Zn structure DNA-binding domain (26). The two Zn$^{2+}$ ions are coordinated by three Cys residues and one His residue,
forming two Zn structures in CCCH (Zn1) and CCHC (Zn2) configurations (Figure S2 of the Supporting Information). We mutated all six highly conserved Cys to Ala and Ser and determined the SBP–DNA binding efficiency by EMSA and SPR. All 12 mutations markedly reduced the AtSPL14–DNA interaction (Figure 2), verifying that all six conserved Cys in the AtSPL14 SBP DNA-binding domain are important for AtSPL14 protein–DNA interaction and consistent with the supposition that these Cys residues participate in Zn2\(^{2+}\) ion binding (26, 28). However, the C164-A and C164-S (corresponding to the second Cys in Zn2) mutant proteins retained some weak binding ability (Figures 2 and S2 of the Supporting Information). The second Cys and the His in Zn1 and the first and fourth Cys in Zn2 were reported to be critical for AtSPL1 DNA binding (28). In that study the second Cys in Zn2 was not mutated, but the His in Zn2 was found to be somewhat dispensable (28). Therefore, we independently determined that all of the Zn1 coordinating residues are essential, but two of the presumed Zn2 coordinating residues (the His and second Cys) are not absolutely required for SBP domain DNA binding. These findings support the conclusions derived from the NMR structure of a truncated SBP domain protein (AtSPL12). The Zn1 structure is critical for overall structure, and removal of part of the Zn2 domain affected DNA binding but had little effect on overall folding (27). It is not yet clear whether proteins with mutations of the Zn2\(^{2+}\) ion coordinating amino acids (His and second Cys) in the Zn2 structure can still bind a second Zn2\(^{2+}\) ion. Another His residue is absolutely conserved in SBP domains (His187 in AtSPL14, Figure S2 of the Supporting Information) and might serve as a substitute fourth ligand. Alternatively, acidic residues or a water molecule might serve as the fourth coordinating ligand (43).

The AtSPL14 DNA-binding kinetics was analyzed by surface plasmon resonance (SPR). The mass transfer limitation due to the rapid association (\(>10^5\)) and dissociation rates (\(<10^{-2}\)) made determination of reliable kinetic constants difficult using conventional SPR (32). Therefore, we also evaluated steady-state binding at equilibrium. The determined equilibrium binding constant, \(K_A (3.8 \times 10^8 \text{ M}^{-1})\), is similar to the reported values (9.6 \times 10^7 \text{ M}^{-1} and \(>5 \times 10^8 \text{ M}^{-1}\)) for the AtSPL4 and AtSPL12 SBP domains, respectively, and 1 order of magnitude greater than the value (2.8 \times 10^7 \text{ M}^{-1}) reported for the AtSPL7 SBP domain (26, 27). Kinetic rate constants were not reported for other SBP domain proteins.

The different \(K_A\) values determined for different SBP domains might be due to the different salt concentrations in the analyte. Our data were generated in HBS–EP buffer (containing 150 mM NaCl), whereas the \(K_A\) of binding for AtSPL4, AtSPL7, and AtSPL12 was assayed in the presence of 100 or 300 mM KCl. The \(K_A\) for AtSPL12 and AtSPL4 at 100 mM KCl (\(>5 \times 10^8 \text{ M}^{-1}\) and \(2.8 \times 10^7 \text{ M}^{-1}\), respectively) was higher than those determined at 300 mM KCl (3.2 \times 10^7 \text{ M}^{-1} and 2.1 \times 10^6 \text{ M}^{-1}), respectively), suggesting that salt concentration affects SBP domain–DNA-binding affinity. Therefore, electrostatic forces contribute to the SBP–DNA interaction, as was also observed in other protein–DNA interactions (44, 45).

Conclusions. In summary, different SBP domain proteins display different binding affinities to the same DNA and different selectivity for DNA targets. Even though they contain the same core consensus binding motif (GTAC), the \(A. \text{ thaliana}\) APETALA1 gene-derived DNA and DNA containing the \(C. \text{ reinhardtii}\) copper-responsive element (CuRE) had different affinities for several SBP domain proteins (28). Our results revealed that AtSPL14–DNA binding is highly sequence selective and allow us to hypothesize which particular amino acids may confer that specificity. Moreover, the well-conserved SBP domains possess diverse DNA-binding affinities for similar DNA sequences. These observations will be useful, in conjunction with additional experimentation, to identify the gene targets for individual SBP domain family members to understand their physiological functions in the context of whole organisms.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

SPR data analysis comparing the subtraction of the signals from a blank reference cell and a reference cell coated with a noncognate DNA indicates that subtracting data from a reference cell coated with noncognate DNA is the preferred control (Figure S1). An amino acid alignment of the SBP DNA-binding domains encoded by the 16 \(A. \text{ thaliana}\) genes, the 2 \(A. \text{ majus}\) genes, and the CuRE-binding \(C. \text{ reinhardtii}\) gene is provided with a schematic of the Zn2\(^{2+}\) ion coordinating residues (Figure S2). The oligonucleotide primers used for site-directed mutagenesis of both the DNA target and the AtSPL14 SBP domain protein are shown in supplementary tables (Tables S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


FIGURE S1: Comparison of surface and noncognate dsDNA-coated reference cell subtraction methods. SPR sensorgrams of different concentrations of the AtSPL14-SBP domain protein (3.125, 6.25, 12.5, 25, 50, 100, 200 and 300 nM; bottom to top) injected onto a SA sensor chip coated with a representative 156 bp cognate dsDNA fragment identified by SELEX that bound in EMSA competition assays. Binding data were collected at a flow rate of 25 μL/min. A) Sensorgram with signals from the control surface reference cell (no DNA) subtracted. B) Sensorgram with signals from the control reference cell (coated with a noncognate dsDNA) subtracted.

FIGURE S2: Multiple sequence alignment of several SBP DNA-binding domains and Zn$^{2+}$ ion coordination. A) SBP domain sequences were aligned using the Clustal method with DNAStar software (Madison, WI). Residues that match the consensus sequence are shaded in black, numbers on the left refer to the starting amino acid, protein designations are indicated on the right (organisms: At, Arabidopsis thaliana; Cr, Chlamydomonas reinhardtii; and Am, Antirrhinum majus), and asterisks and amino acid numbers indicate the positions of the six highly conserved cysteines that were mutated in the AtSPL14 SBP domain. B) The coordinating Cys and His residues for Zn$^{2+}$ ions are indicated for the Zn1 (CCCH) and Zn2 (CCHC) structures.
Table S1. Oligonucleotide primers used for site-directed mutagenesis of SELEX binders

<table>
<thead>
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<td>GCACTGAATTCGCCTACGTACATATTAGCTAATATCTGATCCCG</td>
</tr>
<tr>
<td>M8</td>
<td>CGGGATCAGATAGACTAAGTGTACGTAGGCGAAATTCACTGC</td>
<td>GCACTGAATTCGCCTACGTACATATTAGCTAATATCTGATCCCG</td>
</tr>
<tr>
<td>M11</td>
<td>CGGGATCAGATAGACTAAGTGTACGTAGGCGAAATTCACTGC</td>
<td>GCACTGAATTCGCCTACGTACATATTAGCTAATATCTGATCCCG</td>
</tr>
<tr>
<td>M12</td>
<td>CGGGATCAGATAGACTAAGTGTACGTAGGCGAAATTCACTGC</td>
<td>GCACTGAATTCGCCTACGTACATATTAGCTAATATCTGATCCCG</td>
</tr>
<tr>
<td>M13</td>
<td>CGGGATCAGATAGACTAAGTGTACGTAGGCGAAATTCACTGC</td>
<td>GCACTGAATTCGCCTACGTACATATTAGCTAATATCTGATCCCG</td>
</tr>
</tbody>
</table>

Table S2. Oligonucleotide primers used for site-directed mutagenesis of AtSPL14 SBP domain

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sense oligonucleotide primer (5’→3’)</th>
<th>Antisense oligonucleotide primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C120A</td>
<td>CCGGGAGGGAATTATCCCCATGGCTAGTTGATAATTG</td>
<td>CAATTATCAACCTGAGCCATGGGATAATTCCCTCCCGG</td>
</tr>
<tr>
<td>C125A</td>
<td>CCCATGTGTCAGTTGATAATGTACTGAAAGATTTGTCTCATGC</td>
<td>GCATGAGACAAATCTTCTAGTACATCAACCTGACACATGG</td>
</tr>
<tr>
<td>C142A</td>
<td>CATAGAAGGCATAAAAGTTGCTGAAGTTTCATAGAAGCTA</td>
<td>GTAGCCTTACTATAAGAACTTCTAGCAACTTATGCCTTCTATG</td>
</tr>
<tr>
<td>C161A</td>
<td>CTTAACAGATCGACGGTTGGCTCAACACATGTAGCAGGG</td>
<td>CCTGCTACACTGTGGAGCAACACTTCTGCATCCTTGCC</td>
</tr>
<tr>
<td>C164A</td>
<td>CAGATGAGAGGGTTTGCACCAGTGGCTAGTTTCATCTGC</td>
<td>GCAGATGAAACCTGCTAGCGCTTGTCGAAAACCTCTGCATCTG</td>
</tr>
<tr>
<td>C180A</td>
<td>GAGGGGAAGGAATGCTAGGCGGTAGATTGGCTGG</td>
<td>CCAGCCAATCTACGCGCTAGCTTCTTCTCCCCTC</td>
</tr>
<tr>
<td>C120S</td>
<td>CCGGGAGGGAATTATCCCCATGGCTAGTTGATAATTG</td>
<td>CAATTATCAACCTGAGCCATGGGATAATTCCCTCCCGG</td>
</tr>
<tr>
<td>C125S</td>
<td>CCCATGTGTCAGTTGATAATGTACTGAAAGATTTGTCTCATGC</td>
<td>GCATGAGACAAATCTTCTAGTACATCAACCTGACACATGG</td>
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<tr>
<td>C142S</td>
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<td>GTAGCCTTACTATAAGAACTTCTAGCAACTTATGCCTTCTATG</td>
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<tr>
<td>C161S</td>
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<td>CCTGCTACACTGTGGAGCAACACTTCTGCATCCTTGCC</td>
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<tr>
<td>C164S</td>
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<td>GCAGATGAAACCTGCTAGCGCTTGTCGAAAACCTCTGCATCTG</td>
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<tr>
<td>C180S</td>
<td>GAGGGGAAGGAATGCTAGGCGGTAGATTGGCTGG</td>
<td>CCAGCCAATCTACGCGCTAGCTTCTTCTCCCCTC</td>
</tr>
</tbody>
</table>