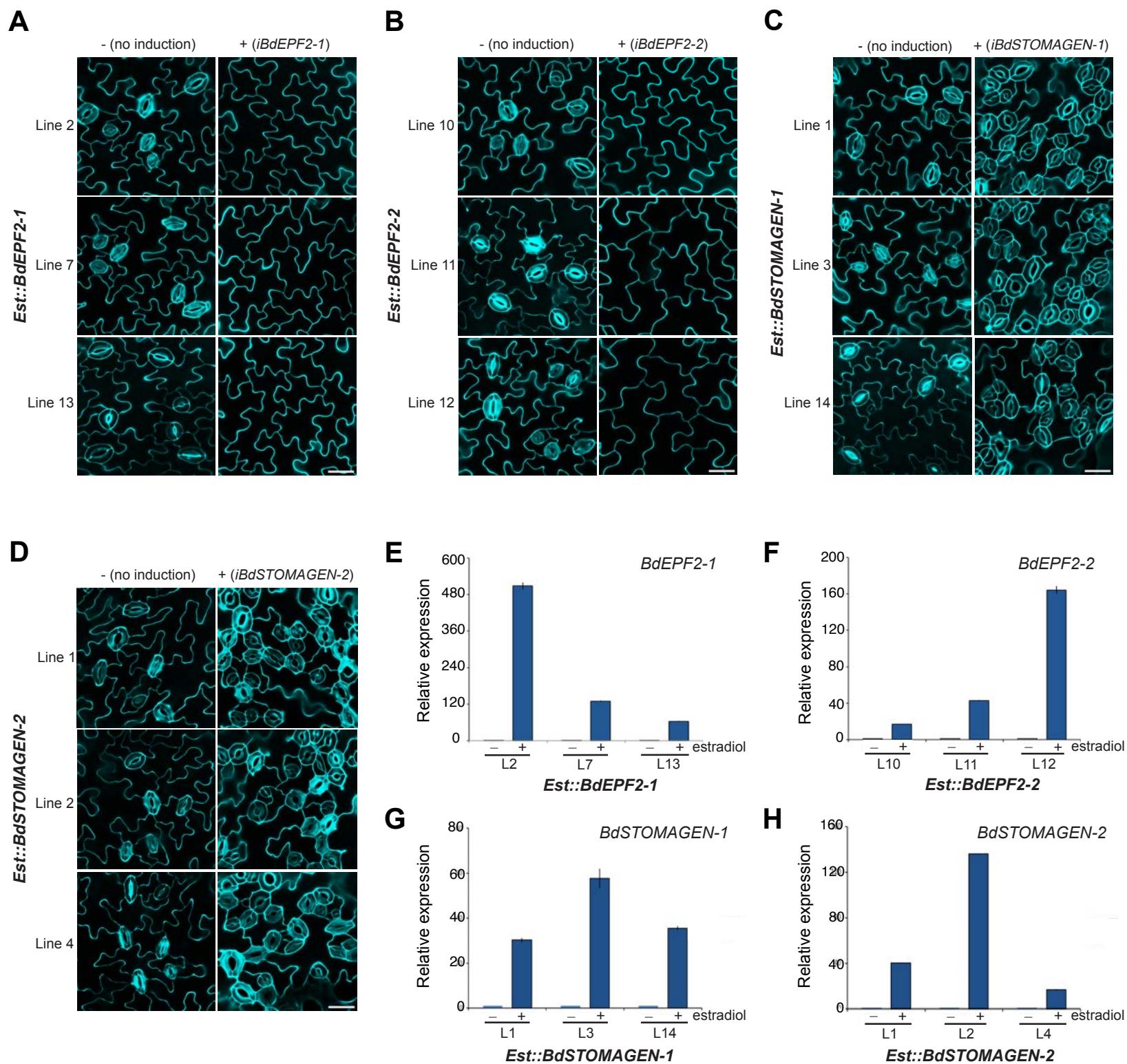
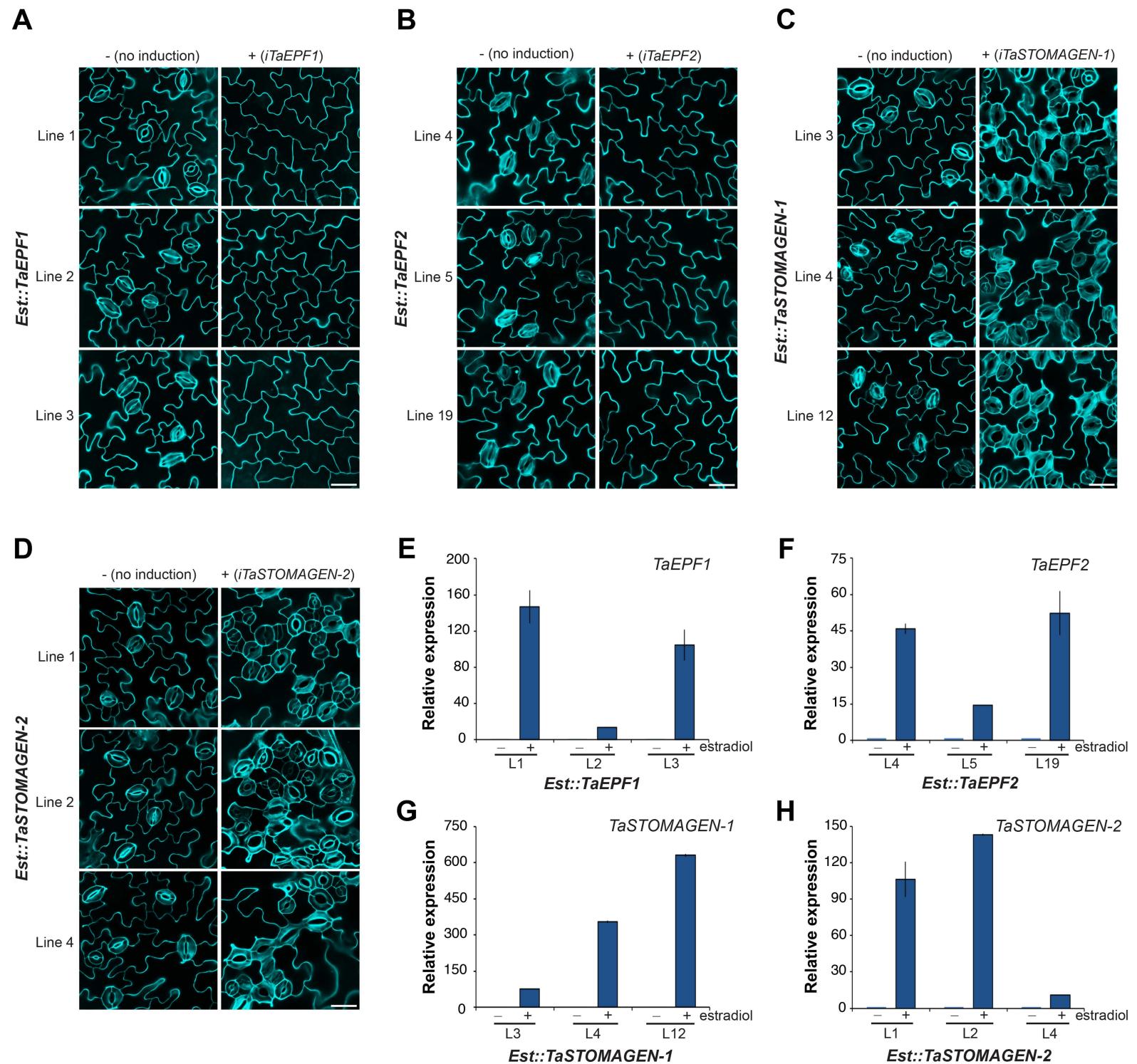
**Fig. S1. Phylogenetic relationships between *Arabidopsis* and grass EPF family members.**

(A) Phylogenetic tree of the EPF family members in *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* (rice), *Hordeum vulgare* (barley), *Sorghum bicolor* (sorghum), *Zea mays* (maize) and *Triticum aestivum* (wheat). The tree was constructed in MEGA7 (Kumar et al., 2016) using the amino acid sequences of the predicted mature C-terminal region of the EPF family members and their grass homologs. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. (B) The sequence alignment of the predicted mature peptide regions of the stomatal EPFs in *Arabidopsis*, AtEPF1, AtEPF2 and AtSTOMAGEN, and their homologs in wheat and the model grass organism, *Brachypodium*. The conserved cysteine residues are highlighted. See also Fig. 1, Table S1-S3.



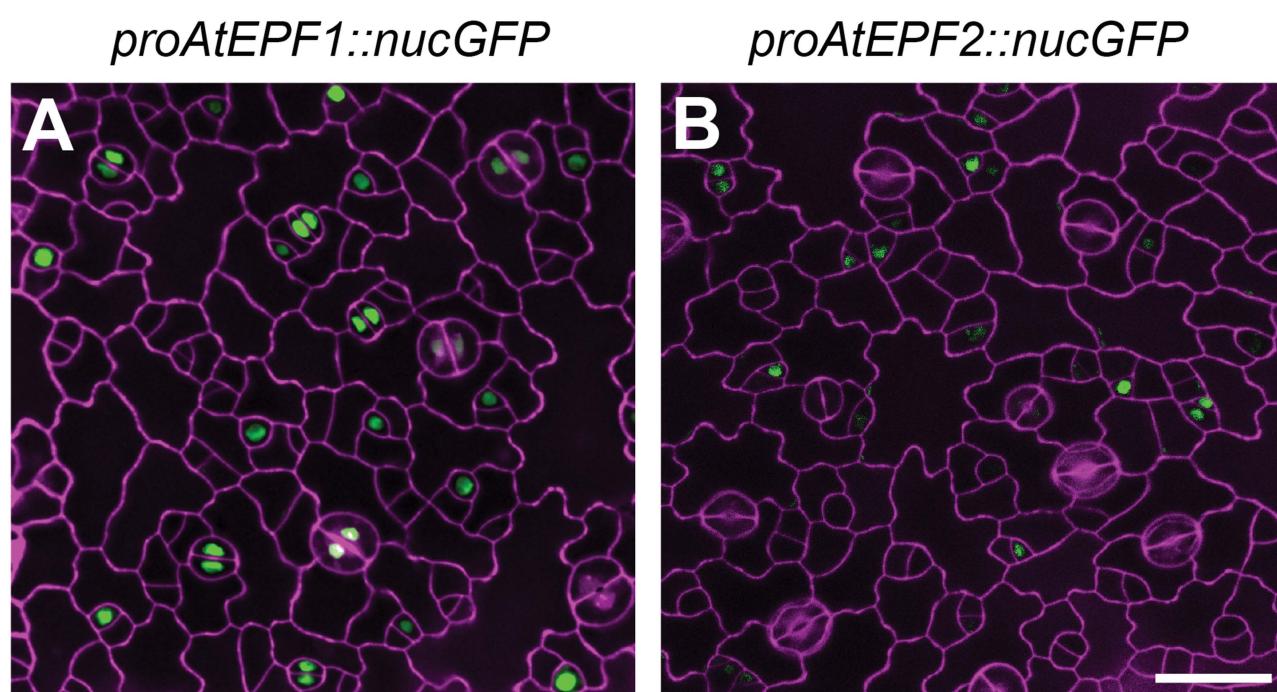
**Fig. S2. Epidermis phenotype of induced overexpression of *Brachypodium* EPF stomatal homologs in multiple independent *Arabidopsis* transgenic lines.**

(A-D) Representative confocal images of abaxial cotyledon epidermis from 10-day-old seedlings of three independent *Arabidopsis* transgenic lines harboring an oestradiol-inducible overexpression construct for each of the four stomatal EPF homologs from *Brachypodium*: (A) *iBdEPF2-1*, (B) *iBdEPF2-2*, (C) *iBdSTOMAGEN-1*, and (D) *iBdSTOMAGEN-2*. Left panels, no induction (control); right panels, oestradiol induction; each row shows representative images from individual lines. Cells were outlined by propidium iodide staining (cyan), and images were taken under the same magnification. Scale bar = 30 µm. (E-H) RT-qPCR analysis of (E) *BdEPF2-1*, (F) *BdEPF2-2*, (G) *BdSTOMAGEN-1*, and (H) *BdSTOMAGEN-2* transgenes in three independent *Arabidopsis* transgenic plants carrying oestradiol-inducible overexpression constructs for each of the four *Brachypodium* stomatal homologs. *eIF4A* was used as an internal control and the data for each uninduced transgenic line was set to 1. Error bars = means with SE ( $n = 3$ ). For primer sequences, see Table S5.



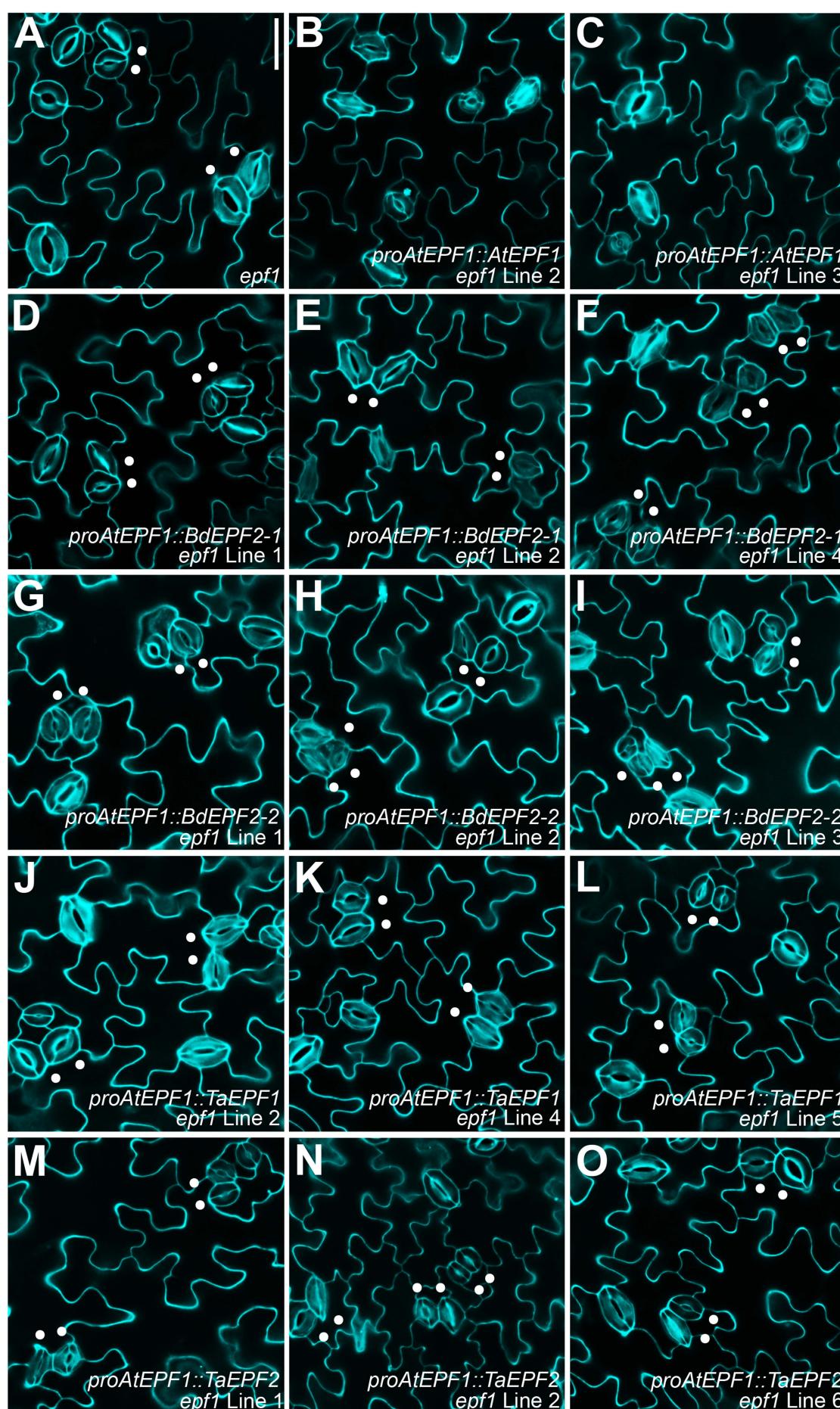
**Fig. S3. Epidermis phenotype of induced overexpression of wheat EPF stomatal homologs in multiple independent *Arabidopsis* transgenic lines.**

(A-D) Representative confocal images of abaxial cotyledon epidermis from 10-day-old seedlings of three independent *Arabidopsis* transgenic lines harboring an oestradiol-inducible overexpression construct for each of four stomatal EPF homologs from wheat: (A) *iTaEPF1*, (B) *iTaEPF2*, (C) *iTaSTOMAGEN-1*, and (D) *iTaSTOMAGEN-2*. Left panels, no induction (control); right panels, oestradiol induction; each row shows representative images from individual lines. Images are taken under the same magnification. Scale bar = 30  $\mu$ m. (E-H) RT-qPCR analysis of (E) *TaEPF1*, (F) *TaEPF2*, (G) *TaSTOMAGEN-1*, and (H) *TaSTOMAGEN-2* transgenes in three independent *Arabidopsis* transgenic plants carrying oestradiol-inducible overexpression constructs for each of the four wheat stomatal homologs. *eIF4A* was used as an internal control and the data for each uninduced transgenic line was set to 1. Error bars = means with SE ( $n = 3$ ). For primer sequences, see Table S5.



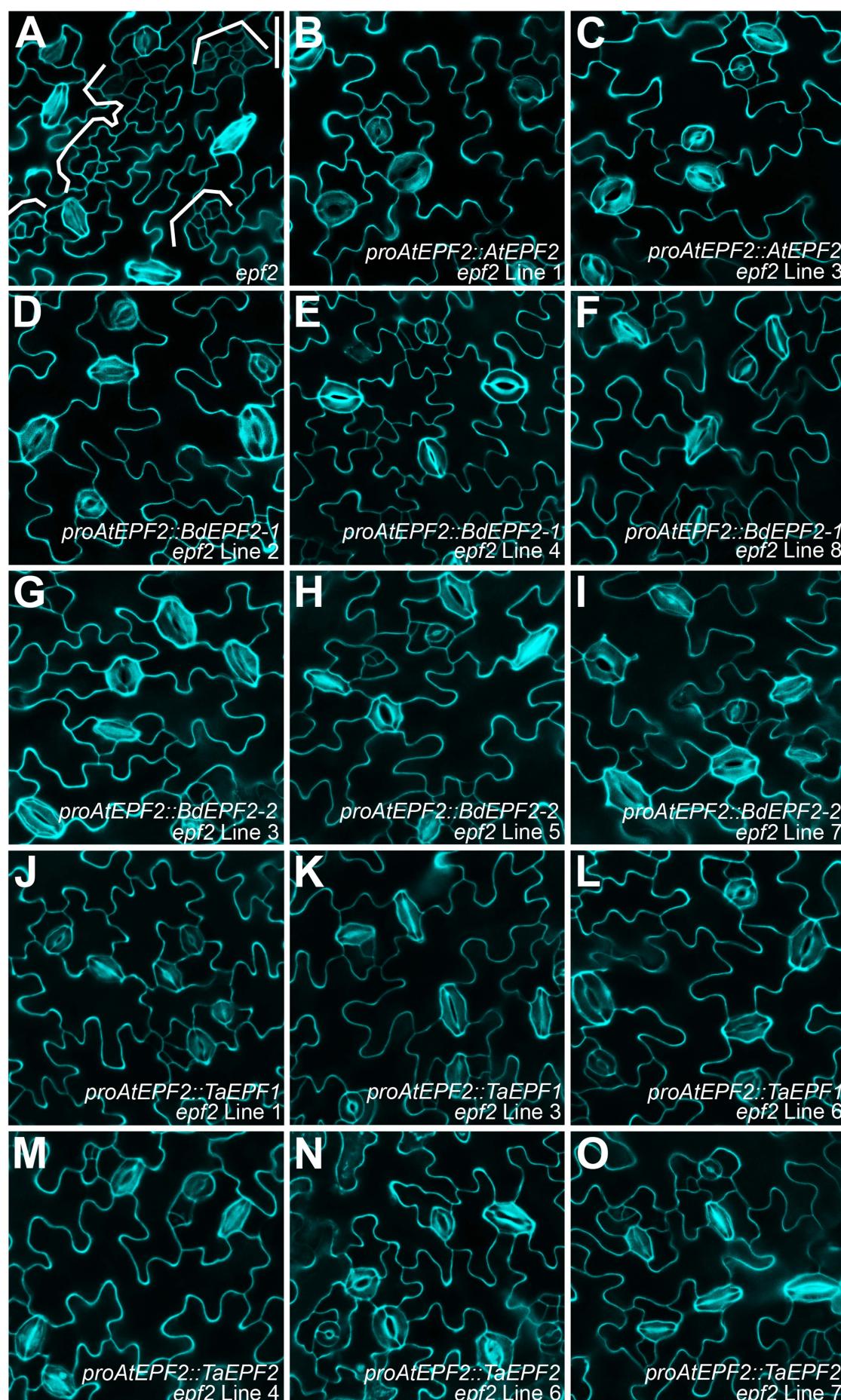
**Fig. S4. Expression patterns of stomatal lineage specific *AtEPF1* and *AtEPF2* promoters used in this study.**

Representative confocal images of the abaxial developing leaf epidermis of 12-day-old wild-type seedlings carrying the green fluorescent protein (nucGFP)-tagged transcription reporters for the promoters of (A) *AtEPF1* and (B) *AtEPF2*. GFP expression is detected in the nuclei of (A) a subset of later stomatal lineage cells (meristemoids, guard mother cells and young guard cells) and (B) early stomatal lineage cells (meristemoid mother cells and meristemoids) indicating that the two promoter fragments used in this study drive correct expression patterns in the stomatal lineage. Cells were outlined by propidium iodide staining (purple), and images were taken under the same magnification. Scale bar = 30  $\mu$ m.



**Fig. S5. Complementation of *Arabidopsis* *epf1* loss-of-function mutants by grass *EPF1/EPF2* homologs.**

Shown are representative confocal images of 10-day-old cotyledon epidermis of the (A) *Arabidopsis* *epf1* mutant, (B,C) two independent transgenic *epf1* plants expressing *proAtEPF1::AtEPF1*, and (D-F) three independent transgenic *epf1* lines expressing *AtEPF1/AtEPF2* homologs from *Brachypodium*: *proAtEPF1::BdEPF2-1*, (G-I) *proAtEPF1::BdEPF2-2*, and (J-L) *AtEPF1/AtEPF2* homologs from wheat: *proAtEPF1::TaEPF1*, and (M-O) *proAtEPF1::TaEPF2*. The *epf1* mutation confers stomatal pairing (dots). Unlike the *proAtEPF1::AtEPF1* construct, *AtEPF1/AtEPF2* homologs from *Brachypodium* and wheat driven by the *AtEPF1* promoter unable to complement the epidermal phenotype of *epf1*. See also Fig. 4A-F,M. All confocal microscopy images were taken under same magnification. Scale bar = 30  $\mu$ m.



**Fig. S6. Complementation of Arabidopsis *epf2* loss-of-function mutants by grass *EPF1/EPF2* homologs.** Representative confocal images of 10-day-old abaxial cotyledons of the (A) Arabidopsis *epf2* mutant, (B,C) two independent transgenic *epf2* plants expressing *proAtEPF2::AtEPF2*, and (D-F) three independent transgenic *epf2* plants expressing *AtEPF1/AtEPF2* homologs from Brachypodium: *proAtEPF2::BdEPF2-1*, (G-I) *proAtEPF2::BdEPF2-2*, and (J-L) *AtEPF1/AtEPF2* homologs from wheat: *proAtEPF2::TaEPF1*, and (M-O) *proAtEPF2::TaEPF2*. The epidermal phenotype of Arabidopsis *epf2* mutants (brackets in A) is rescued by each of the two *AtEPF1/AtEPF2*-like genes from Brachypodium and wheat. See also Fig. 4 G-L,N. All confocal microscopy images were taken under same magnification. Scale bar = 30  $\mu$ m.

**A****MBdEPF2-1, MBdEPF2-2, MBdSTOMAGEN-1, and MBd2g53661 sequence used for bioassays****MBdEPF2-1-mycHis (in pBADg vector)**

MKKLLFAIPLVVVFYSHSHSTMELETGSRLPDCEHACGPCAPCKRVMVSFRCALASESCP  
VAYRCMCRGRFFRVPTLSSAALPPRIRSFLEQKLISEEDLNSAVDHHHHH\*

**MBdEPF2-2-mycHis (in pBADg vector)**

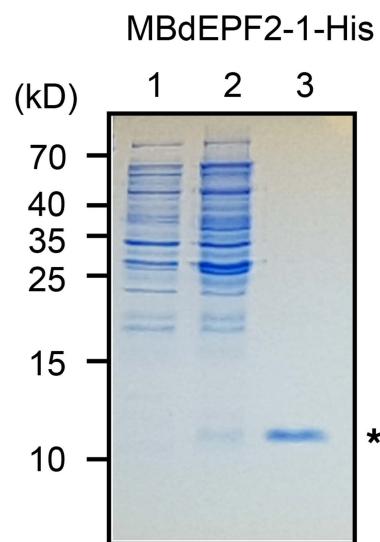
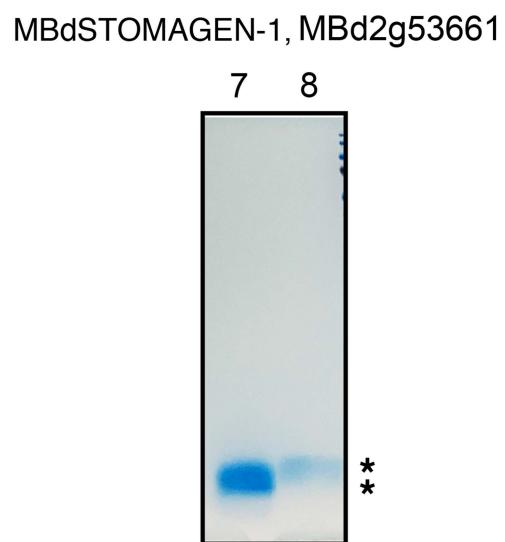
MKKLLFAIPLVVVFYSHSHSTMELETGSSLPDCHACGPCPKPCNRVMVSFKCSIAEPCPM  
VYRCMCKGKCYCVPVSSRIRSFLEQKLISEEDLNSAVDHHHHH\*

**MBdSTOMAGEN-1**

IGSIAPICTYNECRGCRFKCTAEQVPVDANDPMNSAYHYKCVCHR

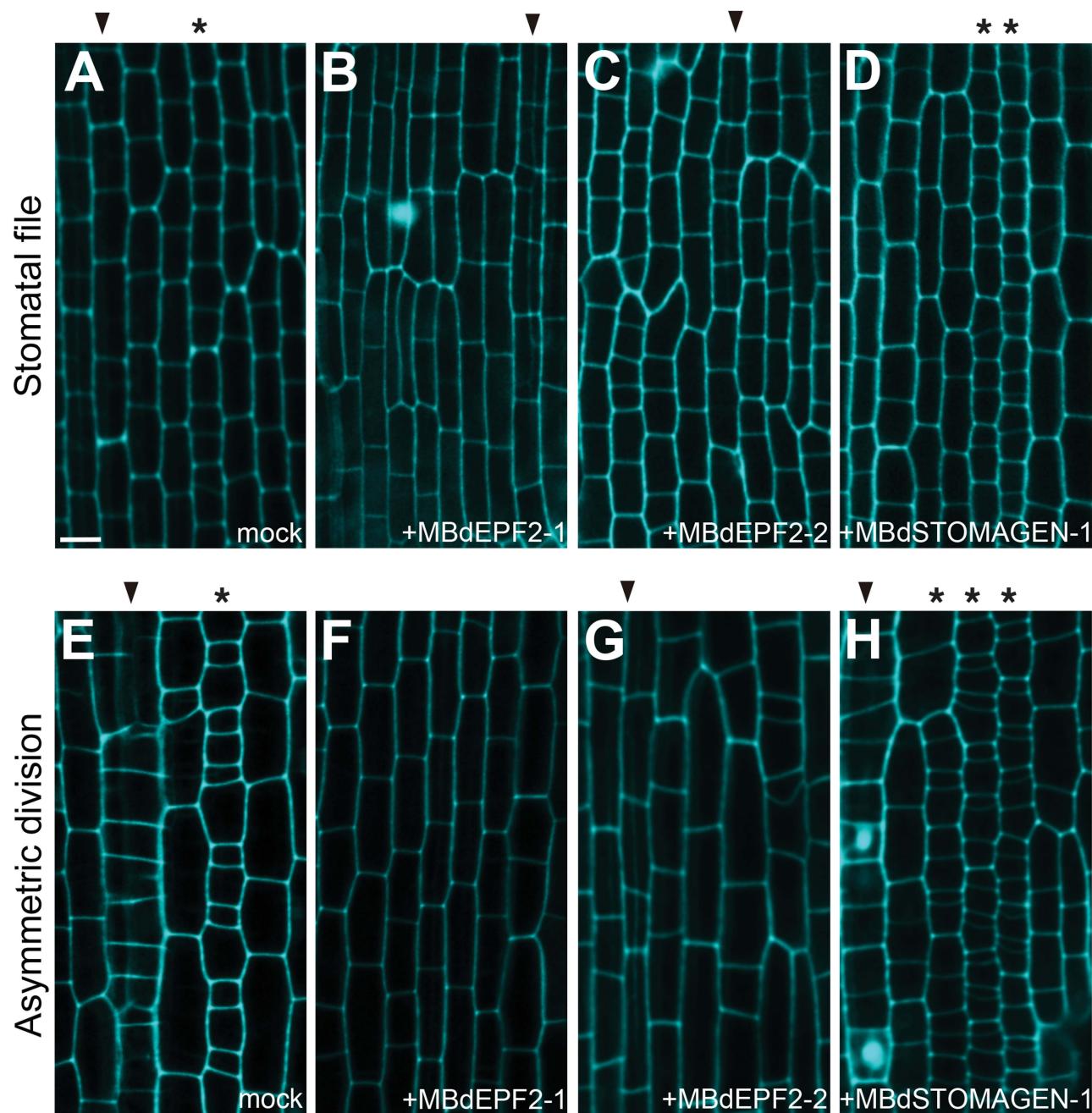
**MBd2g53661**

PGSYPPRCTSCKGSCNPCYPVHVAVPPGPVTAEYYPEAWRCRCGNRLYMP

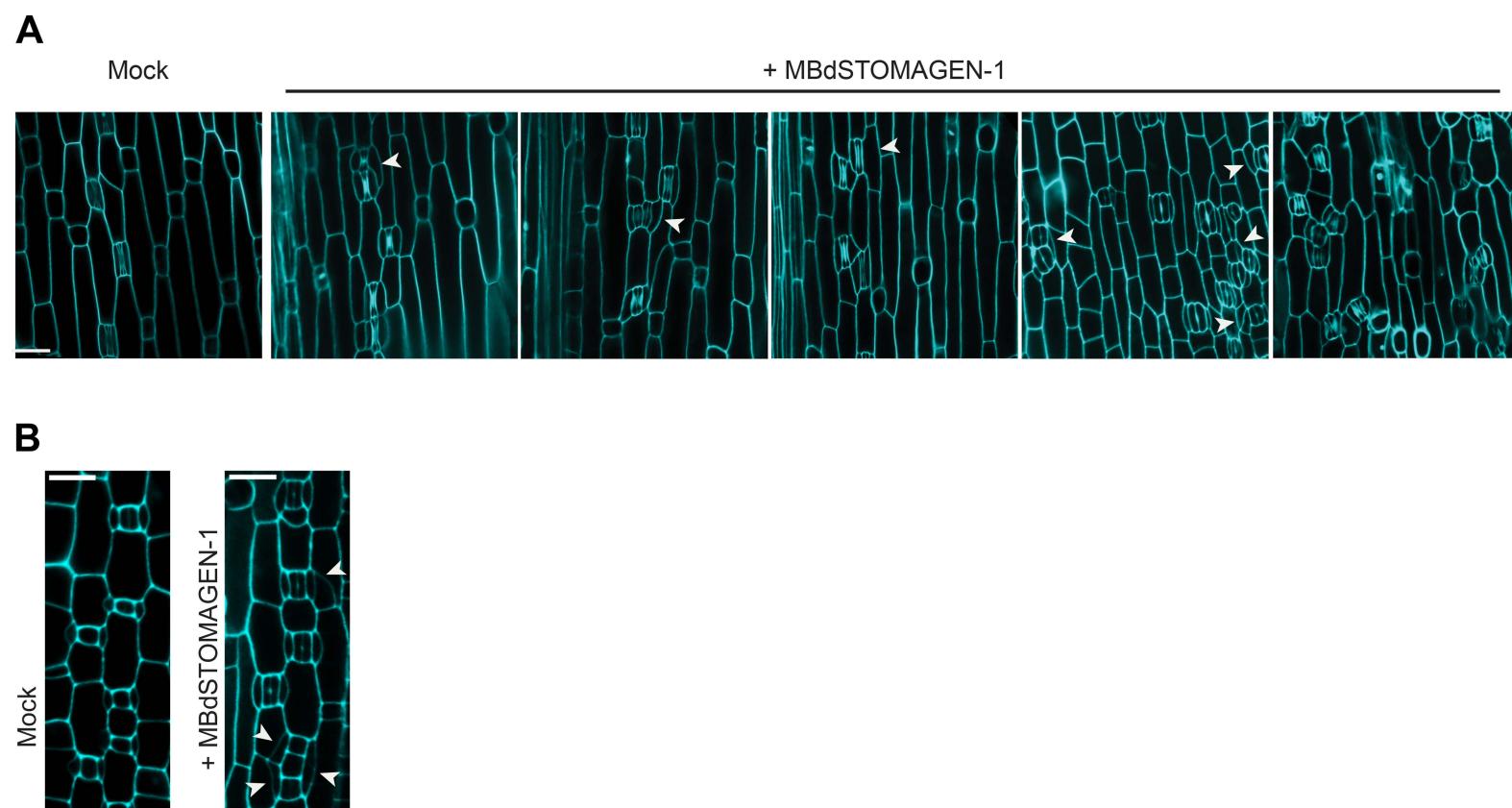
**B****C****D**

**Fig. S7. Amino-acid sequence, expression and purification of bioactive *Brachypodium* peptides used in this study.**

(A) Amino acid sequence of the predicted mature EPF (MEPF) region of MBdEPF2-1, MBdEPF2-2, MBdSTOMAGEN-1, and MBd2g53661 used for bioassays and competition analyses. Underlined: signal sequence from pBADg vector; blue, predicted MBdEPF2-1, MBdEPF2-2, MBdSTOMAGEN-1, and MBd2g53661 sequence; plain, linker, cMyc tag, and His tag. (B-D) Shown are SDS-PAGE gels, stained with Coomassie Brilliant Blue, for expression and purification of bacterially expressed recombinant (B) MBdEPF2-1-His, (C) MBdEPF2-2-His, and (D) synthesized MBdSTOMAGEN-1 and MBd2g53661 after refolding. Lanes 1, 2, 4, and 5: Bacterial lysate carrying MBdEPF2-1 (lanes 1, 2) and MBdEPF2-2 (lanes 4, 5) in the absence (lanes 1, 4) or presence (lanes 2, 5) of L-arabinose for induction; Lanes 3, 6: Purified, dialyzed, and refolded peptide solution of MBdEPF2-1-His (lane 3) and MBdEPF2-2-His (lane 6) and Lanes 7, 8: Refolded synthesized peptide solution of MBdSTOMAGEN-1 (lane 7) and MBd2g53661 (lane 8) used for bioassays and competition analysis in Fig. 5, Fig. 6, Figs. S8-S11. The positions of molecular mass markers in kilodaltons are indicated on the left. Asterisks indicate the size of each His-tagged or synthesized peptide.

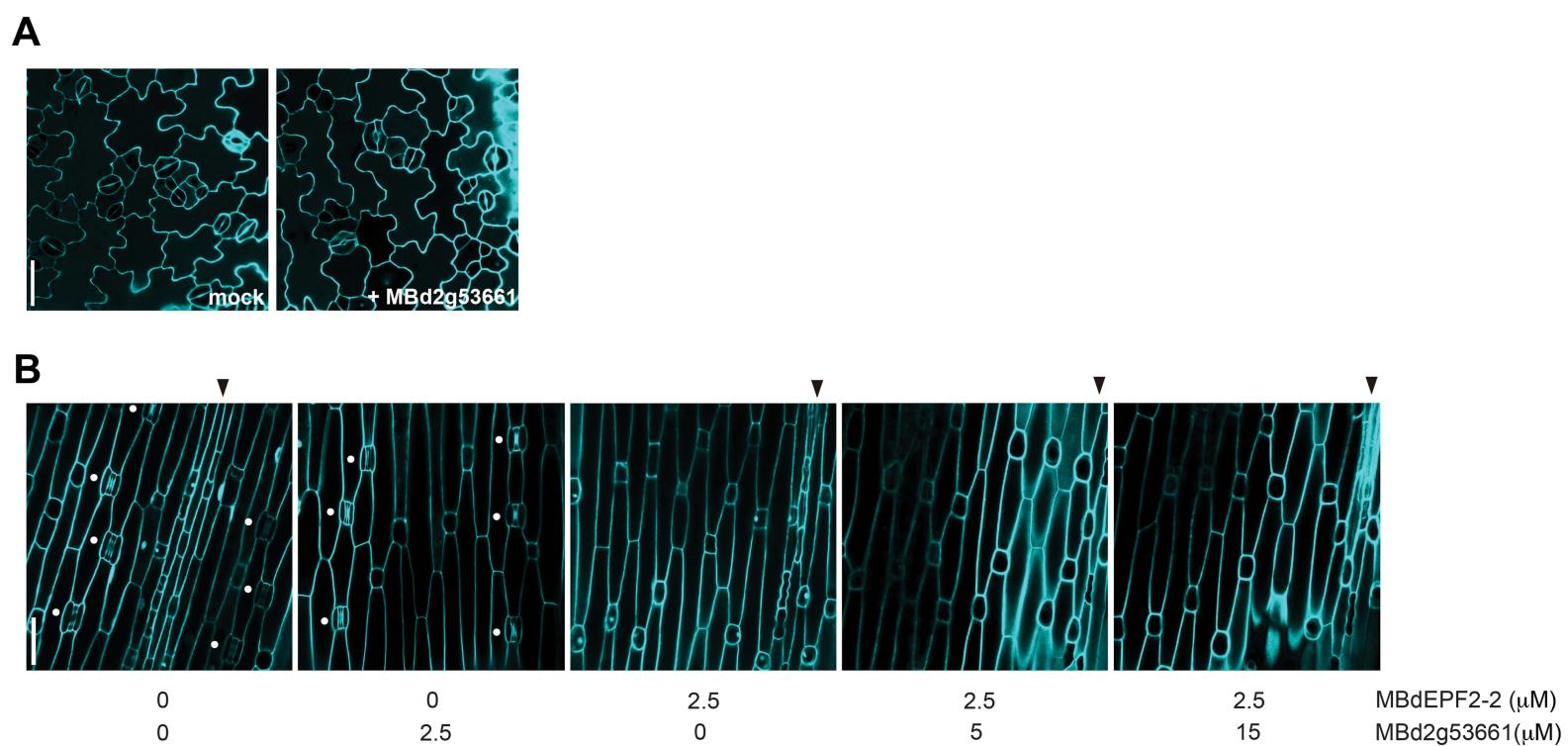


**Fig. S8. Early epidermal phenotypes of *Brachypodium* seedlings treated with bioactive grass EPF peptides.** Confocal images of two early developmental stages of grass stomatal development, stomatal file establishment (A-D) and asymmetric division (E-H) stages, in (A,E) *Brachypodium* wild-type (Bd21-3) seedlings treated with either buffer solution alone (mock) or (B,F) *Brachypodium* MEPF peptides, MBdEPF2-1, (C,G) MBdEPF2-2, (D,H) and MBdSTOMAGEN-1. The epidermis of Bd21-3 seedlings treated with MBdEPF2-1 or MBdEPF2-2 peptide shows neither smaller cell files nor asymmetric entry divisions in the stomatal rows always flanking to the veins, while application of MBdSTOMAGEN-1 to Bd21-3 seedlings results in ectopic smaller cell files and asymmetric divisions. The asterisks indicate smaller cells (A, D) or asymmetric divisions (E, H) in stomatal lineage rows having stomatal fate. Arrowheads, developing leaf veins. All images are from the base of the developing first *Brachypodium* leaf at 7-8 days-post-germination (dpG). Scale bar = 10  $\mu$ m.



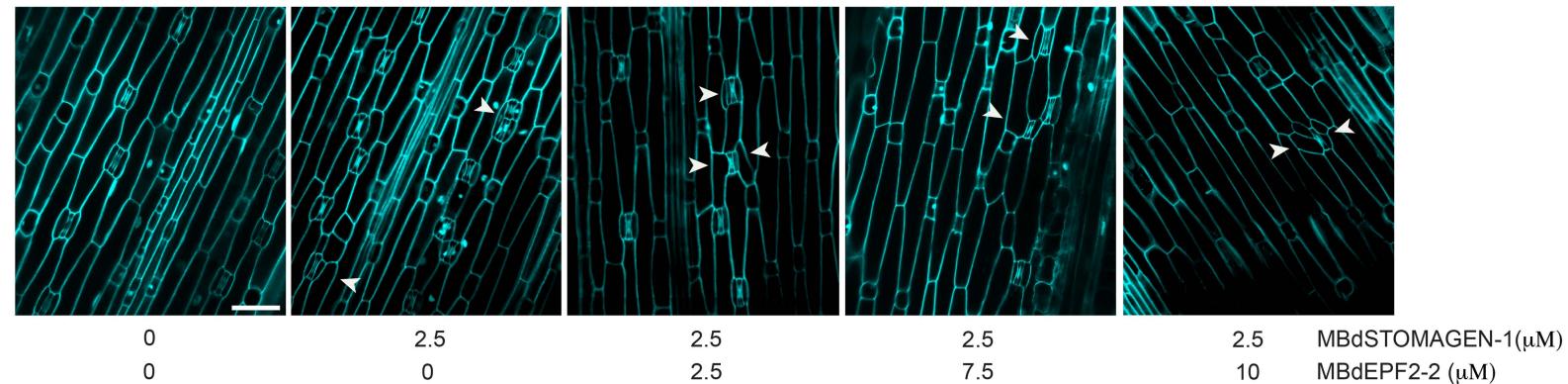
**Fig. S9. Bioactive MBdSTOMAGEN-1 peptide, a 45 amino acid cysteine-rich region of the STOMAGEN-like protein in *Brachypodium*, promotes grass stomatal differentiation and patterning.**

(A) Confocal images of the first leaf epidermis illustrating the phenotypic range observed in *Brachypodium* wildtype (Bd21-3) seedlings treated with 2.5  $\mu$ M MBdSTOMAGEN-1 peptide. All images are shown at the same scale. Scale bar = 30  $\mu$ m. (B) Representative confocal images of cells at subsidiary cell formation and guard mother cell division stages of grass stomatal development in Bd21-3 seedlings treated with either buffer only (mock) or 2.5  $\mu$ M MBdSTOMAGEN-1 peptide. All images are shown at the same scale. Scale bar: 15  $\mu$ m. Bd21-3 seedlings have 4-celled stomatal complexes composed of two guard cells and two subsidiary cells, and they are separated by at least one non-stomatal cell in particular “stomatal” cell files. Application of refolded MBdSTOMAGEN-1 to Bd21-3 seedlings, however, exhibits stomatal patterning defects. Stomatal clusters sometimes have abnormal subsidiary cell morphologies, additional cell divisions, and are dispersed on the epidermis rather than restricted to the specific cell files typical of grass stomata, indicating the formation of ectopic stomatal rows. The arrowheads indicate examples of abnormal subsidiary cells.



**Fig. S10. Unlike BdEPF2 peptides, one of the Brachypodium EPF-family peptides, Bd2g53661, neither controls stomatal development nor competes with BdSTOMAGEN-1.**

(A) Confocal images of the cotyledon epidermis of *Arabidopsis* Col seedlings grown in a buffer solution (mock) or 2.5  $\mu$ M MBd2g53661. Application of refolded MBd2g53661 peptide does not influence the epidermal development in *Arabidopsis*. (B) Bd21-3 seedlings treated with a buffer solution, MBd2g53661, MBdEPF2-2, or MBdEPF2-2 co-treated with increasing concentrations of MBdSTOAMGEN-1. MBd2g53661 does not influence the function of MBdEPF2-2 inhibiting grass stomatal development. Dots indicate stomata which are typically found in specific cell files adjacent to veins (marked by arrowheads). Images were taken under the same magnification. Scale bar = 30  $\mu$ m.



**Fig. S11. Biological activity of BdSTOMAGEN-1 in promoting stomatal initiation, but not the subsidiary cell formation, is antagonized by BdEPF2.**

Confocal images of *Brachypodium* Bd21-3 leaf epidermis treated with a buffer solution, MBdSTOMAGEN-1 alone, or mixtures containing MBdSTOMAGEN-1 plus increasing concentrations of MBdEPF2-2 for 7-8 days. Arrowheads indicate stomata with abnormal subsidiary cell morphologies, such as the spanning of two guard cells by a single subsidiary cell or stomata lacking one subsidiary cell. Images were taken under the same magnification. Scale bar = 50  $\mu$ m.

**Table S1.** EPF genes in Wheat, Sorghum, Rice, Maize, *Brachypodium*, Barley and *Arabidopsis*.

[Click here to download Table S1](#)

**Table S2.** EPF family members in *Triticum aestivum* - notes on annotations in Ensemble Plant genome assembly V1.

[Click here to download Table S2](#)

**Table S3.** Alignment of the C-terminal regions of the EPF family peptides in Wheat, Sorghum, Rice, Maize, *Brachypodium*, Barley and *Arabidopsis*.

[Click here to download Table S3](#)

**Table S4.** List of plasmids constructed in this study.

[Click here to download Table S4](#)

**Table S5.** List of primers and their DNA sequence used in this study.

[Click here to download Table S5](#)