Supporting information for:

Arabidopsis H^+-ATPase AHA1 controls slow wave potential duration and wound-response jasmonate pathway activation

Archana Kumari, Aurore Chételat, Chi Tam Nguyen and Edward E. Farmer*

* Correspondence should be addressed: edward.farmer@unil.ch

This PDF file includes

Materials and Methods

Figs. S1 to S9

References for SI citations

www.pnas.org/cgi/doi/10.1073/pnas.1907379116

SI Materials and Methods

Plant materials, growth conditions and chemicals

Arabidopsis thaliana Columbia (Col) was the wild type. All T-DNA mutants and transformants were in the Col background. Mutants used were: *aha1-7* (salk_065288), *aha1-6* (salk_016325), *ost2-2D* (42), *aha2-4* (salk_082786), *glr3.3* (salk_099757). Seeds were sown individually in 7 cm diameter pots and stratified at 4°C for 2 days then grown in an insect-free room at 21°C under 150 μE m^{-2} s^{-1} light (10 h light, 14 h dark, 70% humidity). Chemicals were purchased from Sigma (Buchs, Switzerland) unless otherwise stated. Fusicoccin was purchased from Santa Cruz Biotechnology, Inc. (Finnell, Dallas, SC-200754)

Transgenic lines

Generation of transcriptional reporter lines

AHA promoter-GUSPlus transcriptional fusions were made for visualization of promoter activity. The *AHA1* promoter (2500 bp) upstream of the translation initiation codon was amplified from wild-type genomic DNA with the following oligonucleotides; (forward: 5'-CGCGggtaccAGATTTTATTAAATAGAGTTGAAGC-3' and reverse: 5'-AGCCcccgggCTTCACCCAGAAGAAATCAACAAAAC-3'), where lower case letters indicate sequences added to create restriction enzyme sites. The amplified DNA was digested with *Kpn*I and *Xma*I restriction enzymes and cloned between the restriction sites of pUC57-L4-KpnI/XmaI-R1 (43) to create pUC57-*AHA1p*. A non-secretable GusPlus in pUL22 (pENTRY-L1-GUSPlus-L2) (44) was used to generate reporter lines. Promoter fragments in pUC57 and GUSPlus in pUL22 were combined in the destination vector pEDO097pFR7m24GW (45) using double Gateway cloning to create *AHA1p-GUSPlus*. This destination vector carries the fluorescence accumulating seed technology (FAST) cassette which was used to select T1, T2, and T3 lines with a MZ16FA fluorescence microscope (Leica, Wetzlar, Germany). Three independent lines were used in experiments.

Generation of translational fusion line

A full-length genomic sequence of *AHA1* was amplified from a transformation-competent bacterial artificial chromosome library the JAtY (46). Primer pairs with suitable Gateway recombination adaptors were used to amplify the genomic fragment; (forward: 5'-aaaaagcaggctATGTCAGGTCTCGAAGATATC-3' and reverse: 5'-agaaagctgggttCACAGTGTAGTGATGTCCTGCT-3'). Lower case letters indicate partial sequences of Gateway adaptors. The amplified gene product was introduced into

pDONR/ZEO-L1/L2. The gene was then recombined with its promotor along with Venus (pEN-L1-VENUS-L2 vector) in a triple Gateway destination vector pB7m34GW creating *AHA1p-AHA1-Venus.* Constructs were introduced into the *aha1-7* mutant background for complementation studies. Plants were transformed by *Agrobacterium tumefaciens*-mediated transformation (47). Transgenics were selected on half-strength MS medium (Duchefa Biochemie, Haarlem, Netherlands) with 20 μg/ml BASTA (PlantMedia, Dublin, OH). Two independent transgenic lines was used for the experiments.

Generation of *SUC2p-PP2C-D1*

The *SUC2* promoter (4685 bp) was amplified from WT genomic DNA with the indicated oligonucleotides, upstream of the start codon ATG of *SUC2* (At1g22710) (forward: 5'-cggggtaccctgctaaaactattccatttcaaaatg-3' and reverse 5'-ttccccccccgggatttgacaaaccaagaaagtaag-3'). The underlined sequences represent *XmaI* and *KpnI* sites respectively. The sequence was cloned after restriction with *XmaI* and *KpnI* into a modified pUC57 (43) to create the pEN-L4-promoter-R1 clone. For *PP2C-D1*, the cDNA was amplified using primer pairs with suitable Gateway adaptors; (forward: 5'-AAAAGCAGGCTGATGGTTAAAC CCTGTTGGAG AAT-3' and reverse: 5'-GAAAGCTGGGTT TCATGATGTTGAATGCATCGGGTA -3'). The amplified cDNA product was recombined into pDONR/ZEO-L1/L2. The *SUC2* promoter in pUC57 and *PP2C-D1* in pUL22 were combined in the destination vector pEDO097pFR7m24GW (45) using double Gateway cloning to create *SUC2p-PP2C-D1*. The construct was introduced into WT plants by *Agrobacterium tumefaciens* mediated transformation. This destination vector carries the fluorescence accumulating seed technology (FAST) cassette and transgenic lines were selected with a MZ16FA fluorescence microscope (Leica, Wetzlar, Germany)

Acidification assays

For root acidification assays, seeds were surface sterilized and grown vertically on one-half-strength solid Murashige and Skoog (MS) medium (0.5x MS, 2.15 g/L, pH 6.0; Duchefa, Haarlem, The Netherlands) supplemented with 0.5 g MES hydrate, 1% sucrose and 0.8% (w/v) agar. 14 day-old seedlings were moved to MS agar medium containing 0.003% (w/v) bromocresol purple (pH 6.5) and changes in the medium acidity (color) were visualized in the root regions (24) after 12-48 h of incubation in long-day conditions (14 h day, 10 h night at 22^0C). Equivalent assays were performed with midveins. In this case the primary vasculature was extracted from leaf-7 or 8 of 6 week-old plants (18) grown on soil. For vein extraction a

transverse incision was made with a razor blade on both sides of petiole approximately 4 mm from the rosette center, leaving midvein intact. The petiole sheath was then manually pulled off to expose the primary vein attached to the plant. The mid-vein was then cut off and placed onto MS agar medium containing 0.003% (w/v) bromocresol purple (pH 6.0). Plates were inspected after 24-36 h of incubation in long-day conditions.

GUS staining and sectioning

The rosettes of 4 week-old plants expressing *AHA1p-GUSPlus* were collected and immediately prefixed in 90% acetone for 20-30 min, washed twice with 50 mM sodium phosphate buffer (pH 7.0), then placed in staining solution (10 mM Na_2EDTA, 50 mM sodium phosphate buffer, 1 mM $K_4Fe(CN)_6$, 1 mM $K_3Fe(CN)_6$, 0.1% (v/v) Triton X-100, 0.5 mg/ml^{-1} X-Gluc (pH 7.0). The staining solution was vacuum-infiltrated into rosettes for 30 min and rosettes were then incubated at 37°C in the dark for 5 h, then cleared with 70% (v/v) ethanol. Images of plants were taken with a VHX-500F digital microscope (Keyence, Mechelen, Belgium). For detailed expression analysis leaf 6 of GUS stained rosettes were further fixed in glutaraldehyde/ formaldehyde/50 mM sodium phosphate (pH 7.2) 2:5:43 (v/v/v) for 30 mins. Sections were made as described in Nguyen and Kurenda *et al.,* (13) and mounted on 20% (v/v) glycerol and photographed with a Leica DM5500 microscope.

Surface potential measurements

For electrophysiology, 5 to 6 week-old plants were used. Surface potentials were recorded with silver/silver chloride electrodes placed on petiole of leaf-8 and leaf-13 and approximately 5 mm away from the rosette center (12). Electrode-leaf connections were made with 10 µl of 10 mM KCl in 0.5% (w/v) agar. A reference electrode was placed in the soil 2-3 cm from the center of the rosette. 50-60% of the apical lamina surface was crushed with plastic forceps. Surface potential recordings were acquired at 100 Hz using LabScribe3 (iWorx Systems, Inc., Dover, NH) software and measurements were analyzed for depolarization amplitude and repolarization duration as described previously (12).

Vein exposure for chemical treatment and surface potential measurements

The primary veins of 5.5 week-old expanded leaves were partially exposed. For this, two shallow transverse incisions were made (approx. 2 mm apart) on the adaxial side of the leaf 13 petiole leaving the midvein intact. Vertical longitudinal incisions were then made on the petiole margins to join the two transversal incisions. Then extravascular tissues were removed gently

from the incised part with forceps leaving the midvein exposed on the adaxial side of the petiole while maintaining vascular tissue continuity while providing support to the exposed vein and preventing it from drying. Plants were kept at saturating humidity for 2-3 h for tissue recovery. For surface potential measurements, silver/silver chloride electrodes were placed on the leaf 8 petiole (approx. 4-6 mm from rosette center) and on the leaf-13, between the exposed vein region and lamina-petiole junction. Chemicals were applied to the exposed vein of leaf 13.

Electrical penetration graph (EPG) recordings

Electrical penetration graphs (EPGs) were used for recording potential changes from sieve elements (SEs) as described previously (31). Experiments were set up in a Faraday cage. To prepare aphid (*Brevicoryne brassicae L.*) electrodes, gold wires (Ø18µm) were carefully attached to the backs of aphids using water-based silver glue (EPG Systems, Wageningen, Netherlands). Aphids were restricted close to the leaf-petiole junction of leaf 13 using a brush. Probing (stylet penetration activity) by aphids was recorded with a Giga-4 EPG amplifier (four-channel DC system; EPG Systems). A reference electrode was placed in the soil. Soon after leaf contact aphids inserted their stylets into the leaf surface, thus closing the EPG circuit. Signals were acquired at 100 Hz using Stylet C software (EPG Systems). Following a several-hour-long probing phase, aphids entered into the phloem phase, characterized by an abrupt voltage drop (~100mV) due to the penetration of the stylet into a SE. In most EPG recordings, the phloem phase was stable and lasted for several tens of minutes. During the aphid ingestion phase (31), 60-70% of the apical lamina of leaf 8 was crushed with plastic forceps and wound signals were recorded from SEs of leaf 13. In order to infer change in voltage from the recordings, a calibration pulse (50 mV) was applied during each ingestion phase after wound signals. On several occasions, the same aphid electrode was used for recordings from a second plant. Measurements were analyzed for depolarization amplitude and repolarization duration as described previously (31). The maximum amplitude was measured from the baseline (*SI Appendix, Fig. S6*). The signal duration was defined as the total time required to depolarize and repolarize to the basal level. See Fig. 4 in (31) for further examples of the full sieve element electrical signal including the fast and slow phases. Only the fast spike signal is shown in Fig. 3*C* of the main text.

Gene expression

For gene expression analysis leaf 8 was wounded with forceps. After 1 h, leaf 8 (wounded) and leaf 13 (distal) were harvested and snap frozen in liquid-N_2. Complementary DNA was

prepared from total RNA, and qPCR was performed as described in Gfeller et al. (48). q-PCR data were standardized to the *ubiquitin-conjugating enzyme21* (*UBC21*) reference gene. Primers for *UBC21* (At5g25760) and *JAZ10* (At5g13220) have been described previously in Gfeller et al. (48). To quantify *AHA1* transcripts in three different regions of *aha1-7*, the following primers were used: *AHA1_32* (forward: 5'-CCGTTGATCTGGAAAAAATTCCG-3' and reverse: 5'-CTTGTTGGGGCCAAATATCAC-3'), *AHA1_3012* (forward: 5'-CGTCAGGTGGTACCCGAGAAAAC-3' and reverse: 5'-TGGTTTCGGCACTGTCGTGTCT-3'), *AHA1_4683* (forward: 5'-CAACTGGTTGCAACTTTGATCG-3' and reverse: 5'-agaaagctgggttGATATACCGAATGGCAAACTTC-3')

Cytosolic calcium transients

The intensometric GCaMP3 sensor (49) was used to detect cytosolic Ca^{2+} transients following wounding. For this, the *aha1-7* mutant was crossed with WT plants expressing GCaMP3 under the control of the *UBQ10* promoter (13). GCaMP3 fluorescence was monitored in plants that were homozygous for the *aha1-7* mutation and for the *UBQ10pro::GCaMP3* transgene. Regions of interest (10 x 10 pixels, corresponding to 0.68 mm x 0.68 mm) at the petiole/leaf junction of leaf 13 were monitored after wounding leaf 8. Data analysis was as in (13).

Serial wounding

The first wound was inflicted on leaf 1 of 2 week-old soil-grown plants. For each wound, 50% of leaf lamina was crushed with forceps. Plants were then rested for three days before the next wound which was applied to leaf 2. In this way, a series of wounds to consecutive leaves was inflicted and stopped after leaf 7 was wounded. Following three more days the fresh weight were measured.

Insect-bioassays

Single plants were grown in individual pots. Eleven pots of 6 week-old plants of each genotype were placed in plexiglass boxes (28.5 x 19 x 19 cm). Four newly hatched *Spodoptera littoralis* larvae were placed on each plant so that each box contained 44 caterpillars. The caterpillars were collected and weighed from individual boxes after 12 days of feeding. Each box was considered as one biological replicate and two replicates were used for the data shown in Fig. 4E.

JA quantification

Measurement of JA, JA-Ile and OPDA was performed by UHPLC-MS/MS as described in Glauser *et al.,* (50). Leaf 8 of 5.5 week-old plants was wounded with forceps and after 45 mins leaf 13 was harvested and snap-frozen in liquid-N_2. As a control, unwounded leaf 13 was analyzed. Frozen tissues were grounded and weighed prior to extraction. The internal standards used were as in Glauser *et al.* (50). For JA, JA-d5: for JA-Ile, JA-Ile-$^{13}C_6$. OPDA quantification used the non-cognate standard JA-Ile-$^{13}C_6$.

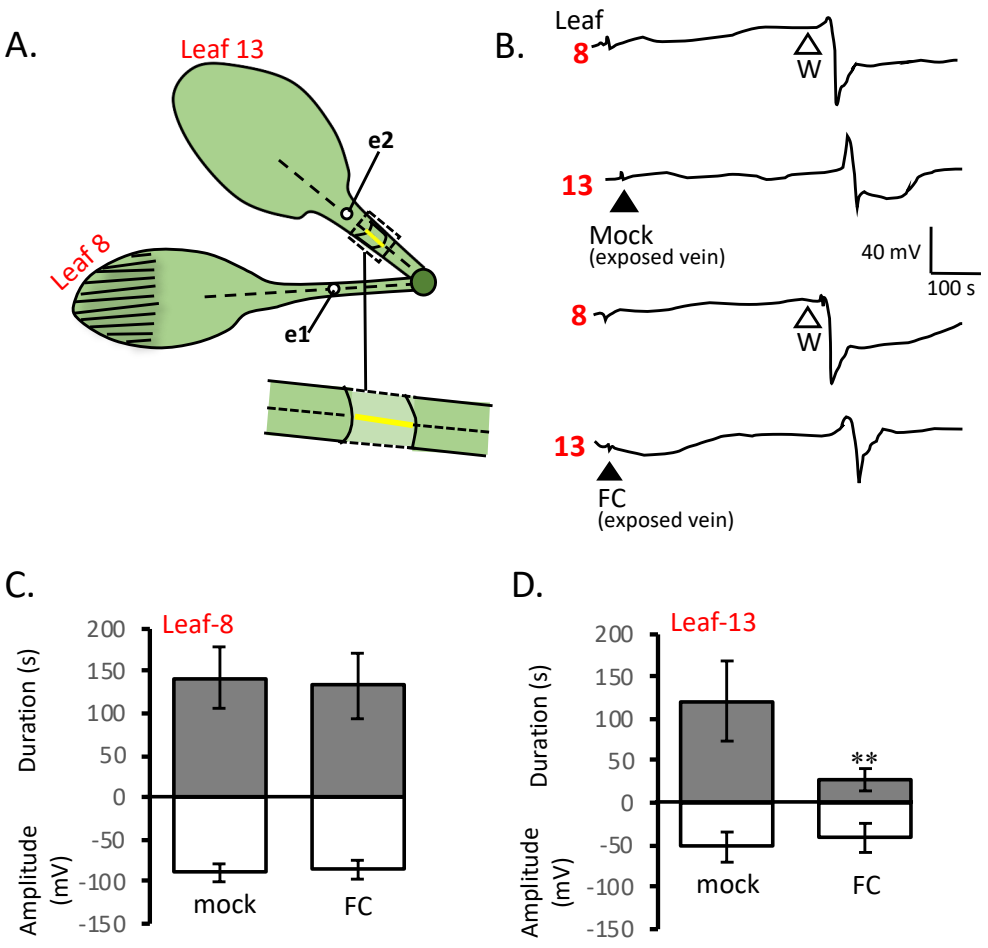

Fig. S1. Effect of modulating H^+-ATPase activity on wound-associated surface potential measurements in 5 week-old wild-type rosettes. (A) Experimental design for surface potential measurement after fusicoccin (FC) treatment (25µM). (B) Surface potential changes recorded on leaf 8 and leaf 13. Solid arrowheads indicate when the mock or FC was applied to the leaf 13 and unfilled arrowheads indicate when leaf 8 was wounded. (C) Surface potential changes on leaf 8 and (D) Surface potential changes on distal leaf 13. Data shown are means +/- SD (n=8). Asterisks refer to data significantly different from wounded WT. Student t-test: * $P < 0.05$, ** $P < 0.001$.

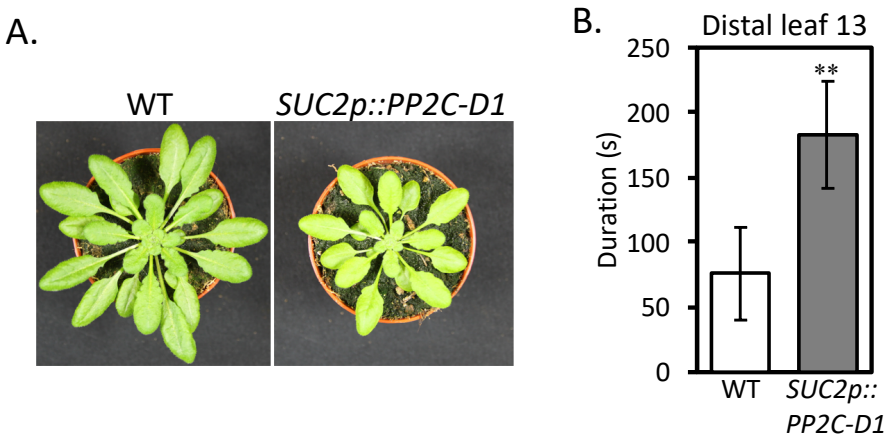

Fig. S2. Effect of expressing protein phosphatase PP2C-D1 on wound-activated surface potential measurements. (A) 5.5 week-old wild type and *SUC2p::PP2C-D1* rosettes. (B) Surface potential changes on leaf 13 in response to wounding leaf 8. Data shown are means +/- SD (n=8-10). Asterisks indicate significant difference with WT. Student t-test: ** $P < 0.01$.

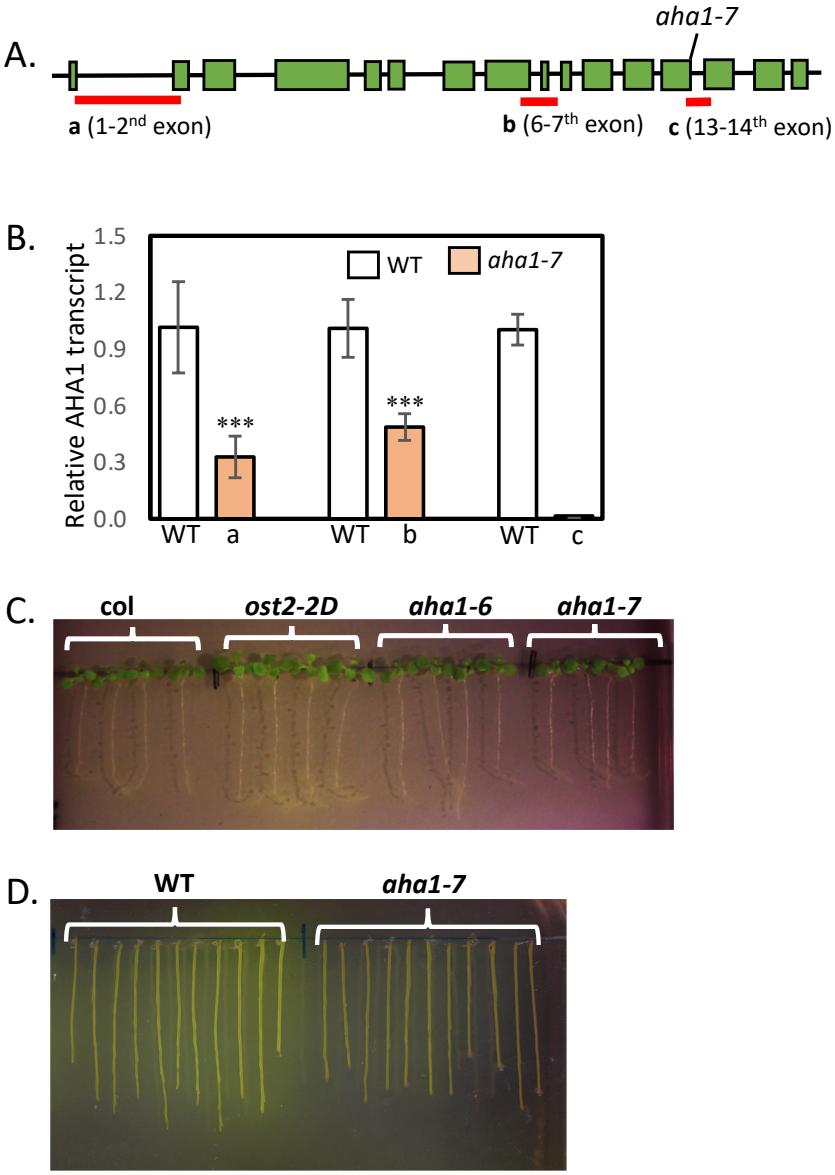

Fig. S3. *AHA1* expression and medium acidification assays in the WT and *AHA1* mutants. (A) Gene model for *AHA1* indicating primer regions a, b and c. (B) *AHA1* transcript levels in unwounded leaves using primer pairs a, b and c. Data shown are means +/- SD. Asterisks indicate significant difference with WT. Student t-test: *** $P < 0.001$. Medium acidification assay with (C) Roots and (D) leaf primary veins. 14 days old seedlings or veins collected from leaves 7/8/9 of 6.5 week old plants were transferred to the plates containing the pH indicator dye bromocresol purple. Color changes were recorded after 24-36 h.

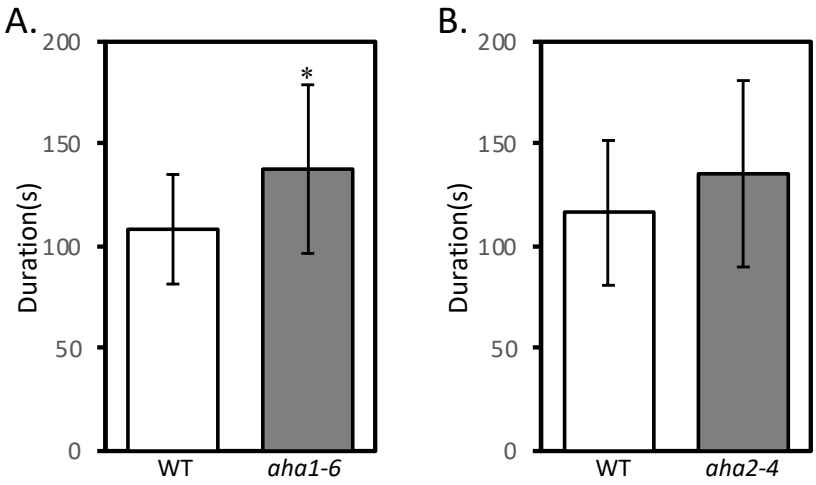

Fig. S4. Wound-activated surface potential changes in *aha* mutants.
Leaf 8 was wounded and leaf 13 was monitored. (A) *aha1-6* and (B) *aha2-4* (n=12). Data shown are means +/- SD. Student t-test: * $P < 0.05$.

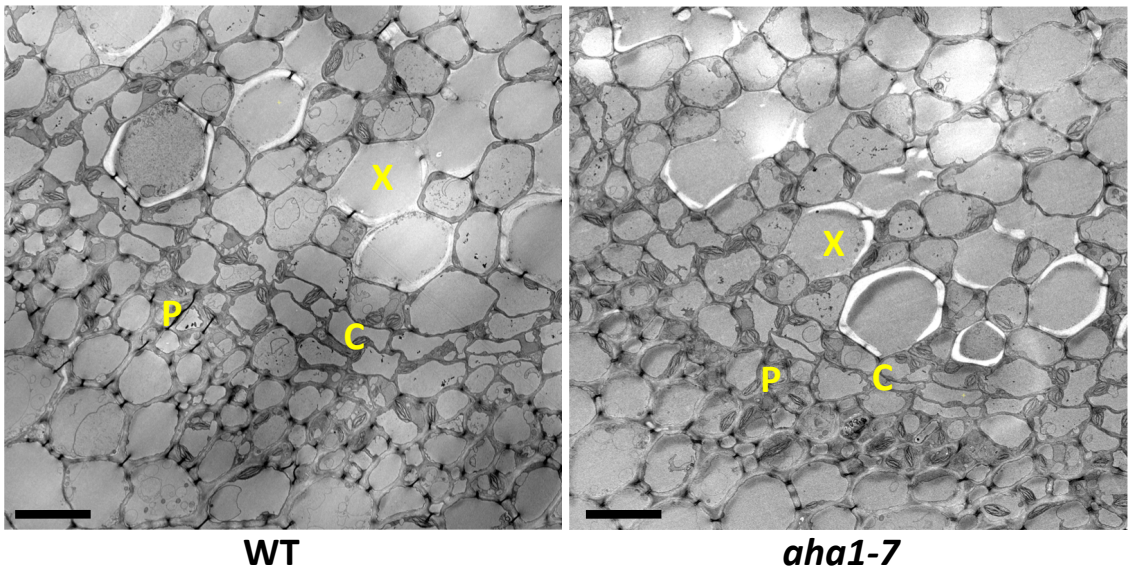

Fig. S5. Transmission electron microscope images of transversal sections of wild-type (WT) and *aha1-7* petioles from leaf 8 of 5.5 week old plants. Scale bars= 10µm. x, xylem; c, cambium and p, phloem regions.

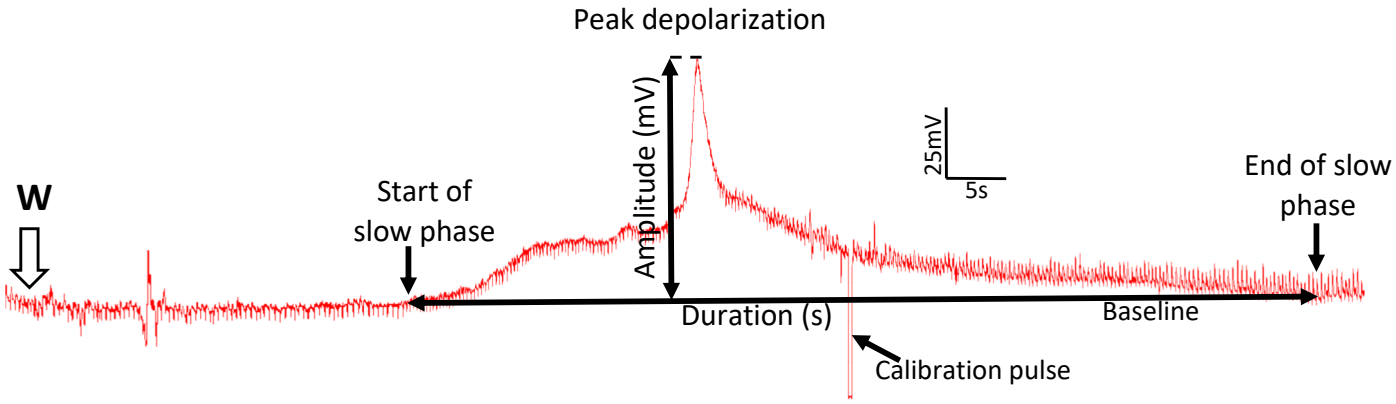

Fig. S6. Quantitation of sieve element electrical signals using living aphid electrodes. A single aphid (*Brevicoryne brassicae*) electrode was placed on leaf 13 of a 5 week-old plant. The circuit was completed with a reference electrode in the soil as detailed in (31). W indicates the moment of wounding leaf 8. The trace was recorded from leaf 13 and two parameters were quantified: duration of the slow depolarization phase and amplitude from baseline of the rapid depolarization spike. The calibration pulse was 50 mV.

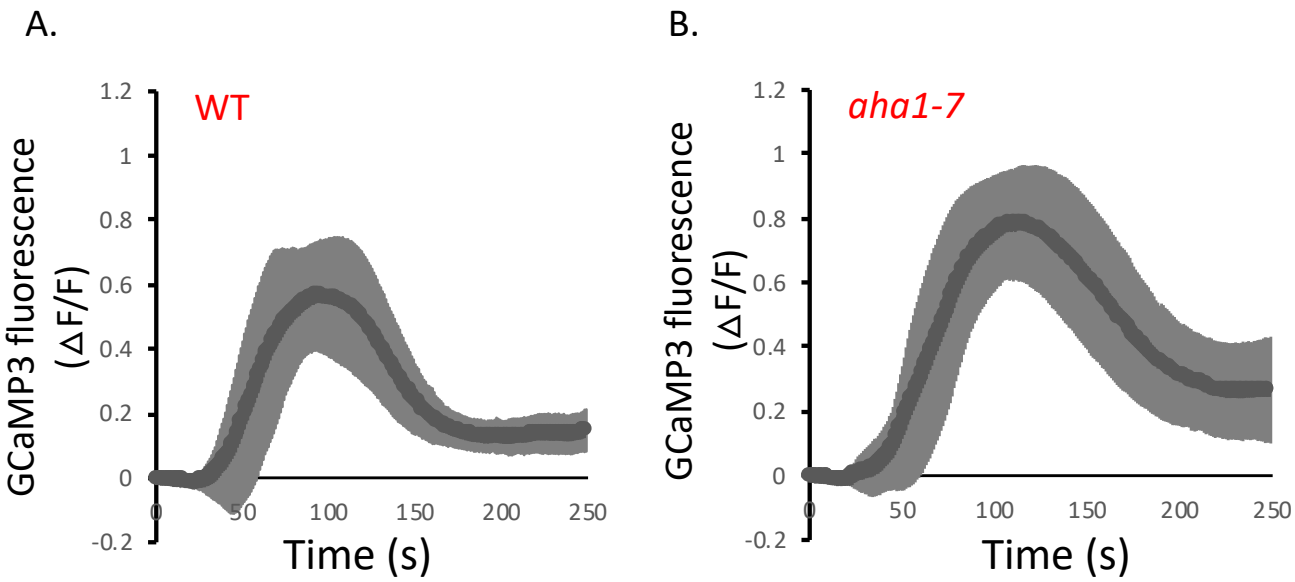

Fig. S7. Wound-activated cytosolic Ca^{2+} changes detected with the genetically encoded sensor GCaMP3 (49). Leaf 8 of the WT or of *aha1-7*, both expressing *UBQ10p::GCaMP3*, was wounded. Regions of interest at the centers of leaf 13 petiole/leaf junctions were monitored. (A) WT, (B) *aha1-7*. The data are from 11 replicates and the envelope shows standard deviations.

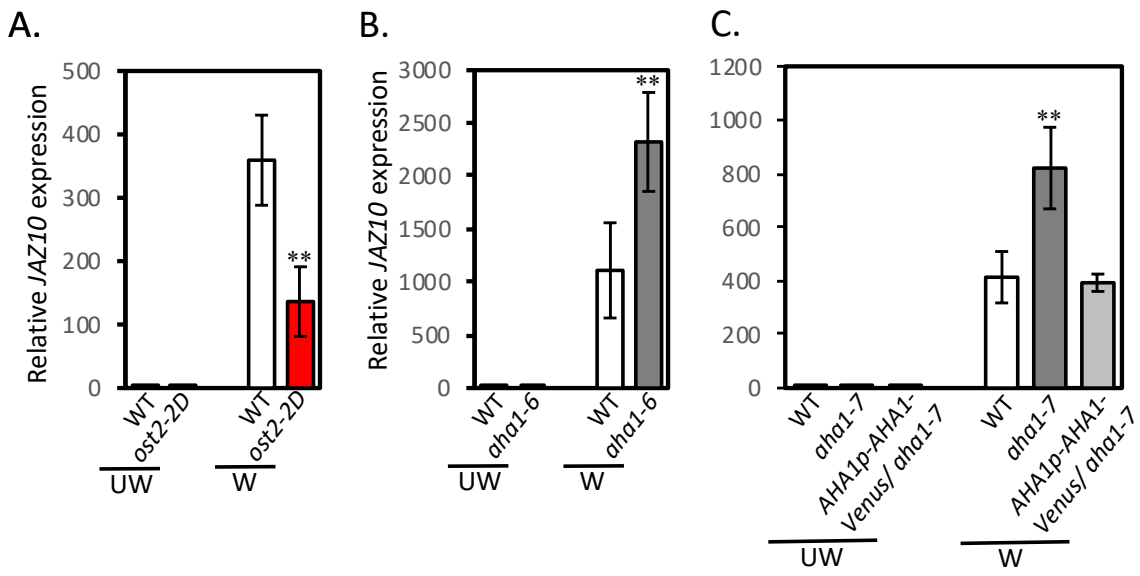

Fig. S8. *JAZ10* transcript levels following wounding.
JAZ10 transcripts in distal leaf 13 one h after wounding leaf 8 of (A) *ost2-2D*, (B) *aha1-6* and (C) complemented *aha1-7* (*AHA1p-AHA1-Venus/ aha1-7*). Data shown are means +/- SD (n=4). Asterisks indicate significant difference with WT. Student t-test: ** $P < 0.01$. UW = unwounded; W = wounded.

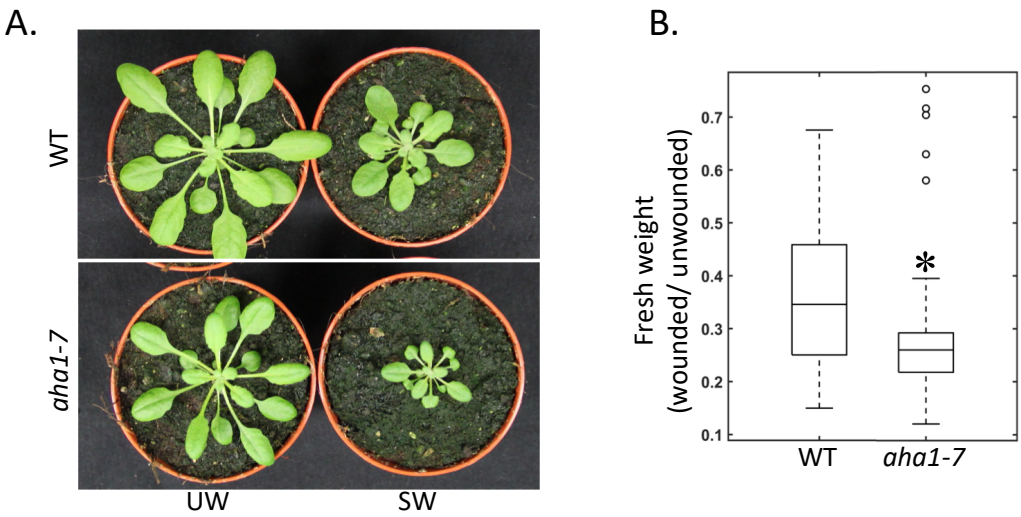

Fig. S9. Enhanced growth inhibition in serially wounded *aha1-7* plants. (A) Rosette morphology of 5 week-old unwounded (UW) and serial-wounded (SW) wild-type (WT) and *aha1-7* plants. (B) Fresh weight ratio of plants before and after serial wounding. For analysis, a two-sided Mann-Whitney test was used to compare weight ratios of SW/UW plants (36 ratios per genotype derived from 6 UW and 6 SW plants). Data shown are means +/- SD. *P = 0.0254.

Supporting References:

42. S. Merlot *et al*., Constitutive activation of a plasma membrane H^+-ATPase prevents abscisic acid-mediated stomatal closure. *EMBO J* **26**: 3216–3226 (2007).
43. A. Chauvin, D. Caldelari, J.-L. Wolfender, E. E. Farmer, Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded Arabidopsis thaliana leaves: A role for lipoxygenase 6 in responses to long-distance wound signals. *New Phytol*., **197**, 566–575 (2013).
44. D. Gasperini *et al*., Axial and radial oxylipin transport. *Plant Physiol*. **169**, 2244-2254 (2015).
45. T. L. Shimada, T. Shimada, I. Hara-Nishimura, A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of Arabidopsis thaliana. *Plant J.* **61**, 519-528 (2010).
46. R. Zhou, L. M. Benavente, A. N. Stepanova, J. M. Alonso, A recombineering- based gene tagging system for *Arabidopsis*. *Plant J.* **66**, 712-723 (2011).
47. S. J. Clough, A. F. Bent, Floral dip: a simplified method for *Agrobacterium*- mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743 (1998).
48. A. Gfeller *et al*., Jasmonate controls polypeptide patterning in undamaged tissue in wounded Arabidopsis leaves. *Plant Physiol*. **156**, 1797–1807 (2011).
49. Tian L. et al. (2009) Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat Methods 6*: 875-881.
50. G. Glauser, A. Vallat, D. Balmer, Arabidopsis Protocols, Methods in Molecular Biology, vol. 1062, DOI 10.1007/978-1-62703-580-4_31.