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Supplementary Information for

# Insect herbivory antagonizes leaf cooling responses to elevated temperature in tomato

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**This PDF file includes:**

Supplementary text

References for SI reference citations

Fig. S1 to S10

Table S1

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## Supporting Information Appendix

### Supplemental Materials and Methods

**Plant materials and growth conditions.** Tomato seeds cv. Castlemart (except where otherwise specified) were germinated at room temperature (22° C) on water-saturated filter paper in the dark for 5 d before transfer to peat pellets (Jiffy-7). Plants were watered daily with 70 ppm nitrogen fertilizer solution. Plants were grown in an environmentally controlled growth chamber maintained at 28° C / 16-h light period and 18° C / 8-h dark period, which represents the control temperature (CT) condition. Temperature-shift treatments were performed within 4-6 hours after dawn. Plants grown for 17 d under CT conditions were split into two groups, which were then transferred to treatment chambers maintained either at CT or the elevated temperature (ET) condition (38° C/16-h light period and 28° C/8-h dark period). Upon transfer, plants were maintained under CT or ET for five days unless otherwise noted (e.g., GDA treatment experiments and Fig. S5). Wounding was performed by pinching the apical 20% of each mature leaflet with a hemostat. Homozygous *jai1-1* plants were selected from a segregating $F_2$ population according to Li *et al.* (1). *jai1-1* was originally isolated in the MicroTom genetic background but was backcrossed into cv. Castlemart as previously described (1). Thus, cv. Castlemart was used as the WT parent for all experiments involving *jai1-1*. *35S::COI1-Myc* transgenic plants expressing an epitope (c-Myc)-tagged COI1 in the MicroTom *jai1-1* genetic background have been described (1, 2). Accordingly, cv. MicroTom was used as the wild-type parent for all experiments involving *35S::COI1-Myc*.

**Growth measurements.** Plant fresh weight (FW) was measured by weighing the total above-ground tissue. For treatments involving insect challenge, we determined experimentally that the FW of tissue consumed by insect feeding was less than the average standard error of shoot biomass. Therefore, the tissue removed by herbivory during the course of the experiment had only a minor effect on plant FW. Petiole angles of leaf 2 (the second oldest true leaf) were determined using ImageJ analysis of whole-plant digital images taken from the side of each plant (e.g., Fig. 2*A*). Petiole angles were expressed relative to the main stem, such that a petiole perpendicular to the main stem was defined as 90°.

**mRNA and proteinase inhibitor quantification.** Seventeen-d-old plants grown and treated under CT or ET conditions as described above were used to quantify wound-induced transcript levels.

Mechanical leaf wounding was performed with a hemostat. Three leaflets per leaf were wounded across the midvein of the leaf apex. Damaged and control (unwounded) leaves were collected at various times after wounding and immediately flash-frozen in liquid nitrogen. RNA was extracted with RNeasy Plant Mini Kit (Qiagen), and cDNA was synthesized using superscript III (ThermoFisher Scientific 2017). *TD2* and *ARG2* transcripts were quantified using real-time quantitative PCR (RT-qPCR) with SYBR Green reagents and primers specified in Supplemental Table 1. Marker gene expression levels were normalized to transcripts for *ACTIN2* using the Δ ΔCt method (3). Wound-inducible proteinase inhibitor II (Inh-II) levels were determined using a radial immunodiffusion assay (4).

**Hormone quantification.** Plants were mechanically wounded across each leaflet as described above for analysis of wound-induced gene expression. Tissue was collected at the indicated times after wounding and immediately flash-frozen in liquid nitrogen. JAs were extracted as previously described (5), with minor modifications. Briefly, frozen tissue was ground in a ball mill and extracted in 80:20 v/v methanol:water containing 0.1% formic acid. $^{13}C_6$-JA-Ile and dihydro-JA were used as internal standards for JA-Ile and JA, respectively. Measurements were performed using a Quattro premier LC-MS-MS (Waters) as previously described (6).

**Insect feeding assays.** Tobacco hornworm (*Manduca sexta*) eggs obtained from Carolina Biological Supply were hatched at 28° C. Neonate larvae were transferred (two larvae per leaf) to leaves of 17-d-old plants using a small paintbrush. Infested plants were either kept under CT conditions or transferred to ET test chamber. Larval weights and consumed leaf area were measured intermittently over the course of the feeding assay using an analytical balance and digital images, respectively. For experiments involving thermal imaging, gas exchange measurements, and petiole angle measurements, two first-instar *M. sexta* larvae were placed on each leaf and were replaced every two days to prevent excessive defoliation.

**Geldanamycin treatment**. Geldanamycin (GDA; Cayman Chemical Company) was vacuum infiltrated into leaves. Approximately 3 h after the beginning of the 16-h photoperiod, 17-d-old plants grown under CT conditions were inverted into beakers filled with 1 L mock solution or a solution containing 0.5 $\mu$M GDA in deionized water with 0.01% Silwet-77. Each plant was infiltrated twice by applying a vacuum for approximately 2 min followed by 10-20 sec re-pressurization until the plants appeared to be water soaked. Plants were allowed to dry on the

benchtop at ambient temperature (22° C) for ~1.5 h or until the leaves showed no signs of water soaking. Infiltrated plants were transferred to either CT or ET test chambers and then allowed to recover overnight prior to mechanical wound or herbivore treatments. Thermal imaging and gas exchange measurements were performed at the indicated times. For Western blot experiments, control samples were collected at ambient temperature. Following transfer of plants to test chambers, tissue was collected and frozen in liquid nitrogen at various times thereafter transfer. Root growth inhibition assays were performed by germinating seeds on filter paper, then saturating with solutions containing GDA, MeJA, or both compounds simultaneously.

**Gas exchange measurements.** Gas exchange measurements were performed with three-week-old WT and *jai1-1* plants (cv. Castlemart) grown with a 16-h light (28° C)/8-h dark (18° C) photoperiod and 400 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD). Measurements were obtained from single mature leaflets (attached) with a LI-6800 system (LI-COR Biosciences, Lincoln, NE, USA) outfitted with a standard leaf chamber (chamber area = 6 $cm^2$). Leaves were acclimated for 40 min or until conductance appeared to be constant, whichever was longer (see *SI Appendix* Fig. S10), at 400 ppm $CO_2$ with 500 μmol $m^{-2}$ $s^{-1}$ PPFD and 1.0 kPa vapor pressure deficit (VPD) and 28 °C leaf temperature as the control temperature or 2.7 kPa VPD and 38° C as the elevated temperature condition. Assimilation rates and stomatal conductance were averaged from three consecutive measurements taken at 30-s intervals and were normalized to projected leaf area as measured by image analysis with ImageJ software.

**Stomatal aperture measurements.** Stomatal aperture size was measured from epidermal impressions (7). Mature leaves were excised and painted with a thin layer of clear nail polish (Kiss Colors) on the lower epidermis, which dried for approximately 5 min, and then peeled away using clear tape. Pictures of the stomata were taken at 400X magnification using a light microscope (AmScope). Stomatal aperture was reported as the stomata width divided by length, using ImageJ software.

**Thermal imaging.** Thermal images were obtained using a FLIR C3 pocket thermal camera (FLIR Systems) having a thermal range of -10° C to 150° C, and thermal sensitivity of < 0.10 °C. Images were acquired approximately 1 m from the sample, using the emissivity value (ε) of 0.98 (8). Average leaf temperatures were determined using FLIRTools software (FLIR). Care was taken to include all genotypes and treatments in a single image.

**Western blot analysis.** Frozen leaves of 3-week-old *35S::COI1-Myc* plants (2) were ground in a TissueLyser (Qiagen). Soluble protein was extracted with 400 µL extraction buffer (50 mM HEPES, 10% glycerol, 2 mM EDTA and 0.5% Triton-X-100) with cOmplete mini protease inhibitor cocktail (Roche). Samples were vortexed for 5 min and then centrifuged at 14,000 x *g* for 5 min to remove insoluble material. Protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad). Ten µg protein per sample was diluted in 6x Laemmli buffer containing 10% v/v β-mercaptoethanol and loaded onto precast MINIPROTEAN 4-20% gradient SDS-PAGE gels (Bio-Rad). Proteins were transferred to PVDF membrane using a Mini Transblot cell (Bio-Rad) at 90 V for 60 min. Blots were blocked overnight at 4° C in 5% w/v dried skim milk in TBS-T buffer. Primary rabbit anti-Myc (Abcam) or rabbit anti-HSP90 (Agrisera) antibodies were diluted 1:5000 in 1% w/v dried skim milk in TBS-T and incubated for 1 h at ambient temperature (22° C), followed by three 5 min washes in TBS-T. Secondary goat anti-rabbit HRP antibody (Agrisera) was diluted 1:10,000 and incubated for 1 h at ambient temperature and then washed three times for 5 min per wash. Immunoreactive signals were visualized by enhanced chemiluminescence, using SuperSignal West Pico substrate (ThermoFisher) and a Biorad Universal Hood Molecular Imaging System (Bio-Rad).

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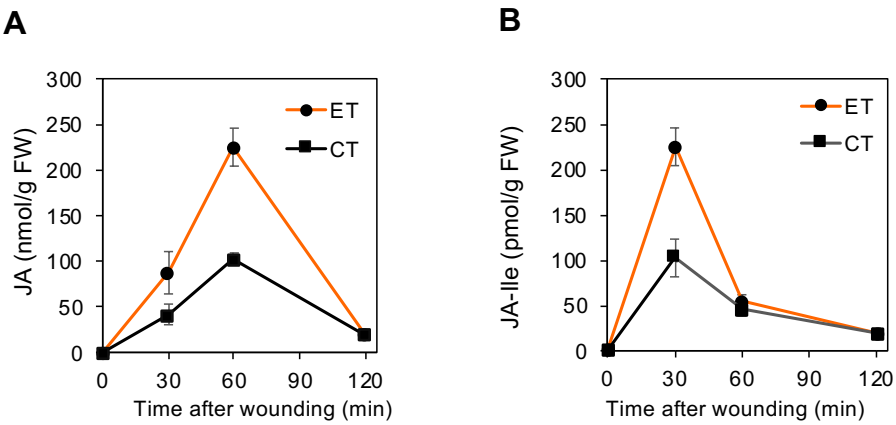



**Fig S1.** Effect of elevated temperature on wound-induced jasmonate levels. Time course of wound-induced jasmonic acid (JA, panel *A*) and JA-Ile (*B*) accumulation in plants grown for 17 d at CT before transfer to CT or ET treatment chambers. Two d after transfer to treatment chambers, leaves were mechanically wounded and harvested at the indicated times (after wounding) for hormone analysis. Data points represent the mean ± SE of four biological replicates, each containing leaves pooled from two plants.

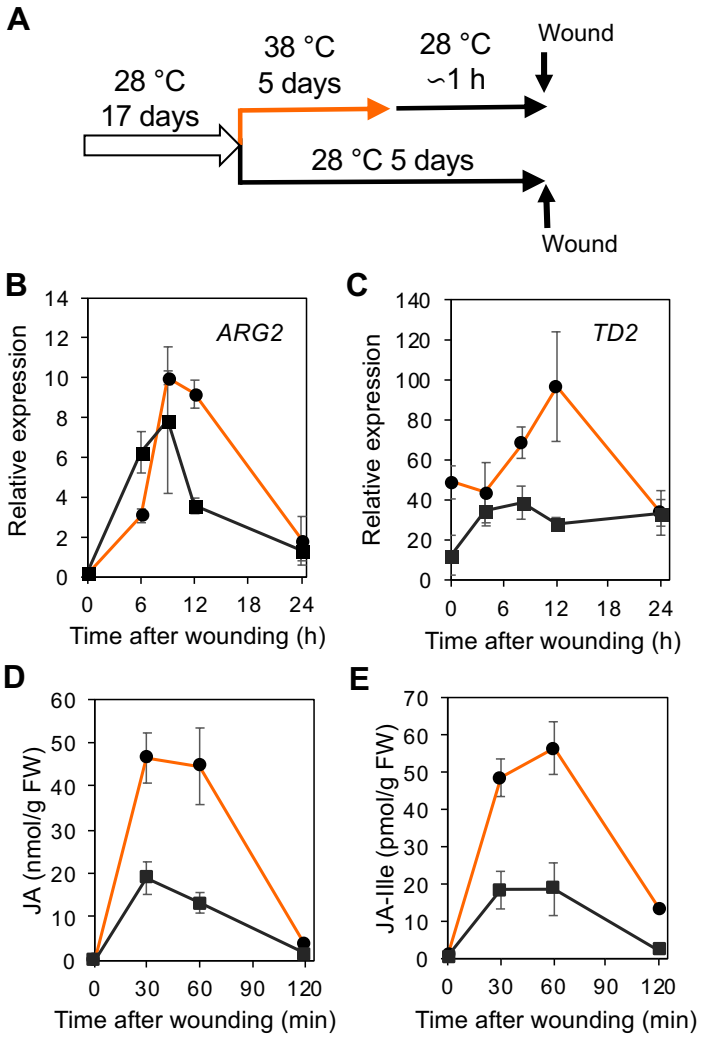



**Fig S2**. Wound responses remain elevated in plants exposed to higher temperature and then reacclimated to control temperature. (*A*) Schematic diagram of experimental treatments. Plants were grown for 17 d under the control temperature (CT) regime (28°C 16 h light/18°C 8 h dark) and then transferred to either CT (black arrow) or ET (orange arrow) treatment chambers for 5 d. Plants were then returned to CT conditions and, following a brief acclimation period of ~1 h, leaves were mechanically wounded with a hemostat. (*B-C*) Time course of wound-induced gene expression in plants grown under continuous CT conditions (black line) or in plants subjected to a 5-d ET treatment and then shifted to CT (orange line). Following wounding, leaf tissue was harvested for RNA extraction at the indicated times after wounding. *ARG2* (*B*) and *TD2* (*C*) mRNA abundance was measured by qPCR, with data normalization to an *ACTIN* housekeeping gene. (*D-E*) Time course of wound-induced JA (*D*) and JA-Ile (*E*) levels in plants grown with (orange line) or without (black line) a 5-d ET pre-treatment as described above. Following wounding, leaf tissue was harvested for hormone extraction at the indicated times after wounding. Data points represent the mean ± SE of four biological replicates containing tissue pooled from two plants.

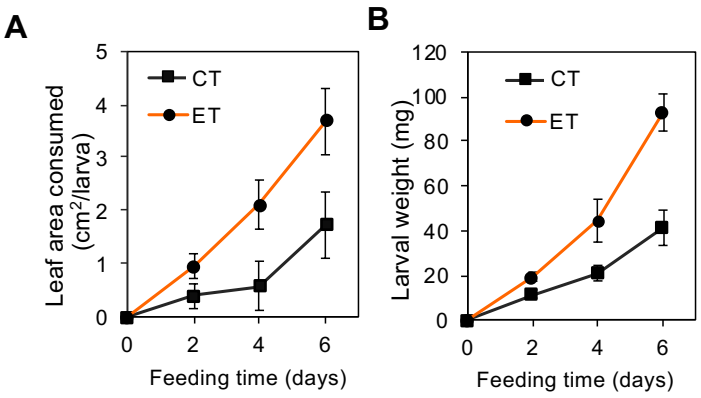



**Fig S3.** Elevated temperature enhances insect feeding on tomato. Seventeen-d-old WT plants grown under CT conditions were transferred to CT (black line) or ET (orange line) treatment chambers and immediately challenged with two *M. sexta* larva per leaf. Feeding proceeded for six d at either CT or ET. The amount of leaf area consumed per larva (*A*) and larval weight gain (*B*) were measured at the indicated times after challenge. Data points represent the mean ± SE of three biological replicates.

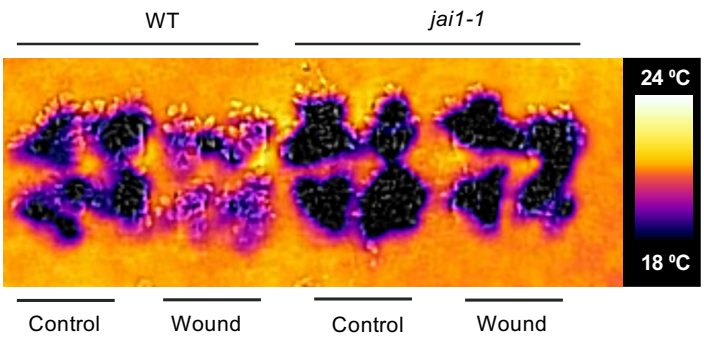
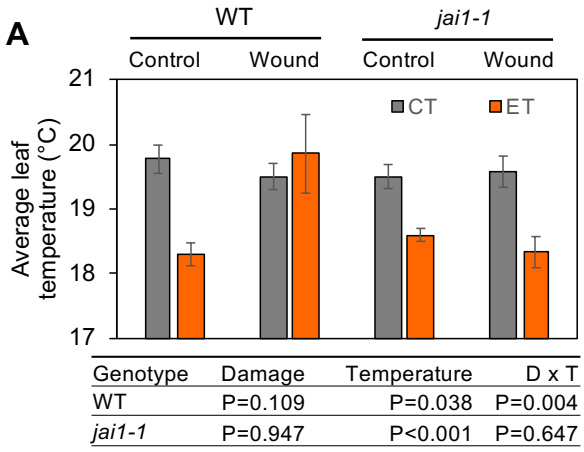
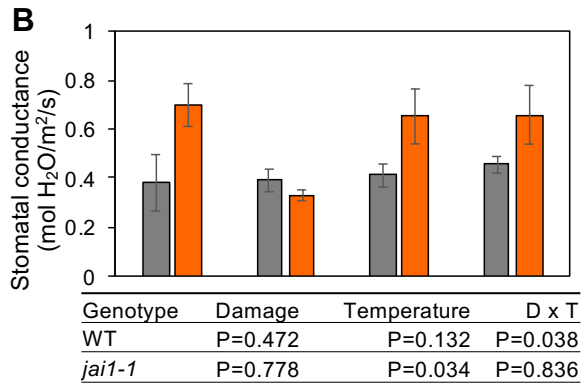


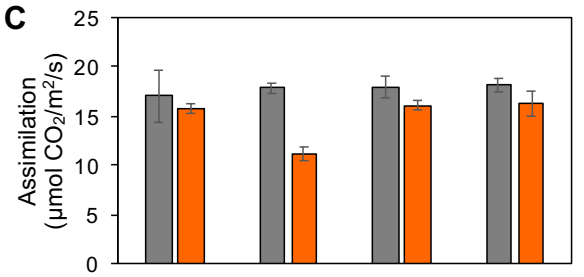

**Fig S4.** Wound-induced jasmonate signaling prevents stomatal-based cooling responses to elevated temperature. WT and *jai1-1* plants were grown for 17 d under CT and then transferred to ET conditions for 5 d. At the time of transfer, one set of WT and *jai1-1* plants received no wounding (Control). A second set of plants was mechanically wounded at the time of transfer and wounding was repeated daily during the 5-d ET period (Wound). After the 5-d ET period, all plants were acclimated to room temperature (22 °C) for approximately 1 h and leaf temperatures were measured with a thermal camera. The image shows four plants per treatment group, imaged from above.




| Genotype | Damage | Temperature | D x T |
|---|---|---|---|
| WT | P=0.109 | P=0.038 | P=0.004 |
| *jai1-1* | P=0.947 | P<0.001 | P=0.647 |

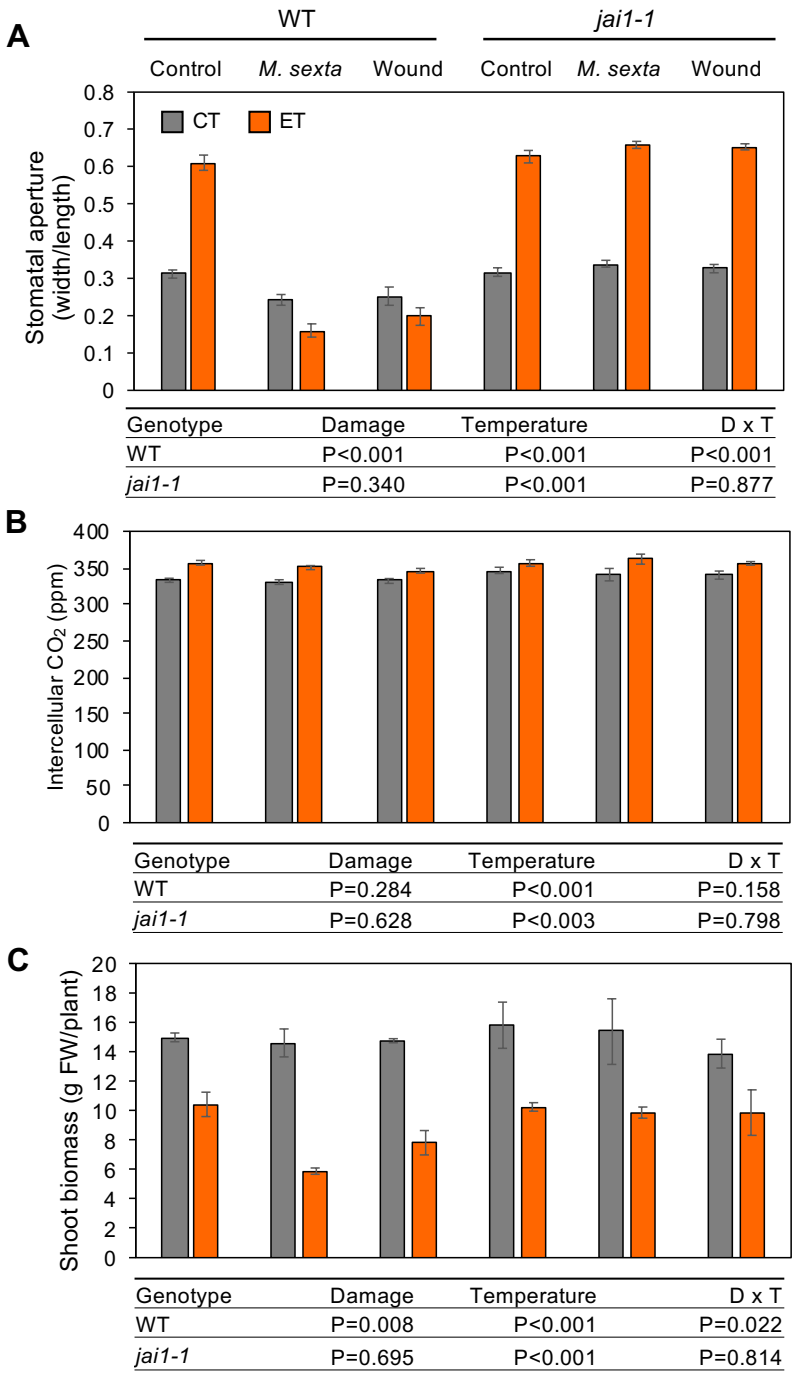



| Genotype | Damage | Temperature | D x T |
|---|---|---|---|
| WT | P=0.472 | P=0.132 | P=0.038 |
| *jai1-1* | P=0.778 | P=0.034 | P=0.836 |




| Genotype | Damage | Temperature | D x T |
|---|---|---|---|
| WT | P=0.216 | P=0.236 | P=0.092 |
| *jai1-1* | P=0.864 | P=0.084 | P=0.986 |

**Fig S5.** Wound-induced jasmonate signaling prevents stomatal-based cooling responses to elevated temperature and represses photosynthesis within 24 h of ET treatment. WT and *jai1-1* plants were grown for 17 d under CT conditions and then transferred to CT or ET treatment chambers for 24 h. Control plants (Con) received no wounding at the time of transfer. Other sets of plants were challenged with daily mechanical damage (Wound) at the time of transfer. (*A*) Average leaf temperatures were measured with a thermal camera after allowing all plants (CT and ET treatments) to acclimate to room temperature (22° C) for approximately 1 h. (*B*-*C*) Stomatal conductance (*B*) and photosynthetic carbon assimilation (*C*) of WT and *jai1-1* plants grown as described in panel *A*. Measurements were made on undamaged areas from damaged leaves using a LI-6800 portable photosynthesis system (LI-COR). Data points represent the mean ± SE of four biological replicates. Two-way ANOVA tables below panels (*A*-*C*) show P values assessing the effect of leaf damage (D), elevated temperature (T), and the interaction of D x T for each of the two genotypes tested (WT and *jai1-1*).




| Genotype | Damage | Temperature | D x T |
|---|---|---|---|
| WT | P<0.001 | P<0.001 | P<0.001 |
| *jai1-1* | P=0.340 | P<0.001 | P=0.877 |

| Genotype | Damage | Temperature | D x T |
|---|---|---|---|
| WT | P=0.284 | P<0.001 | P=0.158 |
| *jai1-1* | P=0.628 | P<0.003 | P=0.798 |

| Genotype | Damage | Temperature | D x T |
|---|---|---|---|
| WT | P=0.008 | P<0.001 | P=0.022 |
| *jai1-1* | P=0.695 | P<0.001 | P=0.814 |

**Fig S6.** Wound-induced jasmonate signaling prevents stomatal-based cooling responses to elevated temperature and represses growth. (*A*) Stomatal aperture measured from epidermal peels of WT and *jai1-1* leaves grown for 17 d under CT before transfer to CT or ET conditions for 5 d without wounding (Control), or transferred in combination with either caterpillar herbivory (*M. sexta*) or daily mechanical wounding (Wound). Stomatal aperture was measured using epidermal peels from four plants. Data points represent the mean ± SE of four plants. (*B*) Intercellular $CO_2$ concentration of WT and *jai1-1* leaves from plants grown as described in panel *A*. Measurements were made on control leaves or undamaged areas from damaged leaves using a LI-6800 portable photosynthesis system (LI-COR). Data points represent the mean ± SE of four biological replicates. (*C*) Shoot biomass of WT and *jai1-1* plants grown as described in panel *A*. Data points represent the mean ± SE of four plants. Tables below panels (A-C) show P values from two-way ANOVA tests of the effect of leaf damage (D), temperature (T), and the interaction of D x T for each of the two genotypes tested (WT and *jai1-1*).

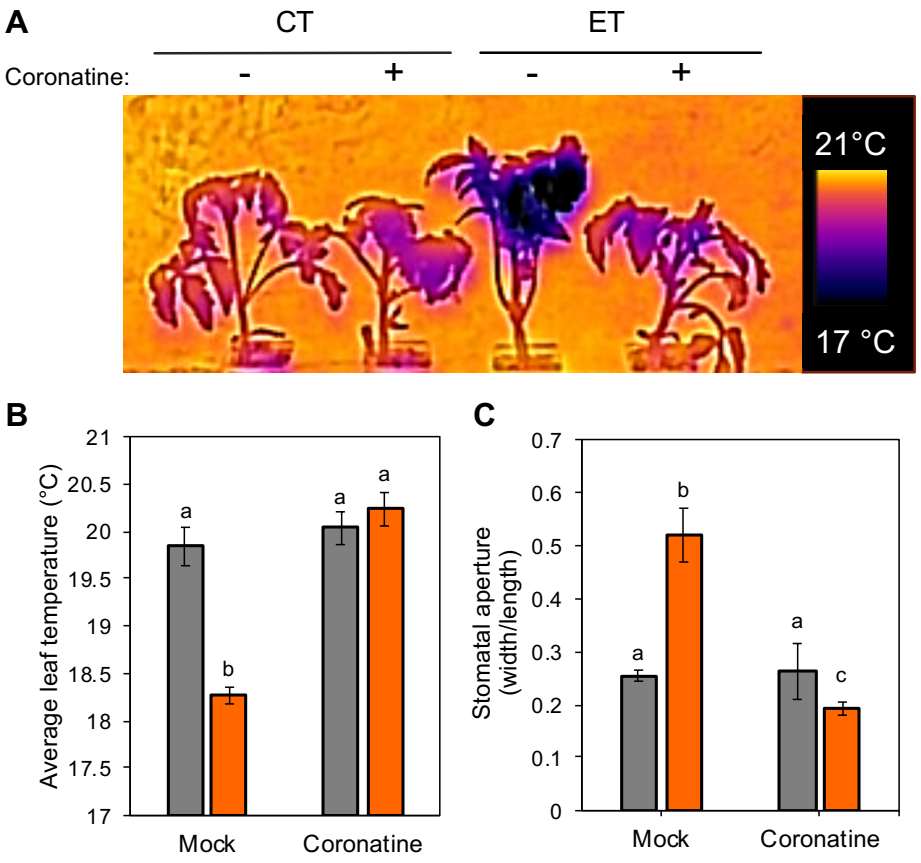
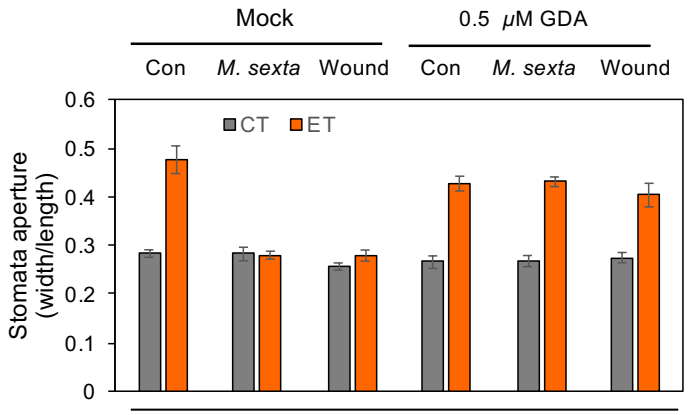



**Fig S7.** Application of the JA-Ile mimic coronatine prevents stomatal-based cooling responses to moderate heat stress. (*A*) Wild-type plants grown under CT conditions for 17 d were sprayed with a solution containing 20 $\mu$M coronatine or a mock control. Replicate sets of coronatine- and mock-treated plants were transferred to either CT or ET treatment chambers for 5 d with re-application of coronatine every 2 d, after which plants were briefly (approximately 1 h) acclimated to room temperature (~ 22° C) for thermal imaging. Representative plants from each treatment are shown. (*B*) Average leaf temperature calculated from thermal images of plants treated as described in panel *A*. (*C*) Stomatal aperture determined from epidermal peels from plants treated as described in panel *A*. Data points represent the mean ± SE of three different plants. Lowercase letters denote significant differences (Tukey HSD test $P < 0.05$).




| Treatment | Damage | Temperature | D x T |
|---|---|---|---|
| Mock | P=<0.01 | P=0.024 | P=0.021 |
| 0.5 μM GDA | P=0.973 | P<0.001 | P=0.919 |

**Fig S8.** The HSP90 inhibitor geldanamycin alleviates wound-induced inhibition of stomatal opening at elevated temperature. Wild-type plants (cv Castlemart) were vacuum infiltrated with a mock solution (Mock) or a solution containing 0.5 μM geldanamycin (GDA). Following a brief drying period at ambient temperature, plants were transferred to either CT or ET treatment chambers overnight. The following day, leaves were wounded either mechanically (Wound) or challenged with insect larvae (*M. sexta*) for 24 h. Stomatal apertures were measured using leaf epidermal peels from four plants per treatment group. Data points represent the mean ± SE of four biological replicates. The table below the panel shows P values from two-way ANOVA tests of the effect of leaf damage (D), temperature (T), and the interaction of D x T for each of the two chemical treatments tested (Mock and GDA).

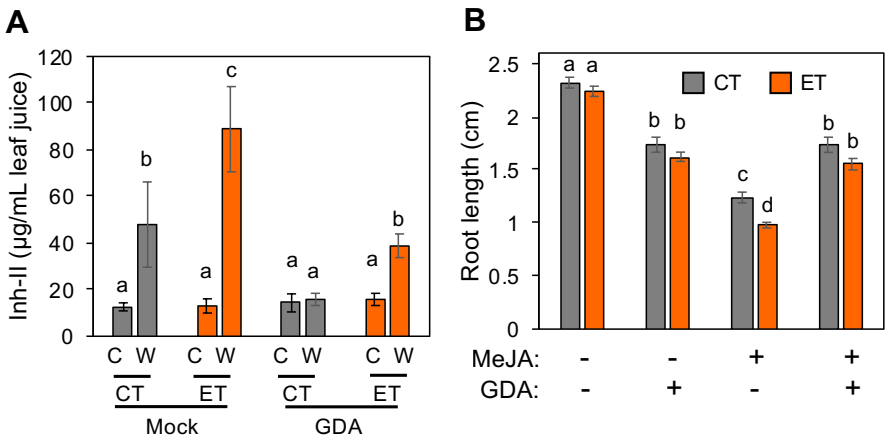



**Fig S9.** The HSP90 inhibitor geldanamycin reduces JA responsiveness. (*A*) GDA inhibits wound-induced Inh-II accumulation. Leaves of WT plants were vacuum infiltrated with a mock solution or 0.5 µM GDA. Plants were allowed to dry on the benchtop before being transferred to either CT (grey bars) or ET (orange bars) treatment chambers overnight. The morning after infiltration, leaves were mechanically wounded (W) and harvested 24-h later for the Inh-II assay. A control (C) set of plants received no wounding. Data points represent the mean ± SE of four biological replicates. (*B*) Effect of GDA on JA-mediated root growth inhibition. WT seeds were germinated at ambient temperature (22 °C) on filter paper. Upon radicle emergence, seedlings were treated with a solution containing 1 mM MeJA, 50 µM GDA, or a combination of both compounds. Treated seedlings were immediately transferred to CT or ET conditions for 2 d, after which time the primary root length was measured. Data points represent the mean ± SE of >30 seedlings. Note that GDA alone had a weak but significant inhibitory effect (~70% of mock control) on root growth at both temperatures.

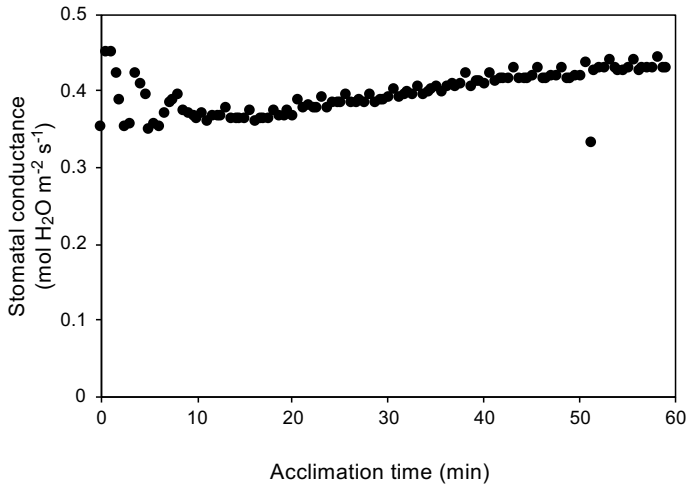



**Fig S10.** Representative curve of the acclimation of stomatal conductance for steady state measurements using a wild-type plant under control temperature (CT) conditions. Leaves were acclimated for 40 min or until conductance appeared to be constant, whichever was longer. Three consecutive measurements taken at 30-s intervals were averaged. The slightly higher-than-average data points at every fifth time point result from a multiphase flash of light used for determination of chlorophyll fluorescence $F_m'$.

Table S1. Oligonucleotide primers used in this study.

| Primer | Sequence 5’ - 3’ |
|---|---|
| Sl_Actin2_F | TTCAACACCCCTGCCATGT |
| Sl_Actin2_R | CCACTGGCATAGAGGGAAAGAA |
| Sl_Arginase2_F | TGGTGAAGGTGTAAAGGGCGTGTA |
| Sl_Arginase2_R | TTACCAGCTTCGCAGCAACCATTG |
| Sl_Threonine Deaminase2_F | CCCTGGGAGTGTATGTAGTTCT |
| Sl_Threonine Deaminase2_R | TCGAATGGTGGGATGTATTTGAT |