SI Materials and Methods

Plant materials and low ψ_w stress treatment

For seedling growth and stress treatments, sterilized seeds were plated on half-strength MS medium with MES buffer (pH 5.7) but without added sugar. Plates were stratified for 4 days at 4°C and the seedlings were grown by placing the plates vertically in a growth chamber (25°C, continuous light at 80–100 µmol photons $m^{-2} s^{-1}$). For ABA measurements seven day-old seedlings were transferred to PEG-infused plates (1) to impose a -1.2 MPa low-ψ_w stress. Samples were collected at 96 h after transfer (or for Col-0 at other times for the time course analysis shown in Figure 1A). Typically, 2-4 genotypes were grown together on a single agar plate and the agar plate was included as a factor in the statistical analysis.

Arabidopsis accessions and T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center and T-DNA mutants genotyped (using primer sets generated by the Signal web resource; http://signal.salk.edu/) to isolate homozygous individuals. We sought to obtain multiple T-DNA lines for each candidate gene, although, but this was sometimes stymied by a lack of suitable T-DNA mutants or inability to verify that mutants lacked, or had reduced level, of the full length gene transcript. Seed harvested from such confirmed homozygous plants was used for further experiments while T-DNA lines which could not be unambiguously genotyped were removed from the analysis. An initial round of T-DNA mutant ABA phenotyping was performed along with reverse transcriptase PCR (RT-PCR) assays of selected lines to test for expression of the target gene (SI Appendix Fig. S3). Primers used for genotyping and RT-PCR analysis of T-DNA lines are given in Dataset S7. Based on the RT-PCR assays, additional T-DNA lines where we could not verify knockout or knockdown of the target gene were removed from the analysis and additional ABA measurements were performed for the remaining lines. ABA phenotyping of T-DNA mutants was performed using the same experimental conditions described above for quantifying ABA in natural accessions. For both the natural accessions and T-DNA mutants, the lines analyzed were randomized into several experimental blocks such that each line was represented in at least two independent experiments. For natural accessions, 4-6 samples from 2 or 3 independent experiments were analyzed, except for a small number of accessions where only one sample per experiment could be collected because of limited seed supply or poor germination. For T-DNA mutants, each T-DNA line was

represented in two or three independent experimental blocks and six to twelve ABA measurements were conducted for each T-DNA line. A combined statistical analysis for all T-DNA mutant ABA data was then performed (see below).

To produce transgenic lines with ectopic expression of *AT3G01130* or *AT1G34300*, full length cDNA was amplified from Col-0 wild type and used for TOPO cloning (primers listed in SI Dataset S7). After sequence verification, the cDNA clones were moved by Gateway recombination into the pEG100 vector (2) for 35S-driven expression. The vectors were transformed into *Agrobacterium tumefacians* strain GV3101 and used to generate transgenic plants in the Col-0 background by floral dip transformation. Transgenic plants were selected based on Basta resistance and homozygous T_3 lines used for ABA analysis.

ABA analysis

Samples were collected in 96 well plate format with typically 72 samples extracted and analyzed together along with standard curve and blank controls (referred to as "ABA analysis plate" below) (3). Seedling samples (20-50 mg) were freeze dried and extracted in 80% methanol with 25 pmol [D_6]ABA (Plant Biotechnology Institute, Saskatoon, Canada) as an internal standard. Extracts were passed through a C_{18} solid phase extraction cartridge (Supelco), evaporated to dryness, resuspended in diethyl ether:methanol (9:1), and derivatized by addition of trimethylsilydiazomethane (TCI). After derivatization, the remaining trimethyldiazomethane was destroyed by addition of 0.5 M acetic acid in hexane (4). The samples were then evaporated, resuspended in small volume of ethyl acetate, injected onto a VF-14MS (Varian/Agilent) column and analyzed by MS/MS. Methanol chemical ionization was used to generate precursor ions of 261 m/z for ABA and 267 m/z for [D_6]ABA. Daughter ions of 229 m/z (ABA) and 233+234 m/z ([D_6] ABA) were used for quantification (5). ABA amount was quantified by calculations based on a standard curve over 2-60 pmol of ABA using the ratio of the 229 and 233+234 peak areas (3).

Gene Expression

Total RNA was extracted from 8 day-old seedlings grown on half-strength MS agar medium using RNeasy plant mini kits (Qiagen). Complementary DNA (cDNA) was synthesized from 1μg of RNA using SuperScript III reverse transcriptase (Invitrogen) according to the

manufacture's protocol. Semi-quantitative RT-PCR for different T-DNA mutants was carried out in most cases using primers (Dataset S7) spanning the site of insertion that was identified from TAIR. *Actin2* or *UBQ10* was used as the reference gene.

Physiological Assays

Leaf water loss was assayed using four-week-old plants grown under long day conditions. Two fully expanded leaves were detached and placed in a tared plastic weighing boat and incubated in a growth chamber 25°C, continuous light at 80–100 μmol photons $m^{-2} s^{-1}$). Water loss was monitored by weighing each hour over the subsequent eight hours. Change in leaf weight monitored over the subsequence 8 hours. Seedling growth and proline content were assayed as previously described (6). For growth assays, five-day-old seedlings were transferred to either fresh control plates (-0.25 MPa) or PEG-infused plates of a moderate stress severity (-0.7 MPa). Root Elongation and seedling dry weight were measured after seven or ten days for the control and stress treatments, respectively. Four to six seedlings of wild type and four to six seedlings of one mutant line were grown in the same plate and growth of the mutant normalized to the wild type seedlings in the same plate. For proline assays, seven-day-old seedlings were transferred to -1.2 MPa PEG-infused plates and sample collected after 96 h of stress treatment. Proline was quantified by ninhydrin assay.

Statistical Analysis

We estimated genetic values for natural variation in ABA accumulation using a linear mixed model applied to the individual plant data. The 'lmer' function in the R package 'lme4' was used to fit a linear mixed effects model with a fixed intercept and random effects for genotype, ABA analysis plate, agar plate, and experiment. We then used random effects for genotype as the phenotype in ABA GWAS. GWAS was conducted using the efficient mixed-model association (EMMA) software package of Kang et al. (7). We similarly followed Kang et al. (7) and used identity-in-state at all SNPs to calculate the kinship matrix among genotypes.

To provide a heuristic guide to identify candidate genes associated with the lowest p-values SNPs or with the clusters of moderately low p-value (T1000) SNPs, each gene was assigned a score as previously described (8). The score was based on how many top 1000 ($p \leq$ 5.6 x 10^{-3}, 1 point each), top 100 ($p \leq 4.9$ x 10^{-4}, 2 points each) or top 20 ($p \leq 9.8$ x 10^{-5}, 3 points

each) were within 5 kb of any part of the gene body (UTR, exons and introns). The list of T1000 SNPs and associated genes is given in SI Dataset S2. Where ever the point total for a gene exceeded three, a region of interest was designated. This identified 116 regions of interest (SI Dataset S3, SI Appendix Fig. S2, regions are numbered 1-116 from chromosome 1 to 5). The total scores for each candidate gene was also ranked (SI Dataset S4). In choosing candidate genes to analyze by reverse genetics we systematically tested multiple genes in each of the five top scoring regions (these regions included the 1st, 2nd and 4th lowest P-value SNPs in the GWAS). Additional candidate genes to test were based on having a high score, clustering of low P-value SNPs in one or two candidate genes and, in some cases, existing gene annotation (for example, genes with previous indication of stress or ABA-related function).

T-DNA mutant ABA data was analyzed using a linear mixed-model approach with Proc Mixed in SAS v9.2 (SAS Institute, Inc). The global model included T-DNA insertion lines, experimental block, and ABA analysis plate (nested within experimental block) as fixed effects and agar plate as a random effect. Significance of each candidate gene was evaluated using planned contrasts of each knockout genotype versus Columbia wild type with the LSmeans statement and the difference option. The Type-1 error rate (alpha=0.05) for multiple testing was controlled using a strict Bonferroni correction. Bonferroni corrections considered each test region an independent hypothesis, and adjusted for either the number of genes or T-DNA lines assays within each region. Thus, the nominal alpha was divided by either the number of genes or T-DNA mutants tested in each region (e.g., 0.05/ number of genes tested in that region).

Climate Association

Climate factors at accession sites of origin were compiled and tested for association with ABA accumulation or with SNPs surrounding the genes with significant effect on ABA as previously described (9, 10). ABA accumulation had a marginal (not significant after accounting for population structure) association with wetter locations (see main text). In addition, ABA accumulation was significantly associated with the sixth (Spearman's rank correlation, rho = -0.15, p = 0.0272), 8th (rho = -0.14, p = 0.0470) and 10th (rho = 0.15, p = 0.0296) climate principal components (PCs). PC6 explained only 3 percent of total climate variation, and was associated with precipitation seasonality and altitude. Specifically, ABA accumulation was greater for values of PC6 with more temporal variability in precipitation and higher altitudes. However, in a

linear mixed model including random effects to control for population structure (7) these ABA-climate PC associations were not significant (all p > 0.07).

A broader scan of 124 individual climate variables [climate variables and data described in (10)], found a significant association of higher ABA accumulation with wetter locations (total growing season precipitation, rho = 0.16, p = 0.0202). Also, lower ABA accumulation was associated with higher May VPD with (rho = -0.15, p = 0.0342). After accounting for population structure in a linear mixed-model, the association with VPD in May was still significant (p = 0.0084); however, the association was largely driven by 6 outliers of high VPD (greater than 0.75 kPa) and low ABA accumulation (p = 0.54519 after removing outliers).

References for SI Materials and Methods

1. Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu JH, & Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant Journal* 45(4):523-539.
2. Earley KW*, et al.* (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant Journal* 45(4):616-629.
3. Verslues PE (2017) Rapid quantitation of Abscisic Acid by GC-MS/MS for studies of abiotic stress response. . *Plant Stress Tolerance: Methods and Protocols, Second Edition,* Methods in Molecular Biology, ed Sunkar R (Springer).
4. Schmelz EA*, et al.* (2003) Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. *Proc. Natl. Acad. Sci. U. S. A.* 100(18):10552-10557.
5. Muller A, Duchting P, & Weiler EW (2002) A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to Arabidopsis thaliana. *Planta* 216(1):44-56.
6. Bhaskara GB, Wen TN, Nguyen TT, & Verslues PE (2017) Protein Phosphatase 2Cs and Microtubule-Associated Stress Protein 1 Control Microtubule Stability, Plant Growth, and Drought Response. *Plant Cell* 29(1):169-191.
7. Kang HM*, et al.* (2008) Efficient control of population structure in model organism association mapping. *Genetics* 178(3):1709-1723.
8. Verslues PE, Lasky JR, Juenger TE, Liu TW, & Kumar MN (2014) Genome-Wide Association Mapping Combined with Reverse Genetics Identifies New Effectors of Low Water Potential-Induced Proline Accumulation in Arabidopsis. *Plant Physiology* 164(1):144-159.
9. Lasky JR*, et al.* (2014) Natural Variation in Abiotic Stress Responsive Gene Expression and Local Adaptation to Climate in Arabidopsis thaliana. *Molecular Biology and Evolution* 31(9):2283-2296.
10. Lasky JR*, et al.* (2012) Characterizing genomic variation of Arabidopsis thaliana: the roles of geography and climate. *Molecular Ecology* 21(22):5512-5529.

SI Appendix Figure S1: QQ plot of observed versus expected p-values for the ABA GWAS analysis.

Note: SI Appendix Figure S2 begins after Fig S9 below.

SI Appendix Figure S3: RT-PCR analysis of selected T-DNA mutant lines. Primers used for RT-PCR and sizes of PCR products are given in SI Dataset S7. Numbers beside the gel picture indicate that number of cycles of PCR amplification for each target gene. *Actin2* was amplified for 28 cycles as a control gene for all samples. Red indicates examples of T-DNA lines that were removed from the ABA analysis (additional discarded lines are not shown here).

SI Appendix Figure S4: ABA content of *prt6* mutants.
ABA accumulation of wild type and *prt6* mutants was measured after 96 h at
-1.2 MPa. Data are means ± S.E. (n = 7) and are representative of results from
three independent experiments. Asterisks indicate significant difference (p >
0.01 by T-test) compared to wild type.

**SI Appendix Figure S5: Expression of surrounding genes for T-DNA lines of
at1g29740 (Region 10)**
Two T-DNA lines with insertion AT1G29740 gave conflicting ABA phenotypic
data (main text Figure 2). Therefore expression of the RLK genes
surrounding *AT1G29740* was tested to see if the T-DNA insertions affected
the expression of these surrounding genes (for example if the promoter
present in the T-DNA sequence activated expression of these neighboring
genes). No differences in expression were found. Upper gel picture for each
gene indicates expression of that gene and the number to the right is the
number of PCR cycles used for amplification. *Actin2* was used as a reference
gene for all RT-PCR reactions. Primer sequences are given in SI Dataset S7.

SI Appendix Figure S6: ABA content of transgenic lines with ectopic expression of *AT3G01130* or *AT1G34300*.

ABA accumulation of wild type and transgenic lines was measured after 96 h at -1.2 MPa. Data are means ± S.E. (n = 3-9). Data are from three independent transgenic lines for each construct. The experiment was repeated with consistent results. Asterisks indicate significant difference ($p > 0.05$ by T-test) compared to wild type. Gel images below the graph shows semi-quantitative RT-PCR assays to verify increased expression of the target gene in each transgenic line. The *AT3G01130* transgenic lines had substantially increased expression. For *AT1G34300*, the transgenic lines had expression only slightly above the wild type and we were unable to recover lines with higher levels of expression (perhaps because of the effect of *at1g34300* on growth, see SI Appendix Figure S8). Gene expression was measured for whole seedlings collected 96 h at -1.2 MPa in the same experiments where ABA quantification was conducted. Ubiquitin10 (UBQ10) was used as a reference gene.

SI Appendix Figure S7: Physiological analysis of selected T-DNA mutants: Detached leaf water loss.

T-DNA mutants with significant effect on ABA accumulation (Figure 2, SI Dataset S5) were grown in soil under long day conditions and two fully expanded leaves from four week old plants were detached, placed in a tared plastic weighing boat and change in leaf weight monitored over the subsequence 8 hours. Data are means ± S.E. (n = 6 for wild type, n = 5 for mutants). Asterisks (*) indicate a significant difference (P ≤ 0.05) compared to wild type at the final time point.

SI Appendix Figure S8: Physiological analysis of selected T-DNA mutants: Root elongation and proline accumulation.

A. Root elongation and seedling dry weight were assayed for the same T-DNA mutants shown in SI Appendix Figure S7. Five-day-old seedlings were transferred from control media to plates of the indicated water potential and root elongation and dry weight measured after 7 days (-0.25 MPa) or ten days (-0.7 MPa). Four to six seedlings of wild type and four to six seedlings of one mutant line were grown in the same plate and growth of the mutant normalized to the wild type seedlings in the same plate. For wild type at -0.7 MPa root elongation and dry weight were decreased by approximated 70% compared to the unstressed control. Data are means ± S.E. (n = 5-9). Asterisks (*) indicates significant differences (P ≤ 0.05, one-sided T-test) of mutant compared to wild type. For *at3g23080*, *at2g17760* and *at1g34300*, the two bars are data from the two T-DNA lines shown in Figure 2 and Supplemental Dataset S5.

B. Proline accumulation measured after transfer of seven-day-old seedlings from unstressed control plates to -1.2 MPa PEG-infused plates for 96 h. Data are means ± S.E. (n = 3). No significant differences between mutants and wild type were found.

SI Appendix Figure S9: Analysis of additional ABA GWAS regions of interest

A. In region 48, T-DNA mutants of *AT3G21600* (which encodes a senescence and dehydration related protein) had marginally non-significant increase in ABA accumulation. In region 43, mutants of *AT3G12490* (which encodes the stress-associated protein CYS6) had no effect on ABA accumulation while a mutant of *AT3G12510* (which encodes a MADS box transcription factor) had marginally non-significant increase in ABA accumulation.

B. For region 36, three T-DNA mutants of *bZIP17* gave contrasting affects on ABA accumulation despite RT-PCR analysis showing that all three had knockout or knockdown (indicated by diagonal hash marks in the graph) of *bZIP14* expression (SI Appendix Figure S3). White asterisk indicates difference in ABA content that was significant based on Bonferonni corrected p-value.

C. None of the *bZIP17* T-DNA mutants affecting expression of the neighboring gene *AT2G40940* (which encodes ETHYLENE RESPONSE SENSOR 1, ERS1). Thus activated *ERS1* expression cannot explain the differing ABA accumulation of bZIP17 mutants.

Region 3

Region 4

Region 6

Region 9

Region 11

Region 14

Region 15

Region 16

Region 17

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Region 23

Region 26

Region 27

Region 42

Region 43

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Region 46

Region 47

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Region 50

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Region 54

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Region 62

Region 64

Region 65

Region 72

Region 74

−ln(p)

bp

ATG09640
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ATG09625
ATG09620
ATG09610
ATG09600
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