Supplementary Information for

Protein Phosphatase 2A promotes stomatal development by stabilizing SPEECHLESS in *Arabidopsis*

Chao Bian[1,2,#], Xiaoyu Guo[1,#], Yi Zhang[3], Lu Wang[1,2], Tongda Xu[3], Alison DeLong[4] and Juan Dong[1,2*],

[1] The Waksman Institute of Microbiology, Rutgers, the State University of New Jersey; Piscataway, NJ

[2] Department of Plant Biology, Rutgers, the State University of New Jersey, New Brunswick, NJ

[3] FAFU-Joint Centre, Horticulture and Metabolic Biology Centre, Haixia Institute of Science and Technology. Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, P. R. China

[4] Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI

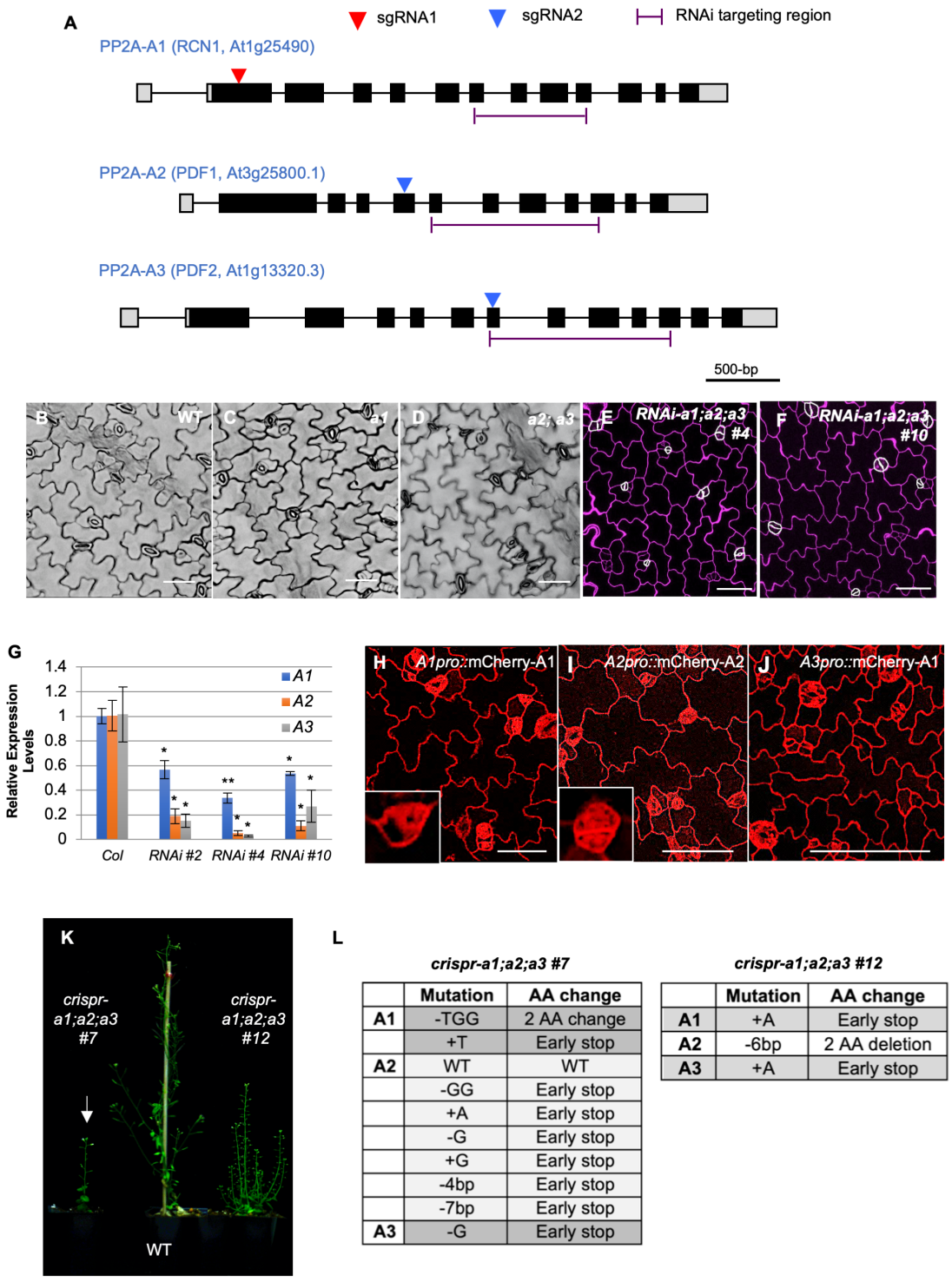
[#] Equal contribution

*Correspondence should be addressed to J.D. (dong@waksman.rutgers.edu)

This PDF file includes:

Figs. S1 to S7

Table S1



crispr-a1;a2;a3 #7

	Mutation	AA change
A1	-TGG	2 AA change
	+T	Early stop
A2	WT	WT
	-GG	Early stop
	+A	Early stop
	-G	Early stop
	+G	Early stop
	-4bp	Early stop
	-7bp	Early stop
A3	-G	Early stop

crispr-a1;a2;a3 #12

	Mutation	AA change
A1	+A	Early stop
A2	-6bp	2 AA deletion
A3	+A	Early stop

Fig. S1. Stomatal phenotypes and genetic characterization of *pp2a-a* mutants

(A) Gene structure of three *PP2A-As* with exons (black boxes), introns (lines) and UTRs (gray boxes). Sites targeted by sgRNA1 and sgRNA2 are indicated by red and blue triangles, respectively. RNAi targeting sites are indicated by purple lines. (B-D) DIC images of 5-dpg adaxial side cotyledons of WT (B), *a1* (C), and *a2;a3* (D)

seedlings. (E-F) Confocal images of 3-dpg cotyledon epidermis in *RNAi-a1;a2;a3 #4* (E) and #10 (F). Guard cells are manually traced with white highlights to improve visibility. Cell outlines are visualized by PI staining (magenta).(G) Quantitative real-time PCR analysis for transcript levels of *A1*, *A2* and *A3* in 5-dpg seedlings. *ACTIN2* was used as an internal reference. Three biological replicates were performed. Data are mean ± SD. *significantly different compared to the WT (Col) values (Student's *t*-test, *P < 0.05, **P < 0.001). (H-J) Confocal images of 3-dpg adaxial epidermis of cotyledons expressing *A1pro*::mCherry-A1 (H), *A2pro*::mCherry-A2 (I), and *A3pro*::mCherry-A1 (J). (K) Comparison of adult plant morphology of Col, T1 individuals of *crispr-a1;a2;a3 #7* and *#12*. (L) Mutations detected (and the resulting predicted amino acid changes) in T2 plants of *crispr-a1;a2;a3 #7* and *#12*. Genotype data for *crispr-a1;a2;a3* plants were obtained as described in the Methods. Scale bars represent 50 μm.

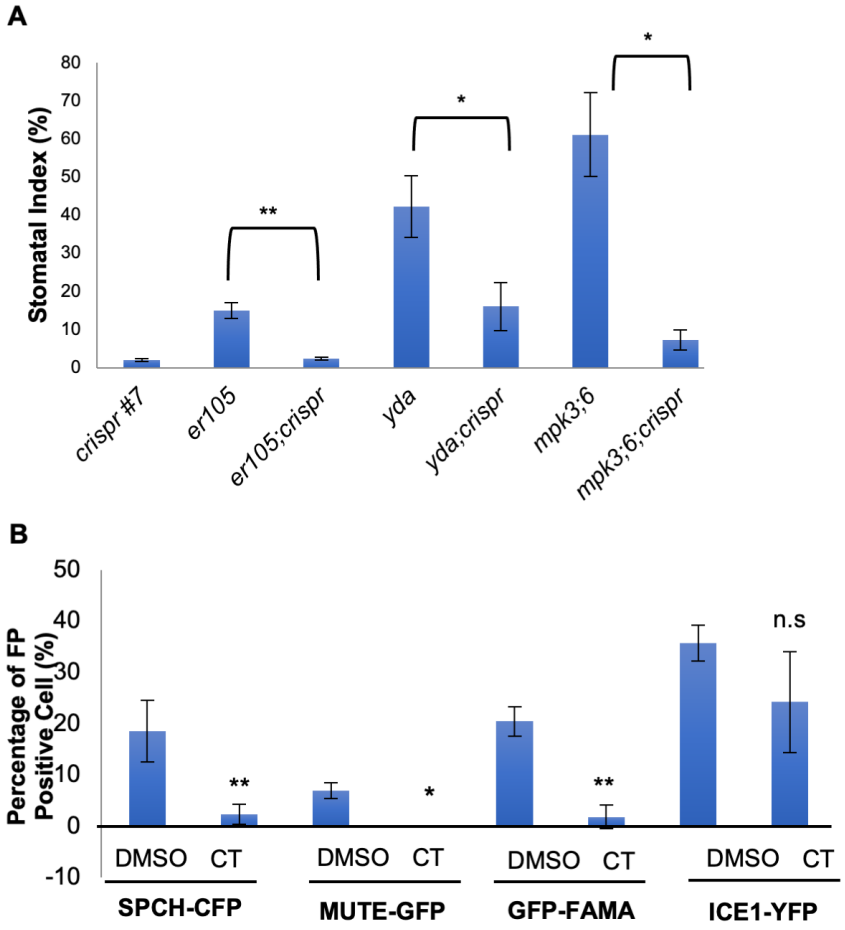


Fig. S2. Quantification of stomatal phenotype and bHLH expression in response to CT treatment.

(A) Quantification of stomatal index (SI) in 3-dpg adaxial side cotyledons of the indicated genotypes. Data are mean ± SD. n=546-3858 cells. Student's *t*-test, *P < 0.05, **P < 0.001. (B) Quantification of percentage of fluorescence-positive cells in 3-dpg cotyledons of SPCH-CFP, MUTE-GFP, GFP-FAMA, and SCRM/ICE1-YFP grown on 1/2 MS medium with DMSO or 50 μM CT. n=774-8112 cells from 10-12 individual plants. Data are mean ± SD. *significantly different from the DMSO control values (Student's *t*-test, *P < 0.05, **P < 0.001). n.s: non-significant.

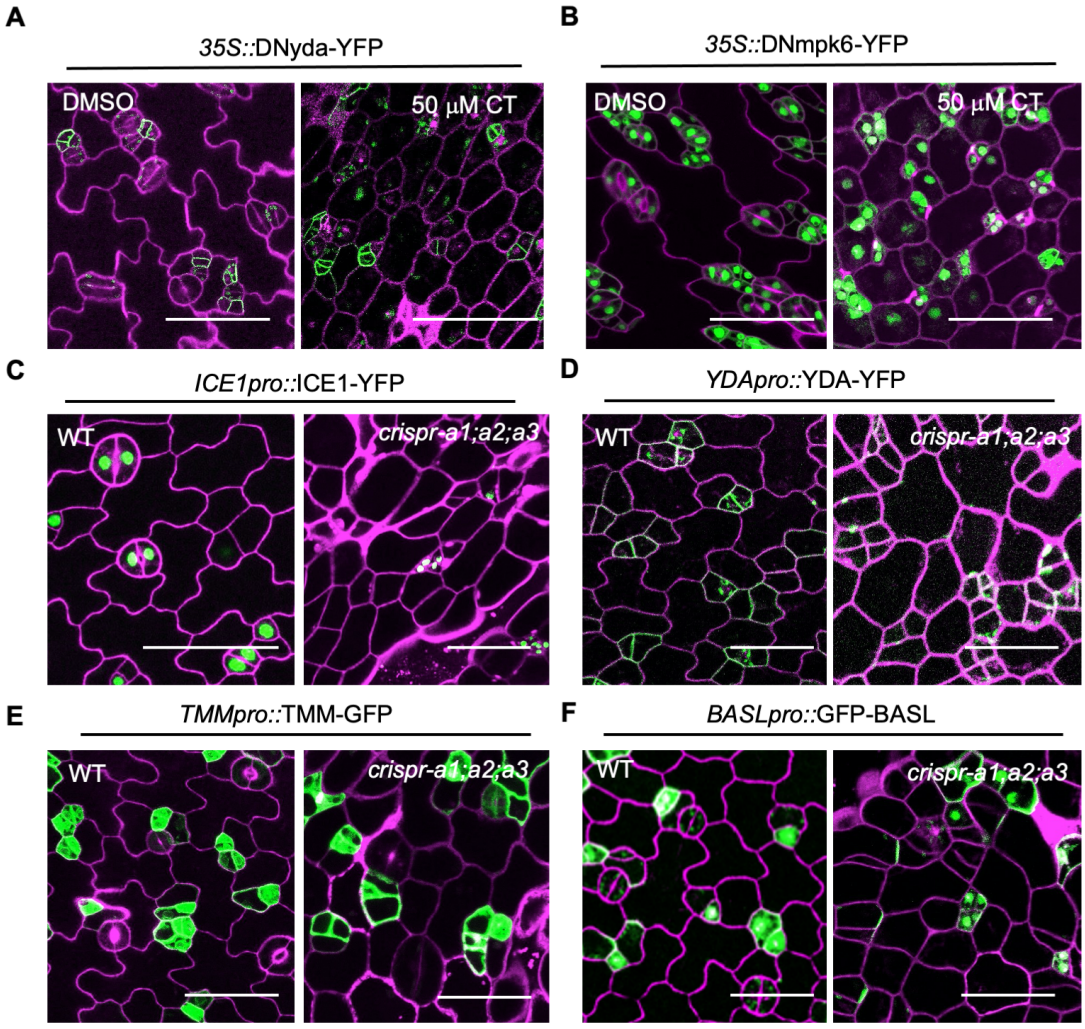


Fig. S3. Expression patterns of stomatal regulators when PP2A activities are defective.

(A-B) Confocal images of 3-dpg cotyledons in *35S::*DNyda-YFP (A) and *35S::*DNmpk6-YFP (B) seedlings grown on 1/2 MS medium with DMSO (left panels) or 50 μM CT (right panels). (C-F) Confocal images of 3-dpg seedlings expressing SCRM/ICE1-YFP (C), YDA-YFP (D), TMM-GFP (E) and GFP-BASL (F) in the WT (left panels) and *crispr-a1;a2;a3* backgrounds (right panels). Cell outlines are visualized by propidium iodide (PI) staining (magenta) and GFP/YFP are shown in green. Scale bars represent 50 μm.

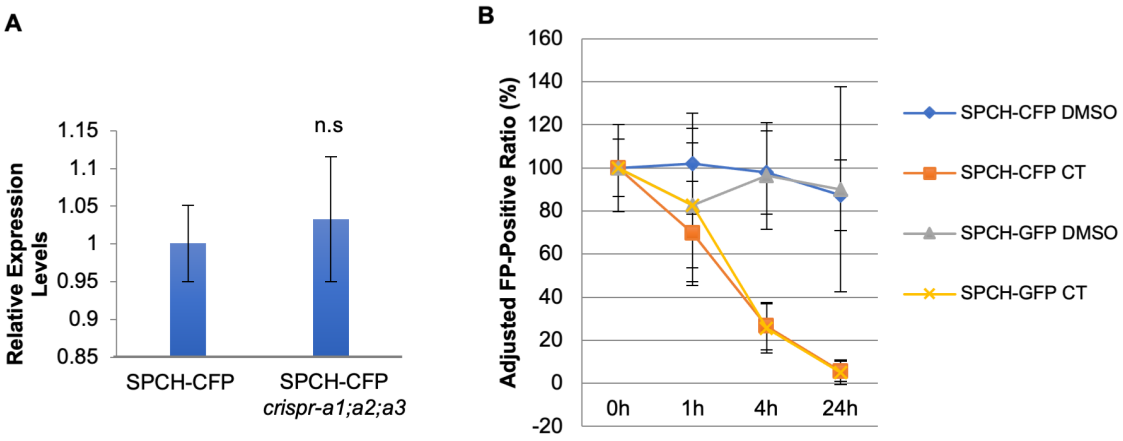


Fig. S4. Differential influences of defective PP2A on SPCH transcripts and proteins.

(A) Quantitative real-time PCR assay for transcript levels of SPCH-CFP in the WT and *crispr-a1;a2;a3*, respectively. *ACTIN2* was used as an internal reference. Three biological replicates were performed. Data are mean ± SD. Student's *t*-test. n.s: non-significant. (B) Quantification of GFP/CFP-positive cells in *SPCHpro::*SPCH-CFP and *SPCHpro::*SPCH-GFP 3-dpg cotyledons. Seedlings were treated with DMSO or 50 μM CT. Confocal images were captured at 0-, 1-, 4-, and 24-hr after treatment. For each sample, n=1247-3571 cells in 10-12 individual seedlings were collected for quantification. Adjusted Ratios were generated by defining the initial percentages of CFP/GFP-positive cells as 100%, then the relative ratios at each time point were calculated accordingly. Data are mean ± SD.

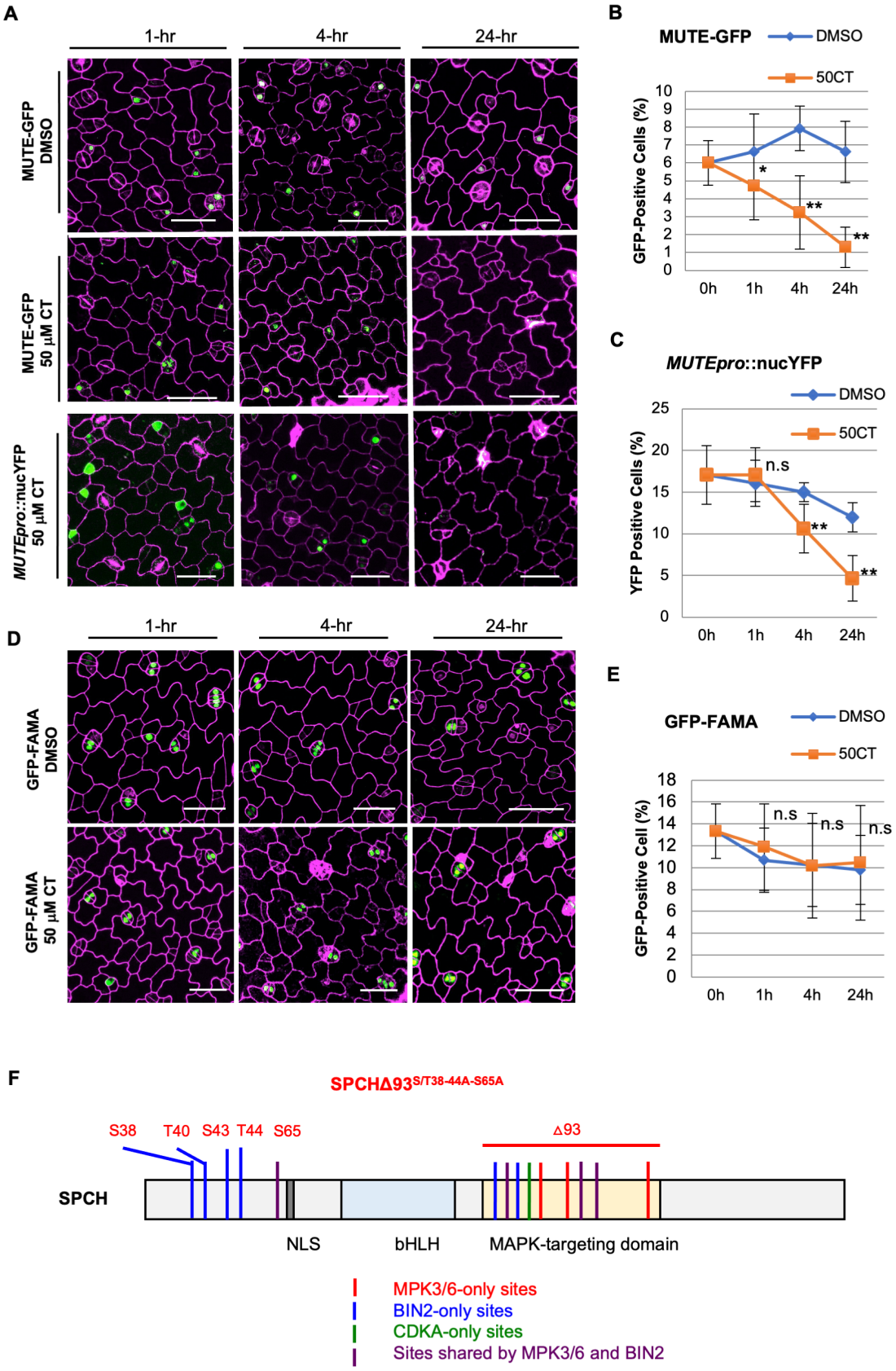


Fig. S5. Differential responses of key bHLH transcription factors to defective PP2A function

(A) Confocal images of 3-dpg adaxial cotyledons expressing translational fusion MUTE-GFP and transcriptional fusion *MUTEpro::*nucYFP. Cell outlines are visualized by PI staining (magenta) and GFP expression are in green. Seedlings were treated with DMSO or 50 μM CT for 1-hr, 4-hr, and 24-hr, respectively. (B-C) Quantification of GFP/YFP-positive cells at different time point in 3-dpg MUTE-GFP, n=965-2888 cells (B) and *MUTEpro::*nucYFP, n=699-2129 cells (C). (D) Confocal images of 3-dpg seedlings expressing translational fusion GFP-FAMA in responding to DMSO or 50 μM CT at 1-hr, 4-hr, and 24-hr, respectively. (E) Quantification of GFP-positive cells in 3-dpg seedlings expressing GFP-FAMA in responding to DMSO or 50 μM CT for 0-hr, 1-hr, 4-hr, and 24-hr. n=794-2256 cells counted for each time point. Quantification data (B, C, and E) are mean ± SD. *significantly different from the DMSO control values (Student's *t*-test, *P < 0.05, **P < 0.001). n.s: not significant. Scale bars in (A) and (D) represent 50 μm. (F) Diagram for SPCH subdomains with identified phosphorylation sites (vertical lines, color coded as indicated). NLS, nuclear localization signal. SPCHΔ93$^{S/T38\text{-}44A\text{-}S65A}$ is a variant with the MAPK-targeting domain deleted (Δ93) and a few N-terminal phosphorylation sites (S/T, S38, T40, T43, and S65) mutated to Ala to make S/T38-44A-S65A.

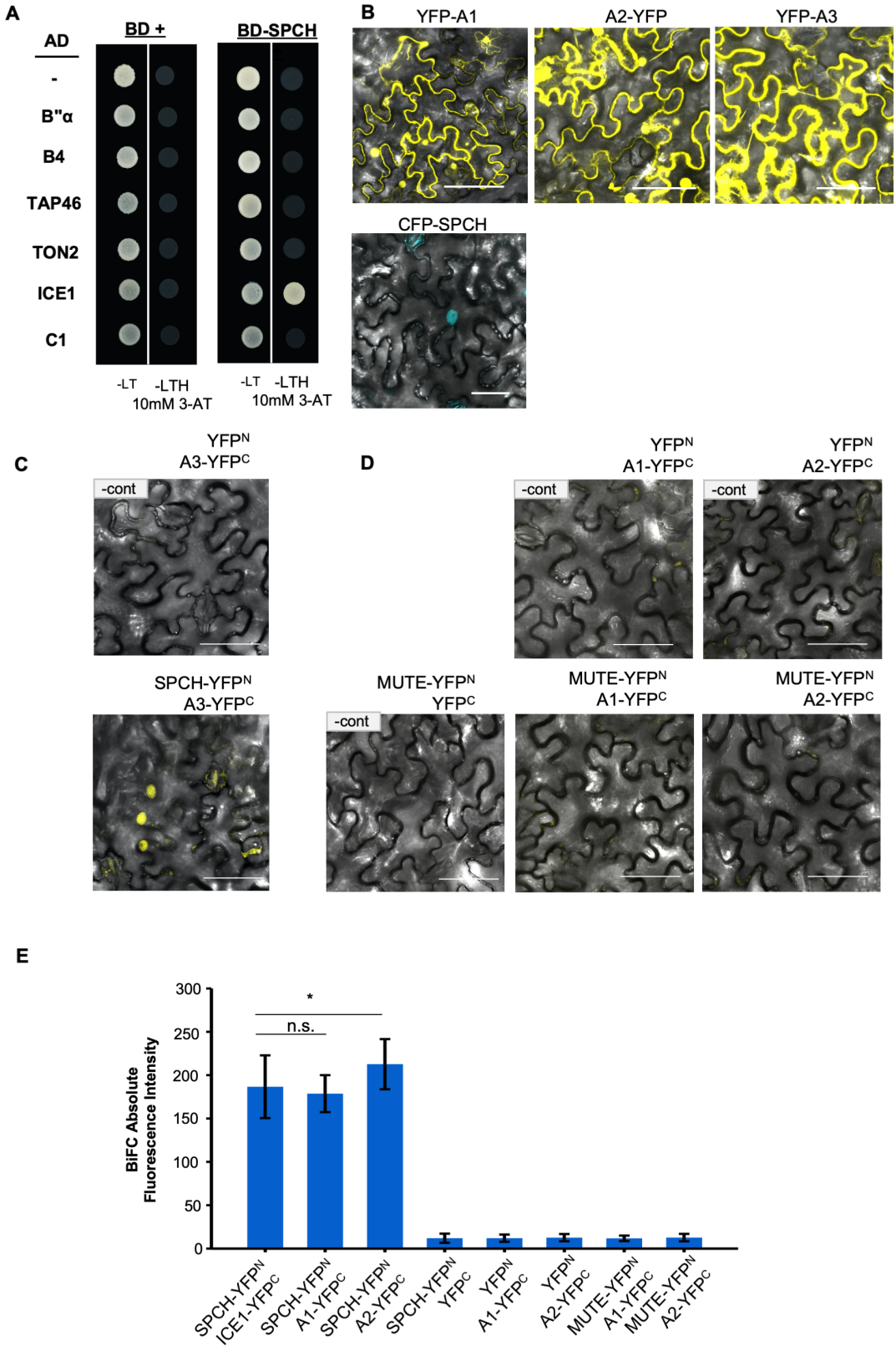


Fig. S6. Interaction assays for SPCH with the PP2A subunits

(A) Yeast two-hybrid assay to test BD-SPCH interaction with AD-B"α/B13, -B'β/B4, -TAP46, -TON2 and -C1. BD and AD empty vectors were used as negative controls,

SCRM/ICE1, a known binding partner of SPCH, was used as positive control. Left, growth controls (-Leu-Trp). Right, interaction tests (-Leu-Trp-His with 10 mM 3-AT). (B) Confocal images to show transient protein expression patterns of YFP-A1, A2-YFP, YFP-A3, and CFP-SPCH in tobacco epidermal cells. (C) Confocal images of BiFC assays. Complemented YFP expression (yellow) was shown when SPCH-YFP^N coexpressed with A3-YFP^C. The YFP^N co-expressed with A3-YFP^C was used as a negative control. (D) Confocal images of BiFC assays to test interactions between MUTE and the PP2A-A subunits. The expression of half YFPs (YFP^N and YFP^C) were used as negative controls. -cont, negative controls in (C) and (D). Scale bars represent 50 μm in (B-D). (E) Quantification of the absolute fluorescence intensity levels of YFP obtained from the same confocal settings, e.g. laser intensity and smart gains, for BiFC assays in (D and Fig. 5B), including positive/negative controls and testing samples. Data are mean ± SD. For each sample, n= 15 cells collected from 3 independent experiments. Student's *t*-test, *P < 0.05, **P < 0.001. n.s: not significant.

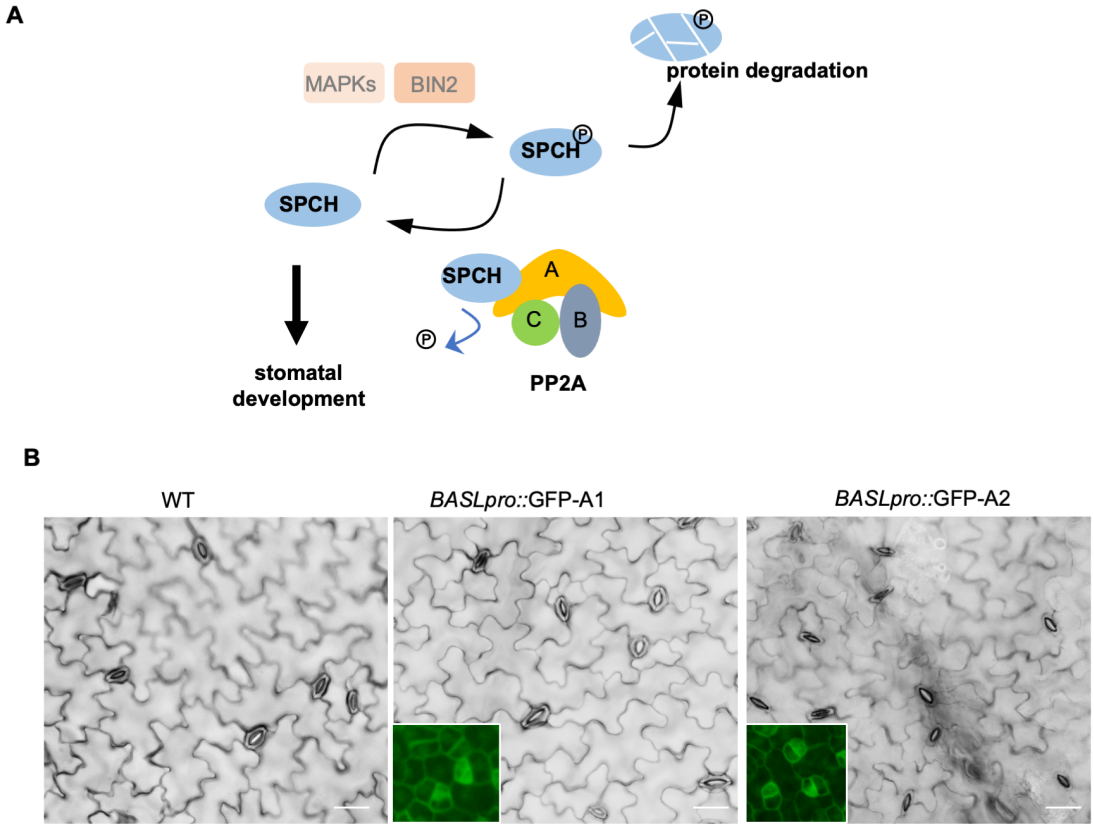


Fig. S7. PP2A promotes stomatal development by stabilizing the SPCH protein
(A) A working model for PP2A promoting stomatal development by stabilizing the SPCH protein. PP2A might function in opposition to the identified kinases (MAPKs and BIN2) to balance the phosphorylation status of SPCH in the initiation of stomatal lineage cells. (B) DIC images of 5-dpg adaxial side cotyledons of the WT, *BASLpro::*GFP-A1, and *BASLpro::*GFP-A2 seedlings. GFP expressions are shown in the insets (green). Scale bars represent 25 μm.

154 **Table S1. Primers used in this study**

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Primer Name	Sequence (5'-3')	Purpose
F-RNAi-a1;a2;a3	TAT CAT CAG ACT CTT CTC AGC ATG TC	*RNAi*-a1;a2;a3
R-RNAi-a1;a2;a3	GGG TTG TTA ACC ATC TCA AGA ACC	
RT-RCN1-F	CCA TAG TCG ACC AAT CAG TG	Real-time PCR for A1
RT-RCN1-R	CAG TTC ATA GCC AGC AAC C	
RT-PDF1-F	CCA ATA GTT GAT CAA TCG GTT G	Real-time PCR for A2
RT-PDF1-R	TAC CGA GGC ACT CTA GTA G	
RT-PDF2-F	ATA GTC GAC CAA GCG GTT G	Real-time PCR for A3
RT-PDF2-R	CCC AAC GAA CAA ATC ACA G	
RT-F-CFP	ACA ACT ACA TCA GCC ACA ACG	Real-time PCR for SPCH-CFP
RT-R-CFP	CTC GTT GGG GTC TTT GCT CAG	
ACTIN2-F	TCT TCC GCT CTT TCT TTC CAA GC	Real-time PCR control
ACTIN2-R	ACC ATT GTC ACA CAC GAT TGG TTG	
RT-SPCH-F	TTC TTT CAC CAT CAA GAT TGG	Real-time PCR for SPCH
RT-SPCH-R	GTA CTG CTC TCT CGT TAA GG	
RT-MUTE-F	GCT ATC TTT TCA AGT TCT TCA CC	Real-time PCR for MUTE
RT-MUTE-R	GTA TCA AGG CTC ATG TAA CG	
RT-FAMA-F	CAA ACC GTC CTC TAC TCC	Real-time PCR for FAMA
RT-FAMA-R	CAC TCT TCC AAA TGC TTG G	
RT-ICE1-F	TGT TCT GTG GTC GTA GAC C	Real-time PCR for SCRM/ICE1
RT-ICE1-R	TCA GGC AGT ATC TCT TGT CC	
F-proSPCH-PmeI	GCC GTTTAAAC CGA AGT ACT GAT CTT TCT GCT C	SPCH promoter cloning
R-proSPCH-KpnI	GGC GGTACC CGT GAT TAG AGA TAT ATC CTT CTC TC	

F-CRISPR-sgRNA1	GATT GGTGCTTCTTGCAATGGCTG	*crispr*-a1
R-CRISPR-sgRNA1	AAAC CAGCCATTGCAAGAAGCACC	
F-CRISPR-sgRNA2	GATT GGCC TCAGTTATAA TGGGAA	*crispr*-a2;a3
R-CRISPR-sgRNA2	AAAC TTCCCATTATAACTGAGGCC	
F-Seq-RCN1	ATG GCT ATG GTA GAT GAA CCG TTG	*crispr* mutant genotyping for A1
R-Seq-RCN1	CGG CGT AAT CTA GTG CTA CAT AAA TC	
F-seq2-PDF1	CTT GAT GAT CCC ATT TAC TTC CTC TTG	*crispr* mutant genotyping for A2
R-Seq-PDF1	TCT GCA ACA TAC ACA ATC CAA CC	
F-Seq-PDF2	GTG GCT GAT GTG TAA TAG GCA AG	*crispr* mutant genotyping for A3
R-Seq-PDF2	CCT AAA GAT GAC AGT GAC TAG GAC G	
F-RCN1-NotI	GCTCC GCGGCCGC ATG GCT ATG GTA GAT GAA CCG TTG	A1 coding sequence cloning
R-RCN1-AscI (NO STOP)	A GGCGCGCC GGA TTG TGC TGC TGT GGA AC	
F-PDF1-NotI	GCTCC GCGGCCGC ATG TCT ATG ATC GAT GAG CCG TTG	A2 coding sequence cloning
R-PDF1-AscI (NO STOP)	A GGCGCGCC GCT AGA CAT CAT CAC ATT GTC AAT AGA TTG	
F-PDF2-NotI	GCTCC GCGGCCGC ATG TCT ATG GTT GAT GAG CCT TTA TAC C	A3 coding sequence cloning
R-PDF2-AscI (NO STOP)	A GGCGCGCC GCT AGA CAT CAT CAC ATT GTC AAT AG	

F-PP2A-C1-NotI	GCTCC GCGGCCGC ATG CCG TTA AAC GGA GAT CTC G	PP2A-C1 coding sequence cloning
R-PP2A-C1-AscI	A GGCGCGCC CAA AAA ATA ATC AGG GGT CTT GCG C	
F-TAP46-NotI	GCTCC GCGGCCGC ATG GGT GGT TTG GCT ATG G	TAP46 coding sequence cloning
R-TAP46-AscI (-STOP)	A GGCGCGCC GCC ACA AGG TGT GAG TTT CTT G	
F-B''α-NotI	GCTCC GCGGCCGC ATG GAA ATC GAT GGT GGA AAC GAT G	B''α coding sequence cloning
R- B''α-AscI (-STOP)	A GGCGCGCC AAA TGG AGA TTC GAG TGG TTC ATC C	
F-TON2-NotI	GCTCC GCGGCCGC ATG TAT AGC GGA TCT AGC GAT GGT G	TON2 coding sequence cloning
R-TON2-AscI (-STOP)	A GGCGCGCC CTG AGA CTC TTC CTC AGG TGG T	
F-B4-NotI	GCTCC GCGGCCGC ATG TTT AAG AAA ATC ATG AAA GGT GGG C	B4 coding sequence cloning
R-B4-AscI (no stop)	A GGCGCGCC GGA AGT GAT CAT ATG ATC TTC TTC TCC	
F-SPCH-38-40-43-44A	GGTGCCGGAGAGATAGCTCCGGCAGCTG CAGCTGCACCTAAAGATGG AACCACAAG	SPCH site-mutation
R-SPCH-38-40-43-44A	CCATCTTTAGGTGCAGCTGCAGCTGCCG GAGCTATCTCTCCGGCACCTTCAAGAC	
F-SPCH-65A	GGATCAAGATTATGAAAACTCAGCTCCTA AGAGGAAAAAGCAAAG	SPCH site-mutation
R-SPCH-65A	CTTTGCTTTTTCCTCTTAGGAGCTGAGTTT TCATAATCTTGATCC	

F-proPP2AA1	GTC TTG TTT TGT TTG TGC TTT CC	A1 promoter cloning
R-proPP2AA1	CTT ATG TGA AAG TTC GAA TCA AAT CAC	
F-proPP2AA2	CGT ATT CAT AGT TCC TGA GAT TGA G	A2 promoter cloning
R-proPP2AA2	CTT CAA CAA CAC CAA CAA CAA AAT TAC	
F-proPP2AA3	GTT GTA CAG TTG CAT ATG TGT GTG	A3 promoter cloning
R2-proPP2AA3	GTC GAT AAG CAC AGC AAT CGG	

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