Natural variation identifies new effectors of water-use efficiency in *Arabidopsis*

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Water-use efficiency (WUE) is the ratio of biomass produced per unit of water consumed; thus, it can be altered by genetic factors that affect either side of the ratio. In the present study, we exploited natural variation for WUE to discover loci affecting either biomass accumulation or water use as factors affecting WUE. Genome-wide association studies (GWAS) using integrated WUE measured through carbon isotope discrimination ($\delta^{13}C$) of *Arabidopsis thaliana* accessions identified genomic regions associated with WUE. Reverse genetic analysis of 70 candidate genes selected based on the GWAS results and transcriptome data identified 25 genes affecting WUE as measured by gravimetric and $\delta^{13}C$ analyses. Mutants of four genes had higher WUE than wild type, while mutants of the other 21 genes had lower WUE. The differences in WUE were caused by either altered biomass or water consumption (or both). Stomatal density (SD) was not a primary cause of altered WUE in these mutants. Leaf surface temperatures indicated that transpiration differed for mutants of 16 genes, but generally biomass accumulation had a greater effect on WUE. The genes we identified are involved in diverse cellular processes, including hormone and calcium signaling, meristematic activity, photosynthesis, flowering time, leaf/vasculature development, and cell wall composition; however, none of them had been previously linked to WUE. Thus, our study successfully identified effectors of WUE that can be used to understand the genetic basis of WUE and improve crop productivity.

WUE plasticity | carbon isotope | genome-wide association mapping | leaf temperature | plant cysteine oxidases

Plants move large amounts of water through the soil–plant–atmosphere continuum by water uptake from roots to loss of water to the atmosphere through stomatal pores. Plants can limit water loss by closing stomata or by having fewer or smaller stomata. However, this must be balanced with the need to maintain sufficient gas exchange for CO_2 to enter the leaf for photosynthesis (1). If gas exchange through the stomata is too restricted, depletion of internal leaf CO_2 can limit photosynthesis and hence, plant biomass and productivity (2). Globally, agriculture accounts for over 80% of freshwater use (3). Therefore, improving water-use efficiency (WUE) or enhancing water productivity (i.e., more yield per unit of water consumed) is important for crop improvement (4). However, efforts to improve WUE have met with limited success thus far (5).

Molecular approaches have demonstrated that reduced stomatal conductance (g_s) can improve WUE without reducing biomass accumulation (4, 6). Since abscisic acid (ABA) is the key inducer of stomatal closure, several studies have targeted ABA signaling genes to reduce g_s. Several transgenic strategies to alter ABA sensitivity led to increased WUE while maintaining high biomass and grain yield under progressive drought (7–9). Ectopic expression or mutation of genes that regulate stomatal development can also lead to increased WUE via effects on stomatal density (SD) (6). While these studies successfully increased WUE by manipulating previously described regulatory genes, other approaches are needed to allow an unbiased and broader search for new effectors of WUE. Particularly, the studies mentioned above focused on manipulating the transpiration side of WUE by changing stomatal density or altering stomatal opening and closing behavior. It is less clear whether changing the other side of WUE, biomass accumulation, can also be a path to increased WUE. Because of the complexity of plant growth regulation, this side of WUE is less amenable to manipulation by targeting a priori candidate genes.

Plants exhibit natural genetic variation in WUE (10–16). This includes both variation in the transpiration (stomatal behavior and density) as well as the biomass (biomass and plasticity of biomass during water limitation) sides of WUE 4). Quantitative trait loci (QTL) mapping has identified many QTL driving variation in $\delta^{13}C$ (the ratio of ^{13}C to ^{12}C), a widely used proxy for integrated WUE (4). One such study found that a single amino acid change in *Mitogen-Activated Protein Kinase 12* (*MPK12*) is

Significance

Sustainable water use is critical for agricultural productivity. Most of the water that plants take up from soil is transpired through stomatal pores while CO_2 enters the leaf. Thus, plants trade water for photosynthetic carbon. Balancing the amount of water lost relative to biomass accumulated, usually referred to as water-use efficiency (WUE), is a critical determinant of crop performance in water-limited environments. We used GWAS and reverse genetics to discover genes underlying WUE variation in *Arabidopsis thaliana*. These genes affected WUE by altering biomass accumulation or altering water consumption (or both in some cases). These results greatly expand the range of genes that may be manipulated to enhance WUE and contribute to sustainable water use in agriculture.

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responsible for variation in stomatal conductance and WUE in *Arabidopsis* accession Cvi-0 (17, 18). Another QTL analysis discovered that *ERECTA*, a gene involved in stomatal development, contributes to variation in $\delta^{13}C$ and transpiration efficiency (19) . Genome-wide association studies (GWAS) using large panels of natural accessions are an attractive alternative to biparental linkage mapping. In *Arabidopsis*, there is substantial natural variation in many water use– and drought-related traits (20–23) and the decay of linkage disequilibrium occurs over a relatively short interval. Thus, once single nucleotide polymorphisms (SNPs) associated with a particular trait are identified, testing only a few candidate genes in the vicinity of strongly associated SNPs, or a cluster of moderately associated SNPs, may find a gene(s) affecting the trait of interest (22, 23). Integrating other information, such as transcriptome data, can further assist in identifying the most promising candidate genes (24). The combination of GWAS and reverse genetic testing of candidate genes allows for an open-ended search for effector genes. This is particularly useful for traits, such as WUE, where it is difficult to apply traditional forward genetic screening. To date, only a few studies have employed GWAS to identify candidate loci affecting WUE (24–28) and there is relatively little data on validation of candidate genes identified by GWAS for drought-related traits (22, 23).

We applied GWAS and reverse genetic testing to discover loci affecting WUE plasticity whereby *Arabidopsis* accessions increase, or fail to increase, WUE in response to soil drying. Such an approach allowed us to find genes that had not been previously implicated in WUE, including genes affecting the biomass accumulation side of WUE rather than the transpiration side. Indeed, the largest effect we observed was in mutants of *Plant Cysteine Oxidase 5* (*PCO5*), which affects WUE mainly by allowing increased biomass production. Our study identified several WUE effector genes that offer routes to manipulate and improve this key trait.

Results

GWAS Analysis to Identify Genomic Regions Associated with Variation in WUE Plasticity. We leveraged the measurements of integrated WUE as $\delta^{13}C$ of above-ground biomass of well-watered plants or plants subjected to terminal drought from Kenney et al. (21). We reanalyzed the core subset of 185 accessions (Dataset S1). Most accessions had a higher WUE in the drought treatment (higher $\delta^{13}C$, F = 42.89, $P < 0.0001$; Fig. 1*A*). Only a few accessions had decreased or stable WUE in the drought treatment compared to the unstressed (wet) control (Fig. 1*A*). GWAS was performed using the plasticity of WUE (Fig. 1*B*; plasticity = drought − well-watered control (wet); mean of −0.585 and range −3.35 to 1.75) as well as the WUE of the well-watered control and drought treatments (*SI Appendix*, Fig. S1). While all three sets of GWAS results identified potentially interesting associations, we focused on the WUE plasticity GWAS for further study, since the ability to change WUE in response to changing environmental conditions may be an important adaptive trait whose regulation is poorly understood. Moreover, we hypothesized that selecting genes from WUE plasticity GWAS would discover genes affecting both sides (transpiration or biomass accumulation) of the WUE ratio. The SNPs most significantly associated with WUE plasticity were distributed across several genomic regions (Fig. 1*C*). As GWAS generates many candidates, selecting the most promising genes for the follow-up functional study is challenging. Thus, we used several layers of data to identify and prioritize a large set of candidate genes for high-throughput testing, rather than studying the molecular mechanisms of any particular gene (*SI Appendix*, Fig. S2*A*). We first generated a list of candidate genes for which any part of the gene body (untranslated regions [UTRs], introns, and exons) was within 5 kb of one or more of the top 500 lowest *P*-value SNPs (nominal $P = 4.37 \times 10^{-5}$ to 4.2×10^{-3}). The 1,058 genes so identified (Dataset S2) were
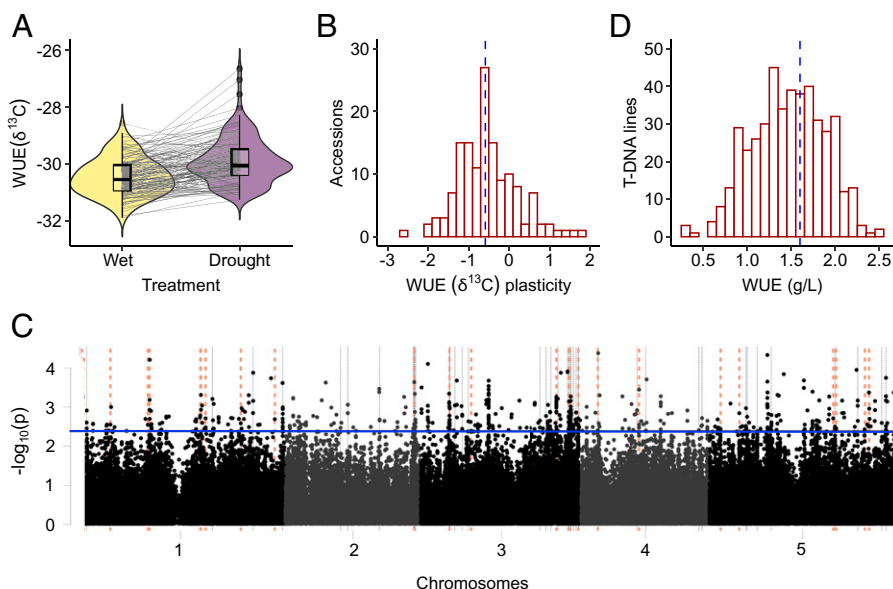

Fig. 1. Natural variation, GWAS analysis, and reverse genetic tests of candidate genes for WUE by gravimetric and $\delta^{13}C$ analyses. (*A*) Plastic response of 185 *Arabidopsis* accessions to terminal drought. WUE ($\delta^{13}C$ of above-ground biomass) values are pooled accession values within each treatment. (*B*) Distribution of WUE ($\delta^{13}C$) plasticity (difference in $\delta^{13}C$ between drought and wet) for 185 *Arabidopsis* accessions. Blue vertical line indicates the median. (*C*) Manhattan plot of SNP *P* values from the GWAS analysis using the WUE plasticity data shown in *A*. The blue horizontal line indicates the cutoff for the top 500 low *P*-value SNPs. The vertical lines indicate the genomic positions for the prioritized GWAS candidates for which we analyzed WUE (biomass produced per unit of water consumed) using reverse genetics. Gray lines indicate location of genes where the T-DNA mutant(s) did not have significantly altered WUE and orange dotted lines indicate genes where the T-DNA mutant(s) did have significant effect on WUE. (*D*) Distribution of gravimetric WUE (g/L, ratio of biomass to water consumed) for 88 T-DNA mutants from high throughput screening using small containers (n = 3 to 10 biological replicates per genotype). Blue vertical line indicates the mean of Col-0 wild type.

then categorized based on their stress-induced gene expression to further determine which genes were most likely to be involved in WUE plasticity. Stress-responsive changes in gene expression were identified by referring to transcriptomic studies (29, 30) (Datasets S3 and S4) that examined gene expression during acclimation to an extended duration of nonlethal drought stress conditions similar to those used to measure WUE plasticity in the current study. This analysis identified 198 genes that were associated with the top 500 WUE plasticity SNPs and that were up-regulated during drought stress (*SI Appendix*, Fig. S2*B* and Dataset S5). These genes were further subjected to Gene Ontology (GO) enrichment analysis, which identified 10 significant functional annotation terms (*SI Appendix*, Figs. S2*C* and S3 and Dataset S6). To incorporate a range of gene functions into our validation experiments, we selected the top 10 genes from each enriched GO term based on the lowest *P* values of their associated SNPs. We also included all 19 genes from the transcriptional regulation GO cluster. This filtering identified 72 candidate genes (Dataset S7). One limitation of this approach is that it may discard poorly annotated genes. We found 29 such genes annotated as proteins of unknown functions associated with the top 500 SNPs and were transcriptionally responsive to stress (Dataset S5, genes highlighted in red). Thus, we also included those 29 genes in our functional study.

Reverse Genetic Analysis of Prioritized Genomic Regions. We isolated 88 homozygous T-DNA insertion lines covering 70 candidate genes (Dataset S9). We were unable to obtain homozygous T-DNA lines for the other 31 prioritized genes. (T-DNA alleles for all prioritized genes are given in Dataset S8). These homozygous lines were tested for their effect on WUE by gravimetric analysis using a high-throughput closed container system with well moistened soil (Fig. 1*D* and *SI Appendix*, Fig. S4*A* and *B*). This first round of screening identified mutants for 27 genes that significantly differed from wild type for WUE (*SI Appendix*, Fig. S4*A* and Datasets S10 and S11). Among these 27 genes, only 9 genes had previously known roles in abiotic stress (Dataset S12).

These mutants were further analyzed for their effect on WUE by two independent gravimetric analyses using larger containers (237 mL) to allow the plants a larger rooting volume and space for rosette biomass (*SI Appendix*, Fig. S4*C*). We also measured $\delta^{13}C$ of above-ground biomass, leaf surface temperatures, SD, and stomatal index (SI) (Dataset S13). This set of measurements included *mitochondrial editing factor11* (*mef11-5*) and *open stomata kinase* (*ost1-3;* OST1 is also referred to as SnRK2.6) mutants as controls known to affect water loss. *mef11-5* is hypersensitive to ABA-mediated stomatal closure and exhibits elevated leaf temperatures and reduced water loss under water stress (31, 32). In contrast, *ost1-3* has reduced ABA sensitivity, leading to reduced stomatal closure, greater leaf water loss, and reduced leaf temperature (33, 34). Note that mutants for two genes, *Cytochrome P 450 family 76* (*CYP76C2*) and *AT3G58660*, did not germinate for unknown reasons, and so we were unable to include them in our further study. We found a significant positive correlation ($R^2 = 0.646$, $P < 0.001$) for WUE between smaller containers (high-throughput system) and larger containers for these mutants (*SI Appendix*, Fig. S5*A*).

These experiments identified genes that altered either side (biomass accumulation or transpiration) of WUE (Fig. 2*A*). For example, *plant cysteine oxidase 5* (*pco5-1* and *pco5-2*) had an increase in biomass (2.8-fold), which gave it a higher WUE despite the fact that it consumed substantially more water

(1.9-fold) than the Col-0 wild type (Fig. 2*A*, purple circles). A few other mutants had no change in biomass but showed moderately reduced water consumption (significant based on nominal P value), leading to increased WUE (Fig. 2*A*, blue circles). Several other mutants had moderately decreased WUE mainly due to decreased biomass accumulation with no significant change in water consumption (Fig. 2, green circles). We also found mutants with strong reduction in biomass as well as water consumption. However, changes in biomass had a relatively larger impact on WUE in these mutants (Fig. 2*A*, orange circles). The two control mutants *mef11-5* and *ost1-3* showed high and low WUE, respectively (Fig. 2*A*), consistent with their effects on ABA sensitivity. Interestingly, *mef11-5* was the only mutant in our analysis where decreased biomass was associated with increased WUE. Across these mutants, there was a strong correlation between above-ground biomass gain and water consumption (Fig. 2*B*; $R^2 = 0.89$, $P < 0.001$). We also found that WUE was moderately correlated with biomass (Fig. 2*C*; $R^2 = 0.628$, $P < 0.001$) and weakly correlated with water consumption (Fig. 2*D*; $R^2 = 0.364$, $P < 0.001$), suggesting that the majority of mutants analyzed primarily altered the biomass side of the WUE ratio. In addition, we found a relatively weak correlation between WUE and SD (Fig. 2*E*; $R^2 = 0.142$, $P < 0.017$), and no significant relationship between WUE and stomatal index (*SI Appendix*, Fig. S5*B*; $R^2 = 0.083$, $P < 0.057$), suggesting that changes in SD or SI were not the main driver of altered WUE in our set of mutants. The correlation between gravimetrically determined WUE and $\delta^{13}C$ was significant and positive (Fig. 2*F*; $R^2 = 0.546$, $P < 0.001$). We discuss sets of discovered genes based on their impact on WUE, biomass, and water consumption below.

Genes That Act as Negative Effectors of WUE. We found that mutants of *Plant cysteine oxidase 5* (*pco5-1 and 2*), *Defective UGE in root* (*dur-1*), *Calcium-dependent protein kinase 23* (*cpk23*), and *Nuclear speckle localized RNAa* (*nsra-1 and 2*) had increased WUE, indicating that the proteins encoded by these genes have a negative effect on WUE (Fig. 3). None of these mutants affected SD (*SI Appendix*, Fig. S6*A*). Two mutant alleles of *PCO5* had a strongly significant increase in gravimetric WUE and $\delta^{13}C$ while also producing more biomass and consuming more water (Fig. 2*A*, purple circles). Thus, mutants of *PCO5* were more water productive despite having significantly reduced leaf temperatures (Fig. 3 and *SI Appendix*, Fig. S7) indicative of a higher transpiration rate. Since *pco5* mutants had no effect on SD, the reduced leaf temperature suggests that stomatal size regulation of stomatal aperture may instead be altered. *PCO5* is one of five cysteine oxidases involved in N-end rule protein degradation and hypoxia response (35).

Mutants of three other genes in this category, *cpk23*, *dur-1*, and both *nsra* alleles, had significantly increased WUE. These mutants had no effect on biomass but showed moderately reduced water consumption, suggesting that they altered the transpiration side of WUE (Figs. 2*A*, blue circles and Fig. 3). Among these, *cpk23* had the strongest effect on WUE. *dur-1* had a marginally nonsignificant ($P = 0.08$) increase in $\delta^{13}C$, consistent with the gravimetric analysis. (For marginally nonsignificant effect, nominal *P* values are listed in the text and in figures throughout.) *cpk23* and *dur-1* had elevated leaf temperatures, whereas *nsra-1* and *nsra-2* had decreased leaf temperatures compared to wild type (Fig. 3 and *SI Appendix*, Fig. S7). The control mutant *mef11-5* had a moderate decrease in biomass and strongly reduced water consumption leading to increased WUE. It had no effect on SD (*SI Appendix*, Fig. S6*A*) but did
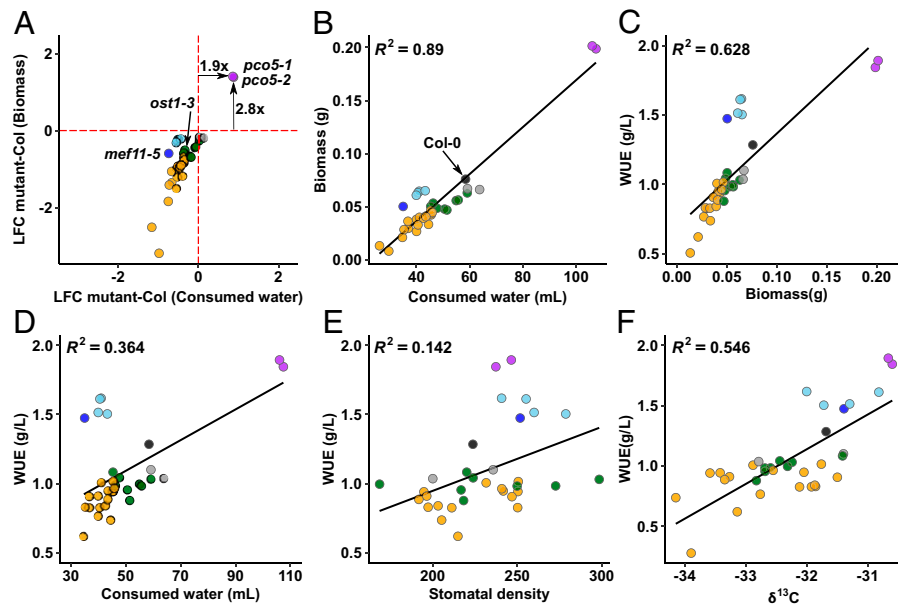
Fig. 2. Reverse genetic tests of candidate genes for WUE by gravimetric analysis using larger containers. (*A*) Relative contribution of biomass and consumed water of a given mutant as a factor leading to altered WUE (g/L, biomass produced per water consumed). Graph shows the Log2 fold changes (LFCs) of above-ground biomass and consumed water for a given mutant compared to the Col-0 reference (dotted red lines). Mean fold change of water consumption and biomass compared to Col-0 was mentioned for *PCO5* mutants at gene level. The altered WUE, changes in biomass, and consumed water is indicated by color-filled circles; high WUE with significant increase in biomass as well as consumed water (purple), high WUE with significant decrease in biomass as well as consumed water (dark blue), high WUE with no significant effect on biomass but reduced water consumption (light blue), low WUE with significantly decreased biomass with no significant effect on consumed water (green), low WUE with significant decrease in biomass as well as consumed water (orange), and low WUE with no significant effect on either side (biomass accumulation or consumed water) of WUE (gray). For the correlation analysis (*B–F*), mean value for Col-0 and individual T-DNA lines of each gene were plotted (*n* = 5 to 10 biological replicates for each data point). (*B*) Association of above-ground biomass with consumed water. (*C*) Association of WUE with above-ground biomass and (*D*) consumed water. (*E*) Association of WUE with stomatal density. Note that, stomatal measurements for a few mutants with reduced growth, the number of samples is less (*n* = 3 to 4 biological replicates from two independent experiments) because of difficulty in obtaining epidermal peels from those mutants. Three other mutants were omitted from this analysis for the same reason (*SI Appendix*, Fig. S6). (*F*) Association of $\delta^{13}C$ with WUE (g/L). $\delta^{13}C$ was measured for the above-ground biomass as shown in *B*. Colors in *B–F* are as in *A* except for the Col-0, which is represented by a black-filled circle.

have elevated leaf temperature (Fig. 3 and *SI Appendix*, Fig. S7), consistent with previous reports (31, 32). The elevated leaf temperatures of *cpk23*, *dur-1*, and *mef11-5* are consistent with their reduced water consumption. However, the decreased leaf temperatures for the *NSRa* mutants contrast with their reduced water consumption. It is possible that although *NSRa* mutants did not have significantly reduced biomass, they may have had reduced leaf area that could explain the apparent mismatch between water consumption and leaf temperature. *DUR* encodes a uridine diphosphate (UDP)-glucose epimerase (UGE) involved in UDP-arabinose biosynthesis, possibly affecting cell wall properties (36). NSRa is an RNA binding protein with no previous information to connect it to stress responses or stomatal development. NSRa, and related NSRs, affect alternative splicing and thus could influence activity of downstream genes leading to increased WUE.

Mutants Related to Hormone, Calcium, or Stress Signaling Had Decreased Growth Leading to Decreased WUE and Water Consumption. Mutants of 13 genes had a strong decrease in WUE mainly due to a strong reduction in biomass even though their water consumption was also reduced (Fig. 2*A*, orange circles and Fig. 4). None of these mutants affected SD (*SI Appendix*, Fig. S6*B*). Mutants of the first five genes in this category *mterf defective in Arabidopsis* (*mda1-3*), *cytochrome P450, family 7070a3* (*cyp707a3-3*), *dehydration responsive element-binding protein 2a* (*dreb2a-1 and 2*), *general control nonrepressible 20* (*gcn20-2*), and a hypothetical protein with unknown function (*at1g49170*) had a strong decrease in biomass as well as strongly reduced water consumption (Fig. 4). However,

biomass was more strongly reduced, leading to a substantial decrease in WUE in these mutants. Decreased $\delta^{13}C$ in these mutants was also consistent with the gravimetric WUE. *MDA1*, *CYP707A3*, and *DREB2a*, were previously reported to affect ABA and water stress signaling, whereas *GCN20* has roles in hormone signaling (37). *MDA1* encodes for mitochondrial transcription termination factor (mTERF). *mda1* was previously shown to enhance salt and osmotic stress tolerance probably due to reduced sensitivity to ABA (38). *CYP707A3* acts as an ABA 8′-hydroxylase involved in ABA catabolism (39). *cyp707a3-3* had elevated leaf temperatures (Fig. 4 and *SI Appendix*, Fig. S8) consistent with its reduced water consumption and with previous observations of lower stomatal conductance and higher basal ABA levels in unstressed *cyp707a3* plants (39). *DREB2a* is a transcription factor that functions in both water and heat-stress responses (40). Of the two *dreb2a* alleles we examined, *dreb2a-3* had a stronger effect than *dreb2a-4*, consistent with *dreb2a-4* being a knockdown, rather than knockout (41). Both mutant alleles of *DREB2a* had decreased leaf temperatures (Fig. 4 and *SI Appendix*, Fig. S8), indicating that they may affect WUE by controlling stomatal aperture. *GCN20* encodes an ABC transporter family protein. Mutants of *GCN20* were defective in MAMP/bacterium-triggered stomatal closure but respond normally to salicylic acid (SA) and ABA (37). *gcn20-2* had a decrease in leaf temperatures and no change in SD, consistent with a previous report (37). The *AT1G49170* mutant also had decreased leaf temperature (Fig. 4 and *SI Appendix*, Fig. S8).

Mutants of the next six genes in this category include *osmo-sensitive calcium-permeable cation channel 3.1/early responsive to dehydration 4* (*osca3.1/erd4*), *jasmonic acid* (JA), *transporter 4*
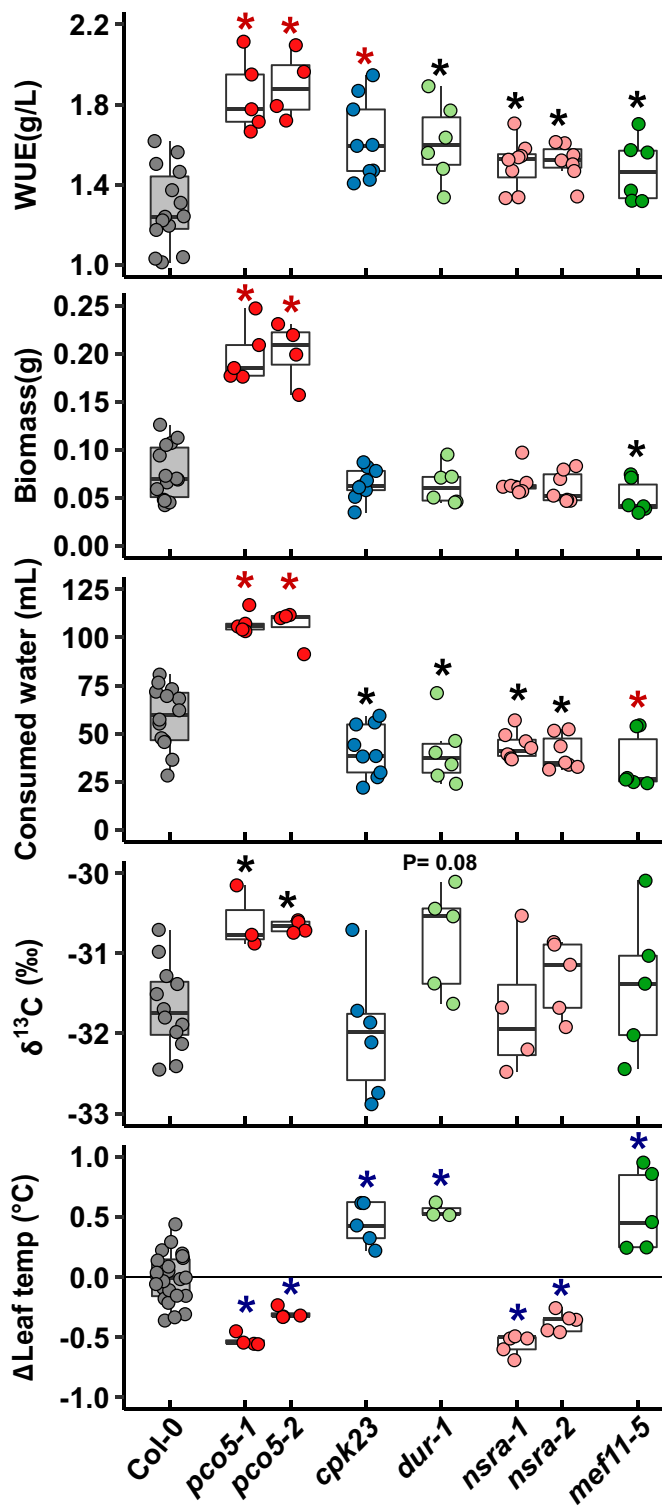
Fig. 3. Mutants of four genes and control mutant *mef11-5* had higher WUE than wild type. WUE, above-ground biomass, consumed water, $\delta^{13}C$ analysis for the above ground biomass and leaf temperatures of the plants for which WUE was measured are presented. The box plots indicate the first and third quartiles with a median line and the whiskers represent the 1.5× interquartile range. Asterisks indicate statistically significant difference for a comparison of mutants against Col-0 wild type (red: strongly significant difference [adjusted $P < 0.05$], black: moderate effect based on nominal P value) (Dataset S12). For other lines, nominal P values are listed. Note that, the leaf temperatures data were collected from the second independent experiment ($n = 3$ to 5 biological replicates). For imaging, biological replicates of a set of mutants were gathered with Col-0 replicates, which generated repeated measures of Col-0. Our statistical analyses are based on comparing each mutant with relevant Col-0 replicate data collected as a block. For mutants, the difference of leaf temperatures (Δ = mutant − Col-0) were plotted and

(*jat4/atjat4*), *histone acetyl transferase* (*hac5*), *suppressor of max2 1–like 5* (*smxl5*), *AT1G03687*, and *grana deficient chloroplast 1* (*gdc1*) had strong decreases in biomass with moderately reduced water consumption, indicating that biomass was the driver for the decreased WUE of these mutants (Fig. 2*A*, orange circles). OSCA3.1 and JAT4 have known or highly probable roles in stress or hormone signaling (42, 43) and the mutants of *OSCA3.1* and *JAT4* showed strong decreases in WUE. Similarly, *hac5-6*, *smxl5-2*, mutants of *AT1G03687*, and *gdc1-2* all had strongly decreased WUE. HAC5 functions in the transcriptional repression of genes related to flowering time and floral development (44). *hac5-6* had a marginally nonsignificant decrease in leaf temperatures ($P = 0.09$) (Fig. 4 and *SI Appendix*, Fig. S9). SMXL5 promotes secondary phloem formation (45) and *smxl5-2* had decreased $\delta^{13}C$ consistent with gravimetric WUE. The altered WUE in *smxl5-2* might be due to up-regulation of several stress-related pathways in this mutant (45). The function for AT1G03687 is unknown. Both mutants of *AT1G03687* had strong decreases in $\delta^{13}C$ consistent with gravimetric WUE. GDC1 is a ankyrin domain containing chloroplast protein essential for grana formation (46). *gdc1-2* had a marginally nonsignificant decrease in water consumption ($P = 0.08$) and $\delta^{13}C$ ($P = 0.057$) and showed increased leaf temperature. Consistent with previous reports, *gdc1-2* exhibited pale green leaves and ceased growth at the vegetative stage (46), indicating that this mutant had decreased WUE because of impaired photosynthesis.

The next gene in this category was *IQ-domain 11* (*IQD11*), another Ca^{2+} responsive protein involved in regulation of microtubule orientation (47). Among two T-DNA alleles, only *iqd11-1* showed a strong decrease in WUE, biomass, and $\delta^{13}C$, while *iqd11-2* had a more moderate effect on these phenotypes and was not significant for $\delta^{13}C$. *iqd11-2* has a T-DNA insertion in the 5′ UTR and thus is likely a knockdown rather than knockout mutant (Dataset S11). Note that *iqd11-1* had a marginally nonsignificant decrease in water consumption ($P = 0.058$). Both mutants of *IQD11* had decreased leaf temperatures (Fig. 4 and *SI Appendix*, Fig. S9). *Parallel 1/Nucleoline-1* (*PARL1/NUC1*) is involved in many aspects of ribosomal biogenesis and affects leaf venation (48). *parl1-2* is the only mutant in this category that had a moderate decrease in WUE. It had decreased $\delta^{13}C$ consistent with gravimetric WUE and showed elevated leaf temperature (Fig. 4 and *SI Appendix*, Fig. S9).

The changes in leaf temperatures for mutants in this category suggest their role in stomatal response (Fig. 4 and *SI Appendix*, Figs. S8 and S9). The elevated leaf temperatures found for *cyp707a3-3*, *gdc1-2*, and *parl1-2* was consistent with their reduced water consumption; however, decreased leaf temperatures found for mutants of *DREB2a*, *GCN20*, *HAC5*, and *IQD11* contrasts with their reduced water consumption. Here again, change in leaf area or rosette architecture may explain the seeming mismatch between leaf temperature and water consumption.

Mutants with Reduced WUE and Biomass but with Little Effect on Total Water Consumption. Mutants for six genes, *With No Lysine* (*WNK11*), *Plethora 1* (*PLT1*), *AT2G45460*, *AT3G62220*, *Basic Penta Cysteine* (*BPC2*), and *Carbon Catabolite Repressor 4b* (*CCR4b*), had decreased WUE and reduced biomass but no

for Col-0, the individual data points relative to the Col-0 mean were plotted. The horizontal solid line denotes Col-0 reference. The blue asterisks denote a significant difference ($P < 0.05$) based on t test performed on original leaf temperature values. Note that plots for original values and thermal imaging block by block are provided in *SI Appendix*, Fig. S7.
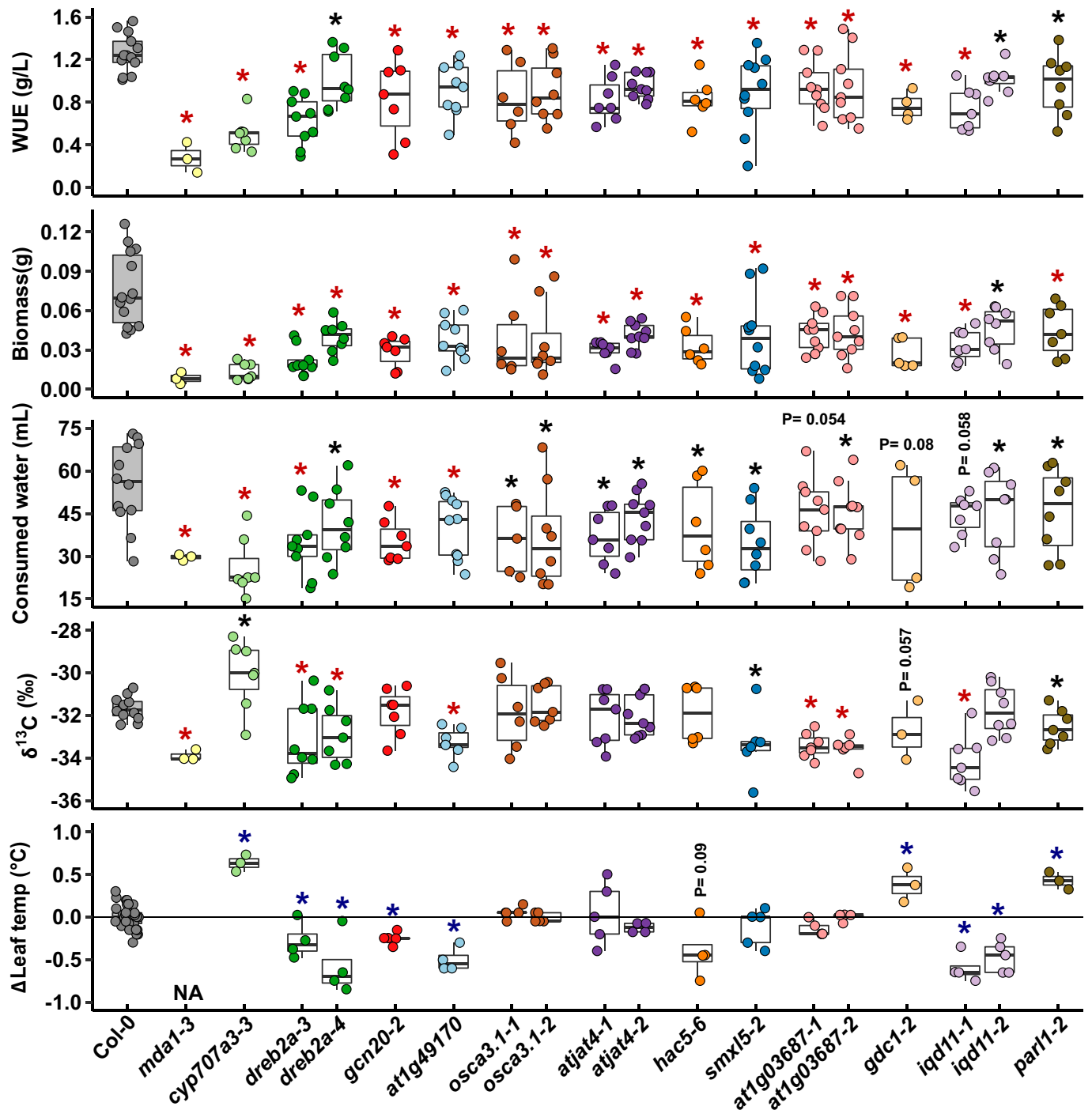
Fig. 4. Mutants of 13 genes had reduced biomass and reduced water consumption as a factor leading to decreased WUE. The data presentation format is the same as described for Fig. 3. Plots for original leaf temperature values and thermal imaging block by block are provided in *SI Appendix*, Figs. S8 and S9. NA denotes missing data ("not available").

significant change in water consumption (Fig. 5 and *SI Appendix*, Fig. 2A, green circles) or SD. WNK11 belongs to the WNK protein kinase family, which is involved in various processes, such as ABA sensitivity, proline accumulation, regulation of flowering time, and intracellular signaling through G protein (49); however, the function of WNK11 itself is unknown. *wnk11-1* had strongly decreased WUE and biomass while others had moderate effects in this category. The decreased δ^{13}C in *wnk11-1* was consistent with gravimetric analysis. PLT1 is a negative regulator in ABA inhibition of root growth (50). *plt1* had a marginally nonsignificant (P = 0.054) decrease in δ^{13}C, consistent with gravimetric

WUE. BPC2 functions in negative regulation of osmotic stress tolerance and it also determines β-1,4-galactan accumulation in response to salt stress (51, 52). *bpc2* also had decreased δ^{13}C consistent with gravimetric WUE. It is possible that WNK11, PLT1, and BPC2 affect WUE through mechanisms related to ABA or osmotic stress signaling.

AT2G45460 and *AT3G62220* encode proteins of unknown function. For *AT3G62220*, we observed disparate results for two independent T-DNA lines; only *at3g62220-1* (promoter insertion) had a significant effect on biomass, while *at3g62220-2* (exonic insertion) had a marginally nonsignificant (P = 0.08)
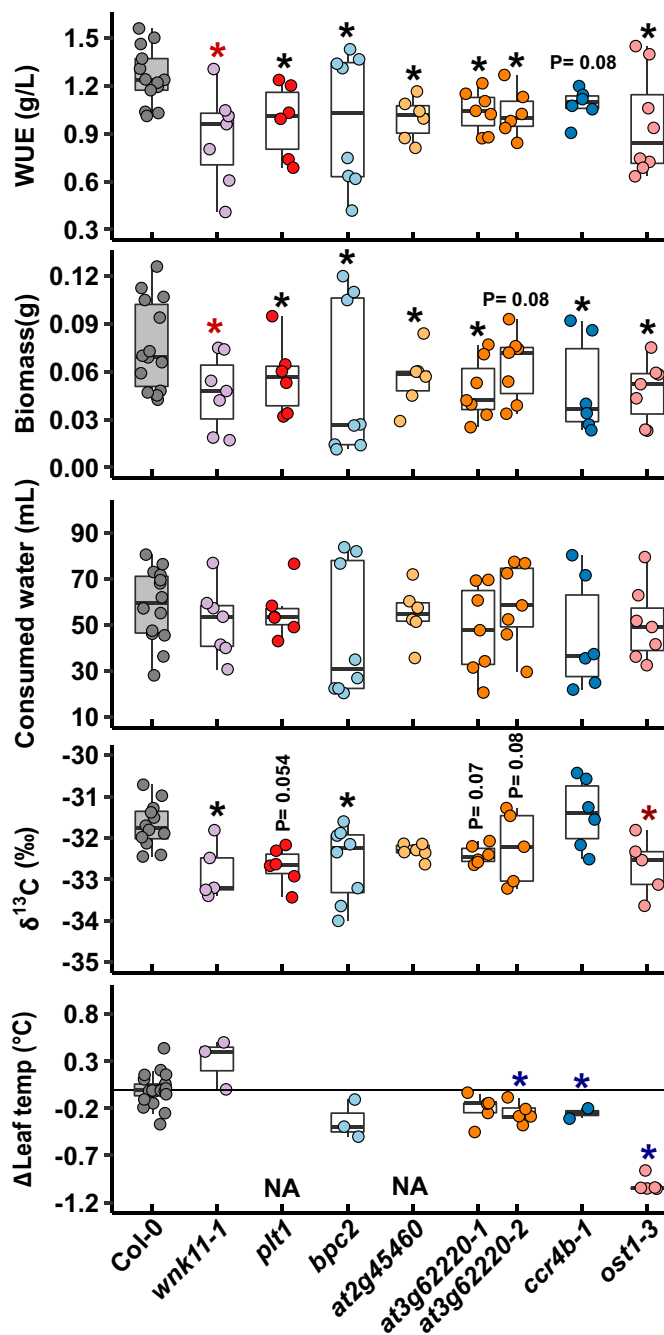
Fig. 5. Mutants of six genes and control mutant *ost1-3* had reduced biomass as a factor leading to decreased WUE. The data presentation format is the same as described for Fig. 3. Plots for original leaf temperature values and thermal imaging block by block are provided in *SI Appendix*, Fig. S10. NA denotes missing data ("not available").

effect. Conversely, *at3g62220-2* had a significant effect on leaf temperature and SD while *at3g62220-1* had no significant effect (Fig. 5 and *SI Appendix*, Figs. S6C and S10). *ccr4b-1* had a marginally nonsignificant decrease in WUE ($P = 0.08$) and it had decreased leaf temperatures. CCR4b determines the poly(A) length of transcripts related to starch metabolism and may also affect degradation of stress responsive RNAs via its interaction with Pumilio RNA-binding protein 5 (53). The control mutant *ost1-3* had a moderate effect on WUE and biomass, a strong decrease in δ13C, and an expected decrease in leaf temperatures (Fig. 5 and *SI Appendix*, Fig. S10). Surprisingly the altered leaf temperatures did not affect the overall water consumption in these mutants and none of these mutants influenced SD except

for *at3g62220-2* that had increased SD (*SI Appendix*, Fig. S6C). There is no prior information for genes in this category to link them to WUE or traits related to plant water relations.

Finally, we found mutants of natural resistance associated macrophage protein 4 (NRAMP4) and carbamoylphosphate synthetase subunit B/Venosa3 (*CarB/VEN3*) had marginally nonsignificant decreases in WUE without significantly affecting final biomass or water consumption (*SI Appendix*, Fig. S11 and Fig. 2A: gray circles). In addition, these mutants did not affect δ13C or SD (*SI Appendix*, Figs. S11 and S6D) and *carb* did not affect δ13C or leaf temperatures (*SI Appendix*, Fig. S11). NRAMP4 is functionally redundant with NRAMP3 in maintaining photosynthesis under cadmium and oxidative stress (54). CarB is involved in the conversion of ornithine into citrulline in arginine biosynthesis; however, the *carB* mutant used in this study is a knockdown mutant that expresses a reduced level of carB (55), suggesting that a *carB* knockout mutant may have greater effect on WUE.

Discussion

Our strategy of combining GWAS results and gene expression data generated a large and broad set of candidates for reverse genetic tests. We found 25 genes with significant differences in WUE (Datasets S9 and S11). As such, the success rate from our candidate gene selection strategy is moderate (positive rate of 0.35 [25/70 discovered candidates]). However, the lack of phenotype in T-DNA mutants cannot conclusively rule out a candidate gene as the source of the GWAS association since knockout mutants might not recapitulate gain-of-function or altered function natural alleles. Also, gene redundancy could be another mitigating factor limiting the impact of knockouts for some of our candidate genes. Construction of higher order mutants, including close homologs or the generation of overexpression lines, might reveal phenotypes for these genes where T-DNA mutants did not alter WUE. Our study also validated genes grown under well-watered conditions. It may be that some of our candidates would show more dramatic responses to water deficit and would have been detected in more comprehensive soil drying screens. Importantly, our goal was to fully use the GWAS data to generate a broader set of candidate genes for high-throughput testing rather than using more stringent statistical testing to identify one or few SNPs followed by detailed studies to decipher the mechanistic role of any particular gene affecting WUE. We focused on the genomic regions with the top 500 lowest P-value SNPs (nominal $P = 4.37 \times 10^{-5}$ to 4.2×10^{-3}) and used gene expression as an additional criterion to prioritize candidate genes for validation. Despite these caveats, a combined GWAS and reverse genetic approach proved to be an effective way to discover genes influencing WUE without relying on assumptions about underlying mechanisms, as it has been for other stress-related traits (22, 23). The candidate genes we validated belong to a range of gene families such as histone acetyl transferases, mitochondrial transcription factors, mechanosensitive ion channels, ABC transporter G family, ankyrin repeat-domain containing proteins, cytochrome P450, and family 707A. Strikingly, a recent GWAS study also identified genes belonging to these families as some of the most promising candidates for WUE-related traits in sorghum (24).

Since WUE is a ratio of biomass accumulated per unit of water consumed, it can be influenced by factors that alter either side of the ratio (or affect one side of the ratio more than the other side). Our approach allowed us to find mutants affecting either side of the WUE ratio and to parse out which aspect of

WUE was most affected by each mutant. Interestingly, we found mutants affecting both sides of the WUE ratio, thus demonstrating that changes in growth are as likely to influence WUE as changes in transpiration. In addition, we did not find any mutants where decreased biomass was associated with increased WUE. Thus, contrary to what may be a common assumption, our data show that simply decreasing biomass was not sufficient to increase WUE. This pattern also supports the hypothesis that our approach identified specific effectors of WUE, as opposed to merely finding plants that cannot grow well. Our data identified more mutants with decreased WUE than with increased WUE, suggesting that WUE is under positive regulation as part of mechanisms balancing net carbon assimilation to water use. None of the genes we identified would likely have been predicted to affect WUE a priori (with the possible exception of *CYP707a3*).

While the WUE effector genes we identified may at first look seem to be a random assortment of genes, they can in fact be grouped into several categories that make their potential roles in WUE clearer. The first group of genes we identified are genes involved, or potentially involved, in stress-related signaling or hormone metabolism but not previously connected to WUE. The most prominent of these is *PCO5*. The *pco5-1* and *pco5-2* mutants had the strongest increase of WUE of any mutants we examined because of their strongly increased biomass production. PCOs are components of the N-end rule pathway, which act as redox status sensors. Several PCOs act as oxygen or redox status sensors that regulate stability of the group VII ethylene response factors (ERF-VII) via the N-end rule pathway of targeted proteolysis in response to hypoxia (56). PCO5 activity toward ERF-VII destabilization was demonstrated in vitro, but its in planta function remains to be established (57). Interestingly, Proteolysis 6 (PRT6) and other N-end rule components were shown to affect ABA accumulation and ABA sensitivity (23, 58). Thus, our data provide another piece of evidence that the N-end rule pathway influences many types of environmental responses in addition to the hypoxia responses where it has been best characterized. Whether or not the increased biomass and WUE of *pco5* mutants is dependent on ERF-VII protein stability or other targets of N-end rule degradation will be of interest for future studies.

Our data also implicate calcium-dependent signaling in WUE. Our finding that OSCA3.1 had strongly reduced WUE and biomass is especially interesting as there have so far been little physiological data to link the short-term calcium responses mediated by OSCAs to longer-term acclimation to water limitation or other environmental conditions. Also, mutants of the calcium responsive protein IQD11 had a similar effect on WUE and biomass as *osca3.1* mutants. As most IQDs are also microtubule binding proteins, IQD11 could also affect WUE via effects on microtubule stability or organization that alter stomatal function or affect biomass (59, 60). Mutation in one of the calcium-dependent protein kinases, *cpk23*, led to increased WUE in our growth conditions. It is surprising that *cpk23* had increased WUE rather than decreased WUE as seen in *ost1* (*snrk2.6*) because CPKs and SnRK2 can both activate the guard cell slow anion channel-associated 1 (SLAC1) by phosphorylating distinct sites on SLAC1 (61). While this suggests a similar function of CPK23 and SnRK2s, our results are consistent with another study that found *cpk23* had slower water loss and reduced stomatal aperture (62). Together, these findings that OSCA3.1, IQD11, and CPK23 all strongly affected WUE, but affected it in different ways, suggest that multiple calcium-dependent changes in guard cell function or regulation of growth (biomass)

can alter WUE. Consistent with this idea, the genes associated with the top 500 GWAS SNPs also included CPK8 and several other types of calcium binding proteins (Dataset S2).

Other signaling genes we found to impact WUE include several genes that may have been expected to have some effect on WUE. Nevertheless, the actual mutant phenotypes we found were still surprising in several ways. CYP707A3 is involved in ABA catabolism and the simplest expectation may be that even a slight increase of endogenous ABA levels of *cyp707a3* would enhance stomatal closure and increase WUE. We saw no evidence of this but instead observed that *cyp707a3* had decreased WUE driven by a strong decrease in biomass accumulation. This suggests that the biomass inhibition caused by disrupted ABA metabolism in *cyp707a3* was too substantial to be overcome by any increase of stomatal closure. Similarly, the *PLT1* mutant had enhanced ABA inhibition of root growth but *plt1* had decreased WUE and biomass similar to *cyp707a3*. The WNK kinase family is known to affect ABA sensitivity but there is no specific information on WNK11 (49). JAT4 is involved in JA translocation and there are a number of indications that JA affects ABA sensitivity (and vice versa) and that the ratio of endogenous JA to ABA accumulation is important for various stress response.

While several of the genes mentioned above have a connection to ABA signaling, it is perhaps surprising that we did not find more ABA-related genes among our GWAS candidates. Moreover, the only mutant that we observed to have decreased biomass and increased WUE was the ABA-hypersensitive mutant *meff11-5*, which was used as a control. This suggests that knockout mutants where stomata remain sufficiently closed to limit photosynthesis are relatively rare compared to other loci with more moderate or indirect effects on WUE. It is also possible that the core ABA signaling components that have strong effects on ABA sensitivity have relatively little natural variation among the accessions used in our GWAS analysis and thus were not detected in our list of top candidate genes. Similarly, another type of gene that may be expected to affect WUE is developmental regulators that determine stomatal density or size. Surprisingly, our GWAS analysis did not find any known regulators of stomatal development that show strong association with δ^{13}C. The only known stomatal development genes among the candidate genes identified by GWAS was *SCREAM1/ICE1* (associated with the 21st ranked SNP) and *CKB1* associated with the 59th ranked SNP (Dataset S2). However, these were not analyzed further as they were not among the stress up-regulated genes in the transcriptome datasets we used to select stress up-regulated candidate genes. Other stomatal regulators may have limited effect on the WUE plasticity that was the basis of our GWAS or may have limited natural variation in the group of accessions studied. The mutants we did find to affect WUE generally had no effect on stomatal density (Dataset S6), further indicating that, at least in the population of *Arabidopsis* accessions used for our GWAS, stomatal development was not a key driver of WUE variation. An alternative explanation would be that key genes involved in hormone responses or core components of cell fate and development are under strong purifying selection due to potential pleiotropic costs (63). It may be that there is more standing genetic variation in more specialized or modular aspects of physiology or growth that lead to the observed natural genetic variation in WUE. Alternatively, it may be that our filtering and candidate gene selection strategy was biased toward certain classes of genes. It would be extremely valuable to compare validation rates for candidate genes identified in GWAS using several different gene selection criteria, including the use of complementary omics

datasets or expert opinion more broadly. The development of tools for leveraging information-rich datasets to prioritize genes for validation, especially in a rigorous statistical framework, are desperately needed.

Maximizing the amount of biomass, or other components of yield, produced per amount of water input can be a beneficial agronomic trait in many environments. However, increasing WUE at the expense of biomass production may not be as useful. Even by focusing solely on reverse genetic, loss-of-function analysis, we could find numerous genes that may be useful for more targeted study and manipulation of WUE without negative effects on biomass accumulation. For example, *pco5* mutants have strongly increased biomass accumulation leading to dramatic increase in WUE. Conversely, we found several mutants that decrease WUE without affecting biomass. These would be good candidates for gain-of-function analysis and for detailed studies under soil drying. In any case, these results uncover several pathways that may be used to influence and understand the molecular basis of WUE.

Materials and Methods

The datasets of WUE (δ^{13}C) covering 185 accessions (Dataset S1) were linked to published genomic data on each accession from the 250K SNP chip and were used in GWAS mapping. GWAS was conducted using a linear mixed-model approach (64) followed by the prioritization of candidate genes. The T-DNA insertion lines were obtained from the *Arabidopsis* Biological Resources Center (ABRC) and screened for the homozygous lines (Datasets S8 and S9). Gravimetric WUE was performed as described previously (65, 66) with few modifications. Carbon isotope composition (δ^{13}C) was analyzed at the University of California Davis Stable Isotope Facility. Further description of all methods, including measurements of SD, SI, leaf temperatures, and statistical methods can be found in *SI Appendix, Materials and Methods*.

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