Variation in MPK12 affects water use efficiency in Arabidopsis and reveals a pleiotropic link between guard cell size and ABA response

David L. Des Maraisa,1, Lisa C. Auchinclossb, Emeline Sukamtoha, John K. McKayc, Tierney Logana, James H. Richardsb, and Thomas E. Juenger.a

aDepartment of Integrative Biology, Institute of Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712; bDepartment of Land, Air, and Water Resources, University of California, Davis, CA 95616-8627; and cDepartment of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523

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Plant water relations are critical for determining the distribution, persistence, and fitness of plant species. Studying the genetic basis of ecologically relevant traits, however, can be complicated by their complex genetic, physiological, and developmental basis and their interaction with the environment. Water use efficiency (WUE), the ratio of photosynthetic carbon assimilation to stomatal conductance to water, is a dynamic trait with tremendous ecological and agricultural importance whose genetic control is poorly understood. In the present study, we use a quantitative trait locus-mapping approach to locate, fine-map, clone, confirm, and characterize an allelic substitution that drives differences in WUE among natural accessions of Arabidopsis thaliana. We show that a single amino acid substitution in an abscisic acid-responsive kinase, AtMPK12, causes reduction in WUE, and we confirm its functional role using transgenics. We further demonstrate that natural alleles at AtMPK12 differ in their response to cellular and environmental cues, with the allele from the Cape Verde Islands (CVI) being less responsive to hormonal inhibition of stomatal opening and more responsive to short-term changes in vapor pressure deficit. We also show that the CVI allele results in constitutively larger stomata. Together, these differences cause higher stomatal conductance and lower WUE compared with the common allele. These physiological changes resulted in reduced whole-plant transpiration efficiency and reduced fitness under water-limited compared with well-watered conditions. Our work demonstrates how detailed analysis of naturally segregating functional variation can uncover the molecular and physiological basis of a key trait associated with plant performance in ecological and agricultural settings.

natural variation | abiotic stress | GxE interaction

Water availability is fundamental to nearly every aspect of plant biology and has likely imposed strong and recurring selective pressure on plant populations, impacting the evolution of plant form and physiology (1, 2). Accordingly, water availability and atmospheric demand—interacting with temperature—are fundamental determinants of plant distribution, abundance, and productivity worldwide (3). Temporal and geographic variations in water availability are therefore predicted to result in adaptation to optimize water use.

Photosynthesis requires both CO2 and water. CO2 is increasingly available from the atmosphere, but must be transported to chloroplasts within cells. Diffusion of CO2 in the gas phase through tiny pores in the surface of leaves called stomata brings CO2 into contact with the wet surfaces of mesophyll cells, where it diffuses in water and across membranes to chloroplasts. Inevitably the wet cell surfaces allow evaporation and water loss by diffusion of water vapor through stomata to the atmosphere. This water loss, transpiration, drives root water uptake and transport through the plant. When soil water is limiting or atmospheric demand high, partially closing stomata reduces water loss but at the cost of reduced CO2 uptake. This trade-off results in a fundamental constraint on land-plant form and physiology (4–6).

At the whole-plant level this trade-off is represented by a plant’s transpiration efficiency (TE), which is measured as the ratio of total biomass to total water consumption. TE is challenging to measure accurately, so more often leaf-level intrinsic water use efficiency (WUE; the ratio of photosynthetic carbon assimilation to stomatal conductance to water), or lifetime integrated proxies of WUE, such as the ratio of Δ13C to Δ12C (Δ13C or Δ12C) in leaf tissue, are used. Considerable within-species variation in WUE and TE has been identified in both crop (e.g., refs. 7–9) and natural plant species (e.g., refs. 4, 10–12). WUE and TE are common targets of artificial selection to optimize yield in water-limited agricultural environments. A variety of wheat with higher yield under rainfed, dry climate conditions in Australia was developed by selecting for low Δ13C as a proxy for high WUE (13). The effects of selection on WUE have also been demonstrated in the natural environment, where there are likely strong interactions between WUE and life-history strategies, particularly flowering time (10, 14, 15). Understanding the molecular, physiological, and developmental determinants of variation in WUE is therefore of critical importance for improving agricultural output with less water input, and for determining the evolutionary consequences of natural variation in plant water relations.

Significance

Water is essential for nearly all aspects of plant biology, though, for many plants, water is a limited resource. Water use efficiency measures the ratio of photosynthetic carbon fixation to water lost via leaf transpiration and is a critical determinant of plant productivity in field environments. We identify a molecular variant that drives variation in water use efficiency between two natural genotypes of Arabidopsis thaliana. We show that two alleles, distinguished by a single substitution in a signaling protein, cause whole-plant differences in plant physiological responses to environmental cues, demonstrating the molecular basis of a gene-by-environment interaction in a trait of interest to plant breeders and ecologists.


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1Present address: Arnold Arboretum and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138.

2To whom correspondence should be addressed. E-mail: tjuenger@austin.utexas.edu.

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Studies on the genetic basis of WUE reveal that quantitative trait loci (QTL) driving variation in WUE colocalize with QTL for leaf length, tiller number, and leaf nitrogen content in rice (16), flowering time, branch number, leaf nitrogen content, and above-ground biomass in Arabidopsis thaliana (17, 18), and leaf transpiration rate in Brassica oleracea (19). Collectively, these data suggest that WUE is a complex trait that may affect and be affected by myriad developmental, physiological, and life-history components in plants. Not surprisingly, laboratory mutants in many genes have been found to affect WUE and TE in A. thaliana and other model plant species. These include mutations affecting stomatal behavior [AtRD20 (20), AtOST1 and AtAABA2 (21), and Nicotiana MPK4 (22)], stomatal size [AtCESA7 (23)], stomatal density [AtERECTA (24)], and cuticular thickness and extent [AtCer9 (25)]. Many of these mutations also confer changes in plant stature and reduce seed yield, suggesting the possibility of functional trade-offs at the loci. It remains unknown if variation in any of these genes underlie WUE or TE QTL in natural populations or if such genes would make suitable targets in breeding programs to improve plant yield in water-limited environments.

In the present study, we fine-mapped and cloned an allelic variant underlying natural variation in WUE in A. thaliana. This variant is at a locus identified previously as delta2.1 in a cross between the temperate climate Landsberg erecta (Ler) accession and subtropical Cape Verde Island (CVI) accession. The CVI allele causes a 16.2% decrease in TE compared with Ler (18). We confirm the functional role played by the cloned variant using transgenic complementation and find that the CVI allele is characterized by a single amino acid substitution in a highly conserved region in MPK2, a protein with a known role in guard cell physiology. We show that the CVI allele confers higher stomatal conductance under well-watered conditions because of larger stomatal aperture and reduced sensitivity to ABA inhibition of stomatal opening. The CVI allele also causes greater sensitivity of stomatal closure to vapor pressure deficit (VPD). However, CVI-MPK2 retains normal function in ABA-mediated stomatal closure, suggesting that the allele does not affect all previously identified functions of MPK2.

Results

Characterization and Map-Based Cloning of the Delta2.1 QTL

Juenger et al. (18) identified five QTL associated with differences in WUE in the Ler × CVI mapping population, which collectively explained approximately 31% of variation in WUE in this cross. Two of these loci colocalized with QTL associated with Ler × CVI flowering time. In the present study we focused on QTL delta2.1, on linkage group 2, which explains the largest fraction of variance in WUE in the Ler × CVI treatment interaction. (ANOVA: F = 9.19, P < 0.0001). We therefore considered MPK2 to be a likely candidate for the gene underlying delta2.1.

An Amino Acid Substitution in MPK2 is the Causal Variant Underlying Delta2.1.

We next studied the Ler and CVI alleles at MPK2 to screen for nucleotide variants that could potentially lead to the observed QTL effects on WUE. The CVI-MPK2 allele contains a point substitution in exon 1 that causes an amino acid change from glycine to arginine at position 53 (Fig. L4). This glycine residue is conserved in all annotated A. thaliana MAP kinases (Fig. 1B) as well as rat ERK2 and FUS3 from Saccharomyces cerevisiae. In yeast, this glycine lies in a highly conserved loop between two β-sheets that form a side of the activation site of the kinase (29).

There are also three SNPs differentiating the Ler and CVI alleles located in introns and one SNP located 200-bp upstream of the MPK2 start codon. The CVI variants are shared with those found in Col-0 at the intergenic SNP and two of the intron SNPs, suggesting that these SNPs do not result in functional changes. We rejected the hypothesis that the intergenic SNP results in gene-expression differences between the Ler and CVI

![Figure 1](image)

Fig. 1. A single nucleotide change in CVI leads to a glycine to arginine substitution at residue 53 of A. thaliana MPK2. (A) Gene model of MPK2 from the Columbia accession (TAIR10) with SNPs differentiating Ler and CVI MPK2 alleles shown as vertical lines. Shaded areas in exons denote predicted untranslated regions. (B) The glycine residue is conserved in all annotated A. thaliana MAPKs. Gene tree adapted from ref. 40.
alleles by comparing MPK12 expression in Ler and the NIL (T test: \( t = 0.39, P = 0.708 \) (Fig. S4).

To test directly the functionality of MPK12 alleles and confirm the role of this gene in driving physiological variation at delta2.1, we cloned a 2.2-kb fragment containing the complete MPK12 coding sequence and upstream intergenic region from Ler into the pMDC162 binary vector. We used Agrobacterium tumefaciens to transform this plasmid into the NIL background and then screened T3 and T4 progeny for WUE. Multiple transgenic lines show complete complementation of the low WUE of the NIL (Tukey HSD test at \( \alpha = 0.05 \): all transgenics significantly higher WUE than the NIL, and five transgenics indistinguishable from Ler) (Fig. 2). In contrast, NIL plants transformed with a vector control retain low WUE. Collectively, these results suggest that the CVI exon 1 amino acid substitution alters MPK12 protein function and thereby drives the allelic difference underlying the delta2.1 QTL.

CVI-MPK12 Drives Higher Stomatal Conductance and Lower Transpiration Efficiency Under Well-Watered Conditions. Because WUE reflects the relationship between photosynthesis and water loss, variation in either of these two factors could lead to differences in WUE. We showed previously that the delta2.1 QTL does not cause differences in photosynthetic rate, suggesting that the lower WUE conferred by the CVI allele at this locus results from greater plant water use (18). Indeed, TE—measured as whole-plant biomass acquisition as a function of water consumption—is strongly and significantly lower in the NIL than in plants with the Ler-MPK12 allele (ANOVA: \( F = 50.44, P < 0.0001 \)) (Fig. 3A). The primary cause of water loss from herbaceous plants is transpiration via stomata. In addition, water can be lost from leaves to the drier atmosphere directly through the leaf cuticle. We tested for an effect on cuticular conductance and found no significant difference between Ler and the NIL (ANOVA: \( F = 0.60, P = 0.66 \)) (Fig. S5). In contrast, the stomatal conductance of the NIL under well-watered conditions is nearly twice that of plants containing the Ler-MPK12 allele (ANOVA: \( F = 51.47, P < 0.0001 \)) (Fig. 3B). The mpk12-1 allele in a Col-0 background likewise has much higher reference stomatal conductance than wild-type (Fig. S6). These data suggest that low WUE in the NIL can, in part, be explained by higher stomatal conductance conferred by CVI-MPK12 under well-watered conditions.

We explored two hypotheses regarding how the CVI-MPK12 allele drives lower WUE compared with Ler. First, CVI-MPK12 might increase stomatal conductance if it affects stomatal size because of perturbation of the stomatal developmental pathway or if it causes constitutively higher guard cell turgor, leading to larger stomatal apertures. Second, CVI-MPK12 might impair the ability of plants to control the aperture of stomata over short time scales via changes in guard cell turgor that result from endogenous or environmental signals.

CVI-MPK12 Plants Have Larger Guard Cells and Stomata. To address the first hypothesis, we measured the size of individual guard cells and also the complete stomatal apparatus in epidermal peels from the abaxial surface of mature leaves. We found that stomata of the NIL have significantly larger guard cells (Fig. 3C) (ANOVA \( F = 48.9, P < 0.0001 \)) and stomata (Fig. 3D) (ANOVA \( F = 43.2, P < 0.0001 \)) compared with plants containing the Ler-MPK12 allele. Two previous studies also found that single MPK12 mutants in a Columbia background had greater stomatal apertures, measured as the ratio of width to length, although the authors did not explicitly test this difference (27, 30).

CVI-MPK12 Shows Normal Guard Cell Closure But Altered Opening Response to ABA. Previous work in the Columbia accession identified MPK12 as a component of a reactive oxygen species (ROS)-mediated ABA signaling cascade in guard cells, in which the MPK12 transcript is highly and constitutively expressed (27). To test the hypothesis that the CVI allele results in lower WUE by impairing ABA-mediated guard cell behavior, we measured stomatal aperture in response to exogenous ABA in the NIL, Ler, and a representative transgenic line with the Ler allele in the NIL background (which displayed Ler-like WUE). In Columbia, as with most plants, exogenous ABA treatment results in the rapid closure of stomata because of turgor change in guard cells (31). We found that the ABA closure response of NIL stomata was indistinguishable from plants with the Ler allele (Fig. 4A; see Table S1 for ANOVA table). ABA also acts to inhibit stomatal reopening (32). We found that experimentally closed stomata of NIL plants reopen in the presence of exogenous ABA, whereas plants with the Ler allele remain closed (Fig. 4B; see Table S2 for ANOVA table). These results suggest that the CVI mutation in MPK12 partially impairs ABA-mediated inhibition of stomatal opening.

The stimulus for stomatal closure is often an ABA signal originating in plant roots, but cues arising at the leaf level are also very important (33). VPD measures the gradient between

**Fig. 2.** WUE, measured as \( ^{81}C_{\text{O}} \), of MPK12 alleles from CVI and Ler. Lower (more negative) values of \( ^{81}C_{\text{O}} \) indicate lower WUE. NIL contains a 45-kb introgression of CVI genome in a Ler background. Light gray bars are seven independent transgenic insertions of the Ler-MPK12 allele in a NIL background. Also shown is an empty vector transgenic control in the NIL background. \( n = 10–12 \) plants for each measurement. Vertical bars indicate 1 SE above and below the mean. Letters indicate results of a Tukey HSD test; bars not sharing letters are significantly different at \( \alpha = 0.05 \).

**Fig. 3.** Plant water relations under well-watered conditions. NIL plants have lower transpiration efficiency (A; \( n = 14 \) plants for each measurement), higher stomatal conductance (B; \( n = 14–17 \) leaves each on separate plants for each measurement across five independent experiments), longer guard cells (C; \( n = 80–160 \) guard cells from separate stomata for each measurement), and larger stomatal complexes (D; \( n = 80–160 \) stomata for each measurement) than plants containing the Ler-MPK12 allele. Vertical bars indicate 1 SE above and below the mean. Letters indicate results of a Tukey HSD test; bars not sharing letters are significantly different at \( \alpha = 0.05 \).
actual atmospheric vapor pressure and the vapor pressure of an atmosphere that is saturated with water, such as the intercellular spaces of leaves. Increasing VPD results in increased transpirational water loss from leaves, unless plants respond by reducing stomatal conductance; in fact, change in transpiration rate, which affects hydration level of guard cells or adjacent cells, may be the proximal cue for a response to VPD. We tested whether the CVI allele alters stomatal response to increasing VPD. Expressed as the change in stomatal conductance as a function of increasing VPD, the CVI-MPK12 allele confers a greater reduction in stomatal conductance than does the Ler allele (ANOVA: F = 22.83, P < 0.0001) (Fig. 4C). The mpk12-1 mutation in the Col-0 background also shows a stronger VPD response than does wild-type Col-0 (Fig. S6). These data suggest that altered MPK12 function does not impair the ability of stomata to respond to changes in VPD and, in fact, may enhance this response compared with the Ler and Col-0 alleles.

Discussion

Plant stomata play a critical role in maintaining plant water balance and in modulating the CO2 available for photosynthesis.

The Functional Basis of WUE. WUE is a complex trait that is affected by many other plant traits and can have a large influence on yield and fitness in the field environment. Evidence from several plant systems suggests an important role for variation in stomatal conductance in driving differences in WUE (34, 35). We show here that lower WUE in the A. thaliana accession CVI compared with Ler is driven in part by variation in stomatal conductance. The stomatal conductance of a leaf is a function of the number and size of stomata, as well as their dynamically controlled aperture, which can respond to environmental and developmental signals, such as light, moisture, circadian rhythms, hormones, and CO2 (33). Our data show that the CVI mutation in MPK12 affects both the size of stomata and their short-term response to environmental cues. One exciting hypothesis is that although stomate size is developmentally controlled, it is not necessarily genetically fixed within a plant and may show plasticity over the lifetime of a plant (36). Signals from mature leaves, exposed to ambient atmospheric conditions and therefore possibly varying in stomatal conductance through time, may be transmitted to newly emerging leaves that can adjust their developmental patterning to better match the current, perceived, local environment (37, 38). It remains to be determined, therefore, whether MPK12 plays a direct role in stomatal development or whether the effect is via altered transpiration rate caused by short-term ABA-mediated modulation of stomatal aperture.

MAP Kinases Play Diverse Roles in Stomatal Form and Function. Previously, Jammes et al. (27) demonstrated that MPK12 is involved in ROS-mediated stomatal closure in response to ABA signaling, although their results suggested that MPK12 was functionally redundant with a second, distantly related MAP kinase, MPK9 (27). By assaying phenotypes that integrate over the lifetime of plant tissues, we show here that MPK9 and MPK12 are not redundant in all MPK12 functions. Single mutations in MPK12—in both a Ler background (the NIL studied here) and Col-0 background (mpk12-1)—cause significant reductions in WUE (Fig. 2 for Ler and Fig. S3 for Col-0) and, for the Ler background, reduced TE (Fig. 34) (we have not assayed TE in Col-0 or mpk12-1). We also show that a single MPK12 mutation in both backgrounds increases stomatal conductance in a well-watered environment (Fig. 3B and Fig. S6). These plant-wide phenotypes are caused by significant increase in stomatal size (Fig. 3 C and D) and the impairment of the ABA inhibition of stomatal opening (Fig. 4B). This latter finding is at odds with prior work by Jammes et al., but the difference may be because of the fact that the substitution in CVI is at a different site, in a different protein domain, than the Col mpk12-1 allele, and that we tested its effect in a different genetic background (Ler).

It is interesting to note that single mutations in MPK12 do not significantly impair the ABA-mediated stomatal closure response but do alter stomatal response to increasing VPD. This pattern is seen in both Col-0 and Ler backgrounds. Two hypotheses, perhaps not independent of each other, might explain this finding. First, MPK12 may be redundant with MPK9 in ABA-mediated closure, as shown by Jammes et al. (27), but may not be functionally redundant in VPD response. A second hypothesis is that there is an ABA-independent pathway of stomatal closure in response to VPD.

Earlier studies have also shown that the stomatal apertures of MPK12 single mutants are larger than wild-type (27, 30), although the authors of those studies did not discuss this observation. Studies of Nicotiana MPK4, an ortholog of AtpMPK12 that, similarly, signals for stomatal closure in response to environmental cues, also found that expression knockdowns of Nicotiana MPK4 conferred larger stomata (22, 39). Additionally, Nicotiana attenuata plants with transgenically reduced expression of MPK4 show higher constitutive stomatal conductance to a similar extent as Cvi-MPK12. Phylogenetically, AtMPK12 and Nicotiana MPK4 are members of a small clade of MAPKs that also includes functionally divergent AtMPK4 and AtMPK12 (22).

MAPKs are ubiquitous enzymes that act in phosphorylation cascades. The 20 MAPKs, 10 MAPKKs, and 60 MAPKKKs predicted in A. thaliana hint at the tremendous combinatorial diversity of function that may be conferred by these proteins (40). A growing body of evidence suggests that MAP kinases...
form a molecular link between short- and long-term responses to the environment. For example, *A. thaliana* MPK3 and MPK6 play diverse roles in response to environmental cues (41, 42), and were recently shown to be components of non–ABA-mediated stomatal closure in response to pathogen exposure (43). MPK3 and MPK6 are also essential for normal stomatal development (44). We show here that natural variation at MPK12 can affect guard cell size in *A. thaliana* and that disrupting MPK12 reduces ABA-inhibition of stomatal opening and increases short-term sensitivity to increasing VPD. Future work should focus on whether MPK12 participates directly in the well-studied stomatal developmental pathway or if its effects are indirect.

### Methods

#### Plant Growth and Initial Phenotyping

Seeds were grown in randomized blocks in growth chambers under 12-h days at 22 °C/18 °C. We fine-mapped the delta2.1 locus by recurrent backcrosses of NIL delta-2.1 (18) to the Ler parent, resulting in a NIL (euB4A8) that represented ~45 kb of CVI genomic material in a homozygous Ler background. NIL-euB4A8 has low WUE compared with the Ler parent. NIL-euB4A8 is referred to as “NIL” throughout this report. For each round of backcrossing, and subsequent phenotyping of mutant and transgenic lines, we scored WUE as carbon isotope composition (δ13C), assayed by the University of California at Davis Stable Isotope Facility (http://stablesisotopelfacility.ucdavis.edu). δ13C is given relative to the Pee Dee Belemnite standard. Based on previous studies of guard cell physiology (27, 28), and our previous work demonstrating a strong role of stomatal conductance as the proximate cause of delta2.1 (18), we identified two genes in the NIL-euB4A8 interval, MPK12 (At2g46070) and At2g46090, as possible candidate genes underlying the delta2.1 QTL. We scored δ13C in two mutants of MPK12 identified by Jammes et al. (27) (mpk12-1 and mpk12-2), a T-DNA knock-in of At2g46090 (SALK 130222C), and Col-0 (CS70000); all three mutants were obtained from the Arabolipos Biological Resource Center. Additional details of plant growth and phenotyping can be found in *3 Methods*.

#### Cloning and Transgenics

We PCR-amplified the Ler genomic region containing MPK12, including the entire upstream sequence proximal to At2g46080, through the 3′ UTR of MPK12. We cloned into a pMDC162 binary vector and then introduced into NIL-euB4A8 via floral-dip transformation. All phenotype measurements were made on homozygous T3 or T4 lines. Initially, seven independent transgenic lines were phenotyped by carbon isotope analysis to confirm complementation. Subsequent assays were made only on representative lines, t.g. 1, t.g. 3, and t.g. 6.

#### Stomatal Characteristics and Cuticular Conductance

We grew plants until rosette leaves were large enough for individual leaf measurements with the fluorescence cuvette of the Li-6400 photosynthesis system (LiCor). Measurements of stomatal conductance (g), photosynthesis (A), and VPD were taken over several midday periods with varying cuvette relative humidity. Measurements were made at a minimum of four different relative humidities. For each plant, the regression of g versus ln(VPD) was calculated and used to determine reference g at VPD = 1 kPa and sensitivity of g to changing VPD (56). Genotypic differences in reference g and sensitivity were analyzed with one-way ANOVA. Relative cuticular conductance was determined by weighing rosettes every 5–10 min in a common temperature and relative humidity environment to determine the steady rate of water loss after complete stomatal closure. Genotypic differences in relative cuticular conductance were analyzed with ANOVA. Details of atmospheric conditions used to calculate stomatal conductance and response to VPD, as well as calculations of intrinsic WUE, can be found in *3 Methods*. Guard cells and stomata from fresh leaf peels of 21-d-old plants were imaged at 400x under a compound microscope, calibrated to an absolute scale, and then measured using ImageJ (57).

#### Stomatal Response to Abscisic Acid Stimulus

ABA-induced stomatal behavior assays were performed on fully expanded rosette leaves from 21-d-old plants. Ten to 20 stomata from three or four plants from each genotype were assayed in each experiment. For stomatal closure, following Pei et al. (58), leaves were detached and floated in buffer under bright light for 2 h. ABA was then added to treatment samples to a final concentration of 50 μM. After 1-h incubation, stomatal peels were visualized under a compound microscope and width and length were estimated using ImageJ. Stomatal aperture is reported as area, calculated as an ellipse. To measure ABA inhibition of stomatal opening, leaves were detached and wrapped in aluminum foil for 2 h to induce closing. The leaves were then floated under light in a solution with or without 20 μM ABA (27). Stomata were imaged and measured as above. To assess the role of ABA treatment and genotype on stomatal behavior, we fit ANOVAs implemented in JMP.

#### Whole-Plant Transpiration Efficiency

We grew 10 plants each of Ler, NIL-euB4A8, t.g. 3, and t.g. 6 in plastic cups filled with Sunshine MVP potting soil. Plants germinated on MS agar were transplanted to soil and then covered with paraffilm with two small holes, one for the plant and one to allow for watering. Water consumption was estimated daily by weighing water content; water was then readded with a pipette. After 20 d of growth, the complete above-ground plant was excised, dried, and weighed. TE was estimated as the ratio of total above-ground biomass to total water consumption (expressed in grams assuming 1 mL = 1 g) during growth. Genotypic differences were estimated by one-way ANOVA.

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The Molecular Basis of Genotype by Environment Interaction. MPK12 in *A. thaliana* shows genotype by environment interaction (GxE), with the CVI allele less sensitive to the effect of ABA on inhibition of stomatal opening, and more responsive to short-term changes in VPD than the Ler allele. This GxE is caused by a single amino acid substitution in a signaling protein that functions late in the ABA-mediated stomatal closure pathway (27, 43). Cloning allelic variants and confirming their functional effects allows us to identify the cellular basis of GxE and thereby allows an assessment of the proximate evolutionary processes that shape local adaptation to the environment. Our study reveals the finding that GxE in plants is driven by variation in many different proteins (45), from proteins that directly sense the environment [e.g., phytochromes (46, 47)] to signal-transduction components (e.g., MPK2) and biosynthetic enzymes [e.g., *PSC51* (48)]. It remains unclear how common these large-effect mutations, often identified in QTL-cloning exercises such as that performed here, are in natural populations.

In the present case, among 510 accessions thus far sequenced by the 1,001 genomes initiative (49), the CVI-MPK12 variant is found only in the CVI accession. However, we have identified three additional amino acid substitutions in the MPK12 kinase domain segregating in *A. thaliana*: S216L, V227L, and P239A. All three of these sites are highly conserved in the *A. thaliana* MPK gene family. A fourth substitution, V331M, is fairly common in *A. thaliana*, although located in a variable domain in the gene family. We cannot, therefore, rule out the hypothesis that additional independent variants at MPK12 drive variation in WUE in *A. thaliana*. The combination of geographical isolation and the unique tropical habitat of the CVI populations of *A. thaliana* present a challenge in distinguishing the role of selection versus random genetic drift. The relatively constant VPD and that disrupting MPK12 reduces ABA-inhibition of stomatal opening and increases short-term sensitivity to increasing VPD. Future work should focus on whether MPK12 participates directly in the well-studied stomatal developmental pathway or if its effects are indirect.
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Supporting Information

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SI Methods

Plant Growth and Initial Phenotyping. Seeds for the dry-down experiment were cold-stratified in water at 4 °C, sown directly onto Sunshine MVP potting soil (SunGro Horticulture), and grown in randomized blocks in the greenhouse with supplemental light (600 PAR) simulating long days (16 h). After establishment, dry-treatment plants received half of the watering applied to wet treatment plants. When plants ceased flowering and siliques were no longer expanding, we harvested all siliques. Fitness is reported as total fruit length (fruit number × average length of 10 random siliques from each plant). Estimating fitness as total biomas, or total fruit length on the log scale produced equivalent results.

Plants for all other experiments were sown as above and then grown in growth chambers under 12 h days at 22 °C/18 °C. Previous quantitative trait loci (QTL) analysis (1, 2) identified a locus on the long end of chromosome two, which underlies a difference in integrated water use efficiency (WUE) between the Landsberg erecta (Ler) and Cape Verde Island (CVI) genotypes. We fine-mapped this locus by recurrent backcrosses of near isogenic line (NIL) delta-2.1 (1) to the Ler parent, resulting in a NIL (NIL-euB4A8) that represented ∼45 kb of CVI genomic material in a homozygous Ler background. NIL-euB4A8 has low WUE compared with the Ler parent. We hybridized genomic DNA from three individuals each of Ler, CVI, and NIL-euB4A8 to the Affymetrix ATH1 microarray and confirmed that there were no other regions of CVI genome in the NIL-euB4A8 germplasm by performing probe-level ANOVA as described previously (3). For simplicity, NIL-euB4A8 is referred to as “NIL” throughout this report.

For each round of backcrossing and subsequent phenotyping of mutant and transgenic lines, we scored WUE as carbon isotope composition (δ13C). The complete rosette from 10 to 12 plants was excised and dried overnight in a drying oven at 80 °C. Samples were ground to a fine powder and assayed by the University of California at Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu/). δ13C is given relative to the PeeDee Belemnite standard, and we report composition rather than discrimination (Δ) because the isotopic composition of CO2 in the ambient air was highly variable in the growth chambers and greenhouses used (4).

Based on previous studies of guard cell physiology (5, 6), and our previous work demonstrating a strong role of stomatal conductance as the proximate cause of delta2.1 (1), we identified two genes in the NIL-euB4A8 interval, MPK12 (At2g46070) and At2g46090, as possible candidate genes underlying the delta2.1 QTL. We scored δ13C in two mutants of MPK12 identified by Jammes et al. (6) (mpk12-1 and mpk12-2), a T-DNA knock-in of At2g46070 (SALK 53022C), and Col-0 (CS70000); all three mutants were obtained from the Arabidopsis Biological Resource Center.

Cloning and Transgenics. We PCR-amplified the Ler genomic region containing MPK12, including the entire upstream sequence proximal to At2g46080, through the 3′UTR of MPK12. We cloned the resulting fragment into the pDONR221 plasmid via the Gateway BP reaction (Invitrogen). The MPK12 fragment was subcloned into a pMDC162 binary vector via the Gateway LR system (Clontech). All phenotype measurements were made on homozygous T3 or T4 lines. Initially, seven independent transgenic lines were phenotyped by carbon isotope analysis to confirm complementation. Subsequent assays were made only on representative lines, t.g. 1, t.g. 3, and t.g. 6.

Stomatal Conductance, Sensitivity to Vapor Pressure Deficit, Cuticular Conductance, and Intrinsic WUE. We grew plants of Ler, NIL-euB4A8, t.g. 1, t.g. 3, t.g. 6, mpk12-1 mutant, and Col in 5 × 5-cm pots with short (5 h), 20 °C days, 250 μmol·m−2·s−1 photosynthetic photon flux density, and 60% relative humidity until rosette leaves were large enough for individual leaf measurements with the fluorescence cuvette of the LI-6400 photosynthesis system (LiCor). Measurements of stomatal conductance (g), photosynthesis (A), and vapor pressure deficit (VPD; based on leaf temperature and cuvette relative humidity and air temperature) were taken over several midday periods with varying cuvette relative humidity (20–70%). All measurements are reported per unit leaf area. Measurements were made at a minimum of four different relative humidities spanning at least 30% relative humidity—and in most cases at six relative humidities spanning 40–50% relative humidity—with leaves approximately at growth temperature (20 ± 1 °C). For each plant, the regression of g versus ln(VPD) was calculated and used to determine reference g at VPD = 1 kPa [i.e., ln(VPD) = 0, which approximately corresponds to atmospheric 60% relative humidity at a leaf temperature of 20 °C] and sensitivity of g to changing VPD (8). Genotypic differences in reference g and sensitivity were analyzed with one-way ANOVA. After these measurements were taken, plants were covered overnight to bring them to full hydration, and rosettes were cut at the root-shoot junction 2 h after artificial “sunrise.” Relative cuticular conductance was determined by weighing the rosettes (every 5–10 min) in a common temperature and relative humidity environment to determine the steady rate of water loss after complete stomatal closure (http://prometheuswiki.publish.csiro.au/tiki-custom_home.php). Leaf water potential did not differ among genotypes when stomatal conductance fell to zero (ANOVA F = 1.04, α = 0.43), and averaged −1.29 MPa (SE 0.03). Genotypic differences in relative cuticular conductance were analyzed with ANOVA in JMP Pro version 10.0 (SAS Institute), including a block factor for the day of experiment, to account for differences in temperature and relative humidity during drydowns. Rosettes were then dried, ground to a fine powder, and analyzed for carbon isotope composition as above. Although not reported here, δ13C of these plants corresponded to the genotypic values given for a larger set of plants in Fig. 2. In addition, δ13C of these plants correlated with single leaf, intrinsic WUE calculated as A/g at VPD of 1 kPa (r = 0.48, P < 0.0001, n = 80).

Stomatal Response to Abscisic Acid Stimulus. ABA-induced stomatal behavior assays were performed on fully expanded rosette leaves from 21-d-old Ler, NIL-euB4A8, and transgenic line 3. Ten to 20 stomata from three or four plants from each genotype were assayed in each experiment. For stomatal closure, following Pei et al. (9), leaves were detached and floated in 20 mM KCl, 5 mM Mes·KOH, pH 6.15, under bright light for 2 h. ABA (Sigma-Aldrich) was then added to treatment samples to a final concentration of 50 μM. After 1-h incubation, stomatal peels were visualized under a compound microscope and width and length were estimated using ImageJ (10). Stomatal aperture is reported as area, calculated as an ellipse (π × 0.5 width × 0.5 length). To measure ABA inhibition of stomatal opening, leaves were detached and wrapped in aluminum foil for 2 h to induce closing. The leaves were then floated under light in a solution of

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5 mM KCl, 10 mM Mes·KOH, 50 μM CaCl₂, pH 6.15, with or without 20 μM ABA (6). Stomata were imaged and measured as above. To assess the role of ABA treatment and genotype on stomatal behavior, we fit ANOVAs with treatment, plant, and genotype*treatment effects, with plant as a random effect, implemented in JMP.

**Whole-Plant Transpiration Efficiency.** We grew 10 plants each of Ler, NIL-euB4A8, t.g. 3, and t.g. 6 in plastic cups filled with 250 mL of Sunshine MVP potting soil. Plants germinated on MS agar were transplanted to soil and then covered with parafilm with two small holes, one for the plant and one to allow for watering. Water consumption was estimated daily by weighing of soil water content; water was then readded with a pipette. After 20 d of growth, the complete above-ground plant was excised, dried for 48 h in an 80 °C drying oven, and weighed. Transpiration efficiency (TE) was estimated as the ratio of total above ground biomass to total water consumption (expressed in grams assuming 1 mL = 1 g) during growth. Genotypic differences were estimated by one-way ANOVA.

**Quantitative PCR.** We removed fully expanded leaves from 21-d-old plants of Ler and NIL euB4A8 and extracted RNA using the Spectrum Plant Total RNA Kit (Sigma Aldrich). We performed quantitative PCR reactions on MPK12 and three control genes (At2g28390, At4g34270, and At3g18780). We used ProbeLibrary (Roche Applied Science) for the experiments with ABgene one-step quantitative PCR reagents and the ViiA7 real-time PCR machine (Applied Biosystems). Three biological replicates were run. Relative mRNA abundance was determined by Ct for each reaction. We performed a one-way ANOVA with genotype as a fixed effect to test the hypothesis that MPK12 expression differed between NIL euB4A8 and Ler.


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**Fig. S1.** Fitness of Ler and NIL-delta2.1 in well-watered and water-restricted environments. Letters indicate results of a Tukey-honest significant difference (HSD) test; bars not sharing letters are significantly different at α = 0.05. n = 9 for each estimate.
Fig. S2. Details of fine-mapping the delta2.1 interval. H011-01, NIL3, AG-2-17-5, and AG1-1-69 are NILs derived from NIL-delta2.1, which was described previously (1). Blue indicates CVI genomic material; red indicates Ler genomic material. Vertical lines along chromosomes represent markers used in genotyping. For visualization purposes, recombination breakpoints are assumed to be equidistant between markers.

Fig. S3. WUE for mutations in two candidate genes underlying delta2.1. mapK12-1 and mapK12-2 were identified by Jammes et al. (6) from a TILLING population. At2g46090 is a putative sphingosine kinase. Letters indicate results of a Tukey HSD test; bars not sharing letters are significantly different at α = 0.05.

Fig. S4. Expression of MPK12 as determined by quantitative PCR, shown as the Ct score of MPK12 minus the geometric mean of three control genes: At2g28390, At4g34270, and At3g18780. n = 3.
Fig. S5. Cuticular conductance of *Ler*, the NIL, and transgenic *Ler-MPK12* lines, as estimated by the rate of water loss following stomatal closure from excised leaves. *n* = 9–26 for different lines.

![Cuticular conductance graph](image)

Fig. S6. (A) Stomatal conductance of wild-type Col-0 plants and *mpk12-1* mutants at a reference VPD. (B) Change in stomatal conductance as a function of increasing VPD. Vertical bars indicate one SEM. *n* = 13 for Col-0, 4 for *mpk12-1*.

![Stomatal conductance graph](image)

Table S1. ANOVA results from the stomatal closure assay

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Table S2. ANOVA results from the stomatal opening assay

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