Supplementary Information

Title:
Stigma plasma membrane H^+-ATPase is involved in pollen hydration and pollen tube penetration in Brassicaceae self-incompatibility

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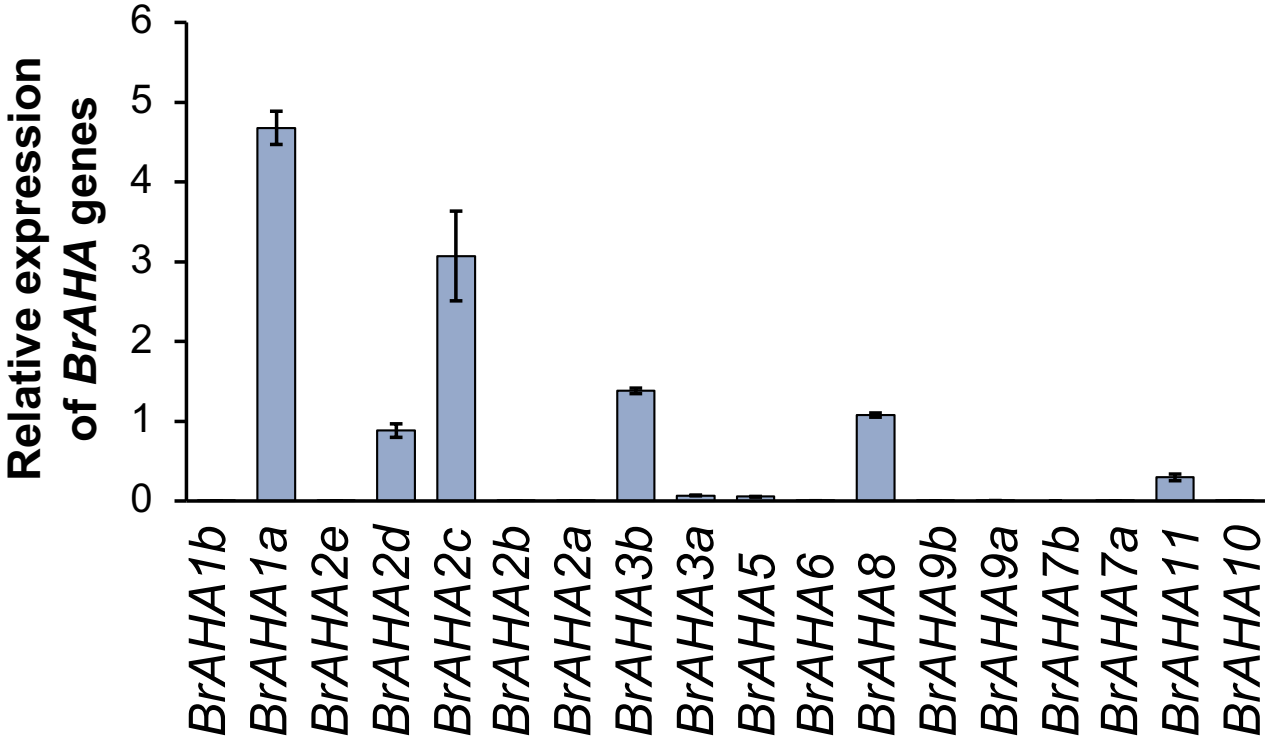
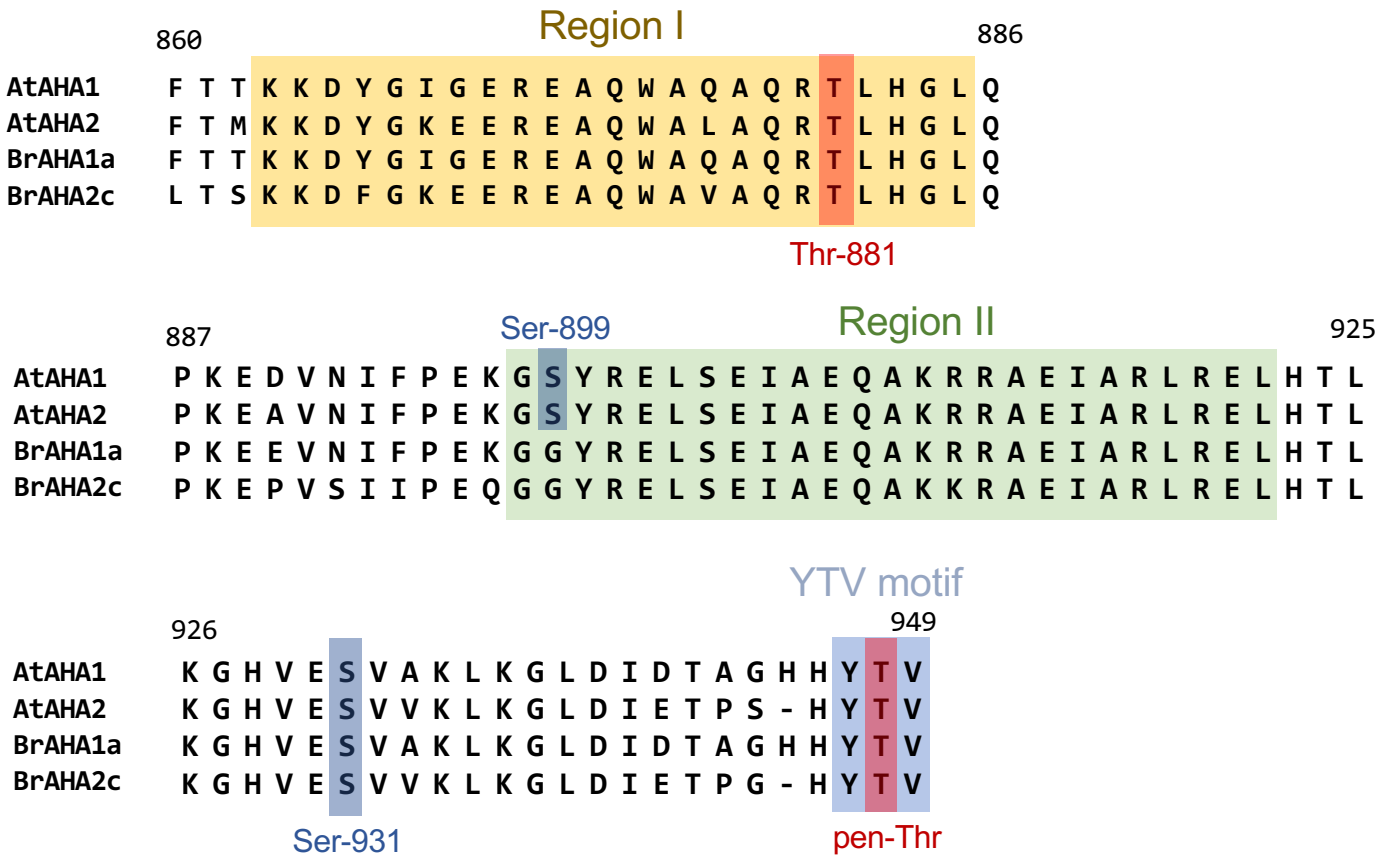
Supplementary Figure S1



Supplementary Figure S1. Expression of *BrAHA* genes in S^8 stigmas by RT-qPCR.

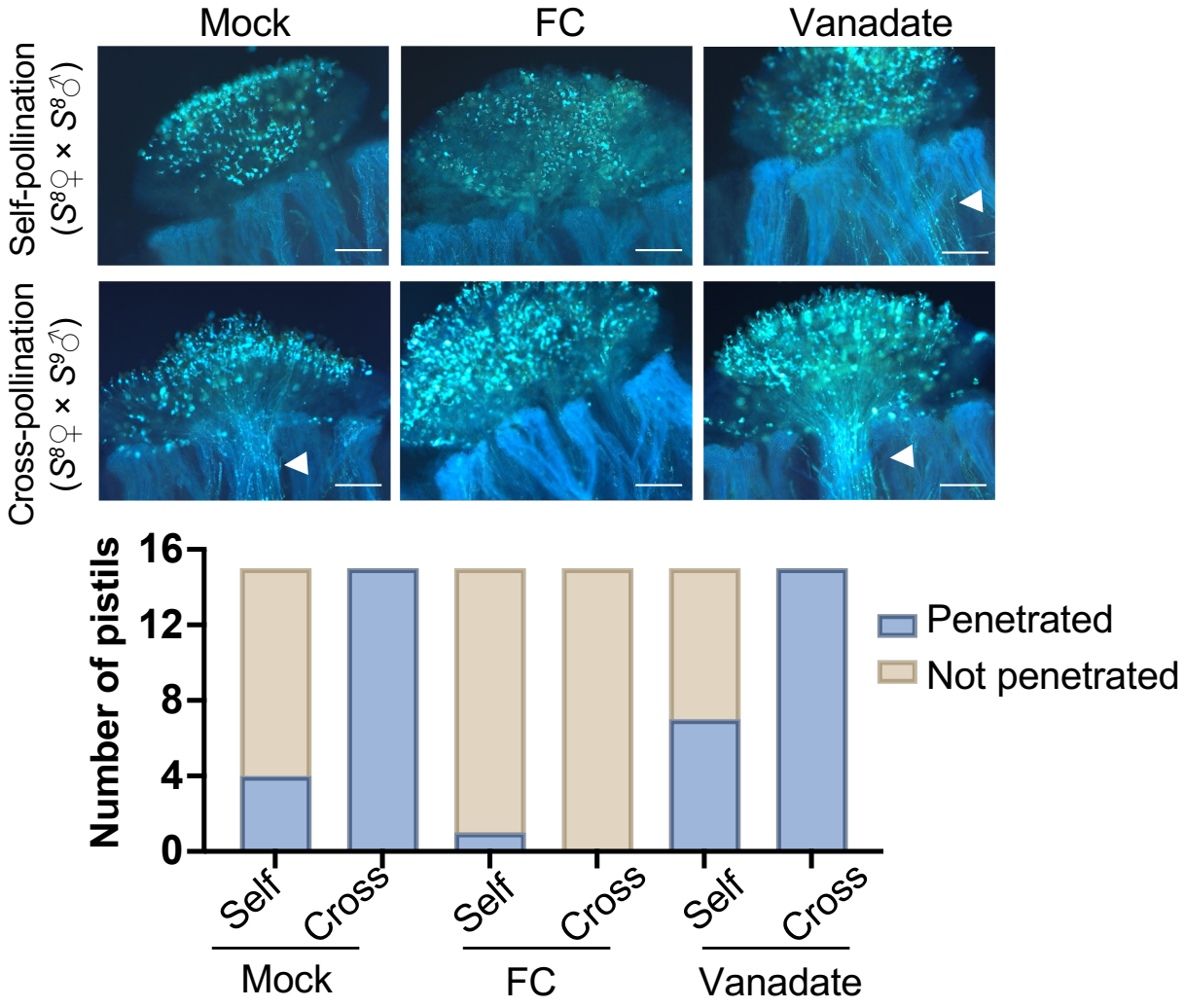
Five stigmas from *B. rapa* S^8 plants were sampled to extract total RNA. Expression of each *AHA* gene relative to that of *ubiquitin-conjugating enzyme 21* (*UBC21*) is shown. Data are mean ± SD (n = 3).

Supplementary Figure S2



Supplementary Figure S2. Multiple alignment of amino acid sequences of the C-terminal region of PM H$^+$-ATPases in *A. thaliana* and *B. rapa*. The amino acid sequences of the C-terminal cytosolic regions of AtAHA1, AtAHA2, BrAHA1a and BrAHA2c are shown. The highly conserved Region I, Region II, and the YTV motif are indicated in color. Amino acid residues highlighted in red and blue indicate those associated in the activation and inactivation of AHA by phosphorylation, respectively.

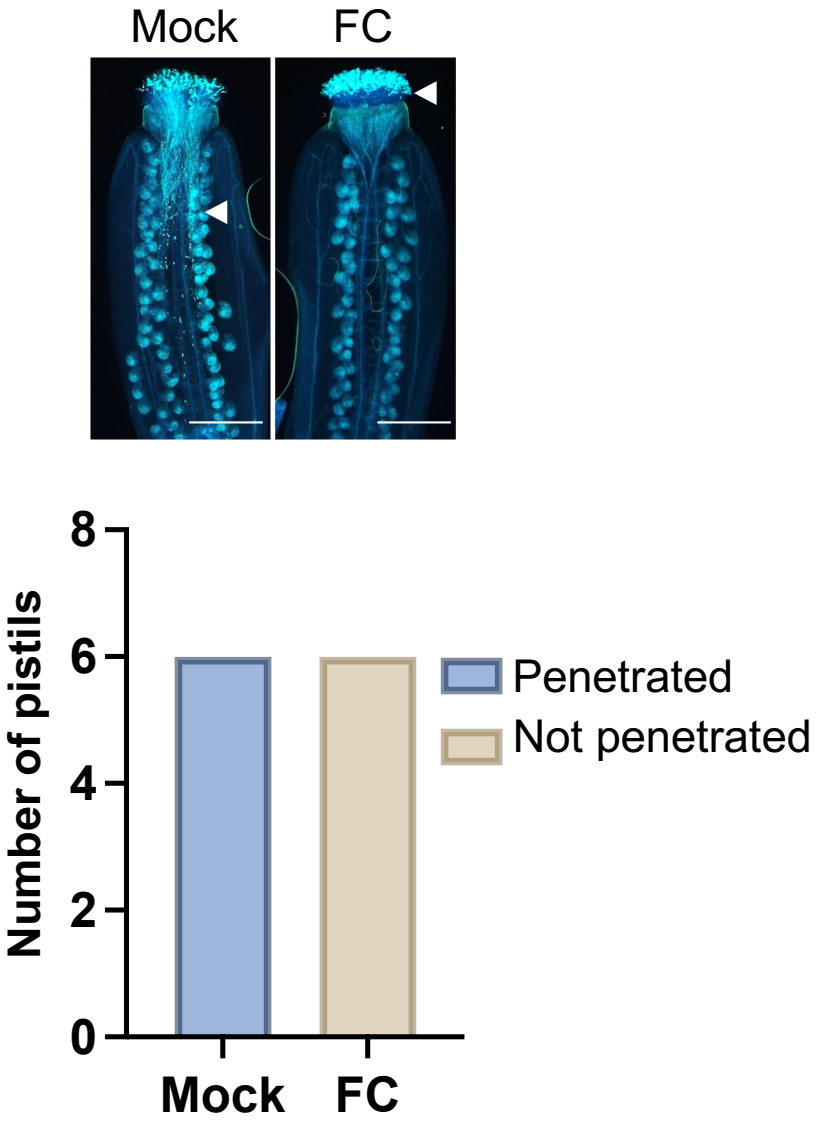
Supplementary Figure S3



Supplementary Figure S3. Confirmation of the effect of stigma PM H$^+$-ATPase activity on pollen tube penetration into the stigmas of *B. rapa S^8* strain.

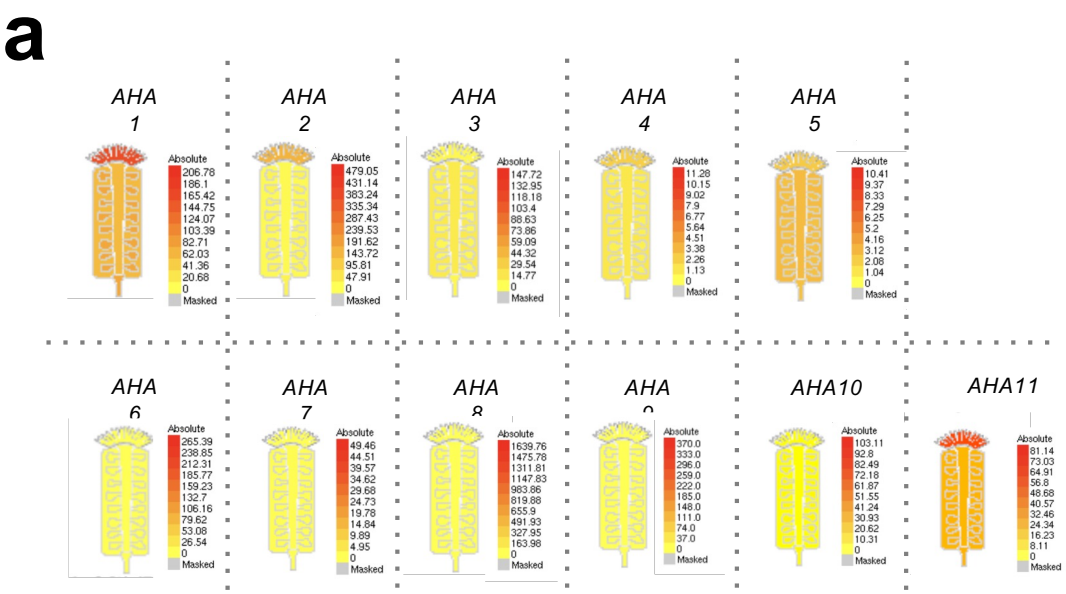
Pollen tube penetration into the pistil of a self-incompatible *B. rapa S^8* strain was essentially performed as described in Fig. 3. In Fig. 3, *S^9* strain of *B. rapa* was used as the female parent to understand the effect of stigma PM H$^+$-ATPase activity on pollen tube penetration. In this experiment, *S^8* strain was used as the female parent. White arrowheads indicate the positions of the tip of the pollen tubes that penetrated into the pistil. Scale bars indicate 200 μm. A stacked bar graph shows the number of pistils penetrated/not penetrated by the pollen tube (n=15). “Penetrated” and “Not penetrated” are indicated as in Figure 3b.

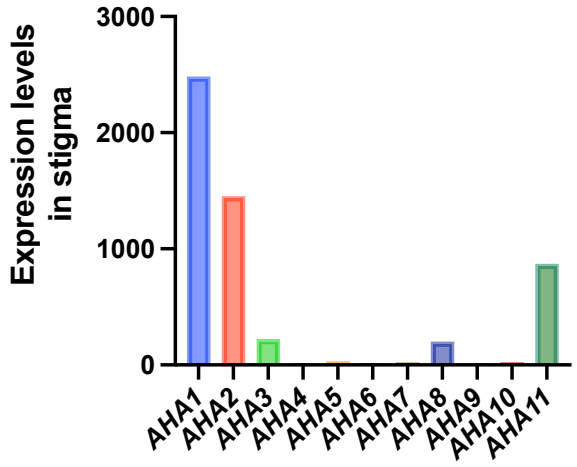
Supplementary Figure S4

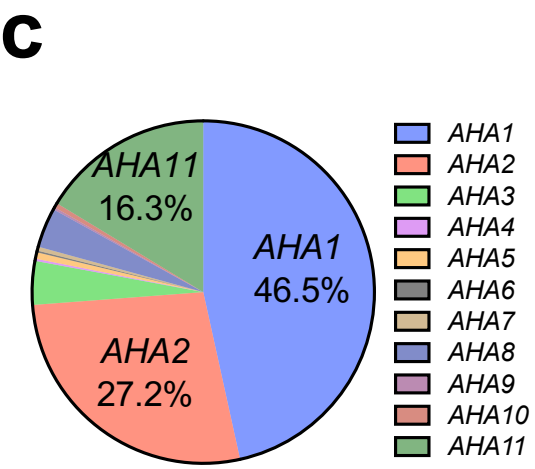


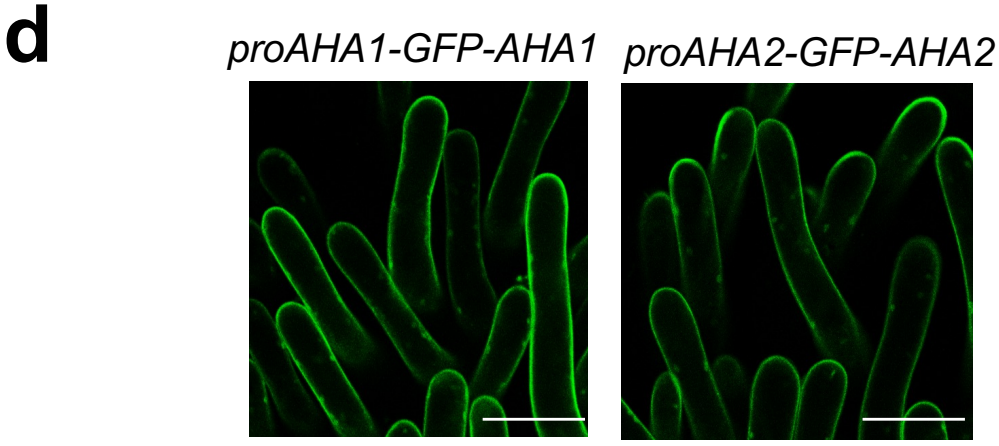
Supplementary Figure S4. Pollen tube penetration in FC-treated pistils *of A. thaliana*.
Stigmas of WT plants were treated with FC at 30 μM for 2 days. Pollen from the WT was pollinated onto the FC-treated stigmas. Pollen tubes elongated overnight, and then pistils were fixed for observation. White arrowheads indicate the positions of the tip of the pollen tubes. Scale bars indicate 200 μm. A stacked bar graph shows number of pistils penetrated/not penetrated by the pollen tube (n=6), as in Fig. 3 and Supplementary Figure S3.

Supplementary Figure S5



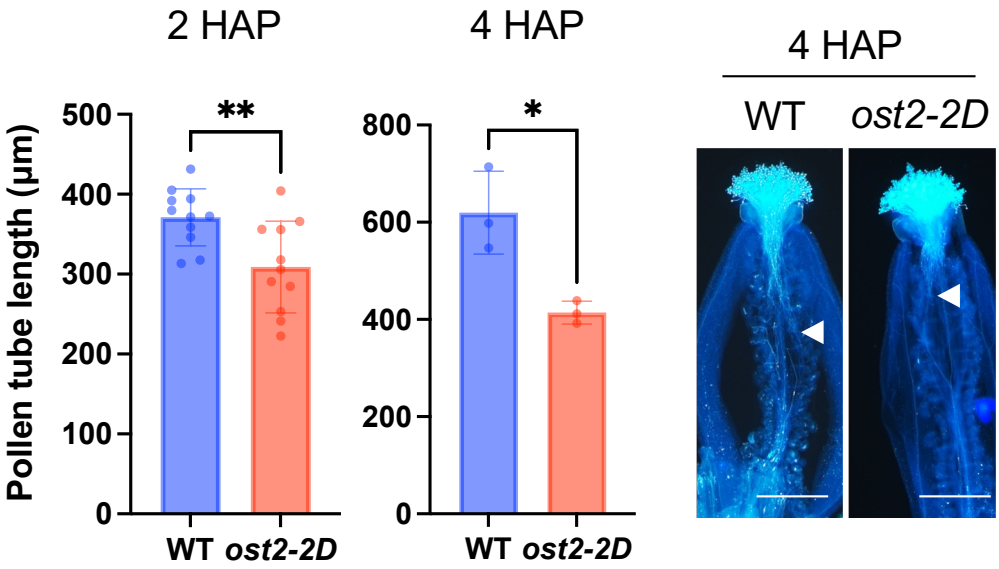






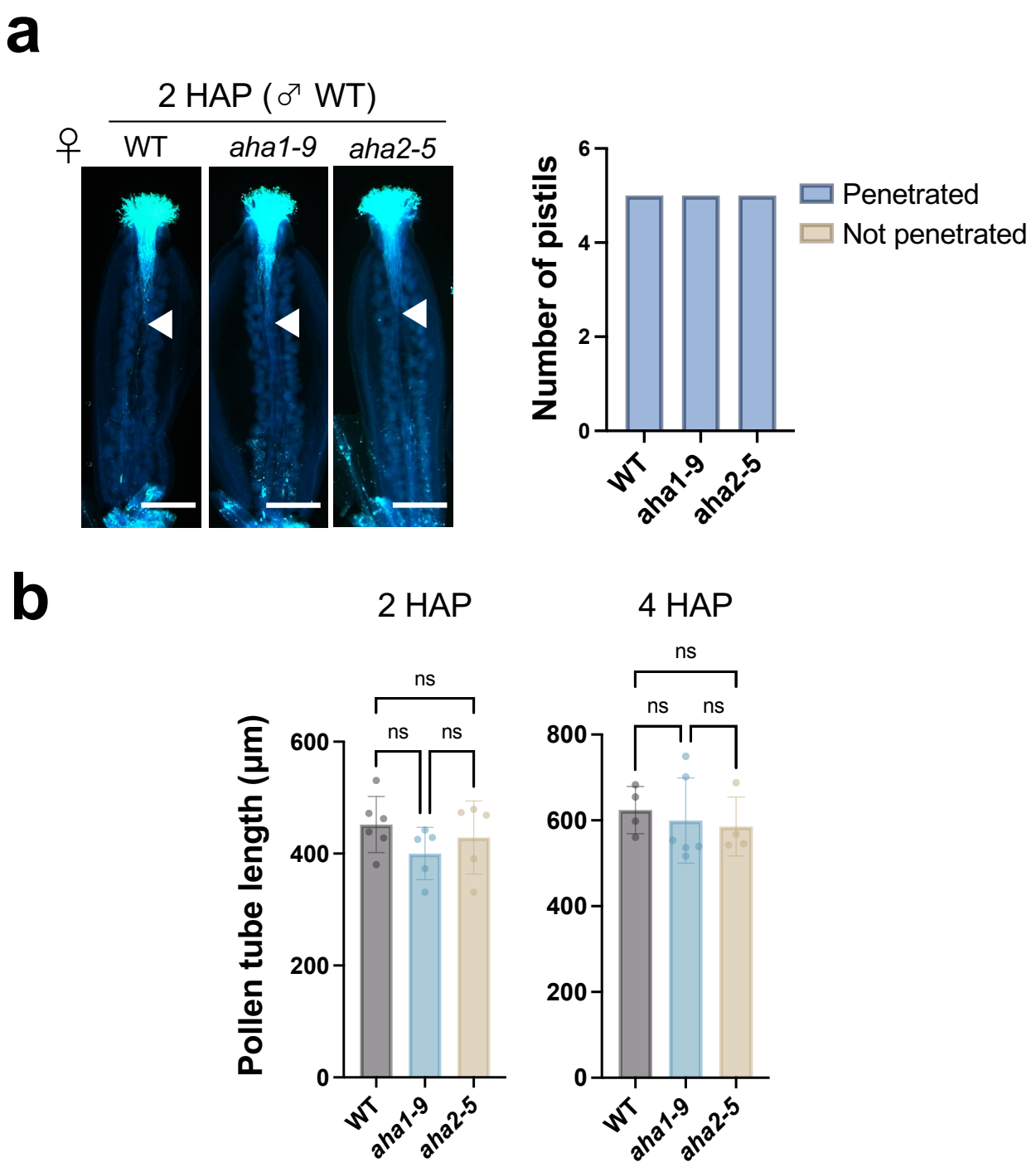
Supplementary Figure S5. Expression of *AtAHA* genes in *Arabidopsis thaliana* stigma.
(a) Expression pattern of each *AtAHA* in pistil tissue was obtained from the Klepikova atlas in the *Arabidopsis* eFP browser (https://bar.utoronto.ca/efp//cgi-bin/efpWeb.cgi?dataSource=Klepikova_Atlas).
(b) Expression levels of each *AtAHA* were obtained from the tissue-specific stigma data in the eFP browser (https://bar.utoronto.ca/efp//cgi-bin/efpWeb.cgi).
(c) Relative expression levels of *AtAHAs* in stigma, based on the values in (b).
(d) Subcellular localization of AtAHA1 and AtAHA2 proteins in papilla cells. Fluorescence images were obtained from papilla cells of 4-week-old transgenic *A. thaliana* plants expressing GFP-fused AtAHA1 and AtAHA2 under the control of their respective native promoters. Scale bars indicate 50 μm.

Supplementary Figure S6



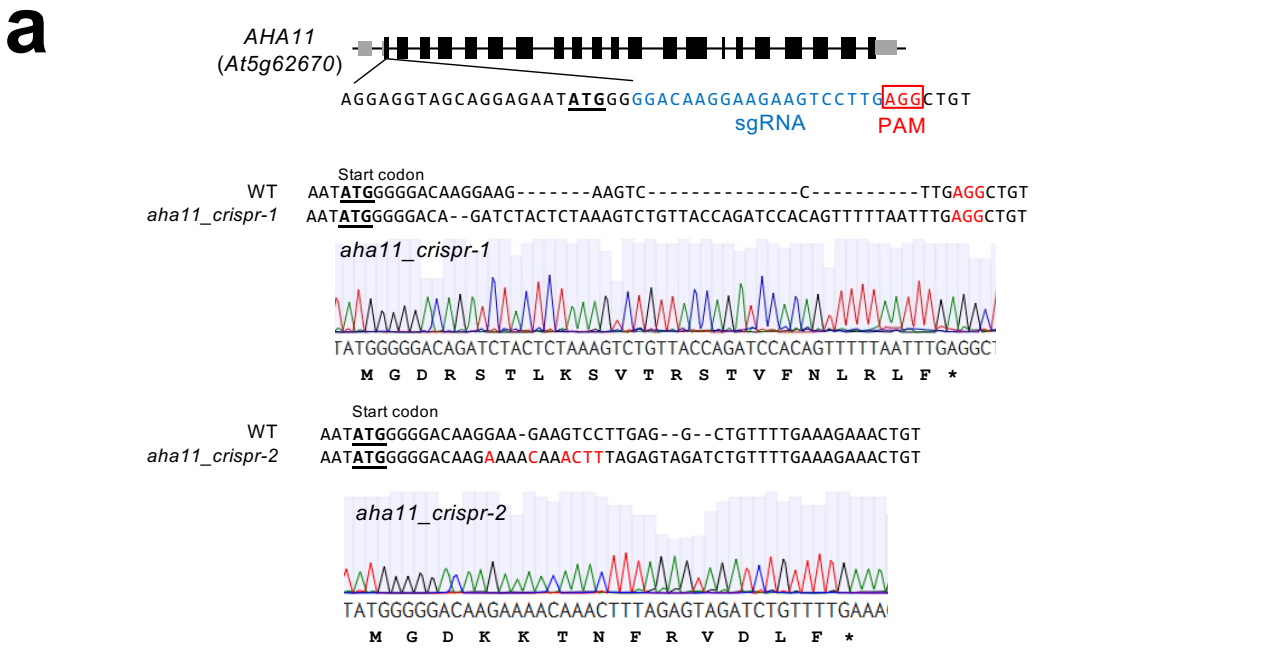
Supplementary Figure S6. **Elongation of WT pollen tubes in pistils of the *Arabidopsis ost2-2D* mutant.**
Pollen from the WT were pollinated onto the stigma of the WT and *ost2-2D*, and the tube length was observed using aniline blue staining 2 and 4 h after pollination (HAP). Graphs show the length of pollen tubes in the pistils 2 and 4 HAP. Data are mean ± SD (2 HAP n=11; 4 HAP n=3). Asterisks indicate statistically significant differences from the corresponding WT using Student's *t*-test (*$p < 0.05$, **$p < 0.01$). White arrowheads indicate the positions of the tip of the pollen tubes that penetrated the pistil. Scale bars indicate 200 µm.

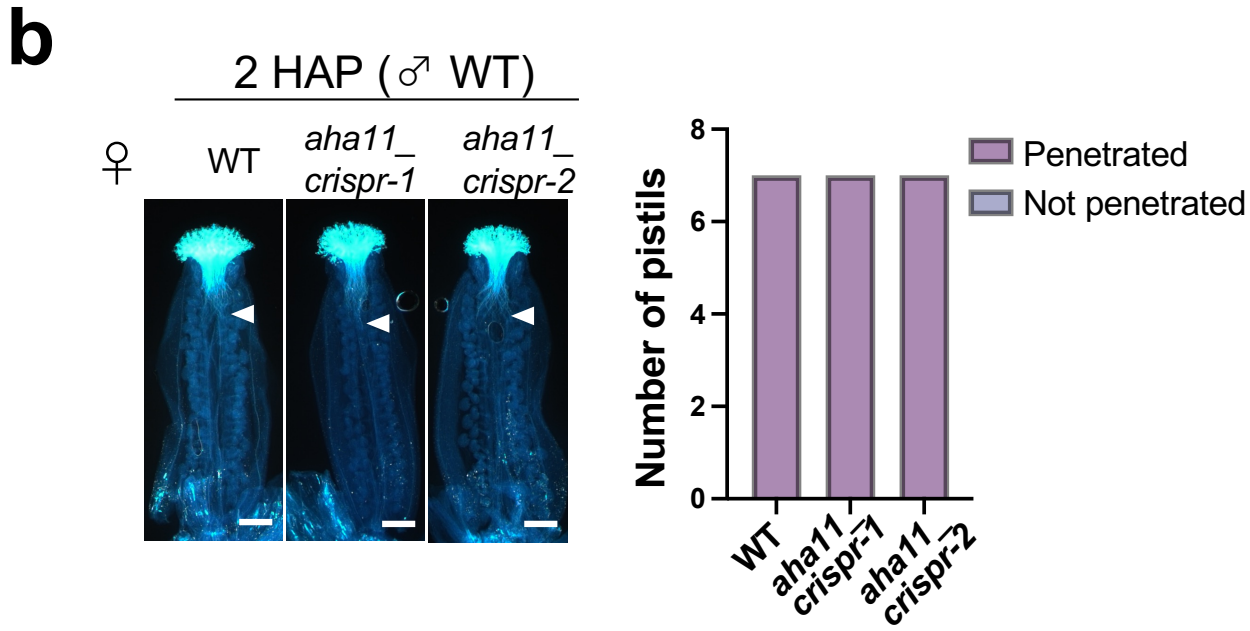
Supplementary Figure S7

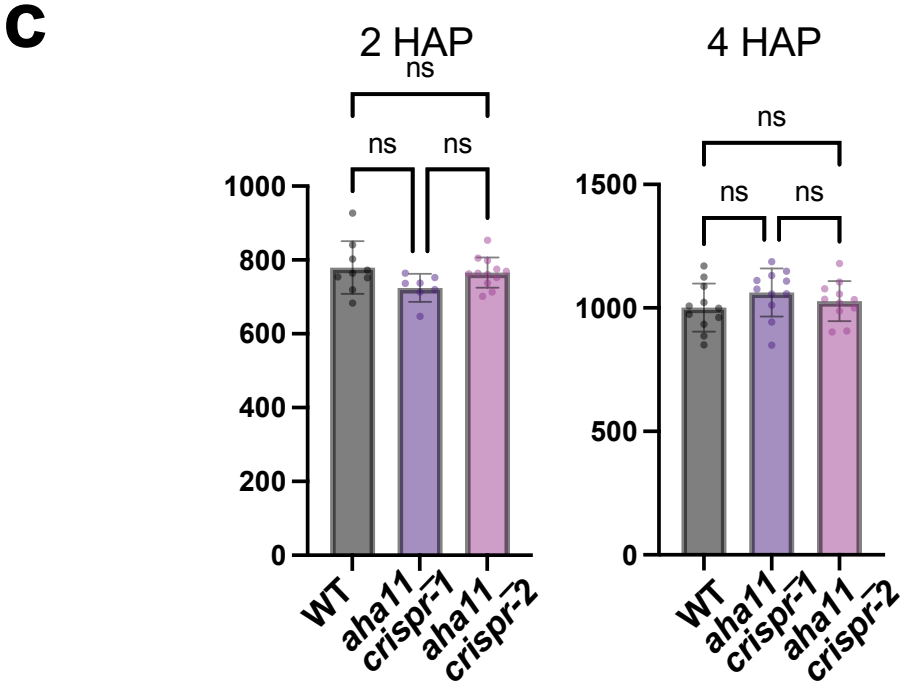


Supplementary Figure S7. Penetration and elongation of WT pollen tubes in pistils of *AtAHA* T-DNA insertion mutants.
(a) Pollen from the WT were pollinated onto the stigma of the WT, *aha1-9*, and *aha2-5*. Images show pollen tube penetration in the pistils. A stacked bar graph shows the number of pistils penetrated/not penetrated by the pollen tube (n=5). White arrowheads indicate the positions of the tip of the pollen tubes that penetrated the pistil. Scale bars indicate 200 μm.
(b) Pollen tube length was measured at 2 h and 4 h after the pollination (HAP), and shown as in Supplementary Figure S6. “ns” indicates no statistically significant difference using Bonferroni’s multiple comparisons test (n=3-6; ns, not significance).

Supplementary Figure S8







Supplementary Figure S8. Penetration and elongation of WT pollen tubes in pistils of *AtAHA11* genome editing mutants.
(a) Diagram of the AHA11 gene and the locations of the single guide RNA (sgRNA) and PAM sequences. The *aha11_crispr-1* and *aha11_crispr-2* mutants have different mutations generated by the same sgRNA. Both mutations induced to appearance of earlier stop codon.
(b) Pollen from the WT were pollinated onto the stigma of the WT, *aha11_crispr-1*, and *aha11_crispr-2.* Images show pollen tube penetration in the pistils. A stacked bar graph shows the number of pistils penetrated/not penetrated by the pollen tube (n=7). White arrowheads indicate the positions of the tip of the pollen tubes that penetrated the pistil. Scale bars indicate 200 μm.
(b) Pollen tube length was measured at 2 h and 4 h after the pollination (HAP), and shown as in Supplementary Figure S6. “ns” indicates no statistically significant difference using Bonferroni’s multiple comparisons test (n=7-12; ns, not significance).

Supplementary Table S1

Quantitative phosphoproteomic analysis of other phosphorylation sites in the C-terminal cytosolic region of BrAHAs. Data were averaged from three independent experiments, and the $\log_2$ fold changes and adjusted *p*-values of Cross vs Self were calculated using FragPipe-Analyst (http://fragpipe-analyst.nesvilab.org/). Sequences of the detected peptide fragments were shown. The phosphorylated amino acids are shown in red color.

Protein	Detected peptides	Phosphorylation site in BrAHA1a	Cross vs Self $\log_2$ fold change	Cross vs Self adjusted *p*-value
BrAHA1a	EAQWAQAQRpTLHGLQPK	Thr881	0.277	0.998
	pTLHGLQPK	Thr881	-0.26	0.958
BrAHA2c	EAQWAVAQRpTLHGLQPK	Thr881	-0.0822	1
BrAHA3a	LRELHpTLK	Thr924	0.478	0.99
BrAHA11	pTLHGLQAPDAK	Thr881	-0.488	1

Supplementary Table S2

Primers used in this study

Primer name		Sequence (5' to 3')
For BrAHA RT-qPCR		
Bra024452	F	TAGGCAGAAGGCACTCGCTA
	R	ACGAGCTACTGCCAATGACC
Bra038835	F	AGGCCTGACCTTCGGAAAAA
	R	CATGGTCCACCAGGGCTTTC
Bra010299	F	AAAAACACATGTTCTGTGCAG
	R	TTGGTCCAAAGATCTGGATCC
Bra010298	F	ATGAGTCTCGAGGATATCAAGAACG
	R	TTGGTCCAAAGATCTGGATCC
Bra011172	F	ATTGAGGCCATTGTCATCGC
	R	GGTTTGTGCTGTCCACAAGG
Bra024101	F	TTTGTCGGTGACAATGGCTATTGG
	R	ACATCCATCCCGGCCAATTCTTCG
Bra024100	F	TTCTTACTCCTTTCTTGTCGGAATCC
	R	CATCTCTCCCTTTCTTCACGTAAGAAA
Bra020447	F	ATAGTGACCGTTGTTTTCTTCTGG
	R	ACGTGTGACGAAGATAAGAGCTTGG
Bra002733	F	ATGGTGGGTGGTGGTGGTTCTCTTGAGG
	R	TTCTGCTCCAGATAGTCCATCTCTGC
Bra007845	F	TAGATTGTCTCTGCAGGGAGCTGTGACG
	R	AATAACTCCACTAAGTTCTTATCAACGG
Bra013168	F	TTGCTCGAAAACAAAGATGATGCCACC
	R	TATATGTTTCTTTTCTTGGAGTCTCC
Bra008288	F	ATCCACTTCACTCCTTGGAAATTC
	R	AATGTGCTTCCTCTCTTGAAGC
Bra003576	F	TTCTTACAGAGATGGTATAGACAACC
	R	AGAGACGGTGAGAACCAATGGCCATG
Bra031563	F	TCTTCTTGTGCTTCTCATCGGAGGG
	R	AGTGTAAGAGTCCCAGTCTTGTCGC
Bra014496	F	AAAGAGATTACAGTCCAGAAACC
	R	TAGCATCATCTACAGCTATTCC
Bra007524	F	ATGAAGCTAAGAGACGTGCAGAACTTGC
	R	TTGTTGTTAGCATCTTCAAGATCAAGG
Bra029249	F	TAACCTCCTTGTCCTTCTCATTGGTG
	R	TTCCTCAATAGCTGTCATCCTCTTGG
Bra016610	F	ATGGCTGAGGATTTAGACAAGCCATTGC
	R	TTCAGCATCTCCAGATAAAAGCCCTGTAGG
UBC21	F	CCTCTGCAGCCTCCTCAAGT
	R	CATATCTCCCCTGTCTTGAAATGC
For Subcellular localization in *N. benthamiana*		
pRI IPCR	F	GGATCCGAATTCTGAATCAACAACTC
pRI IPCR	R	GTCGACGGGCATATGTATCAACAGTG
GFP-Bra011172 IF	F	CATATGCCCGTCGACATGGTGAGCAAGGGCGAGGAG
GFP-Bra011172 IF	R	TCAGAATTCGGATCCTTAGACAGTGTAGTGTCCTGGAG
GFP-Bra038835 IF	F	CATATGCCCGTCGACATGGTGAGCAAGGGCGAGGAG
GFP-Bra038835 IF	R	TCAGAATTCGGATCCCTACACAGTGTAGTGATGCCCAGC
pC1302 AtPIP2a-mcherry IF F	F	TGACCATGGTAGATCATGGCAAAGGATGTGGAAGCCGTTCC
pC1302 AtPIP2a-mcherry IF R	R	ATTCGAGCTGGTCACCTACTTGTACAGCTCGTCCATGCC
For subcellular localization in *A. thaliana*		
gAHA1 F IF HindIII		GGCCAGTGCCAAGCTCTACTACACATACATGAGTC
gAHA1 R IF BamHI		GGTACCCGGGGATCCCCATATCTTTGGACGTG
GFP F IF 5UTR gAHA1		TTCTTCTGGGTGAAGATGGTGAGCAAGGGCGAGGAGC
GFP R IF gAHA1 START		TTCGAGACCTGACATTCCCTTGTACAGCTCGTCCATGCC
AHA1 5UTR IPCR R		CTTCACCCAGAAGAAATCAACAAAACG
AHA1 START IPCR F		ATGTCAGGTCTCGAAGATATCAAG
gANA2 F IF XbaI		GCTCTAGAAATCTTAGGTGGTGAAATTAGAATATTCGG
gANA2 R IF XbaI		GCTCTAGACTGCCTAAAATAAAACTCTTATGTTGTC
GFP F IF 5UTR gAHA2		AAGGAGTGGTGAGAGATGGTGAGCAAGGGCGAGGAGC
GFP R IF gAHA1 START		TTCGAGACTCGACATTCCCTTGTACAGCTCGTCCATGCC
AHA2 START IPCR F		ATGTCGAGTCTCGAAGATATCAAGAACG
AHA2 5UTR IPCR R		CTCTCACCACTCCTTCACTGAG
For generation of AHA11 mutant		
AHA11_sgRNA		GGACAAGGAAGAAGTCCTTG