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

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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

**W**ater 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 CO<sub>2</sub> and water. CO<sub>2</sub> is increasingly available from the atmosphere, but must diffuse to chloroplasts within cells. Diffusion of CO<sub>2</sub> in the gas phase through tiny pores in the surface of leaves called stomata brings CO<sub>2</sub> 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 CO<sub>2</sub> 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 <sup>13</sup>C to <sup>12</sup>C ( $\Delta^{13}\text{C}$  or  $\delta^{13}\text{C}$ ) 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  $\Delta^{13}\text{C}$  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 water relations via inducible and constitutive mechanisms. Furthermore, we show that the alleles respond differently 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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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 *AtABA2* (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 residue in MPK12, 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-MPK12 retains normal function in ABA-mediated stomatal closure, suggesting that the allele does not affect all previously identified functions of MPK12.

## 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 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 and did not colocalize with any detected loci associated with flowering time.

We used a previously generated near isogenic line (NIL) containing a small introgression from the CVI genome in a *Ler* background (NIL-delta2.1) to fine-map the delta2.1 locus. We used  $\delta^{13}\text{C}$ , the ratio of tissue  $^{13}\text{C}$  and  $^{12}\text{C}$ , a widely-used proxy for WUE (26), to estimate WUE throughout this study. NIL-delta2.1 has lower WUE (more negative  $\delta^{13}\text{C}$ ) (18) and has no difference in fitness under well-watered conditions compared with *Ler*, but shows significantly lower fitness under water-limited conditions (Fig. S1) (ANOVA testing genotype × treatment interaction:  $F = 10.01$ ,  $P = 0.003$ ). Using recurrent backcrosses of NIL-delta2.1 to the *Ler* parent, we created a NIL (NIL-euB4A8; hereafter “NIL”), representing an introgression of ~45 kb of CVI material in a *Ler* background, which discriminates against  $^{13}\text{C}$  to the same extent as CVI (Fig. S2).

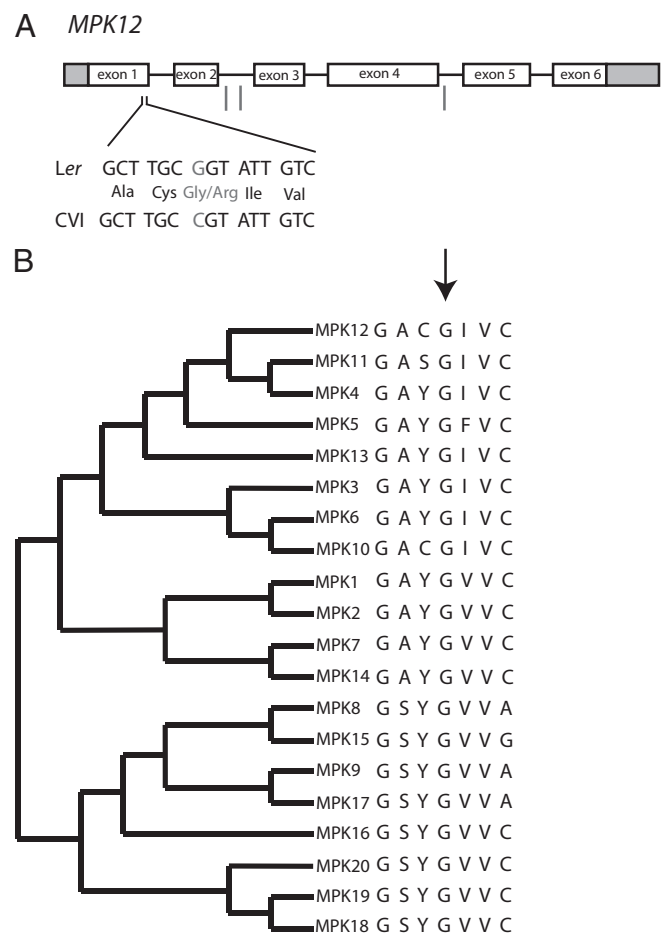
The 45-kb introgression interval is predicted to encode 16 ORFs in the Columbia reference sequence (TAIR10). Because we showed previously that the delta2.1 QTL affects stomatal conductance to a much greater extent than photosynthetic rate (18), we focused our efforts on two candidate genes in this interval with possible signaling roles in guard cells, *MAPK12* [At2g46070 (27)] and a putative sphingosine kinase [At2g46090

(28)]. Although a SALK T-DNA insertion in At2g46090 has no apparent effect on WUE [results of the Tukey honest significant difference (HSD) test at  $\alpha = 0.05$ ] (Fig. S3), either of two mutations in *MPK12* identified by Jammes et al. (27) cause a significant decrease in WUE compared with Col-0 (ANOVA:  $F = 9.19$ ,  $P < 0.0001$ ). We therefore considered *MPK12* to be a likely candidate for the gene underlying delta2.1.

### An Amino Acid Substitution in MPK12 Is the Causal Variant Underlying Delta2.1.

We next studied the *Ler* and CVI alleles at *MPK12* to screen for nucleotide variants that could potentially lead to the observed QTL effects on WUE. The CVI-MPK12 allele contains a point substitution in exon 1 that causes an amino acid change from glycine to arginine at position 53 (Fig. 1A). 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  $\beta$ -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 *MPK12* 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



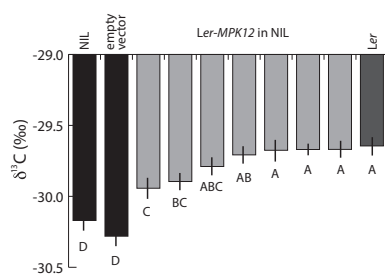
**Fig. 1.** A single nucleotide change in CVI leads to a glycine to arginine substitution at residue 53 of *A. thaliana* MPK12. (A) Gene model of *MPK12* from the Columbia accession (TAIR10) with SNPs differentiating *Ler* and CVI *MPK12* alleles shown as vertical lines. Shaded areas in exons denote 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).

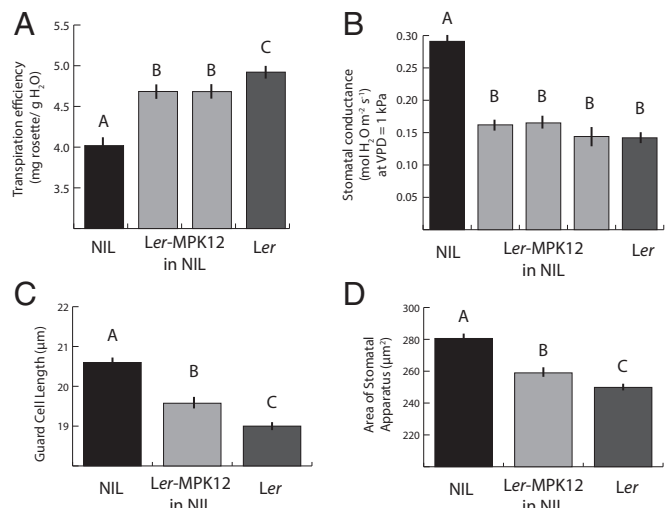
To test directly the functionality of *MPK12* alleles and confirm the role of this gene in driving physiological variation at delta2.1, we cloned a 2.2-kb fragment containing the complete *MPK12* coding sequence and upstream intergenic region from *Ler* into the pMDC162 binary vector. We used *Agrobacterium 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



**Fig. 2.** WUE, measured as  $\delta^{13}\text{C}$ , of *MPK12* alleles from CVI and *Ler*. Lower (more negative) values of  $\delta^{13}\text{C}$  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  $\alpha = 0.05$ .

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



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  $\text{CO}_2$  available for photosynthesis. It is not surprising, then, that their size, shape, distribution, and behavior are controlled dynamically and that genetic variation in these parameters has strong effects on WUE. Our data show that natural alleles at *MPK12* in *A. thaliana* affect WUE via the determination of stomatal size and via the inducible modulation of stomatal aperture. As such, *MPK12* may serve as an integration point between short-term and long-term needs of the plant to balance uptake of  $\text{CO}_2$  and leaf water status. Our work also identifies the molecular genetic basis of ecophysiological variation in nature, providing an example of a protein coding-sequence variant that underlies genotype by environment interaction.

**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  $\text{CO}_2$  (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.

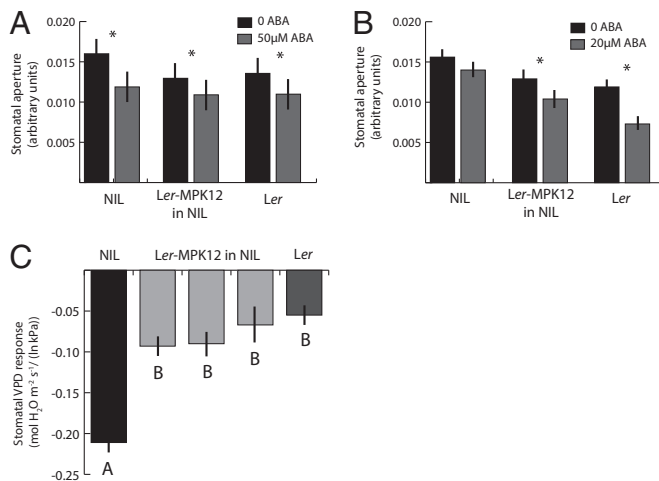
## MAP Kinases Play Diverse Roles in Stomatal Form and Function.

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. 3A) (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. 3C 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 *AtMPK12* 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 *AtMPK11* (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



**Fig. 4.** Response of *MPK12* alleles to external stimuli. (A) ABA induces stomatal closure in plants containing either the *Ler* or CVI *MPK12* allele. Asterisk indicates significant effect of treatment at  $\alpha = 0.05$ . (B) ABA inhibits opening of chemically closed stomates in *Ler* and *Ler-MPK12* transformed NIL, but does not inhibit opening in the NIL.  $n = 40$ –50 stomata across three or four plants for each value in stomatal response assays. Asterisk indicates significant effect of treatment at  $\alpha = 0.05$ . (C) NIL shows a greater stomatal closure response to increasing VPD than *Ler* and *Ler-MPK12* transformed plants. Letters indicate results of a Tukey HSD test; bars not sharing letters are significantly different at  $\alpha = 0.05$ .  $n = 14$ –17 for each measurement,  $n = 5$  for the third transgenic line. Vertical bars indicate 1 SE above and below the mean in all three panels.

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.

**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 reinforces the finding that GxE in plants is driven by variation in many different molecular components (45), from proteins that directly sense the environment [e.g., phytochromes (46, 47)] to signal-transduction components (e.g., *MPK12*) and biosynthetic enzymes [e.g., *P5CS1* (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 moderate temperatures of the CVI environment may permit the persistence of the CVI-*MPK12* allele. Ultimately, field studies and reciprocal transplants could address whether the CVI mutation represents an adaptive response to the local climate. Additional sampling from the CVI will reveal the frequency of the CVI-*MPK12* allele in its natural habitat and may clarify its genetic interaction with other functional variants identified in the CVI accession [e.g., *CRY2* (50), *DOG1* (51), *CBF2* (52), and vacuolar invertase (53)].

The phenomenon of a genotype producing different phenotypes in response to different environmental conditions is a ubiquitous aspect of biology (54). Over the past century, three major conceptual approaches have been used to analyze phenotypic plasticity: physiology, molecular biology, and quantitative genetics (55). Each field aims to describe mechanisms underlying how different environments alter the phenotype of individual genotypes or determine how genotypes differ in response to the same environment. These approaches vary in organismal scale, the degree to which they are biologically mechanistic, and the way in which the system is perturbed. Here we combined these perspectives, using natural variation as the source of perturbation, molecular approaches to isolate the effect of the individual QTL, and prior information on the physiological pathway to dissect interactions underlying the variation. This work advances our understanding of the agriculturally and evolutionarily important trait of WUE in plants.

## 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 ( $\delta^{13}\text{C}$ ), assayed by the University of California at Davis Stable Isotope Facility (<http://stableisotopefacility.ucdavis.edu>).  $\delta^{13}\text{C}$  is given relative to the PeeDee 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  $\delta^{13}\text{C}$  in two mutants of *MPK12* identified by Jammes et al. (27) (mpk12-1 and mpk12-2), a T-DNA knock-in of At2g46090 (SALK 53022C), and Col-0 (CS70000); all three mutants were obtained from the *Arabidopsis* Biological Resource Center. Additional details of plant growth and phenotyping can be found in *SI 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  $\ln(\text{VPD})$  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 *SI 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  $\mu\text{M}$ . 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  $\mu\text{M}$  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 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, 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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