Supporting Information

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SI Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana* ecotype Columbia 0 plants were grown in an air-conditioned room at 22 °C under 16-h light/8-h dark cycles. Plant growth conditions and *Agrobacterium*-mediated transformation were as described previously (1). The transgenic lines were selected on 0.5-X Murashige–Skoog medium (Sigma–Aldrich) containing 50 μg/mL kanamycin (for pMD1-GFP or pBI101 vectors) or 30 μg/mL hygromycin B [for pCAMBIA 1300 (pCAMBIA) vectors]. Seeds for the T-DNA insertion lines were purchased from the Arabidopsis Biological Resource Center. The T-DNA insertion sites were confirmed by PCR. The sperm cell markers pHTR10-HTR10-RFP and LIG1-GFP were kindly provided by Frédéric Berger (Temasek Life Sciences Laboratory, National University of Singapore, Singapore). For crosses with dehiscent anthers, closed flower buds were emasculated 24–48 h before pollination.

CLSM. CLSM of ovules and seeds was performed as described previously (2). Influorescences were fixed in 4% (vol/vol) glutaraldehyde in 12.5 mM cacodylate buffer (pH 6.9), dehydrated through a conventional ethanol series, and subsequently cleared in a 2:1 mixture of benzyl benzoate/benzyl alcohol. Siliques were then opened, and ovules were mounted with immersion oil. The sample was then viewed with a Zeiss LSM510 META laser scanning microscope.

Molecular Cloning. The plasmid 35S-ANK6-GFP was prepared by cloning the *ANK6* coding region without the stop codon into pMD1-GFP vector in the *Xba*I and *Bam*HI sites downstream from the 35S promoter. To study the promoter activity of the *ANK6* gene, a 4,683-bp genomic DNA fragment of the *ANK6* gene (−4624 to +59) was amplified by PCR and inserted into the *Xba*I and *Sma*I sites of pBI101.2, forming the pANK6-GUS construct for transformation. A 1,937-bp genomic DNA fragment of the *SIG5* gene (−1937 to −1) was amplified by PCR and inserted into the *Sal*I and *Xba*I sites of pBI101.2 to produce the pSIG5-GUS construct. For genetic complementation, the *ANK6* cDNA with a terminator fragment was amplified from the WT genomic DNA using the primer pairs ANK6HBF and ANK6HBR. The *ANK6* promoter was amplified using the primer pairs Pro-HBF and Pro-HBR. Restriction enzyme pairs *Kpn*I and *Sac*I as well as *Xba*I and *Kpn*I were used to isolate the cDNA terminator and promoter fragment, respectively, and were then subcloned into binary vector pCAMBIA 1300, resulting in the transcriptional fusion construct P_{ANK6}-cDNA-T_{ANK6}. The *ANK6* complementation construct was transformed into the +/*ank6* mutant plant with the floral dipping method, and the transformants were selected on 30 μg/mL hygromycin. All primers used in this study are included in Table S2. All constructs were verified by DNA sequencing.

GUS Assays. Pistils and siliques were opened and incubated in GUS staining solution [5 mg/mL X-Gluc, 2 mM Fe^{2+}CN, 2 mM Fe^{3+}CN, 10 mM EDTA, 0.1% Triton X-100, 100 mg/mL chloramphenicol in 50 mM sodium phosphate buffer (pH 7.0)] for 2–3 d at 37 °C. For examining ovules, stained pistils were cleared in 20% lactic acid and 20% glycerol, observed with a Zeiss microscope, and documented with a Nikon 4500 camera. For GUS staining of the whole seedling, plants were first decolorized with 95% ethanol and then examined and photographed with an Olympus SZX12 microscope equipped with a camera.

Y2H Screen. We performed the screen essentially as described previously (3). AH109 yeast cells were first transformed with pBD-ANK6, a plasmid containing the ANK6 (residues 20–174) coding sequence fused to the GAL4 DNA-binding domain in the pGBKT7 vector (with a TPR1, selection marker). The transformants were subsequently transformed with the *Arabidopsis* cDNA library cloned in the prey vector pACT, which was obtained from Joe Ecker (Salk Institute, La Jolla, CA). The transformed cells were plated on synthetic dropout selection medium that lacked Trp, Leu, and His supplemented with 20 mM 3-AT to reduce the growth of false-positive colonies. The plates were incubated at 30 °C for 3–12 d. The prey plasmid DNAs were isolated from yeast, transformed and isolated from *Escherichia coli*, and retransformed into yeast cells containing either the empty vector or the vector with bait to verify growth further. The clones that continued to grow in the −His and +3-AT medium after retransformation were selected for DNA sequencing. The full-length *SIG5* cDNA was PCR-amplified from *Arabidopsis* cDNA, sequenced, and cloned into pGADT7 (Clontech) for further analyses.

Recombinant Protein Expressions and in Vitro Pull-Down Assays. Recombinant His-SIG5 (encoding SIG5 amino acids 65–542 with an N-terminal 6× His tag) was generated in pET28A expression vector (Novagen). Recombinant GST-ANK6 was generated in pGEX4T-1 (Amersham Biosciences) expression vector. *E. coli* BL21 star (DE3) (Stratagene) cells harboring ANK6-GST or GST expression constructs were grown to an OD_{600} = 0.6. Cultures were shifted to 15 °C, and expression of fusion proteins was induced for 20 h with 0.6 mM isopropylthio-β-galactoside (IPTG). The IPTG (0.5 mM) was applied to the cultures and incubated for 10 h at 15 °C to generate the His-SIG5 fusion protein.

The pull-down assay was performed as described previously (4). Briefly, *E. coli* BL21 star (DE3) cells expressing GST-ANK6 or GST were harvested by centrifugation, resuspended in 4.0 mL of binding buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1× Roche protease inhibitors] and sonicated. After centrifugation at 17,000 × *g*/min for 30 min, 80 μL of glutathione beads (Sigma–Aldrich) was added to the supernatant and incubated with rotation for 1 h at 4 °C. The beads were washed four times with the binding buffer.

Cell extracts containing the His-SIG5 fusion protein were prepared by sonication in buffer [20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA 1 mM phenylmethylsulphonyl fluoride, 1× Roche protease inhibitors]. After treatment with Nonidet P-40 (0.5%) and glycerol (10%) as well as centrifugation as above, 4.0 mL of supernatant was applied to the glutathione beads coated with either GST-ANK6 or GST. Following a 2.5-h incubation on a rocking platform, the samples were washed four times with NETN buffer [20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 1× Roche proteinase inhibitors]. The bound proteins were eluted by boiling the beads in sample buffer and visualized using Coomassie brilliant blue staining or Western blot analysis.

BiFC Assays. We performed BiFC assays in vivo as described earlier (5). The ORF sequence of *ANK6* was amplified by PCR with primers ANK6BIF and ANK6BIR and was cloned into the *Nco*I and *Bam*HI sites of the plasmid pE3449, resulting in the plasmid pE3449-ANK6, which was called the ANK6-C-terminal CFP fusion protein. Coding sequences of *SIG5* were prepared in the same way and cloned into the plasmid pE3308 in the *Nco*I and

*Bam*HI sites to give rise to the plasmid pE3308-SIG5, referred to as SIG5-N-terminalVenus fusion protein.

Protoplasts were isolated from 5-wk-old *Arabidopsis* rosette leaves as described previously (6). Plasmids (20–30 μg) were cotransformed into protoplasts, followed by incubation under continuous light at 23 °C for 18 h. For fluorescence detection, excitation light (488 nm) was used to elicit fluorescence emission between 498 and 561 nm. To visualize mitochondria, protoplasts were stained with 5 μM MitoTracker Red CMXRos (Invitrogen) and observed under a Leica TCS-SP2 confocal laser scanning microscope (Leica Microsystems) (5) with excitation at 543 nm, and the emission signal was collected at 589–621 nm.

1. Chen J, et al. (2009) Magnesium transporter AtMGT9 is essential for pollen development in Arabidopsis. *Cell Res* 19:887–898.
2. Christensen CA, King EJ, Jordan JR, Drews GN (1997) Megagametogenesis in Arabidopsis wild type and the Gf mutant. *Sex Plant Reprod* 10:49–64.
3. Liu HT, et al. (2008) Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. *Science* 322:1535–1539.
4. Jiang DH, Gu XF, He YH (2009) Establishment of the winter-annual growth habit via FRIGIDA-mediated histone methylation at FLOWERING LOCUS C in Arabidopsis. *Plant Cell* 21:1733–1746.
5. Lee LY, Fang MJ, Kuang LY, Gelvin SB (2008) Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. *Plant Methods* 4:24.
6. Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc Natl Acad Sci USA* 97:2940–2945.

Table S1. Segregation analysis of the *ank6* mutation by PCR-based genotyping

Female × male	WT	*+/ank6*	*ank6/ank6*	% *+/ank6*	%TE
+/ank6 × *+/ank6*	73	147	0	66.8	ND
WT × *+/ank6*	142	143	0	50.1	100.7
+/ank6 × WT	247	202	0	45.0	81.8

ND, not determined; TE, transmission efficiency.

Table S2. Primers for ANK6 and SIG5 molecular analysis

Name	Primer sequence	Purpose
ANK6F	5-TTCGAAGCTAGTTCTTGTTTAG-3	Screening T-DNA mutant of *ank6*
ANK6R	5-GTTGCAAAATAACACACTGAAG-3	
ANK6-Y2HF	5-GCGAATTCAATGTCGATGACAGAG-3	Bait construction for Y2H of ANK6
ANK6-Y2HR	5-CGTCGACCGAGAAACAACCAAGA-3	
ANK6-PDF	5-GAATTCAGACCCAAAATGCTCCAAGAACC-3	Expression for GST-ANK6 fusion proteins
ANK6-PDR	5-GTCGACCCAAGAAGAAGTTCAGTTGTCAGA-3	
Pro-ANK6F	5-GCGATTGTTTCGTTCCAGAG-3	Cloning of promoter of ANK6
Pro-ANK6R	5-GATCGGCGTCTGAATGAGTT-3	
Pro-ANK6SF	5-TCTAGA GCGATTGTTTCGTTCCAGAG-3	Subcloning of promoter of ANK6
Pro-ANK6SR	5-CCCGGG ATCGGCGTCTGAATGAGTTACG-3	
ANK6-GFPF	5-CTCTAGAATGCTCCAAGAACCGTCGGCTG-3	Expression for 35S-ANK6-GFP fusion proteins
ANK6-GFPR	5-CGGATCCGTTGTCAGAACTGGAAGATGTG-3	
ANK6-BIF	5-TCCATGGAAATGCTCCAAGAACCGTCGG-3	Construction for BiFC of ANK6
ANK6-BIR	5-CGGATCCAGTTGTCAGAACTGGAAGATGTGTC-3	
SIG5-PDF	5-GGATCCTCCAGCAATTCAGCTTTGATC-3	Expression for HIS-SIG5 fusion proteins and screening T-DNA mutant of *sig5-3*
SIG5-PDR	5-GTCGACGTTTGGTTTTAGACGATGTATTG-3	
Pro-SIG5F	5-CTTCAACTATGTAACTCCGACTC-3	Cloning of promoter of SIG5
Pro-SIG5R	5-GGCTTTCTCCTCGTTACTTC-3	
Pro-SIG5SF	5-GTCGAC CTTCAACTATGTAACTCCGAC-3	Subcloning of promoter of SIG5
Pro-SIG5SR	5-TCTAGA ATAAGTATAAAAAATGTCTCAGG-3	
SIG5-Y2HF	5-CAGATCTATTCCAGCAATTCAGCTTTGATC-3	Construction for Y2H of SIG5
SIG5-Y2HR	5-GAGCTCTTAGACGATGTATTGACGAAGG-3	
SIG5-FD2F	5-CAGATCTTCAATATGACTGCGGGTGAAG-3	
SIG5-FD3R	5-CGAGCTCAGTCCAAGCTCACTATATCAT-3	
SIG5-FD4F	5-CAGATCTTTGAAGATGGCGGCTCTAAAG-3	
SIG5-FD4R	5-CGAGCTCTCACCCGCAGTCATATTGATC-3	
SIG5BIF	5-TCCATGGCCATGAGATATGTTTCTGCTTGC-3	Construction for BiFC of SIG5
SIG5BIR	5-CGGATCC AGACGATGTATTGACGAAGGTAA-3	
ANK6HBF	5-GGTACC ATTCTTATTTCGTCAATGTTTTTC-3	Construction for rescue construct of ANK6
ANK6HBR	5-GAGCTC CGCAGATCAGAAGTAGCTCACAG-3	
Pro-HBF	5-TCTAGA GCGATTGTTTCGTTCCAGAG-3	Construction for rescue construct of ANK6
Pro-HBR	5-GGTACC CCAACGATGAATCCGAAACG-3	