GABA signalling modulates stomatal opening to enhance plant water use efficiency and drought resilience

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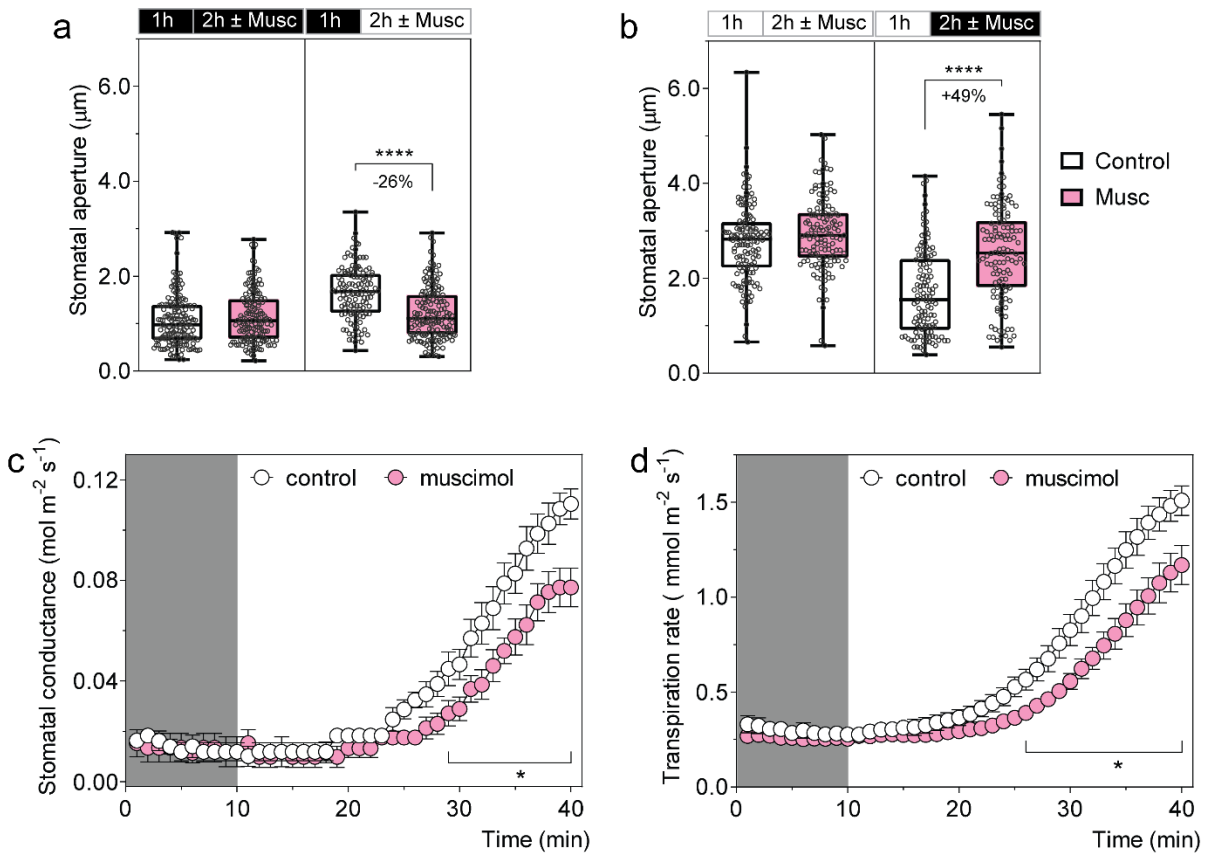
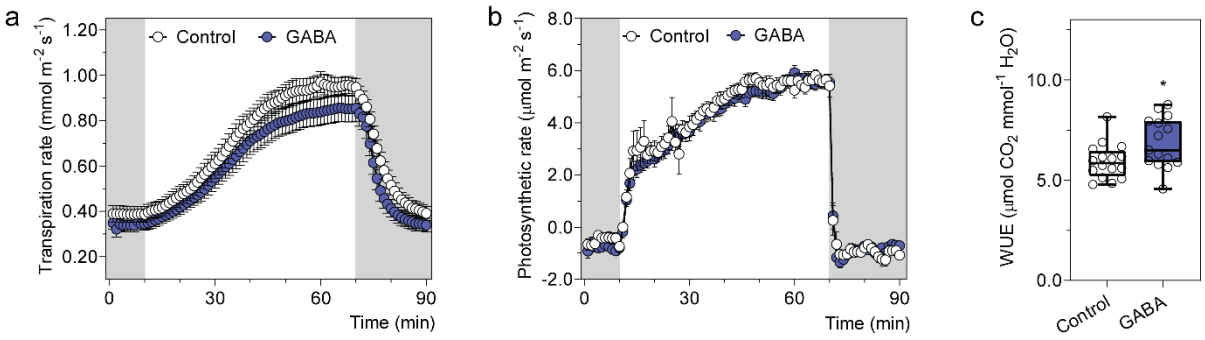
SUPPLEMENTARY INFORMATION

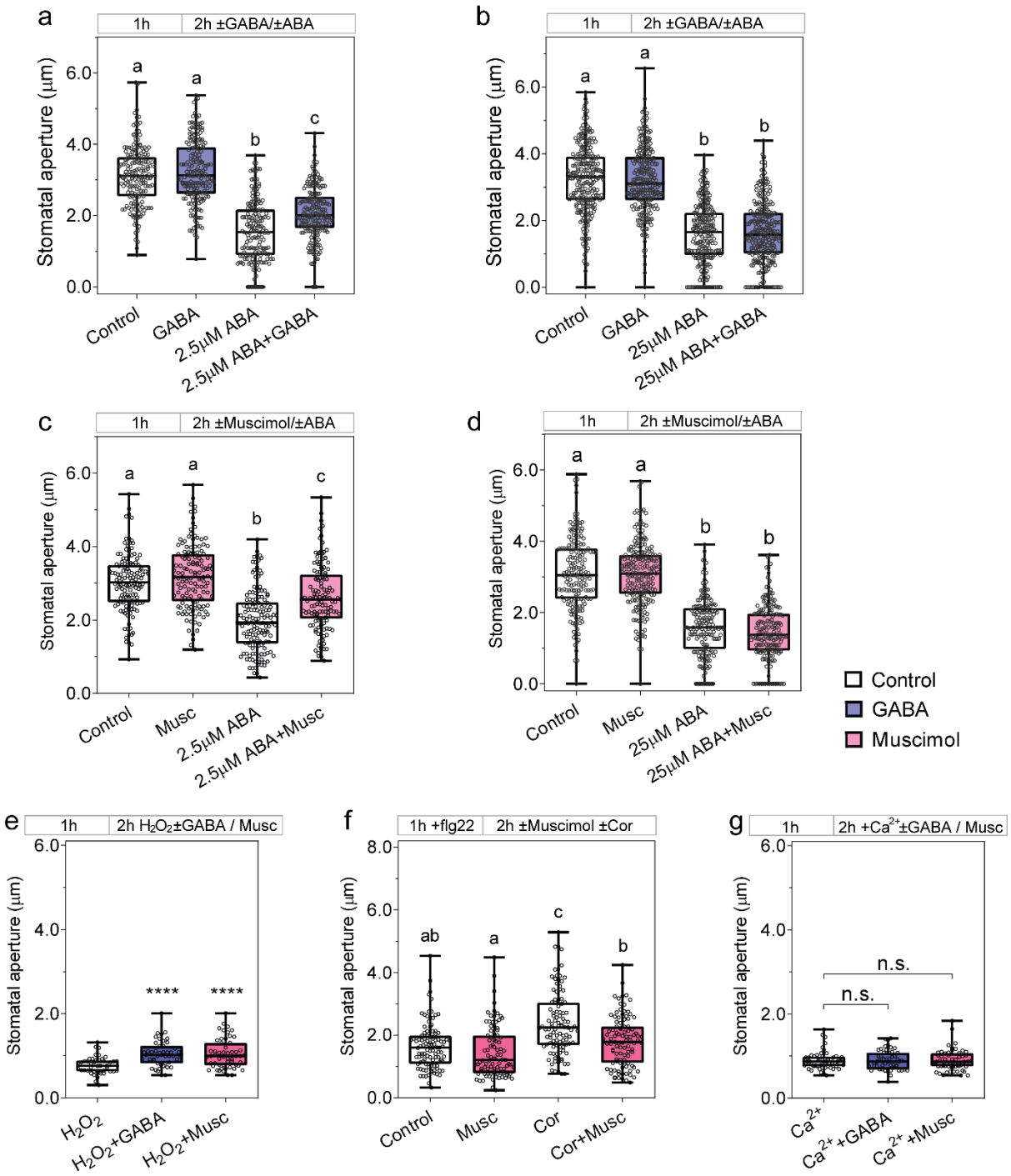


Supplementary Figure 1. Muscimol antagonises stomatal aperture changes initiated by light and dark treatments a-b, Exogenous muscimol application reduces stomatal pore movement in response to light or dark. Epidermal strips were pre-incubated in stomatal pore measurement buffer for 1 h under dark (**a**) or light (**b**), followed by 2 h incubation under constant dark (**a**), light (**b**), dark-to-light transition (**a**) or light-to-dark transition (**b**) as indicated above graphs by black (dark) or white (light) bars, together with the application of 10 µM muscimol (Musc); n = 156 for control (constant dark), n = 151 for muscimol (constant dark), n = 127 for control (dark-to-light transition) and n = 151 for muscimol (dark to light transition) (**a**); n = 134 for control (constant light), n = 132 for muscimol (constant light), n = 118 for control (light-to-dark transition) and n = 120 for muscimol (light-to-dark transition) (**b**). **c-d**, Muscimol feeding reduces stomatal conductance, transpiration rate of detached leaves. Stomatal conductance (**c**), transpiration rate (**d**) of detached leaves of wildtype Arabidopsis plants determined by LCpro-SD Portable Photosynthesis System was fed by artificial

xylem sap solution with or without 10 μM muscimol supplement; n = 7 for control and n = 8 for muscimol (**c, d**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**a, b**) or data are represented mean ± s.e.m (**c, d**); statistical difference was determined by Two-way ANOVA (**a, b**), or two-sided Student's *t-test* (**c, d**), **P*<0.05 and *****P*<0.0001.

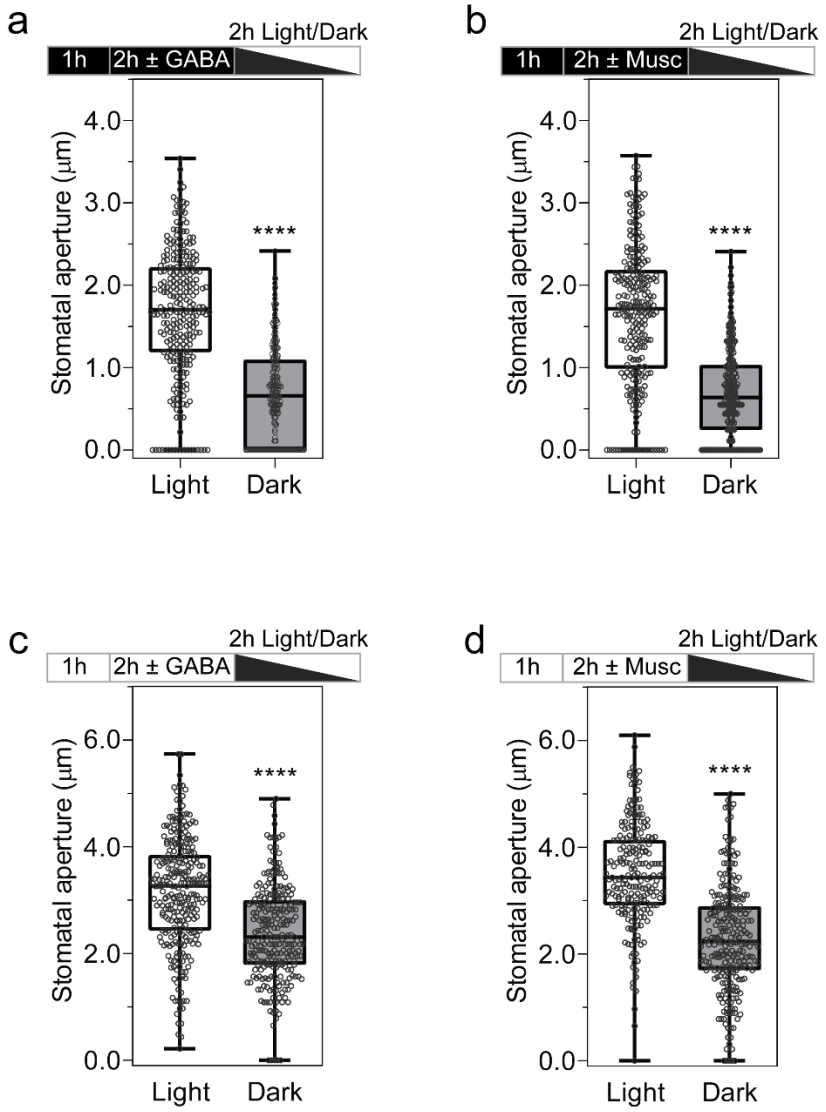


Supplementary Figure 2. GABA feeding increases WUE of detached leaves. GABA feeding reduces transpiration rate and increases water use efficiency (WUE) of detached leaves. Transpiration (**a**) and photosynthetic rate (**b**) of 5-6 week-old leaves of 5-6 week-old *A. thaliana* wildtype plants was recorded using a LiCor LI-6400XT in response to dark (shaded region) and 200 µmol m^{-2} s^{-1} light (white region), fed with artificial xylem sap solutions ± 4 mM GABA. **c**, WUE of detached leaves was calculated based on the ratio of photosynthetic rate (**b**) versus transpiration rate (**a**), n = 16 independent leaves for control and n = 15 for GABA (**a-c**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**c**) or data are represented mean ± s.e.m (**a, b**), statistical difference was determined by two-sided Student's *t-test*, **P*<0.05



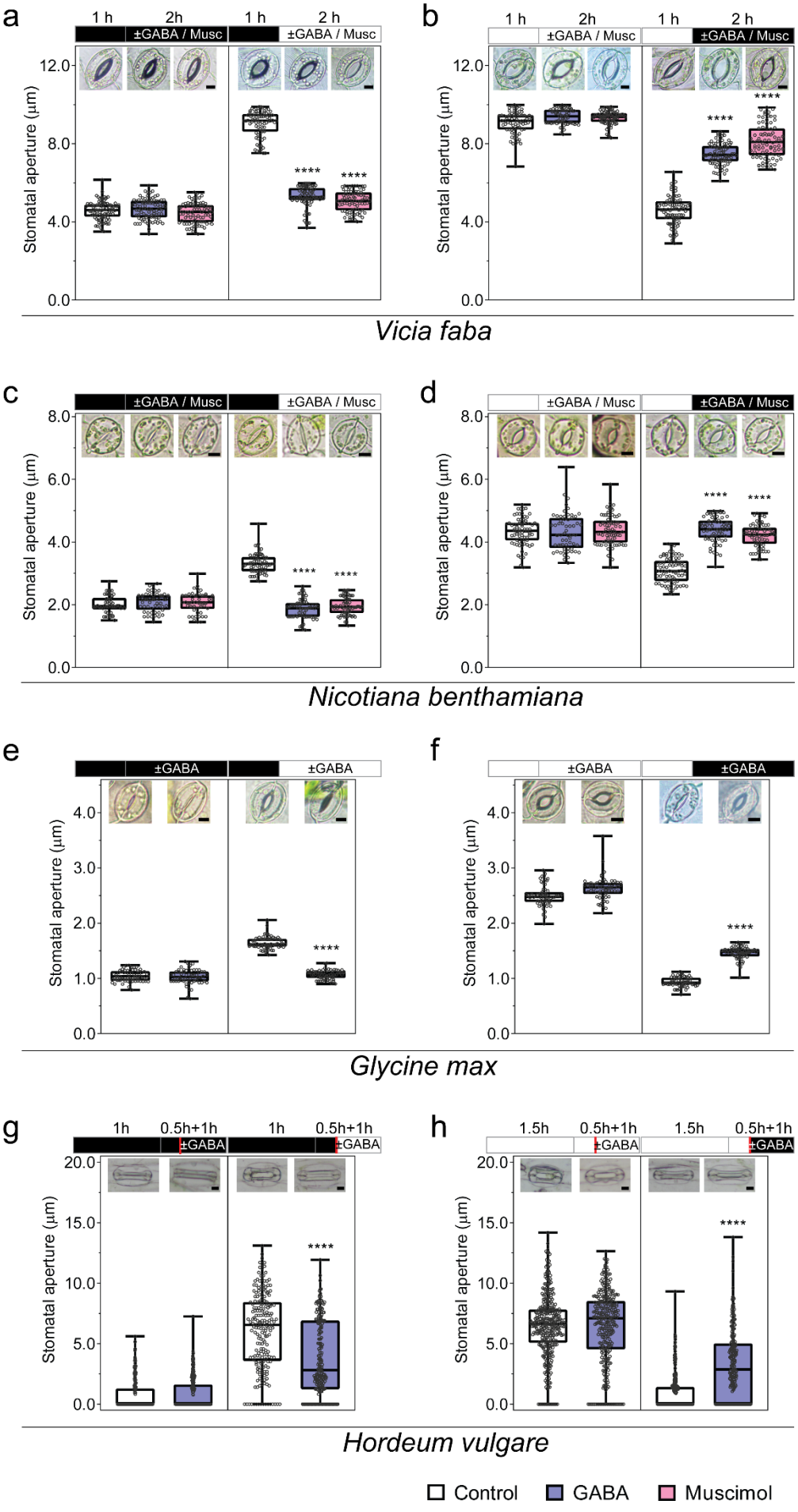
Supplementary Figure 3. GABA and muscimol inhibit stomatal aperture changes triggered by signaling molecules. a-g, Exogenous GABA or muscimol application reduces stomatal closure in response to 2.5 µM ABA (**a, c**), 50 µM H_2O_2[1] (**e**) and stomatal opening to 0.5 µg ml^{-1} coronatine[2] (**f**), but not to 25 µM ABA (**b, d**) and 2 mM $CaCl_2$[1] (**g**). Epidermal strips were pre-incubated in stomatal pore measurement buffer for 1 h under light, followed by 2 h treatment under light with or

without combination of ABA ± 2 mM GABA (**a, b**) / 10 µM muscimol (**c, d**), H_2O_2 ± 2 mM GABA/10 µM muscimol (Musc) (**e**), coronatine (Cor) ± 10 µM muscimol (**f**), and $CaCl_2$ (Ca^{2+}) ± 2 mM GABA/10 µM muscimol (**g**) as indicated; n = 183 for control, n = 191 for GABA, n = 171 for ABA and n = 201 for ABA+GABA (**a**); n = 249 for control, n = 249 for GABA, n = 243 for ABA and n = 261 for ABA+GABA (**b**); n = 133 for control, n = 137 for muscimol, n = 142 for ABA and n = 131 for ABA+muscimol (**c**); n = 180 for control, n = 222 for muscimol, n = 176 for ABA and n = 179 for ABA+muscimol (**d**); n = 44 for H_2O_2, n = 47 for H_2O_2+GABA and n = 54 for H_2O_2+Musc (**e**); n = 103 for control, n = 103 for Cor, n = 101 for Cor+muscimol and n = 91 for muscimol (**f**);); n = 52 for Ca^{2+}, n = 52 for Ca^{2+}+GABA and n = 54 for Ca^{2+}+Musc (**g**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile; statistical difference as determined by One-Way ANOVA (**a-g**), **** P<0.0001, a, b and c represent groups without significant difference, P< 0.05 (**a-d, f**).



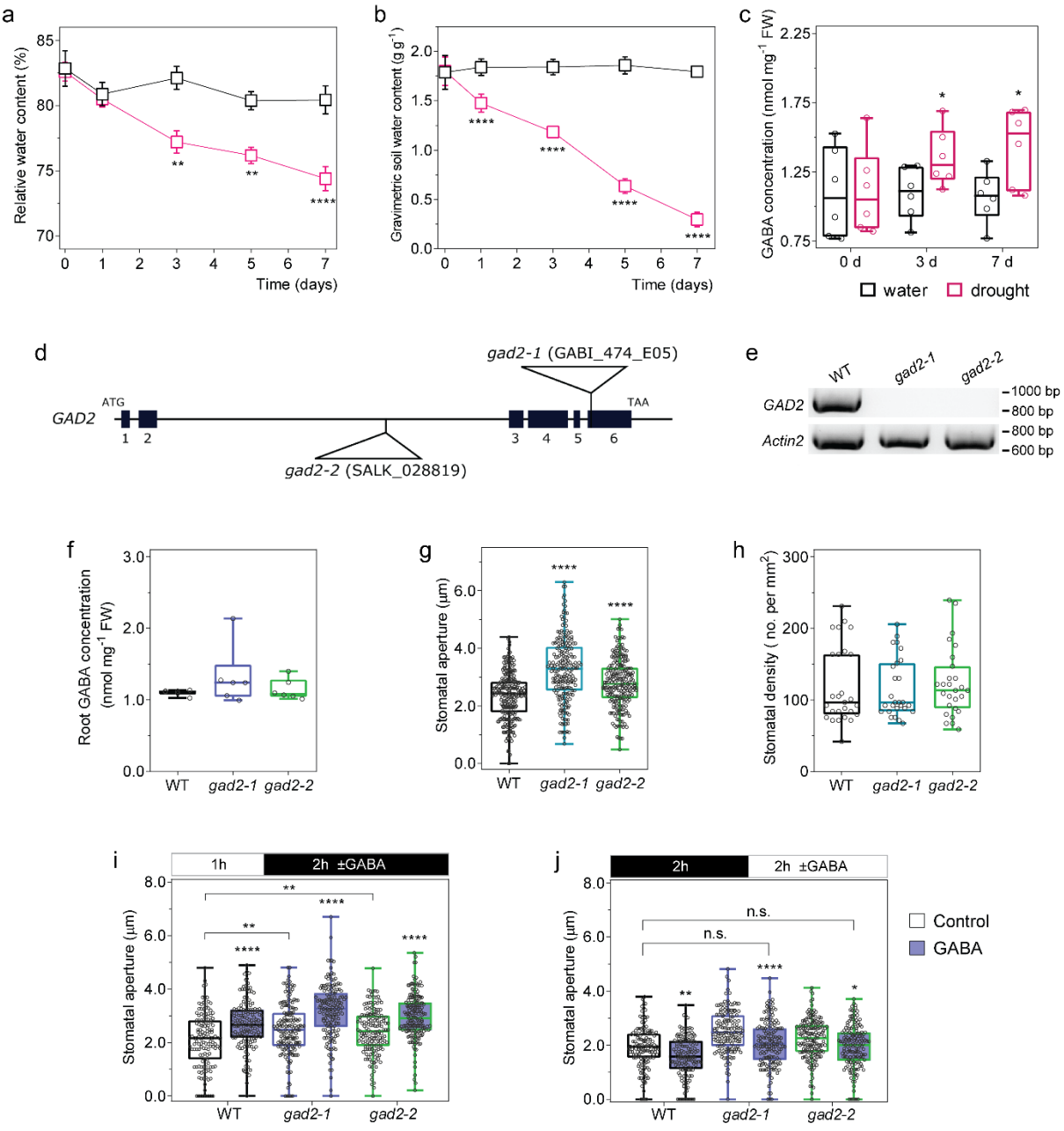
Supplementary Figure 4. Guard cells are viable after treatment with GABA and muscimol. a-d, Guard cells were competent in movement after removal of GABA or muscimol treatments, as closed pores opened when exposed to light (**a-b**) or open pores closed when exposed to dark (**c-d**) following removal of GABA or muscimol. Epidermal strips were incubated under dark (**a-b**) or light (**c-d**) for 1 h, followed by 2 h treatment of 2 mM GABA (**a, c**) or 10 μM muscimol (**b, d**), then epidermal strips were transferred into fresh stomatal measurement buffer with 2 h light or dark treatment before measurement; n = 272 for light and n = 247 for dark (**a**); n = 251 for light and n = 251 for dark (**b**); n = 275 for light and n = 248 for dark (**c**); n = 217 for light and n = 261 for dark (**d**). All data are plotted with box and whiskers plots:

whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile; statistical difference as determined by two-sided Student's *t*-test (**a-d**), **** $P<0.0001$.


a
b
c
d
e
f
g
h
1 h
2h
1 h
2 h
±GABA / Musc
±GABA
1h
0.5h+1h
1.5h
Stomatal aperture (µm)
****
Vicia faba
Nicotiana benthamiana
Glycine max
Hordeum vulgare
Control
GABA
Muscimol

Supplementary Figure 5. **GABA and muscimol inhibit stomatal aperture changes in response to light and dark in *Vicia faba* (broad bean), *Nicotiana benthamiana* (tobacco), *Glycine max* (soybean) and *Hordeum vulgare* (barley).** Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h under dark (**a, c, e**) or light (**b, d, f**), followed by 2 h incubation under constant dark (**a, c, e**), light (**b, d, f**) as illustrated by black (dark) or white (light) bars, ± 2 mM GABA or 10 μM muscimol (Musc) as indicated; barley leaf samples were first detached and bathed in a modified measurement buffer under 2h dark (**g**) or light (100 μmol m^{-2} s^{-1}) (**h**), then pre-treated in the fresh buffer ±1 mM GABA for 0.5 h as indicated by black or white bars; after this pre-treatment (break by red line), leaf samples were incubated in continuous dark (**g**), light (**h**), dark-to-light (**g**) or light-to-dark (**h**) transition for additional 1 h before the epidermal strips were peeled for imaging. n = 88 for control (constant dark), n = 85 for GABA (constant dark), n = 82 for muscimol (constant dark), n = 78 for control (dark-to-light transition), n = 106 for GABA (dark-to-light transition) and n = 76 for muscimol (dark-to-light transition) (**a**); n = 73 for control (constant light), n = 65 for GABA (constant light), n = 76 for muscimol (constant light), n = 89 for control (light-to-dark transition), n = 85 for GABA (light-to-dark transition) and n = 84 for muscimol (light-to-dark transition) (**b**); n = 50 for control (constant dark), n = 52 for GABA (constant dark), n = 50 for muscimol (constant dark), n = 63 for control (dark-to-light transition), n = 65 for GABA (dark-to-light transition) and n = 64 for muscimol (dark to light transition) (**c**); n = 73 for control (constant light), n = 60 for GABA (constant light), n = 78 for muscimol (constant light), n = 78 for control (light-to-dark transition), n = 67 for GABA (light-to-dark transition) and n = 65 for muscimol (light-to-dark transition) (**d**); n = 61 for control (constant dark), n = 60 for GABA (constant dark), n = 63 for control (dark-to-light transition) and n = 62 for GABA (dark-to-light transition) (**e**); n = 59 for control (constant light), n = 59 for GABA (constant light), n = 60 for control (light-to- dark transition) and

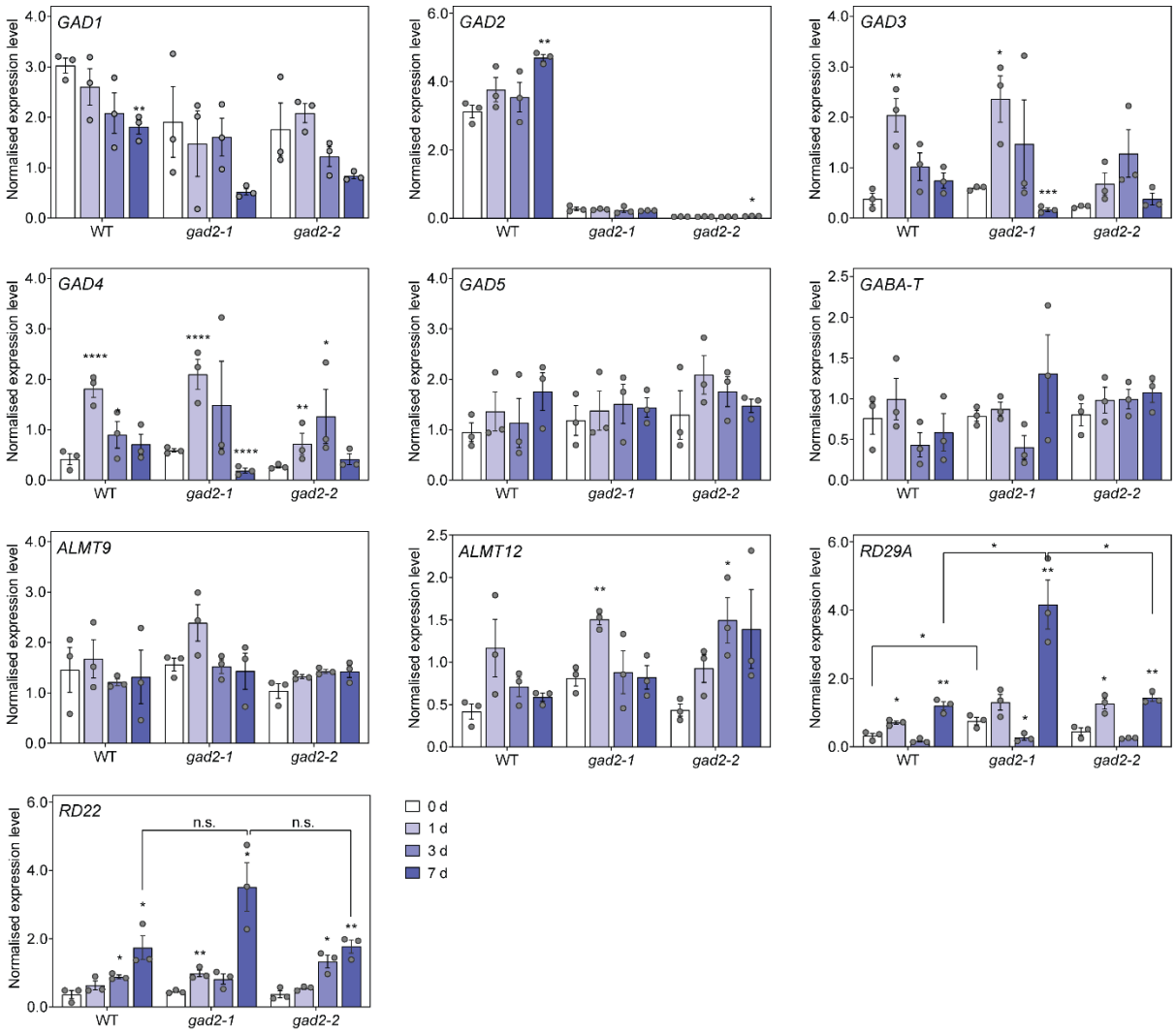
n = 60 (dark-to-light transition) (**f**); n = 177 for control (constant dark), n = 220 for GABA (constant dark), n = 201 for control (dark-to-light transition) and n = 203 for GABA (dark-to-light transition) (**g**); n = 350 for control (constant light), n = 301 for GABA (constant light), n = 289 for control (light-to-dark transition) and n = 228 for GABA (light-to dark-transition) (**h**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile; statistical difference was determined by Two-way ANOVA, ****$P< 0.0001$; scale bars = 5 μm (**a-h**).



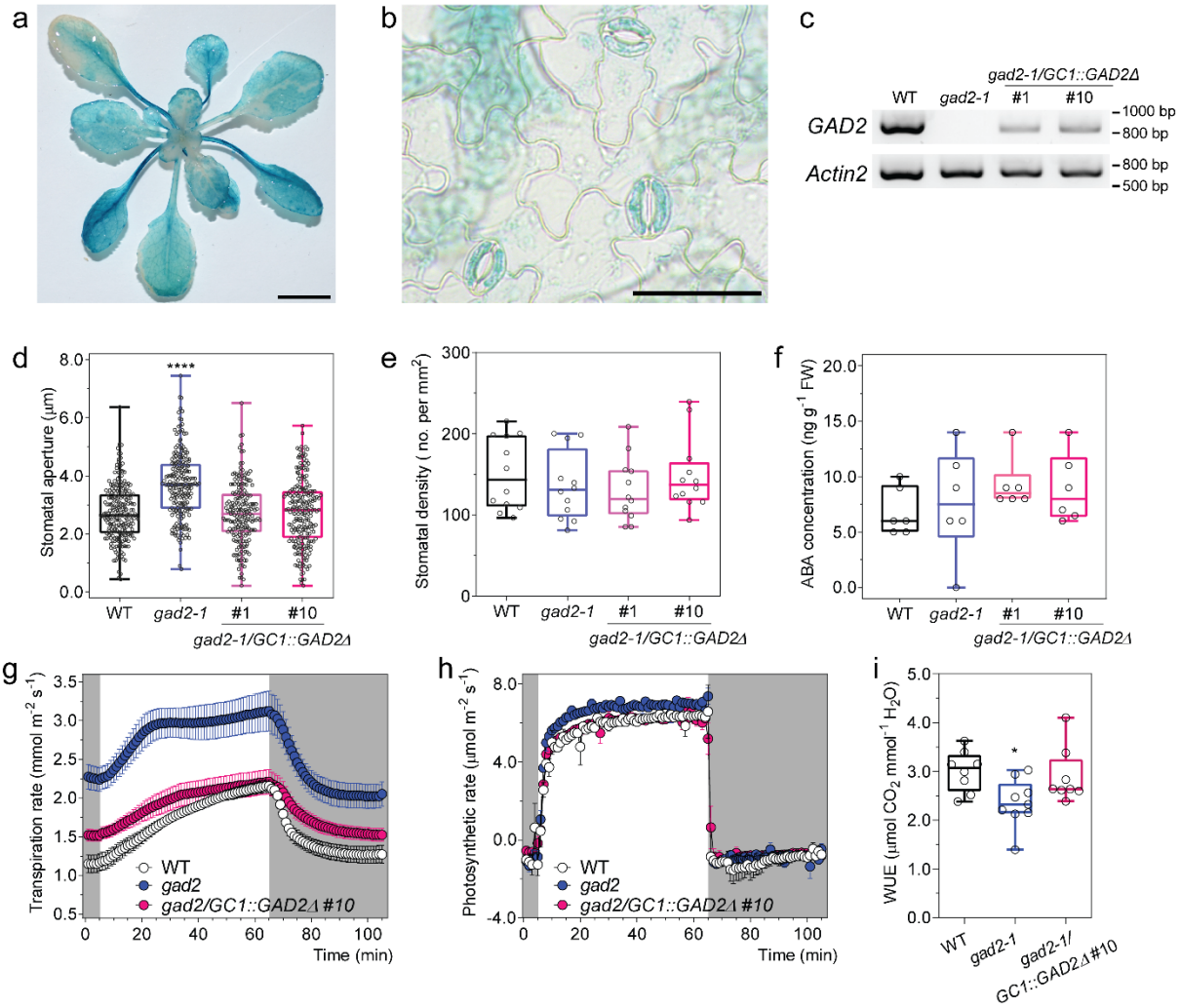
Supplementary Figure 6. **GABA accumulates in leaves of Arabidopsis upon drought, and *gad2* knockout plants have greater stomatal apertures but show wildtype (WT)-like responses to exogenous GABA and root GABA accumulation.** **a,** Relative water content in wildtype Arabidopsis leaves under well-water (black) or drought (red) treatments as indicated. **b,** Water content in the potted soil corresponding to the plants sampled in (**a**). **c,** Leaf GABA concentration of wildtype Arabidopsis following well-watered control treatment (black) or drought (red). 2-3 leaves per plant were sampled for relative water content measurement, as shown in

(**a**); the whole rosette from the sampled plant in (**a**) was harvested and snap frozen in liquid nitrogen for later GABA measurement, as shown in (**c**); and the pot soil of corresponding plants harvested in (**a, b**) was sampled to determine gravimetric soil water content, as shown in (**b**); n = 6 (**a-c**). **d,** A diagram of *GAD2* T-DNA insertional mutant alleles in the Arabidopsis genome. **e,** Reverse transcriptional PCR semi-quantification of *GAD2* transcripts in Arabidopsis WT and *gad2* knockout plants, *Actin2* used as an internal control, similar results were obtained from three independent biological replicates. **f,** Root GABA concentration of WT, *gad2-1* and *gad2-2* plants. Roots were harvested from 5-6 week-old plants grown hydroponically in basal nutrient solution (2 mM NH_4NO_3, 3 mM KNO_3, 0.1 mM $CaCl_2$, 2 mM KCl, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.6 mM KH_2PO_4, 1.5 mM NaCl, 50 µM NaFe(III)EDTA, 50 µM H_3BO_3, 5 µM $MnCl_2$, 10 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.1 µM Na_2MoO_3, pH = 5.6 by KOH)[3], n = 6 plants. **g-j,** Stomatal aperture and density on the leaf abaxial side of Arabidopsis WT and *gad2* knockouts; epidermal strips were peeled and incubated in stomatal measurement buffer for 1 h under light before measurement n = 254 for WT, n = 215 for *gad2-1* and n = 226 for *gad2-2* (**g**); n = 27 sampling areas (0.57 x 0.42 mm) consisting of three areas per leaf, three leaves per plant and three plants per line sampled (**h**). **i-j,** Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h under light (**i**) or 2 h dark (**j**), followed by 2 h incubation dark (**i**) or light (**j**) as indicated by black (dark) or white (light) bars ± blind treatment of 2 mM GABA or control; n = 135, 166 and 157 for WT, *gad2-1* and *gad2-2* with control treatment, n = 146, 162 and 174 for WT, *gad2-1* and *gad2-2* with GABA treatment (**i**); n = 139, 155 and 160 for WT, *gad2-1* and *gad2-2* with control treatment, n = 136, 155 and 153 for WT, *gad2-1* and *gad2-2* with GABA treatment (**j**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**c, f-j**); or data are represented

by mean ± s.e.m. (**a, b**); statistical difference was determined by two-sided Student's *t*-test (**c**), One-way ANOVA (**f-h**) or Two-way ANOVA (**a, b, i, j**), **P*< 0.05, ***P*< 0.01 and *****P*< 0.0001.



Supplementary Figure 7. ***gad2*** **knockouts have similar transcriptional profiles to wildtype plants of other *GADs*, *GABA-T*, *ALMT9*, *ALMT12* or ABA responsive genes under drought stress.** Quantitative real time PCR of *GAD1*, *GAD2*, *GAD3*, *GAD4*, *GAD5, GABA-T*, *ALMT9*, *ALMT12* and ABA marker genes –*RD29A* and *RD22*[4] in the leaves of Arabidopsis wildtype (WT), *gad2-1* and *gad2-2* plants following drought treatment for 0, 1, 3 and 7 days as indicated, expression levels were normalized against three control genes –*Actin2*, *EF1α* and *GAPDH-A*; data are represented by means ± s.e.m; n = 3, statistical difference as determined via the comparison of genes from drought-treated plants (1, 3 and 7 days) with non-droughted (0 day) plants within the same genotype by two-sided Student's *t-test*, *$P< 0.05$ **$P< 0.01$, ***$P< 0.001$ and ****$P< 0.0001$.

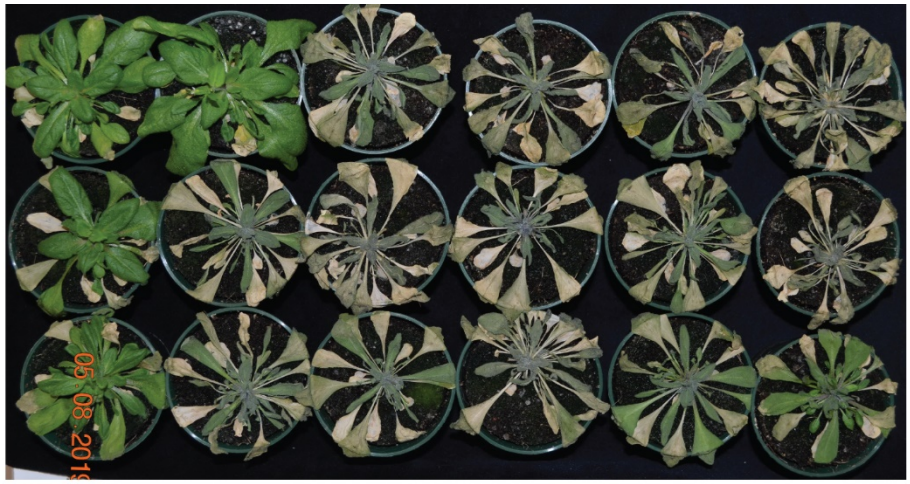


Supplementary Figure 8. *GAD2* is highly expressed in leaves and guard cells, and guard-cell cell complementation of *GAD2Δ* restores stomatal aperture and WUE without modifying stomatal density. a-b, Representative GUS histochemical staining of *pGAD2::GUS* whole rosette**;** image of 3-4 week-old *pGAD2::GUS* plants after staining in histochemical staining buffer, GUS staining of the guard cells was observed in all plants examined that were expressing *pGAD2::GUS*, scale bar = 5 mm (**a**) and epidermal peels from 3-4 week-old *pGAD2::GUS* leaves (**a**), scale bar = 50 μm (**b**). **c,** Reverse-transcriptional PCR quantification of *GAD2* transcripts in Arabidopsis wildtype (WT), *gad2-1*, *gad2-1/GC1::GAD2Δ* #1 and #10 plants, similar results were obtained from three independent biological replicates. **d-e,** Stomatal aperture (**d**) and density (**e**) on the leaf abaxial side of Arabidopsis WT, *gad2-1*, *gad2-*

1/GC1::GAD2Δ #1 and #10 plants; epidermal strips were peeled and incubated in stomatal measurement buffer for 1 h under light before measurement, n = 223 for WT, n = 212 for *gad2-1*, n = 197 for *gad2-1/GC1::GAD2Δ* #1 and n = 224 for *gad2-1/GC1::GAD2Δ* #10 (**d**); n = 12 leaf areas (0.57 x 0.42 mm); two areas per leaf, two leaves per plant and three plants per line were sampled (**e**). **f**, ABA accumulation in rosette leaves of 5-6 week-old Arabidopsis WT, *gad2-1*, *gad2-1/GC1::GAD2Δ* #1 and #10 plants, n = 6. **g-h**, Transpiration (**g**), photosynthetic rate (**h**) of 5-6 week-old Arabidopsis WT, *gad2-1* and *gad2-1/GC1::GAD2Δ* #10 plants in response to dark (shaded region) and 150 µmol m^{-2} s^{-1} light (white region), measured using a LiCor LI-6400XT, n = 8 for WT, n = 9 for gad2-1 and n = 8 for *gad2-1/GC1::GAD2Δ* #10 (**g-h**). **i,** WUE of of 5-6 week-old Arabidopsis WT, *gad2-1* and *gad2-1/GC1::GAD2Δ* #10 plants was calculated based on the photosynthetic rate (**h**) against transpiration rate (**g**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**d-f, i**) or data are represented mean ± s.e.m (**g, h**); statistical difference was determined by One-way ANOVA (**d-f, i**), **P* <0.05 and *****P* <0.0001.

a

WT
(22.2%)



GC1::GAD2Δ #2
(41.7%)

GC1::GAD2Δ #5
(78.6%)

b

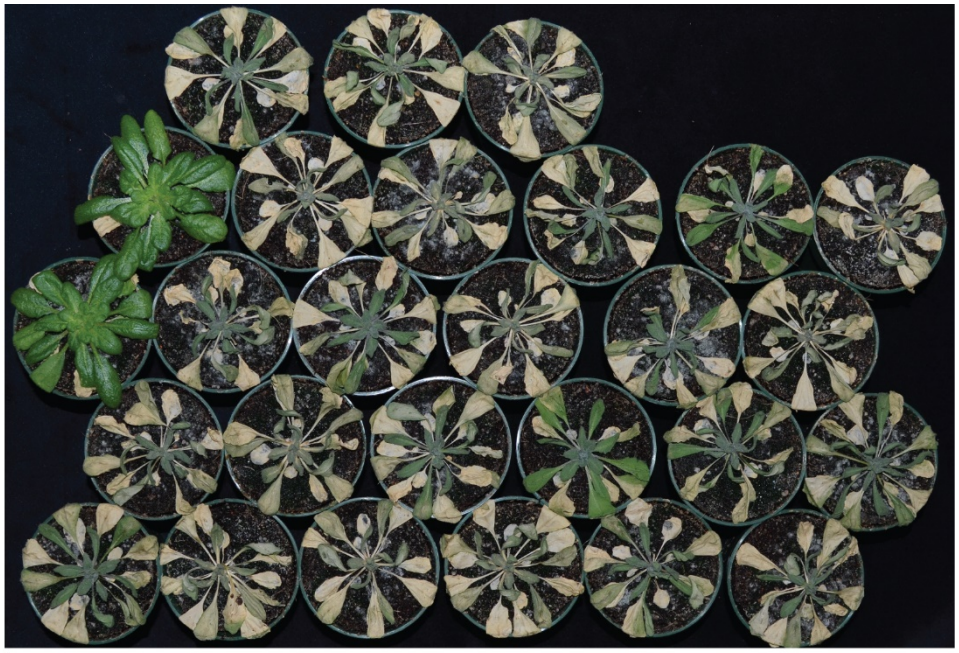
WT
(77.8%)

GC1::GAD2Δ #2
(88.9%)

GC1::GAD2Δ #5
(88.9%)

c

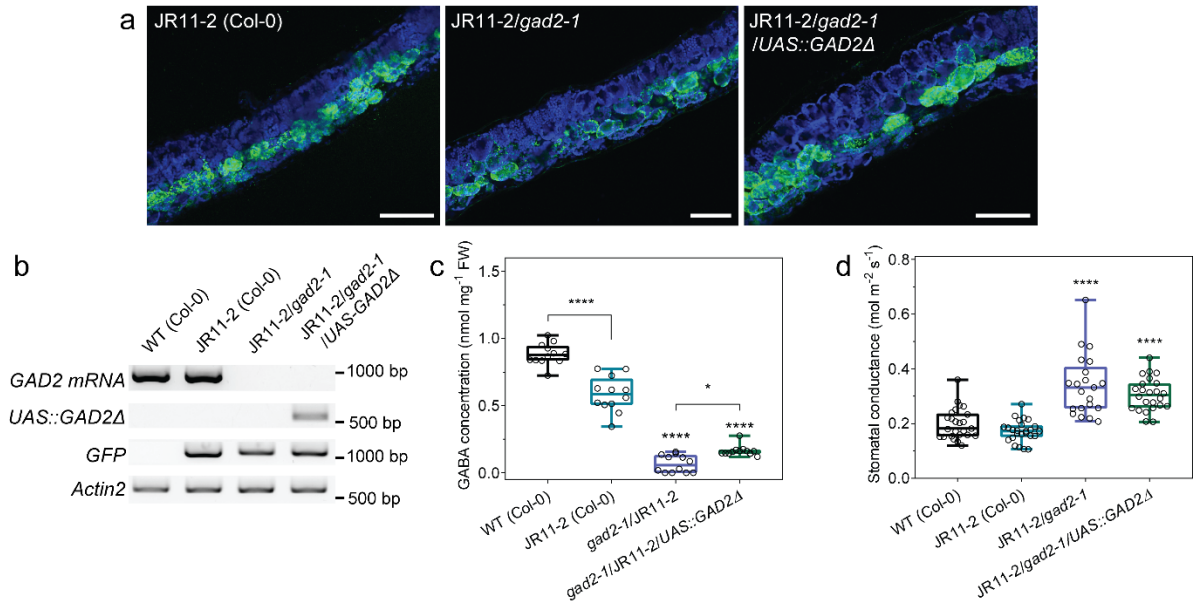
WT
(11.1%)



GC1::GAD2Δ #2
(19.0%)

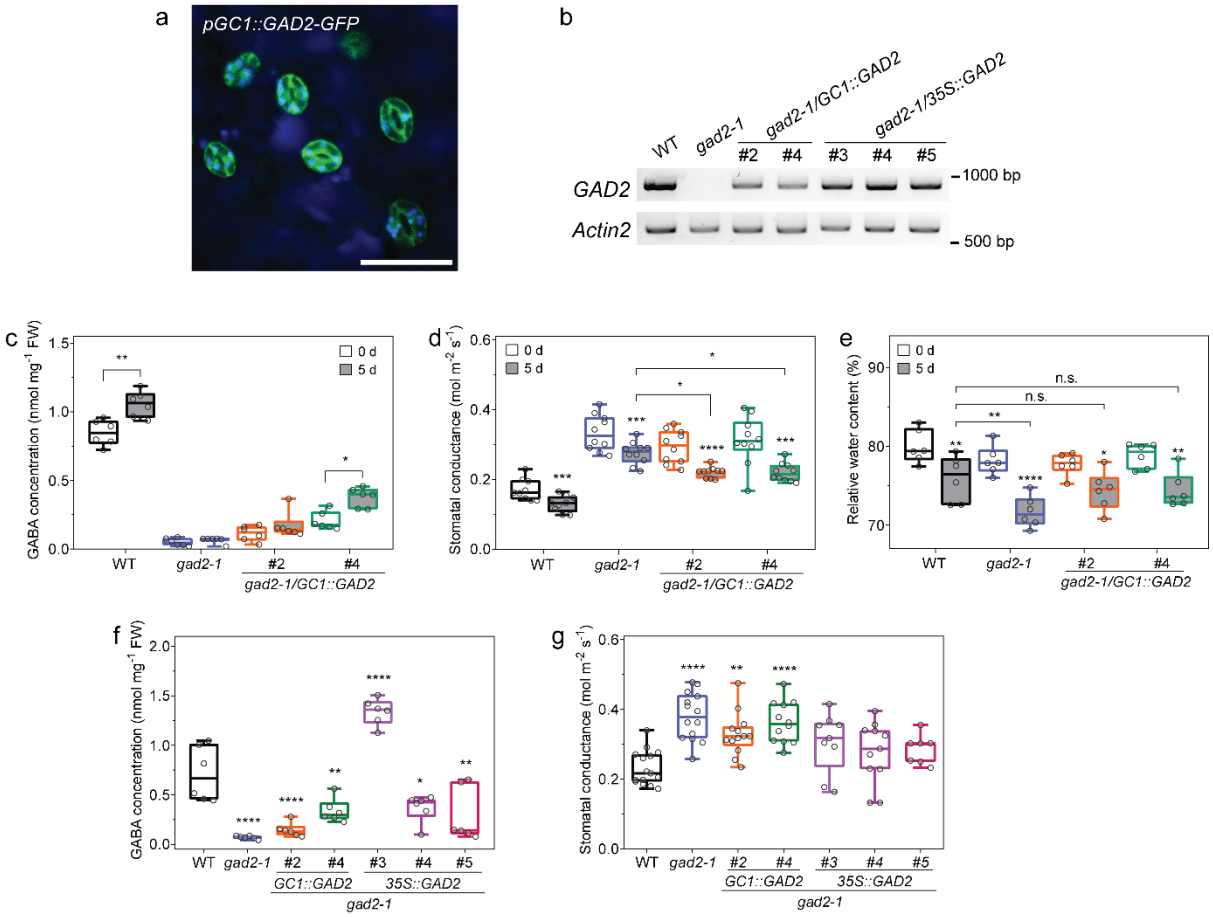
GC1::GAD2Δ #5
(35%)

Supplementary Figure 9. Recovery of re-watered Arabidopsis WT, *GC1::GAD2Δ* #2 and #5 from drought treatment. a-c, Re-water recovery of wildtype, *GC1::GAD2Δ* #2 and #5 plants from drought in three different batches of experiments, plants were re-watered at 2 days after all plant wilting by drought. A higher recovery rate of *GC1::GAD2Δ* #2 and #5 plants than WT was observed from re-watering in all three experiments (**a-c**); 4 out of 18 (22%) wildtype, 5 out of 12 (41.7%) *GC1::GAD2Δ* #2 and 11 out of 14 (78.6%) *GC1::GAD2Δ* #5 plants were recovered from re-water in the first trail (**a**); 21 out of 27 (77.8%) wildtype, 24 out of 27 (88.9%) *GC1::GAD2Δ* #2 and 16 out of 18 (88.9%) *GC1::GAD2Δ* #5 plants were recovered from re-water in the second trail (**b**); 3 out of 27 (11.1%) wildtype, 4 out of 21 (19.0%) *GC1::GAD2Δ* #2 and 7 out of 20 (35%) *GC1::GAD2Δ* #5 plants were recovered from re-water in the third trail (**c**).



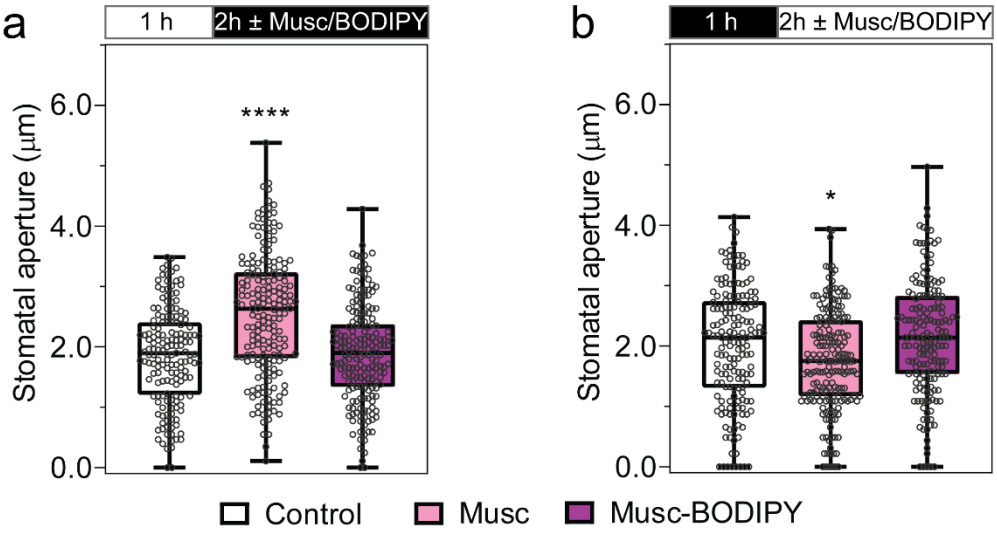
Supplementary Figure 10. **Spongy mesophyll-cell specific expression of *GAD2Δ* in *gad2-1* fails to restore stomatal conductance back to wildtype (WT) levels. a,** Representative images of leaf transverse cross-sections (30 µm thickness) of 3-4 week-old segregated mesophyll-specific enhancer-trap line JR11-2[5] backcrossed into Arabidopsis Col-0 background[6], JR11-2 in *gad2-1* background (JR11-2*/gad2-1*) and JR11-2*/gad2-1* expressing *UAS::GAD2Δ* (JR11-2*/gad2-1*/*UAS::GAD2Δ*), similar images were obtained from all examined lines, scale bars = 100 µm. GFP fluorescence shown in green indicates cells in which *GAD2* expression will be activated by the yeast transcription factor GAL4, blue indicates chlorophyll autofluorescence. **b,** Reverse-transcriptional PCR quantification of native *GAD2* mRNA (*GAD2mRNA*), *GAD2Δ* driven by *UAS* element (*UAS::GAD2Δ*), *GFP* and *Actin2* transcripts in Arabidopsis WT (Col-0), JR11-2 (Col-0), JR11-2*/gad2-1* and JR11-2*/gad2-1*/*UAS::GAD2Δ* plants, *Actin2* used as an internal control; similar results were obtained from three independent biological replicates. **c-d,** Leaf GABA concentration (**c**) and stomatal conductance (**d**) of 5-6 week-old Arabidopsis WT (Col-0), JR11-2 (Col-0), JR11-2*/gad2-1* and JR11-2*/gad2-1*/*UAS::GAD2Δ* plants, stomatal conductance was measured by AP4 Leaf Porometer (**d**); n = 12 (**c**); n = 25 for WT and JR11-2, n = 21

for JR11-2*/gad2-1* and n = 24 for JR11-2*/gad2-1*/*UAS::GAD2Δ*, data collected from two different batches of plants (**d**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**c, d**); statistically differences were determined by One-way ANOVA by comparing with JR11-2 (**c, d**), *P< 0.05 and ****P< 0.0001.

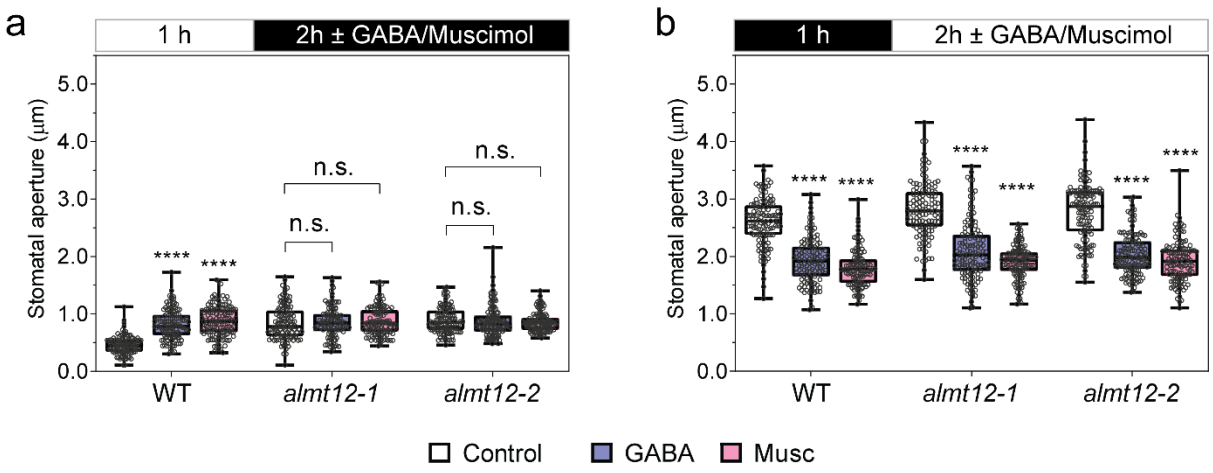


Supplementary Figure 11. Guard-cell specific expression of full-length *GAD2* only reduces the stomatal conductance of *gad2* knockout plants under drought. **a,** Representative confocal image of *gad2*-1 leaves expressing full-length *GAD2* tagged with *GFP* driven by *GC1* promoter (*GC1::GAD2-GFP*), similar images were obtained from all *gad2-1*/ *GC1::GAD2-GFP* plants examined, scale bar = 50 μm. **b,** Reverse-transcriptional PCR quantification of *GAD2* transcripts in wildtype (WT), *gad2-1* and *gad2-1* complementation with full-length *GAD2* driven by guard-cell promoter *GC1* (*gad2-1/GC1::GAD2* #2 and #4) or by a pro35S-CAMV constitutive promoter (*gad2-1/35S::GAD2* #3, #4 and #5), *Actin2* used as an internal control; similar results were obtained from three independent biological replicates. **c-e,** Leaf GABA concentration, stomatal conductance and relative water content of Arabidopsis WT, *gad2-1*, *gad2-1/GC1::GAD2* #2 and #4 plants; n = 6 plants for GABA measurement before (0 d) and after drought treatment for 5 days (5 d) as indicated

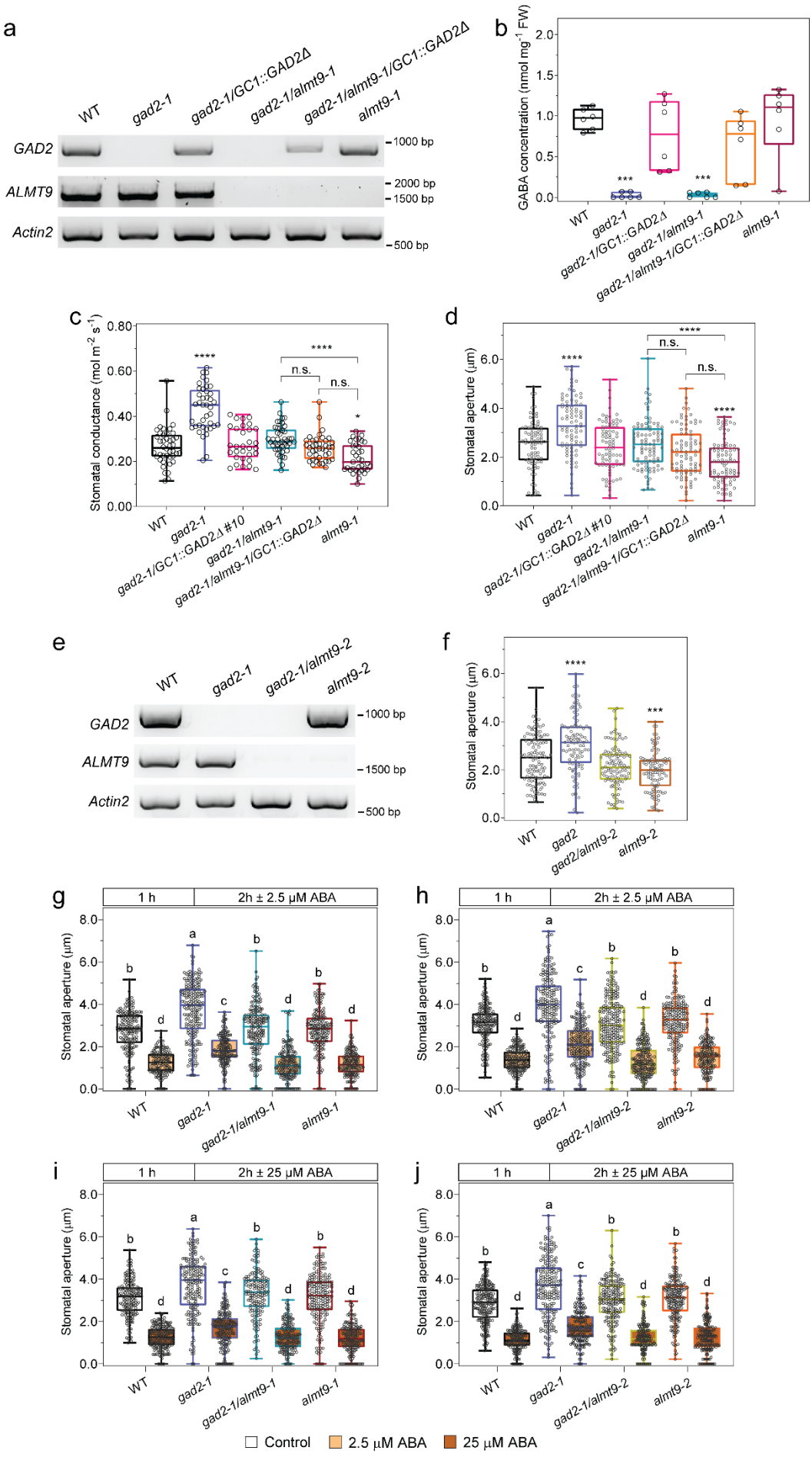
(**c**); the stomatal conductance of 5-6 week-old plants was determined by AP4 Leaf Porometer at 0 d and 5 d after drought treatment, n = 9 for WT and n = 10 for *gad2-1*, *gad2-1/GC1::GAD2* #2 and #4 (**d**); relative leaf water content of corresponding leaf samples at 0 d and 5 d after drought treatment, as shown in (**e**). **f-g,** Leaf GABA concentration and stomatal conductance of WT, *gad2-1*, *gad2-1/GC1::GAD2* #2, #4, *gad2-1/35S::GAD2* #3, #4 and #5 plants; n = 6 plants (**f**); the stomatal conductance of WT, *gad2-1*, and *gad2-1* complementation lines; stomatal conductance of 5-6 week-old plants was determined by AP4 Leaf Porometer in normal conditions, n = 15 plants for WT, n = 14 for *gad2-1*, n = 13 for *gad2-1/GC1::GAD2* #2, n = 12 for *gad2-1/GC1::GAD2* #4, n = 9 for *gad2-1/35S::GAD2* #3, n = 11 for *gad2-1/35S::GAD2* #4 and n = 7 for *gad2-1/35S::GAD2* #5 (**g**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**c-g**); statistically differences were determined by One-way ANOVA by comparing with WT (**f, g**), or within genotypes or treatment by Two-way ANOVA (**c-e**), **P*< 0.05, ***P*< 0.01, ****P* < 0.001 and *****P*< 0.0001.



Supplementary Figure 12. Membrane impermeable muscimol does not antagonises stomatal movement initiated by light and dark treatments a-b, Exogenous muscimol-BODIPY application does not affect stomatal movement. Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h under light (**a**) or dark (**b**), followed by 2 h incubation under light-to-dark transition (**a**) or dark-to-light transition (**b**) as indicated above graphs by black (dark) or white (light) bars, together with the application of 10 µM muscimol (Musc) or muscimol-BODIPY (Musc-BODIPY); n = 161 for control, n = 185 for muscimol and n = 188 for muscimol-BODIPY (**c**); n = 168 for control, n = 190 for muscimol and n = 175 for muscimol-BODIPY. All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**a-b**); statistical difference was determined by One-way ANOVA, *P<0.05 and ****P<0.0001.

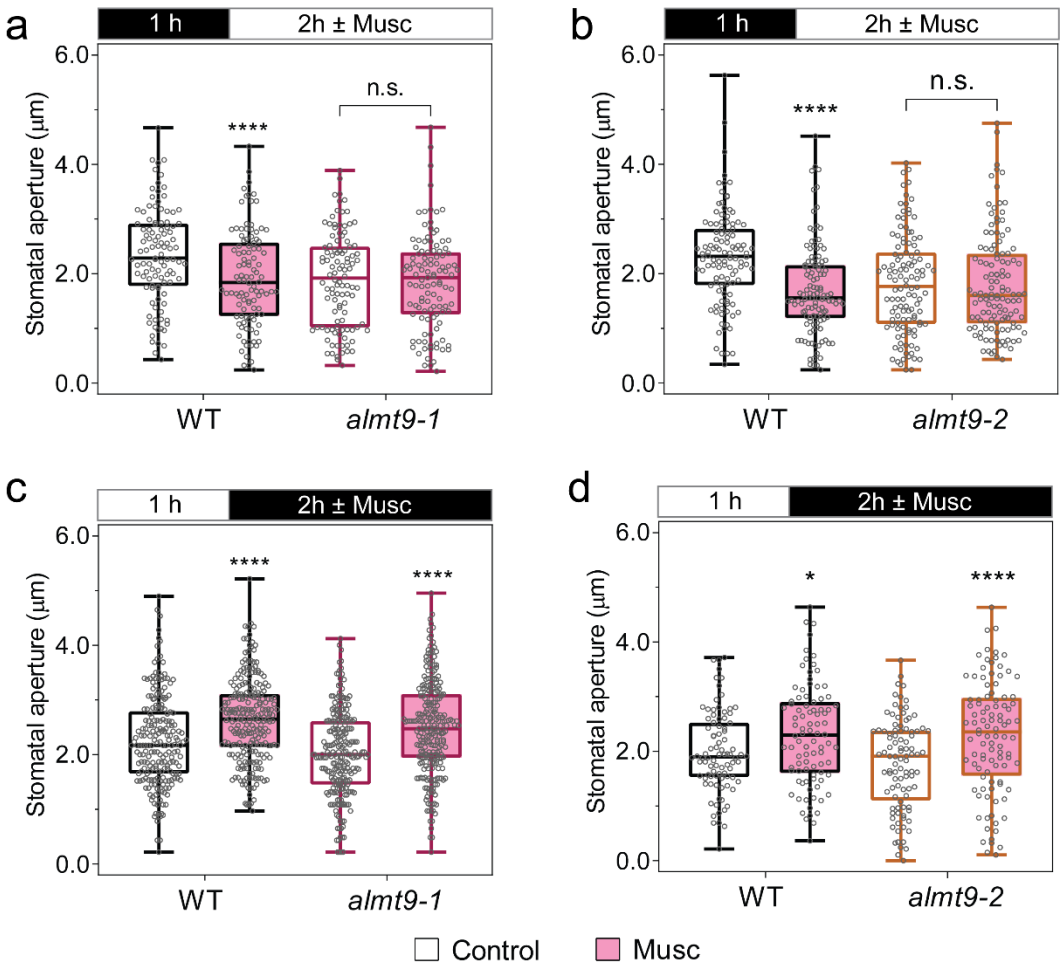


Supplementary Figure 13. Stomatal aperture measurement of WT, *almt12-1* and *almt12-2* knockout plants in response to dark or light. Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h in the light (**a**) or dark (**b**), followed by 2 h incubation in the dark (**a**) or light (**b**) as indicated by black (dark) or white (light) bars above the plots ± 2 mM GABA or 10 µM muscimol (Musc); n = 105 for WT (control), n = 115 for *almt12-1* (control), n = 122 for *almt12-2* (control), n = 122 for WT (GABA), n = 100 for *almt12-1* (GABA), n = 131 for *almt12-2* (GABA), n = 122 for WT (Musc), n = 107 for *almt12-1* (Musc) and n = 118 for *almt12-2* (Musc) (**a**); n = 116 for WT (control), n = 119 for *almt12-1* (control), n = 120 for *almt12-2* (control), n = 113 for WT (GABA), n = 123 for *almt12-1* (GABA), n = 124 for *almt12-2* (GABA), n = 117 for WT (Musc), n = 122 for *almt12-1* (Musc) and n = 116 for *almt12-2* (Musc) (**b**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile; statistical difference was determined using Two-way ANOVA, ****$P < 0.0001$; all experiments were repeated at least twice from different batches of plants with blind treatments.


a
WT
gad2-1
gad2-1/GC1::GAD2Δ
gad2-1/almt9-1
gad2-1/almt9-1/GC1::GAD2Δ
almt9-1
GAD2
ALMT9
Actin2
1000 bp
2000 bp
1500 bp
500 bp
b
GABA concentration (nmol mg-1 FW)
c
Stomatal conductance (mol m-2 s-1)
d
Stomatal aperture (μm)
gad2-1/GC1::GAD2Δ#10
e
gad2-1/almt9-2
almt9-2
f
gad2
gad2/almt9-2
g
1 h
2h ± 2.5 μM ABA
h
i
2h ± 25 μM ABA
j
Control
2.5 μM ABA
25 μM ABA

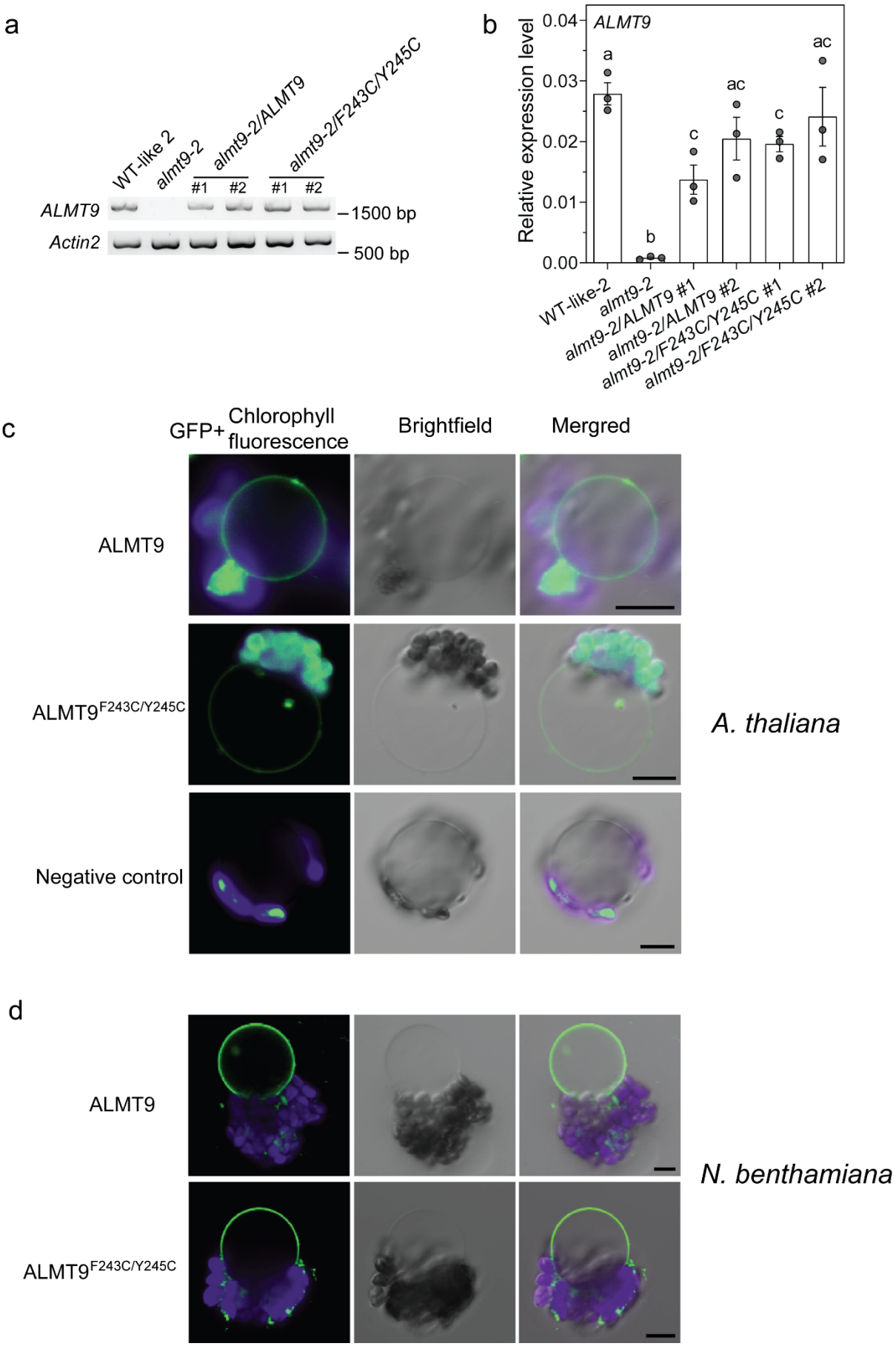
Supplementary Figure 14. **The loss of *ALMT9* rescues the larger stomatal aperture and ABA response of *gad2* knockout plants. a,** Reverse-transcriptional PCR quantification of *GAD2*, *ALMT9* and *Actin2* transcripts in Arabidopsis wildtype (WT), *gad2-1*, *gad2-1/GC1::GAD2Δ* #10, *gad2-1/almt9-1*, *gad2-1/almt9-1/GC1::GAD2Δ* and *almt9-1* plants, *Actin2* used as an internal control; similar results were obtained from three independent biological replicates. **b-d,** Leaf GABA accumulation, stomatal conductance and aperture of WT, *gad2-1*, *gad2-1/GC1::GAD2Δ* #10, *gad2-1/almt9-1*, *gad2-1/almt9-1/GC1::GAD2Δ* and *almt9-1* plants. The whole rosette leaves of 5-6 week-old plants were harvested for GABA measurement (**b**) and used for stomatal conductance measurement, as determined by AP4 porometer; n = 6 plants (**b**); n = 42 for WT, n = 40 for *gad2-1*, n = 31 for *gad2-1/GC1::GAD2Δ* #10, n = 45 for *gad2-1/almt9-1*, n = 40 for *gad2-1/almt9-1/GC1::GAD2Δ* and n = 35 for *almt9-1*, data collected from four independent batches of plants (**c**). Epidermal strips were peeled and incubated in stomatal measurement buffer for 2 h under light before measurement; n = 86 for WT, *gad2-1*, *gad2-1/GC1::GAD2Δ* #10 and *almt9-1*, n = 95 for *gad2-1/almt9-1* and n = 87 for *gad2-1/almt9-1/GC1::GAD2Δ* (**d**). **e,** Reverse-transcriptional PCR quantification of *GAD2*, *ALMT9* and *Actin2* transcripts in WT, *gad2-1*, *gad2-1/almt9-2* and *almt9-2* plants, *Actin2* used as an internal control; similar results were obtained from three independent biological replicates. **f,** Stomatal aperture of WT (n = 115), *gad2-1* (n = 100), *gad2-1/almt9-2* (n = 106) and *almt9-2* (n = 104) plants; epidermal strips were incubated under light for 2 h before measurement. **g-j**, Stomatal response to ABA of Arabidopsis wildtype (WT), *gad2-1*, *gad2-1/almt9-1*, *almt9-1*, *gad2-1/almt9-2* and *almt9-2* plants. Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h under light, followed by 2 h treatment under light with or without 2.5 μM or 25 μM ABA as indicated; n = 189 (control) and n = 145 (ABA) for WT, n = 208 (control)

and n = 192 (ABA) for *gad2-1*, n = 200 (control) and n = 183 (ABA) for *gad2-1/almt9-1*, n = 182 (control) and n = 181 (ABA) for *almt9-1* (**g**); n = 184 (control) and n = 186 (GABA) for WT, n = 188 (control) and n = 207 (ABA) for *gad2-1*, n = 222 (control) and n = 224 (ABA) for *gad2-1/almt9-2*, n = 197 (control) and n = 182 (ABA) for *almt9-2* (**h**); n = 172 (control) and n = 196 (ABA) for WT, n = 190 (control) and n = 182 (ABA) for *gad2-1*, n = 162 (control) and n = 183 (ABA) for *gad2-1/almt9-1*, n = 192 (control) and n = 181 (ABA) for *almt9-1* (**i**); n = 215 (control) and n =178 (ABA) for WT, n = 197 (control) and n = 174 (ABA) for *gad2-1*, n = 189 (control) and n = 174 (ABA) for *gad2-1/almt9-2*, n = 195 (control) and n =180 (ABA) for *almt9-2* (**j**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**b-d, f, g-i**); statistically differences were determined by One-way ANOVA, **P* < 0.05, ****P* < 0.001 and *****P*< 0.0001 (**b-d, f**), or by Two-way ANOVA, a, b, c and d represent data groups that are not statistically different, *P* < 0.05 (**g-i**).

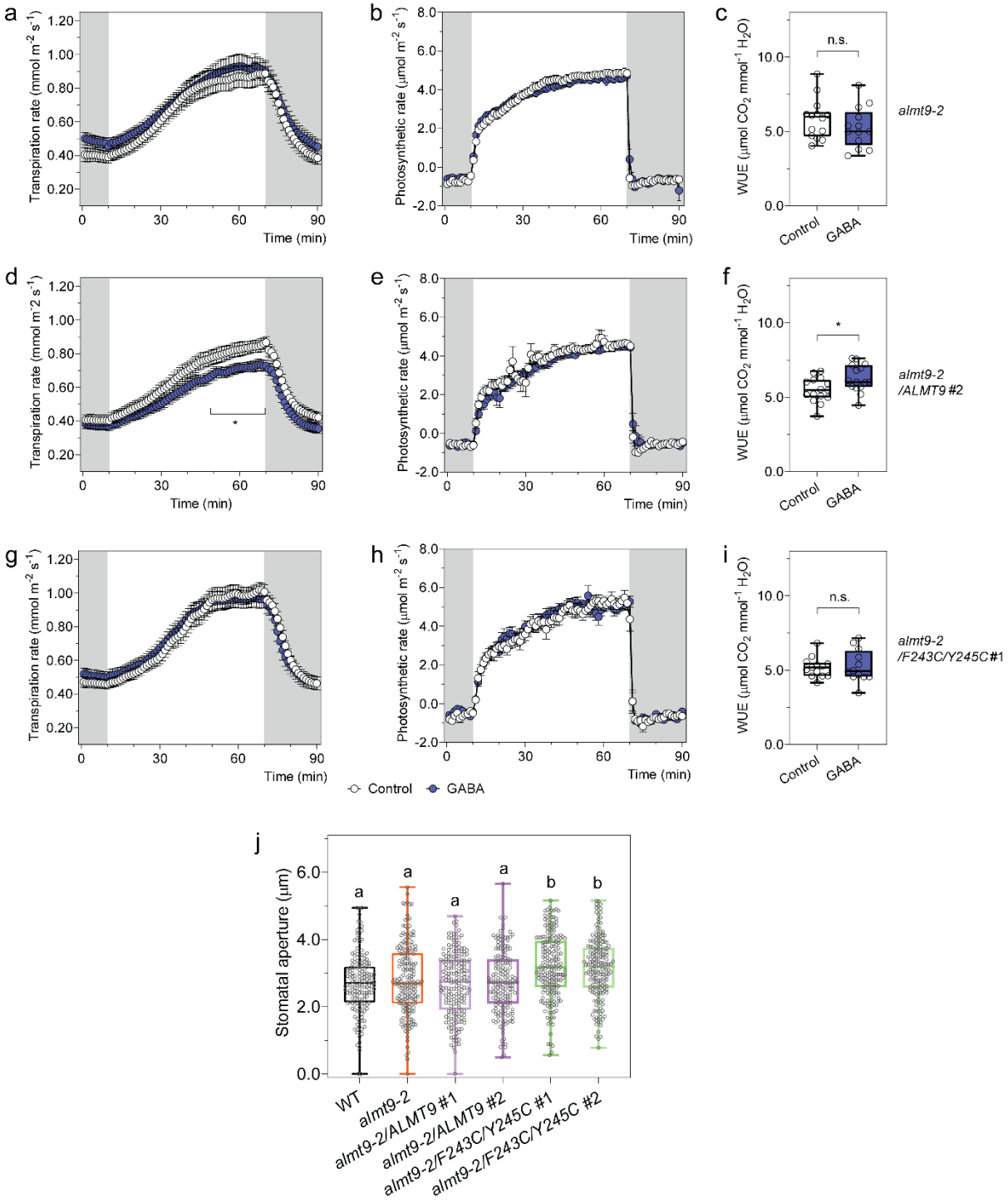


Supplementary Figure 15. *almt9* knockouts abolish muscimol-inhibition of stomatal opening but not affect closure. a-d, Stomatal aperture of wildtype (WT) and *almt9* knockout plants in response to dark or light. Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h under dark (**a-b**) or light (**c-d**), followed by light (**a-b**) or dark (**c-d**) for 2 h as indicated by black (dark) or white (light) bars above graphs, ± 10 µM muscimol (Musc); n = 105 for WT and n = 106 for *almt9-1* with control treatment, n = 106 for WT and n = 111 for *almt9-1* with muscimol treatment (**a**); n = 88 for wildtype (WT) (control); n = 108 for WT (control), n = 116 for *almt9-2* (control), n = 119 for WT (muscimol) and n = 121 for *almt9-2* (muscimol) (**b**); n = 210 for WT and n = 233 for *almt9-1* with control treatment, n = 240 for WT and n = 245 for *almt9-1* with muscimol treatment (**c**); n = 88 for WT (control), n = 96 for *almt9-2* (control), n = 90 for WT (muscimol) and n = 100 for *almt9-2* (muscimol) (**d**); all

experiments were repeated at least twice from different batches of plants with blind treatments (**a-d**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**a-d**); statistically differences were determined by Two-way ANOVA (**a-d**), *P < 0.05 and ****P< 0.0001.

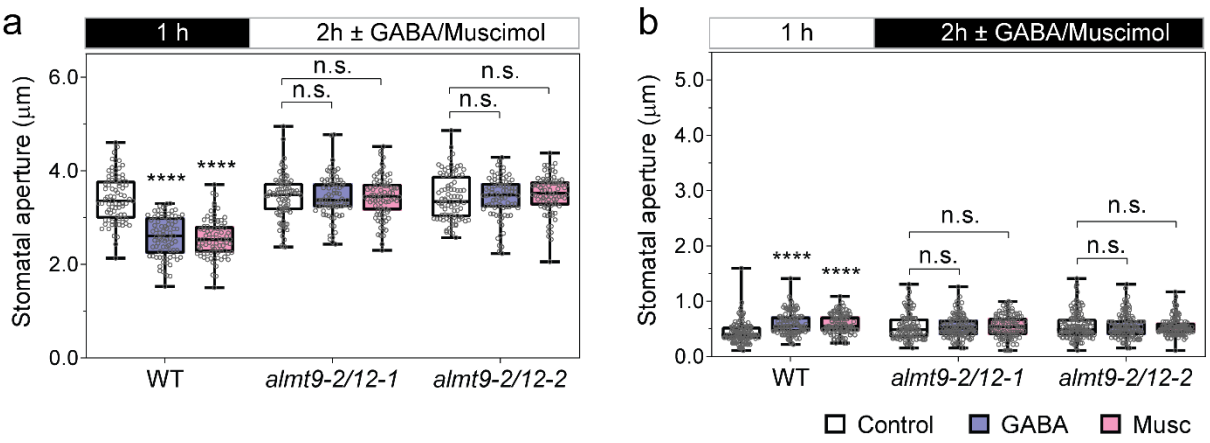

a
WT-like 2
almt9-2
almt9-2/ALMT9
almt9-2/F243C/Y245C
#1
#2
#1
#2
ALMT9
Actin2
1500 bp
500 bp
b
ALMT9
Relative expression level
0.04
0.03
0.02
0.01
0.00
a
b
c
ac
c
ac
WT-like-2
almt9-2
almt9-2/ALMT9 #1
almt9-2/ALMT9 #2
almt9-2/F243C/Y245C #1
almt9-2/F243C/Y245C #2
c
GFP+
Chlorophyll fluorescence
Brightfield
Mergred
ALMT9
ALMT9F243C/Y245C
Negative control
A. thaliana
d
ALMT9
ALMT9F243C/Y245C
N. benthamiana

Supplementary Figure 16. *ALMT9* and *ALMT9*$^{F243C/Y245C}$ show similar expression in *almt9-2* complementation lines and both are present on the tonoplast membrane. a-b, Reverse-transcriptional PCR (**a**) and quantitative real-time PCR (**b**) of *ALMT9* and *Actin2* transcripts in WT-like 2, *almt9-2*, *almt9-2/ALMT9* and *almt9-2/*F243C/Y245C plants. Similar results were obtained from three independent biological replicates (**a**); n = 3 plants and data represented by mean ± s.e.m, statistical difference was determined by two-sided Student's *t*-test, a, b and c represent data groups that are not statistically different, $P < 0.05$ (**b**). **c-d,** Subcellular localisation of ALMT9 and ALMT9$^{F243C/Y245C}$ in Arabidopsis (**c**) and *N. benthamiana* (**d**). Representative confocal image of ALMT9 and ALMT9$^{F243C/Y245C}$ tagged with GFP in the mesophyll protoplasts of *almt9-2/ALMT9* and *almt9-2/*F243C/Y245 complementation lines, repeated on 3 occasions with consistent results (**c**), or transiently expressed in *N. benthamiana* by Agrobacterium infiltration, repeated on 3 occasions with consistent results (**d**); the mesophyll protoplasts of wildtype (WT) Arabidopsis leaves were imaged as control (**c**), using the exact same setting to capture the fluorescence of GFP-tagged ALMT9 and ALMT9$^{F243C/Y245C}$. The mesophyll protoplasts were prepared and lysis as described[7, 8], GFP fluorescence (green) and chlorophyll autofluorescence (purple) captured by Nikon A1R Laser Scanning Confocal (**c, d**).



Supplementary Figure 17. *ALMT9* complementation but not by *ALMT9*$^{F234C/245C}$. **a-i**, Transpiration, photosynthetic rate and WUE of detached leaves from Arabidopsis *almt9-2* (**a-c**), *almt9-2/ALMT9* #2 (**d-f**) and *almt9-2*/F243C/Y245C #1 (**g-i**) fed with artificial xylem sap solution ± 4 mM GABA using a LiCor LI-6400XT. The WUE of *almt9-2* (**c**), *almt9-2/ALMT9* #2 (**f**) and *almt9-2*/F243C/Y245C #1 (**i**) was calculated the ratio of photosynthetic rate (**b, e, h**) versus transpiration rate (**a, d, g**); n = 14 (control)

and n = 13 (GABA) for *almt9-2* (**a-c**); n = 15 (control and GABA) for *almt9-2/ALMT9* #2 (**d-f**); n = 13 (control) and n = 12 (GABA) for *almt9-2/*F243C/Y245C #1 (**g-i**). **j**, Stomatal aperture of WT, *almt9-2* and complementation lines. Epidermal strips were peeled and incubated in stomatal measurement buffer for 2 h under light before measurement; n = 164 for WT, n = 185 for *almt9-2*, n = 190 for *almt9-2/ALMT9* #1, n = 169 for *almt9-2/ALMT9* #2, n = 198 for *almt9-2/*F243C/Y245C #1 and n = 186 for *almt9-2/*F243C/Y245C #2 (**j**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile (**c, f, i, j**); or data are represented by means ± s.e.m (**a, b, d, e, g, h**); statistically differences were determined by two-sided Student's *t*-test (**a-i**), $*P < 0.05$; or by One-way ANOVA, a and b represent data groups that are not statistically different, $P < 0.05$ (**j**).



Supplementary Figure 18. The loss of *ALMT9* and *ALMT12* impairs both stomatal opening and closure sensitivity to GABA. Epidermal strips were pre-incubated in stomatal measurement buffer for 1 h in the dark (**a**) or light (**b**), followed by 2 h incubation in the light (**a**) or dark (**b**) as indicated by black (dark) or white (light) bars above the plots ± 2 mM GABA or 10 μM muscimol (Musc); n = 78 for WT (control), n = 82 for *almt9-2/12-1* (control), n = 83 for *almt9-2/12-2* (control), n = 77 for WT (GABA), n = 77 for *almt9-2/12-1* (GABA), n = 81 for *almt9-2/12-2* (GABA), n = 75 for WT (Musc), n = 81 for *almt9-2/12-1* (Musc) and n = 81 for *almt9-2/12-2* (Musc) (**a**); n = 114 for WT (control), n = 104 for *almt9-2/12-1* (control), n = 120 for *almt9-2/12-2* (control), n = 113 for WT (GABA), n = 114 for *almt9-2/12-1* (GABA), n = 123 for *almt9-2/12-2* (GABA), n = 107 for WT (Musc), n = 106 for *almt9-2/12-1* (Musc) and n = 127 for *almt9-2/12-2* (Musc) (**b**). All data are plotted with box and whiskers plots: whiskers plot represents minimum and maximum values, and box plot represents second quartile, median and third quartile; statistical difference was determined using Two-way ANOVA, ****P < 0.0001; all experiments were repeated at least twice from different batches of plants with blind treatments.

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