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# **Interaction of Arabidopsis Membrane Proteins with SPIRAL1**

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Received 7 September 2004 ; accepted 5 October 2004 (Edited by Y. Hotta)

#### Abstract

Directional growth of plant cells requires proper control of cortical microtubule organization, for which a novel *Arabidopsis* microtubule-localizing protein SPIRAL1 plays an important role. To better understand cellular functions of SPIRAL1, we here identified two highly homologous *Arabidopsis* membrane proteins (SPI1 and SPI2) that interact with SPIRAL1 in a yeast two-hybrid assay. Disruption of either *SPI1* or *SPI2* genes enhanced the right-handed helical growth phenotype of *spiral1*, whereas a double mutant of null *spi1* and *spi2* alleles appeared to be embryonic lethal. The SPI proteins potentially link SPIRAL1 - dependent microtubule functions to endomembrane compartments.

Key words: Arabidopsis, membrane proteins, microtubule, SPIRAL1

# Introduction

Longitudinal elongation of rapidly expanding plant cells requires ordered arrangement of cortical microtubule arrays, which are generally distributed transverse to the organ axis (Gidding and Staehelin, 1991). When proper microtubule organization is disrupted by mutations or by treatment with microtubule-targeting drugs, anisotropic growth is compromised, leading to helical growth phenotypes (Hashimoto, 2002; Thitamadee et al., 2002). In the spiral1 (spr1) mutant of Arabidopsis thaliana, cortical microtubule arrays in root epidermal cells are arranged in shallow left-handed helices, instead of normal transverse arrays, and the mutant roots grow toward the right side of the culture plates when viewed from above (Furutani et al., 2000). The SPR1 gene encodes a member of the novel plantspecific low-molecular-weight protein family, and the SPR1 protein is localized to microtubules in Arabidopsis cells, although it does not directly bind to microtubules in vitro (Nakajima et al., 2004; Sedbrook et al., 2004). It has been postulated that microtubule localization of SPR1 in vivo may be mediated by unidentified interaction partners.

In this study, we identified two potential SPR1interacting proteins by a yeast two-hybrid screening. A genetic interaction study suggests that these novel membrane proteins may function in the same pathway as SPR1.

### **Materials and Methods**

Yeast strains, media, plasmids and cDNA libraries.

The Saccharomyces cerevisiae strain Y166 (MaT -a, gal4, gal80, his3, trp1-901, ade2-101, ura3-52, leu2-3, URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-LacZ, LYS2::GAL1<sub>UAS</sub>-HIS3<sub>TATA</sub>-HIS3, GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub> GAL-URA3), the pAS1 Gal4 DNA Binding Domain (GBD) vector, and the pACT Gal4 Activation Domain (GAD) vector, harboring as transformation markers the S. cerevisiae TRP1 and LEU2 genes, respectively (Durfee et al., 1993), were kindly provided by Prof. Elledge at Baylor College of Medicine in Houston, USA. Yeast YPD and synthetic minimal SC media were prepared as previously described (Durfee et al., 1993).

Two Arabidopsis cDNA libraries were constructed in lambda-ACT (Durfee et al., 1993), using either mRNA isolated from 3-day-old etiolated Arabidopsis seedlings (Kim et al., 1997) or mRNA isolated from Arabidopsis mature roots and leaves (NSF/DOE/USDA collaborative research in plant biology program, USDA 92-37105-7675), were kindly provided by the Arabidopsis Biological Resource Center at Madison, USA.

#### Yeast two hybrid assay and cDNA library screening

A cDNA sequence coding for the SPR1 Open Reading Frame (ORF) was subcloned into the pAS1 vector and transformed into the yeast strain Y166 using the lithium acetate method as described by Durfee et al. (1993). In order to identify cDNAs encoding *SPR1* interacting proteins (SPI), Y166 yeast cells expressing SPR1 were then transformed with plasmid DNAs derived from two different *Arabidopsis* lambda-ACT cDNA libraries. Approximately 1.5 x10<sup>6</sup> transformants were screened for the activation of three reporter genes, *HIS3* for growth on SC-lacking histidine but supplemented with 25 mM 3-aminotriazol, *URA3* for growth on a medium lacking uracil, and *LacZ* for  $\beta$ -galactosidase activity.

Expression of the  $\beta$ -galactosidase reporter gene was evaluated by filter assays. About a dozen of colonies from each transformant strain were grown on the SC medium lacking tryptophan and leucine for 2-3 days, then transferred to Whatman No.5 filter and frozen in liquid nitrogen twice for 10 seconds. Subsequently, the filter was placed onto another filter presoaked with Z buffer (60 mM Na<sub>2</sub>PO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub> pH7.0, 50 mM  $\beta$ -mercaptoethanol) containing X-gal (0.5 mg/mL). The  $\beta$ -galactosidase activity was estimated according with the color reaction observed from 1 to 16 hours.

SPI full-length clones, as well as SPR1 and SPI truncated versions, were generated by PCR, using appropriate primers, and subcloned into pACT and/or pAS1 as described by Sambrook *et al.*, (1989). All the PCR clones and the fusion junctions in the vector were verified by sequencing, using standard methods.

### Plant materials and growth conditions

A. thaliana seeds were surface sterilized in a mixture of 17% sodium hypoclorite (v/v) and 4% triton-X (v/v), and were allowed to germinate on plates containing 0.5x Arabidopsis nutrient solution (Haughn and Somerville, 1986), 2% sucrose and 1.5% agar. After 2 days at 4oC, plates were incubated in a near vertical position at 22°C with a 16 hour light/8 hour dark cycle. Day 0 of grow is defined as the time when plates were transferred to  $22^{\circ}C$ .

### Isolation of spi T-DNA insertion mutants

The *spi1-1*, *spi1-2*, and *spi2-1* mutants were isolated from a 60,480 *Arabidopsis* transgenic plant collection (ecotype WS) available for screening at the University of Wisconsin Biotechnology Center Knockout Facility (http://biotech.wisc.edu). The transformed *Arabidopsis* lines were searched for the presence of T-DNA inserts within *SPI* genes via a PCR screening strategy which involves the use of T-DNA. LB (JL270: 5'-TTTCTCCATATTGAC-CATCATACTCATTG-3') and *SPI*-specific primers (SPI.1-5: 5'-AACTGCGTCGATAAAGTAA-

CGGAGA-3'; SPI.2-5: 5': GTTCCTCTGTTGAT-CTATCCAATCGAATC-3'; and SPI.3: 5'-CGTT-TGGCCTGAAACTTACCAAGCTTGTC-3'). The T-DNA insertion point was determined by sequencing the putative T-DNA-SPI chimeric DNA fragments identified during the screening.

The *spi1-3* and *spi2-2* mutants were identified by an SPI DNA homology search of the Salk Institute Genomic Analysis Laboratory database (http://signal.salk.edu/cgi-bin/tdnaexpress), which displays the genomic DNA sequences flanking the T-DNA integrated in a collection of *Arabidopsis* transgenic lines (ecotype Columbia) (Alonso *et al.*, 2003). The genotype of the mutants was confirmed by genomic PCR amplification, using T-DNA.LB ROK.LB primer (5'-GCGTGGACCGCTTGCTGC-AACT-3'), SPI1.5, SPI2.5, and SPI3 primers, and DNA sequencing of the corresponding T-DNA-SPI chimeric PCR products.

#### Genetic Crosses

spi1spr1, spi2spr1 and spi1sp12 mutants were selected in F2 populations, and the homozygous double or triple mutants in the F3 generation were used for phenotypic analysis. The 0.6-kb deletion in the SPR1 gene was used to confirm the spr1-4 mutation by genomic PCR (Nakajima *et al.*, 2004). The presence of *spi* mutations was confirmed by genomic PCR analysis using T-DNA.LB (JL202 and ROKLB) and SPI specific primers (SPI1.5, SPI2.5 and SPI3).

# Plant RNA Isolation and RT-PCR expression analysis

Total RNA was isolated using RNeasy mini kit (Qiagen) following manufacturer's instructions. First strand cDNA was synthesized from about 1  $\mu$ g total RNA from 7-day-old *Arabidopsis* seed-lings, using Superscript<sup>TM</sup> First Strand Synthesis System (Gibco BRL) as described by the manufacturer.

PCR was carried out under standard conditions, using 3.2 pmole of SPI.5 (5'- ATGAGATCGGTT CAAGCAC-3') and SPI.N3H (5'-ATCAAGTG-GTGAAGGAAATGGT-3') primers, which leads to the amplification of both a 545-bp SPI1 and a 557-bp SPI2 N-terminal cDNA fragments. PCR products were digested with KpnI and fractionated on 1.8% agarose gels in order to differentiate the unrestricted SPI1 cDNA from the SPI2 230-bp and 327-bp KpnI fragments.

# Construction of GVG::myc-SPI1 Arabidopsis transgenic plants

A DNA fragment containing two copies of the c-

myc epitope was produced by PCR from existing plasmids. Then, in order to generate an N-terminal myc-SPI1 fusion, it was subcloned into a pBSK<sup>-</sup> plasmid harboring a cDNA coding for the SPI1 ORF. The myc-SPI1 DNA fusion junction was sequenced to exclude the possibility of PCR induced errors, and the resulting myc-SPI1 chimeric gene subcloned into the glucocorticoid-inducible expression GVG system (Aoyama and Chua, 1997). Arabidopsis plants (Columbia ecotype) were transformed by floral dipping (Clough and Bent, 1998) into Agrobacterium tumefaciens PMP90 strain containing the GVG::myc-SPI1 plasmid, and transgenic plants were selected using the hygromycinresistant marker present in the binary vector. Induction of the myc-SPI1 was done using dexamethazone (Sigma), a strong synthetic glycocorticoid, as described by Aoyama and Chua (1997).

#### Production of anti-SPI polyclonal antibodies

We amplified a cDNA fragment coding for the putative SPI1 160-amino-acid C-terminal region and cloned the resulting PCR product into the pProEX<sup>TM</sup>HTa vector (Gibco BRL). The resulting Hisx6-SPICT recombinant protein was purified from the bacterial insoluble fraction using nickel-nitriloacetic acid agarose (Qiagen) according to the manufacturer's instructions. Polyclonal rabbit antisera were produced using Hisx6-SPICT as an antigen.

#### Plant protein extraction and Western analysis

Total protein extracts were prepared from 2-week -old Arabidopsis seedlings sliced into small pieces by razor blades and ground in ice-cold extraction buffer (50 mM Hepes-KOH pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol, 0.25 mM sucrose, 20  $\mu$  g/mL of pepstatin A, and 20  $\mu$  g/mL of leupeptin) containing 0.1 mg/ml of butylated hydroxytoluene and 10% (v/v) polyclar AT. After filtration through two layers of Miracloth (Calbiochem), the insoluble debris was removed by centrifugation at 7000 g for 15 min at 4°C. Microsomal and soluble protein fractions were separated by centrifuging the protein extracts twice at 50,000g for 30 min at 4°C. Inmmunoblot analyses using antipolyclonal antibodies (1/2000 dilution), SPI or anti-MYC monoclonal antibodies (0.5  $\mu$ g/mL) (Oncogen), were performed using Hybond-P membrane and the ECL-plus kit (Amershan Biosciences), according to the manufacturer's instructions.

### **Results and Discussion**

# Homologous membrane proteins SPI1 and SPI2 interact with SPR1 in a yeast two-hybrid assay

To identify potential SPR1-interacting proteins. we screened Arabidopsis cDNA libraries in a yeast two-hybrid vector for clones that interact with SPR1 in yeasts. A yeast clone was identified that grew without histidine and uracil supplements in the medium, and showed clear LacZ activity. The interaction of this clone was specific to SPR1 since it did not interact with control proteins (human lamin, yeast SNF1 kinase, and human CDK2 kinase) in yeasts (data not shown). Since the Arabidopsis cDNA insert in this clone lacked the 5' part, a fulllength cDNA was subsequently obtained and the encoding protein named SPIRAL1-INTER-ACTING 1 (SPI1). The Arabidopsis genome contains a close homologue of SPI1, which was named as SPI2. A tobacco homolog of SPI1 and SPI2 has been reported to be transiently down-regulated after wounding to the leaf (Hara et al., 2000). These three proteins share considerable homology throughout their entire lengths (Fig. 1). A search of different Gene Bank databases revealed the existence of EST sequences encoding SPI homologs in a variety of plants, but no homolog sequences were found outside the plant kingdom. The hydrophobicity plot analysis identified one potential membrane-spanning region in the middle part of these proteins (underlined with a solid line). Restricted regions of SPI1 and related proteins have weak homologies to UVR endonucleases (underlined with a dotted line; Moolenaar et al., 1995), and a conserved domain of unknown functions in the database (not shown). Their intracellular localization may be predicted to chloroplasts, with the modest or high scores of TargetP (http://www.cbs.dtu.dk/services/TargetP/) and Predotar (http://ww.inra.fr/Internet/Produits/Predotar/); 0.522/0.912 (SPI1), 0.611/0.966 (SPI2), and 0.932/0.713 (tobacco homolog).

To study the intracellular localization, an antibody was raised against a C-terminal part of SPI1. This antibody did not detect specific SPI1 signals in wild-type *Arabidopsis* seedlings (**Fig. 2**, left two lanes). Our attempts to overexpress SPI1 in *Arabidopsis* transgenic plants under the CaMV35S promoter also failed for unknown reasons. We therefore expressed SPI1 (tagged with two copies of the Myc epitope at the N-terminus) under the glucocorticoid-inducible gene expression system (Aoyama and Chua, 1997) in transgenic *Arabidopsis* plants. When Myc-SPI1 expression was



Fig. 1 Amino acid sequence alignment of SPI1, SPI2, and their tobacco homolog. Amino acids conserved in three proteins are highlighted by black boxes. A putative membrane-spanning region and a region with weak homology to UVR endonucleases are underlined by a solid bar and a dotted bar, respectively. The SPI1 amino acid positions used to generate truncated proteins in Fig. 3 are also shown.



Fig. 2 Western blotting analysis of SPI1 in Arabidopsis seedlings. Crude proteins were extracted from whole seedlings and separated into soluble (S) and microsomal (M) fractions. The expression of Myc-SPI1 was induced by growing the transgenic line in the presence of 10  $\mu$ M dexamethasone. The protein blot was probed with an SPI1 antibody. The level of endogenous SPI1/2 proteins was too low to be detectable in this assay, but the ectopically induced Myc-SPI1 was detected in the microsomal fraction. The asterisk indicates a cross-reacting band which serves as a loading control. Each lane was loaded with 20  $\mu$ g protein.

induced by the DEX treatment, the SPI1-antibody recognized a 45-kDa protein in the microsomal

fraction (Fig. 2). This protein was also recognized by a Myc-antibody (data not shown), confirming its identity as Myc-SPI1. These results indicate that SPI1 (and probably SPI2 as well) is localized to membranes, and that SPI1 is not abundant in seedlings.

# SPI1 and SPI2 interact with an N-terminal part of SPR1 and with themselves in yeasts

We next analyzed, in the yeast two-hybrid system, the regions of SPI1, SPI2, and SPR1 that are necessary for their interactions. Several N-terminal, C-terminal, and internal deletions of SPI1 and SPI2 were tested (Fig. 3A). Full-length SPI1 and SPI2 proteins interacted with SPR1. The membranespanning domain and the C-terminal 142 amino acid region of SPI1 were necessary for SPR1 interaction. In contrast, the SPI1 N-terminal 66 amino acid region was dispensable for molecular association with SPR1. On the other hand, the N-terminal half of SPR1 was sufficient for interaction with SPI1. Similar results were obtained when several SPI2 deletions were tested for their interactions with SPR1. Therefore, no clear differences were found for SPI1 and SPI2 with regard to their interactions with SPR1 in yeasts.

In the yeast assay, we also detected interactions between SPI1 and SPI1, and between SPI2 and SPI1 (**Fig. 3B**). We found that the SPI membrane-spanning domain and the C-terminal 142 amino acid



Fig. 3 Interactions among SPI1, SPI2, and SPR1 in yeast two-hybrid assays. (A) Various truncations of SPI1 or SPI2 were expressed in the yeast strain Y166 as Gal4 Activation Domain (GAD) fusions, while SPR1 proteins were expressed as fusions to the Gal4 DNA Binding Domain (GBD). (B) Interaction analysis of different truncations of SPI1 fused to GAD with the full-length SPI1 protein fused to GBD. Asterisks indicate that corresponding GAD-SPI2 constructs were tested and the results were similar to those obtained with GAD- SPI1. The first and last amino acid positions of the SPI1 and SPR1 derivatives are indicated. The predicted membrane-spanning hydrophobic domain (HD) is shown as a hatched box. The strength of interactions was measured by LacZ enzymatic activity and shown in minus and plus signs. Minus (-) indicates no interaction, and stronger interactions are shown with larger numbers of plus (+) signs.

region were necessary for SPI self-association. In contrast, SPI1 N-terminal 66 amino acid region and SPI2 N-terminal 70 amino acid region were dispensable for SPI self-association. These results indicate that the regions of SPI proteins necessary for self-interaction and association with SPR1 overlap significantly.

We tried to provide evidence for interactions between SPI1/SPI2 and SPR1 in plant cells using immunoprecipitation methods, but could not demonstrate clear interactions *in vivo* possibly because of technical difficulties dealing with membrane proteins. In the next step, we sought for possible genetic interactions between SPI1/SPI2 and SPR1.

# Disruption of either SPI1 or SPI2 enhances spr1 phenotypes

Both SPI1 (At1g75380) and SPI2 (At1g19660) are expressed in all Arabidopsis organs (data not shown). To examine relative expression levels of SPI1 and SPI2, we simultaneously amplified corresponding regions of both cDNAs using common PCR primers and distinguished individual cDNAs by specifically cleaving SPI2 cDNAs at the unique *Kpn*I site (**Fig. 4B**). Similar amounts of *SPI1* and *SPI2* cDNAs were detected when RNAs in seedlings were analyzed, indicating that both *SPI1* and *SPI2* are expressed at similar levels in seedlings.

We obtained three T-DNA insertion alleles of *SPI1* and two insertion alleles of *SPI2* (Fig. 4A). spi1-2 and spi2-2 alleles had T-DNA inserted at the 5'-regulatory regions, whereas the T-DNAs in spi1-1, spi1-3, and spi2-1 were found in the non-coding first *SPI1* exon, in the second *SPI1* intron, and in the third *SPI2* exon, respectively. In the spi1-1 and spi1-2 alleles, *SPI1* transcript levels were reduced but still detectable while no expression of *SPI1* was observed in the null spi1-3 allele (Fig. 4B). Neither spi2-1 nor spi2-2 alleles had detectable levels of *SPI2* transcripts, and both alleles thus appeared to be null alleles. These spi1 and spi2 mutant alleles all grew normally and were indistinguishable in morphology from wild-type plants.

We next crossed spr1-4 with the spi1-1 reducedfunction allele and the spi2-1 null allele (Fig. 5). The roots of spr1-4 seedlings grew sharply skewed to the right side of the hard agar plates. The root skewing angles were exaggerated in the spr1-4spi1



Fig. 4 Expression of SPI1 and SPI2 genes in T-DNA insertion lines. (A) Gene organization of SPI1 and SPI2, and positions of T-DNA insertions. Exons are shown in boxes while introns and 5' - flanking regions are shown in lines. Protein coding regions are indicated by black boxes. The orientation of T-DNA left borders (LBs) is shown for each T-DNA insertion. Arrows indicate the PCR primers used in RT-PCR expression analysis in (B). The unique KpnI site in the amplified SPI2 cDNA is also shown. (B) RT-PCR analysis. SPI1 and SPI2 cDNAs were simultaneously amplified by common PCR primers and subsequently distinguished by digesting with KpnI. Seedlings of wild type and five T-DNA insertion lines were analyzed for gene expression.

-1 and spr1-4spi2-1 double mutants, and especially in the spr1-4spi1-1spi2-1 triple mutant. In these mutants, seedling roots often grew toward the upper right corner of the plates. These results present genetic evidence for the interaction between *SPR1* and *SPI1/SPI2*.

# Complete disruption of both SPI1 and SPI2 leads to embryonic lethality

To examine phenotypes of the spi1spi2 double null mutant, we crossed the null spi1-3 allele with the null spi2-2 allele and obtained SPI1/spi1-3 spi2-2/spi2-2 plants in which the SPI1 locus was heterozygous and the SPI2 locus was mutant homozygous. These plants grew normally with no abnormal phenotypes and were self-pollinated. When the selfed progeny was analyzed by genomic PCR for the genotype at the SPI1 locus, we found 27 plants with the wild-type SPI1 allele and 35 spi1-3 heterozygous plants (Fig. 6A). However, no spi1-3 homozygous plants were found in the presence of the spi2-2 homozygous allele. If spi1-3spi2-2 double mutant does not develop to viable seeds, we should expect *spi1-3* heterozygous plants in 66.6% of the selfed progeny. The observed frequency of the spi1-3 heterozygous plants was somewhat lower (56.5%), suggesting that reproductive functions

of *spi1-3spi2-2* gametes and/or *SPI1/spi1-3 spi2-2/spi2-2* embryo development may be also affected in these plants.

When elongating siliques of the selfed SPI1/spi1-3 spi2-2/spi2-2 plants were cut open before maturity, we found undeveloped ovules or seeds, and aborted transparent seeds among viable immature green seeds (**Fig. 6B**). The aborted seeds may be spi1-3spi2-2 homozygous mutants. These results indicate that the residual activity of SPI1/SPI2 is required for plant development, at least in the embryo stage.

The high amino acid sequence homology, similar interaction patterns in the yeast two-hybrid assay, similar genetic interaction with *spr1*, and the phenotypes of single and double mutants suggest that SPI1 and SPI2 act redundantly in most *Arabidopsis* cells. Although physical *in vivo* interaction between SPI1/2 and SPR1 needs to be demonstrated, these novel membrane proteins are promising candidates for SPR1- interacting partners that link SPR1 activity to membrane-associated functions. Endogenous and exogenously expressed SPR1 proteins were found mostly in the microsomal fractions (Nakajima *et al.*, 2004). When expressed in *Arabidopsis* plants, a part of the SPR1-GFP fluorescence was observed in association with endomembranes (Sedbrook,



Fig. 5 Root skewing phenotypes. Seedlings of the indicated genotypes were grown on hard agar plates for seven days and pictured from above the plates. Mutant roots twisted in right-handed direction and grew toward the right side of the plates.

A Genotype	n	% observed	% expected*
SPII/SPII (wild type)	27	43.5	33.3
SPI1/spi1-3 (heterozygous)	35	56.5	66.6
spil-3/spil-3 (homozygous)	0	0	0





Fig. 6 spi1spi2 double null mutant phenotype. (A) Segregation analysis of the spi1-3 allele in the presence of the spi2-2 mutation. The expected ratio (\*) is based on the assumption that spi1-3spi2-2 is embryonic lethal. (B) Immature seeds in a silique of a self-pollinated SPI1/spi1-3spi2-2/spi2-2 plant. 2004). Biological significance of the putative SPR1 – membrane association should be determined in future studies.

# Acknowledgments

We would like to thank the Arabidopsis Biological Resource Center for providing T-DNA mutant alleles and DNA materials, T. Aoyama for providing the GVG vector, S.J. Elledge for providing yeast strains and vectors, and the members of the Hashimoto lab for technical assistance. R. P. was supported by fellowships from Japan Society for the Promotion of Science, European Commission, and Novartis Foundation. This study was in part supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to T. H.

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