Arabidopsis *ECERIFERUM9* involvement in cuticle formation and maintenance of plant water status

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Arabidopsis ECERIFERUM9 involvement in cuticle formation and maintenance of plant water status

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ABSTRACT

Mutation of the ECERIFERUM9 (CER9) gene in Arabidopsis causes elevated amounts of 18 carbon-length cutin monomers, and a dramatic shift in the cuticular wax profile (especially on leaves) toward the very-long-chain free fatty acids (VLCFAs) tetracosanoic acid (C24) and hexacosanoic acid (C26). Relative to wild type, cer9 mutants exhibit elevated cuticle membrane thickness over epidermal cells, and cuticular ledges with increased occlusion of the stomatal pore. The cuticular phenotypes of cer9 are associated with delayed onset of wilting in plants experiencing water deficit, lower transpiration rates, and improved water use efficiency (WUE) measured as carbon isotope discrimination. The CER9 protein thus encodes a novel determinant of plant drought-tolerance-associated traits; one whose deficiency elevates cutin synthesis, redistributes wax composition, and suppresses transpiration. Map-based cloning identified CER9, and sequence analysis predicted it encodes an E3 ubiquitin ligase homologous to yeast Doa10 (previously shown to target ER proteins for proteasomal degradation). To further elucidate CER9 function, the impact of CER9 deficiency on interactions with other genes was examined using double mutant and transcriptome analysis. For both wax and cutin, cer9 showed mostly additive effects with cer6, lacs1, and lacs2, and revealed its role in early steps of both wax and cutin synthetic pathways. Transcriptome analysis revealed that the cer9 mutation affected diverse cellular processes, with primary impact on genes associated with diverse stress responses. Discovery of CER9 lays new groundwork for developing novel cuticle-based strategies for improving the drought tolerance and WUE of crop plants.
INTRODUCTION

Climatological drought is a historic problem for agriculture worldwide as it limits crop production, and is now increasing as a threat due to climate change, as well as dwindling ground and surface water resources. Genetic alterations that reduce overall transpirational water loss by crops are expected to conserve soil moisture and confer drought tolerance by delaying the onset of cellular dehydration stress during prolonged water deprivation (Nobel, 1999; Chaves et al., 2003; Kosma and Jenks, 2007). Stomata play a major role in regulating transpirational water loss through guard cell behavior (regulating stomatal aperture) and/or stomatal density over the leaf surface (Schroeder et al., 2001; Sirichandra et al., 2009; Chaerle et al., 2005; Nilson and Assmann, 2007; Kim et al., 2010). Transpiration is also controlled by the lipidic and hydrophobic plant cuticle, which coats the aerial surfaces of plants. The cuticle controls plant water loss associated with non-stomatal epidermal transpiration, as well as transpiration through the stomatal pore via its role in forming the stomatal ledges (lips), and the cuticular coating that covers the mesophyll surfaces of substomatal chamber (Kerstiens, 2006; Kosma et al., 2009; Xiao et al., 2004; Lü et al., 2009). The cuticle is composed primarily of two lipid classes, the non-polymerized cuticular waxes and the cutin polyester. Most waxes are very-long-chain (C_{20}-C_{34}) saturated lipids that occur as epi- and intra-cuticular lipids, whereas the more hydrophilic cutin polyester consists of C_{16} and C_{18} fatty acid derivatives (e.g. hydroxy fatty acids and dicarboxylic acids) linked primarily by ester bonds.

Multiple sources of evidence suggest that both waxes and cutin are important in maintaining plant water status. These include mutants defective in the composition of waxes but not cutin, such as the tomato cer6 and ps mutants (Leide et al., 2007; Leide et al., 2011); cutin monomer mutants having wild-type waxes such as att1, hth, and gpat4 gpat8 (Xiao et al., 2004; Kurdyukov et al., 2006a; Li et al., 2007); and mutants conferring alterations in both waxes and cutin monomers, such as lacs1, lacs2, bdg, gsd1 (Lü et al., 2009; Chen et al., 2003; Kurdyukov et al., 2006b; Lü et al., 2011), and wax2 (Parsons, unpublished); all were shown to exhibit elevated cuticle permeability.
Additionally, Arabidopsis plants exposed to water deficit conditions induce synthesis of cuticular waxes and cutin monomers, especially waxes, which is often associated with improved plant tolerance to water deficiency (Kosma et al., 2009; Cameron et al., 2006; Seo et al., 2011). When expressed ectopically, the transcription factors MYB96 and WIN1/SHN1 from Arabidopsis, and the AP2 domain-containing transcription factors WXP1 and WXP2 from *Medicago truncatula*, activate cuticular wax and cutin biosynthesis and thereby enhance drought tolerance (Seo et al., 2011; Aharoni et al., 2004; Zhang et al., 2005; Zhang et al., 2007). Activation of cuticular wax biosynthesis by water deficiency in fact requires the MYB96 protein (Seo et al., 2011). Taken together, it is clear that the physical-chemical properties of both waxes and cutin play important roles in establishing the water-barrier properties of the cuticle. How waxes and cutin interact to establish the water permeability barrier of the cuticle is still unclear. Preliminary evidence suggests that cutin polyester forms a matrix (or framework) that guides precise intercalation of waxes and the location of water diffusion pathways (Kosma and Jenks, 2007; Kosma et al., 2009).

In previous reports, the Arabidopsis wax mutant *eceriferum*9 (*cer9*) was shown to have a semi-glossy inflorescence stem (Koornneef et al., 1989). In addition, *cer9* mutants showed extreme alteration in wax profiles, exhibiting dramatic elevations of the very-long-chain fatty acids (VLCFAs), tetraicosanoic (C24) and hexacosanoic (C26) acids, which are constituents at near trace amounts on wild type (Jenks et al., 1995; Goodwin et al., 2005). The effect of the *cer9* mutation on cutin monomer composition is reported here for the first time, revealing a major increase in the amount of this lipid class. We describe isolation of the *CER9* gene and shed new light on a role for *CER9* as a major regulator of plant water use efficiency (WUE) and overall plant stress response. *CER9* is the first described cuticle biosynthesis gene whose deficiency improves both plant response to water deficit and improves WUE, indicating that *CER9* may encode an important new cuticle-associated drought-tolerance determinant.
RESULTS

Molecular identification of CER9

The **cer9-1** mutant was first reported in 1989 (Koornneef et al., 1989), and was later rough mapped to 118.2cM on chromosome 4 using the recessive EMS generated **cer9-1** allele (Rashotte et al., 2004). An outcross to Arabidopsis Columbia-0 (Col-0) was made to fine map the causal locus of **cer9-1**. We initially mapped the **cer9-1** mutation to a region of 400 kb between simple sequence length polymorphic (SSLP) markers Fo15.9M and Fo16.5M using 96 (**cer9-1/Col-0**) F2 plants (Fig. 1A). An enlarged population containing 2,000 F2 plants was then used for further fine mapping.

In the rough mapping interval, we developed six PCR-based markers, including SSLPs, cleaved-amplified polymorphic sequence (CAPS), and dCAPS, and narrowed the location of the **cer9-1** mutation to a 63-kbp interval between markers Fo16.296M and Fo16.359M (Fig. 1B). DNA sequencing of this 63-kbp region revealed a G-to-A single nucleotide polymorphism in **cer9-1** that causes a conversion of a highly conserved cysteine to tyrosine in the predicted RING-variant domain of At4g34100 protein (Fig. 1C).

To establish that the mutation of At4g34100 caused the glossy phenotype observed in **cer9-1**, we obtained a T-DNA insertion allele, GABI_588A06, which was designated as **cer9-2**. The insertion site of GABI_588A06 was confirmed to be in the sixth exon, and located 3,107 bp downstream from the start codon of At4g34100 (Fig. 1C). Semi-quantitative RT-PCR using RNA isolated from leaves showed that the total abundance of the At4g34100 transcript in **cer9-1** was similar to wild type, suggesting that the point mutation in **cer9-1** does not effect mRNA transcript levels (Fig. 1D). The At4g34100 transcript in **cer9-2** was found to be truncated, as a reverse primer (RT-R1) upstream of the T-DNA insertion, but not a reverse primer (RT-R2) downstream of the insertion (Fig. 1D), generated an amplification product with upstream forward primers RT-F1 and RT-F2, respectively. This indicated that the transcript of At4g34100 in **cer9-2** is severely truncated, and would preclude
translation of a full protein. *cer9-2* also exhibited a semi-glossy stem, and stem and leaf wax and cutin monomer composition essentially identical to that of the *cer9-1* allelic mutant (Fig. 3; Fig. 5; Supplemental Fig. S3; Supplemental Fig. S4). Allelism tests showed that *cer9-2* did not complement *cer9-1*, indicating that the visible mutation is caused by defects of the same gene (data not shown). To further confirm that the mutation in At4g34100 is responsible for the *cer9-1* mutant phenotype, a whole cDNA sequence of *At4g34100* driven by a 35S promoter was used to complement the *cer9-2* mutant. Multiple transgenic lines were obtained and two independent transformants were selected for further analysis using visual assessment as well as scanning electron microscopy (SEM). Both *cer9-2* transgenic complementation lines harboring the 35S-*CER9* construct reverted to wild-type glaucous stems, and exhibited wax crystallization patterns identical to wild type, further verifying *CER9* (*At4g34100*) control over the associated phenotypes (Supplemental Fig. S1, A, B, C, and D). PCR-based genotyping confirmed the *cer9-2* background of both transgenic lines using the GABI_588A06-LP, GABI_588A06-RP and o8409 primers (Supplemental Fig. S1E), and RT-PCR analysis of these two lines confirmed the expression of the introduced wild-type *CER9* allele (Supplemental Fig. S1F).

**Organ- and tissue-specific expression of CER9**

We constructed a CER9pro::GUS construct for transformation of wild-type Arabidopsis plants to monitor expression patterns of *CER9* at different developmental stages. *CER9* was constitutively expressed throughout development (Fig. 2), showing high expression in the cotyledon but almost undetectable expression in root radicles of 2-day-old seedlings (Fig. 2A). Relatively strong signals were detected in roots, cotyledons, and true leaves of 5-day-old and 10-day-old whole seedlings (Fig. 2, B and C). In mature plants, the expression levels of *CER9* varied along the whole stem length, with highest expression at the top of the stem, and weakest expression at the base of the stem (Fig. 2D). *CER9* was also highly, but not specifically, expressed in
the epidermal layer (Fig. 2E). *CER9* was strongly expressed in cauline leaves, rosette leaves, inflorescence and siliques (Fig. 2, D, F, G and H).

**The predicted *CER9* transcript encodes a Doa10-like protein**

The *CER9* transcript encodes a predicted polypeptide of 1,108 amino acids. Analysis with the protein domain prediction tool SMART (http://smart.embl-heidelberg.de/) revealed that the predicted CER9 protein has a RING-variant domain and 14 putative transmembrane domains (Supplemental Fig. S2). The conserved sequence of the RING variant domain is C-x (2) -C-x(10-45)-C-x (1) -C-x (7) -H-x(2)-C-x(11-25)-C-x(2)-C, which is different from that of PHD domain [C-x(1-2) -C-x (7-13)-C-x(2-4)-C-x(4-5)-H-x(2)-C-x(10-21)-C-x(2)-C] and Classical RING domain [C-x (2) -C-x (9-39)-C-x(1-3)-H-x(2-3)-C-x(2)-C-x(4-48) -C-x(2)-C]. Domain similarity analysis showed that the CER9 RING-variant domain shares high similarity to that of Doa10 and TEB4, with 49% and 57% identity, respectively (Supplemental Fig. S2). All Doa10 orthologs are characterized by an N-terminal RING-CH domain (as noted above) and an internal conserved segment of ~130 residues called TD (TEB4-Doa10; Kreft and Hochstrasser, 2011). The TD domain (transmembrane 5, 6 and 7) of CER9 has 31% and 45% identity to that of Doa10 and TEB4, respectively (Supplemental Fig. S2).

**Cuticular waxes of the cer9 mutants**

As reported in Jenks et al. (1995), cer9-1 mutant leaf waxes have significantly increased amounts of very-long-chain fatty acids (VLCFAs) relative to wild type. Our leaf wax chemistry data confirmed this result, and showed that C<sub>22</sub>, C<sub>24</sub> and C<sub>26</sub> VLCFAs increased by 90, 60 and 23 fold, respectively (p<0.0001) (Fig. 3A), while aldehydes, 1-alcohols and *n*-alkanes decreased by 70, 84 and 92%, respectively (p<0.0001), relative to wild type. This led to an overall increase of total wax amount on cer9-1 of 27% (p<0.05) over the wild-type parent Ler-0 (Fig. 3A; Table I). Leaf waxes of cer9-2 were similarly altered with an overall 57% (p<0.0001) increase of
total wax relative to the Col-0 parent (Supplemental Fig. S3B).

The total amount of all wax chemical constituents on cer9-1 inflorescence stems was decreased to 44% of wild-type levels (Fig. 3B; Table I). The amounts of stem C\textsubscript{24} and C\textsubscript{26} fatty acids however increased to 689% and 453%, respectively, of wild type. The alkane, secondary alcohol, and ketone wax classes on stems were the most reduced wax classes relative to wild type. Of these classes, these reductions could be attributed primarily to 76%, 65%, and 62% reductions of the C\textsubscript{29} alkane, the C\textsubscript{29} secondary alcohol, and the C\textsubscript{29} ketone, respectively. Stem aldehydes were also changed in cer9-1, with the C\textsubscript{26} aldehydes being increased to 221% of the wild-type amounts, whereas the C\textsubscript{30} aldehydes decreased to 15% of the wild-type amounts. The amount of stem C\textsubscript{26} and C\textsubscript{28} primary alcohols increased to 245% and 137% of the wild-type amounts, respectively, whereas the C\textsubscript{30} primary alcohols decreased to 42% of the wild-type levels. A significant increase was observed for all detected stem ester constituents except C\textsubscript{40} esters, resulting in mutants having over 120% more total esters than the wild type. Scanning electron microscopy (SEM) was used to demonstrate that cer9-1 and cer9-2 stems had a similar wax crystal pattern to each other with markedly lower density of wax crystals than stems of their corresponding wild-type ecotypes (Fig. 4A-D).

**Cutin monomers and cuticle membrane ultrastructure of the cer9 mutant.**

Analysis of the cutin depolymerization products, cutin monomers, likewise exhibited significant changes in cer9 mutants (Fig. 5). The total leaf cutin monomers on cer9-1 were increased by 59% (p<0.0001) over the Ler-0 parent (Fig. 5A). The major cutin monomer, the C\textsubscript{18:2} dioic acid, was increased to 187% (p<0.0001) above wild-type levels, whereas 18-OH-C\textsubscript{18:1} acid was increased by 124% (Fig. 5A). There was no significant difference observed in 16-OH-C\textsubscript{16:0}, 10,16-OH-C\textsubscript{16:0}, and 18-OH-C\textsubscript{18:0} acids, or the C\textsubscript{18:0} dioic acids, between cer9-1 and Ler-0. The C\textsubscript{16:0} dioic acids were reduced to 61% (p< 0.01). Stem cutin monomers of cer9-1 showed comparable proportional changes to those in the leaf. The major leaf cutin monomer, the C\textsubscript{18:2}
dioic acid, was increased to 192% (p< 0.0001) of wild-type levels, whereas C18:0 dioic acid was increased by 43% (p< 0.01) (Fig. 5B). The C16:0 dioic acids were reduced to 54% (p< 0.01). Overall, there was an increase of 67% (p< 0.01) in total cutin monomers on cer9-1 stems compared to Ler-0 stems (Fig. 5B). Altered ultrastructure of the cuticle membrane in cer9-1 leaves and stems was clearly evident using transmission electron microscopy (TEM) (Fig. 6). The cer9-1 cuticle membrane was much thicker, more osmiophilic, and structurally irregular relative to wild type in both leaves (both adaxial and abaxial surfaces) and stems (Fig. 6A - F). The cuticle membrane that forms the cuticular ridge over the outside of the stomatal pore (forming the anti-chamber) was larger in both leaf and stem of cer9-1 (Fig. 6G - J).

**cer9 interactions with other mutants on leaf cuticle lipid metabolism**

As reported by Jenks et al. (1995), waxes of cer8-1 leaves exhibited significant increases in C26, C28 and C30 VLCFAs, which increased relative to wild type by 108, 77 and 191%, respectively (p< 0.05), while other wax classes did not differ significantly (Supplemental Fig. S3A). Relative to Ler-0, the cer6-1 mutant showed an increase in the C22, C24 and C26 VLCFAs of 92, 150 and 38% (p<0.05), and a 4-fold increase in C24 primary alcohols (p<0.001), but a 75% reduction in C28 primary alcohols (p<0.01), 79% less n-alkanes (p<0.001) and 57% less total wax (p<0.001), which is similar to that reported by Jenks et al. (1995). As reported in Lü et al. (2009), the lacs2-3 mutant has lower C28-C30 constituents for each wax class examined (except acids, for which only C30 fatty acid was reduced, and this by 33%, p<0.05) resulting in 38% lower aldehydes (p<0.01), 45% lower 1-alcohols (p<0.05), 21% lower n-alkanes (p<0.05) and 30% lower ketones (p<0.01) (Supplemental Fig. S3B). The cer8-1 cer9-1 double mutant had a leaf wax phenotype very similar to cer9-1, except the acid pool in the double mutant was slightly higher. These results indicate an additive effect on acid amount produced by the wax biosynthesis pathway. The cer6-1 cer9-1 double mutant had a more extreme leaf wax phenotype than the cer9-1 single mutant, having significantly higher fatty acids. In cer6-1 cer9-1, the C22, C24
and C_{26} fatty acids increased by 292, 217 and 20 fold, respectively, relative to wild-type (p<0.0001) (Supplemental Fig. S3A). Aldehydes, 1-alcohols, and n-alkanes decreased by 65, 72 and 96%, respectively (p<0.0001). This led to an overall increase of total wax load in cer6-1 cer9-1 of 159% (p<0.0001) over Ler-0 (Supplemental Fig. S3A). As with the cer8 mutant, cer9 also appears to have overlapping function with cer6. The lacs2-3 cer9-2 double mutant showed similar leaf wax phenotype to the cer9-2 single mutant, except that all wax classes besides the acid class were slightly reduced. cer9 also showed general additive interactions with lacs2 in wax production (Supplemental Fig. S3B).

Even though the cer6-1 mutant exhibits major alterations in its waxes (Supplemental Fig. S3A), the cer6-1 mutant did not differ in leaf cutin monomers from its isogenic wild-type Ler-0, and no gene interaction with cer9 was observed (Supplemental Fig. S4A). As CER6 encodes a component of fatty acyl-CoA elongase, 3-ketoacyl-CoA synthase (KCS6) (Millar et al., 1999), the lack of the cer6 mutation impact on cutin was not, after all, unexpected since cutin monomers do not undergo elongation. As reported (Lü et al., 2009), the cer8-1 mutant allele had 40% more leaf C_{18:2} dioic acid (p<0.001), while all other cutin monomers were not significantly different. There was an overall increase in total-identified cutin monomers of 23% (p<0.01) on cer8-1 leaves over those of the isogenic wild-type Ler-0 (Supplemental Fig. S4A). The cer8-1 cer9-1 double mutants had similar cutin monomer patterns as the cer9-1 single mutant, except a slight suppression of 16 carbon monomers, indicating that cer9 is fairly epistatic to cer8 in cutin biosynthesis. As reported by Lü et al. (2009), leaves of lacs2-3 had lower amounts of all detected cutin monomers than wild type, including a 53% reduction in C_{16:0} dioic acid (p<0.0001), a 50% reduction in 18-OH-C_{18:0} acid (p<0.0001), a 16% reduction in the C_{18:0} dioic acid (p<0.01), a 45% reduction in the C_{18:1} dioic acid (p<0.001), an 83% reduction in the C_{18:2} dioic acid (p<0.0001), and an overall decrease of total cutin monomers by 58% (p<0.0001) relative to Col-0 (Supplemental Fig. S4B). The cutin monomers profile detected in the lacs2-3 cer9-2 double mutant is similar to that of single mutant lacs2-3, indicated that
*lacs2-3* impact is relatively epistatic of *cer9-2* in the cutin biosynthesis pathway (Supplemental Fig. S4B).

**Effect of the *cer9* mutation on plant response to water deficiency**

Whole plant wilting tests conducted in pots showed that both *cer9-1* and *cer9-2* mutants are more resistant to water deficit in the potting mix than their corresponding wild type, wilting earlier and reaching lower leaf relative water contents more quickly than the wild types (Fig. 7). The transpiration rates of whole growing plants were assessed by gravimetric methods over diurnal light/dark periods to show that the *cer9-1* and *cer9-2* allelic mutants exhibited lower transpiration rates than the near-isogenic wild types when grown under water-sufficient conditions (Fig. 8A). Reduced transpiration rates from growing *cer9* plants apparently enhanced the capacity of *cer9* mutants to delay the onset of leaf wilting as water in the potting mix became increasingly deficient (Fig. 7). Further, the differences in transpiration rate were much greater in light than dark conditions, implicating *cer9* effects on stomatal water loss (Fig. 8, A and B). Interestingly, the lower transpiration rate of the *cer9-2* mutant was overcome when *cer9-2* is placed together with the *lacs2-3* mutation in the *lacs2-3 cer9-2* double mutant, supporting our interpretation of the cuticle’s chemical composition that *lacs2-3* is epistatic to *cer9-2* (Fig. 8B). All these effects appear due to *cer9*’s impact on the cuticle phenotype since stomata density, pavement cell density, and stomatal index of *cer9-2*, *lacs2-3*, and *lacs2-3 cer9-2* double mutant are unchanged, being similar to that of wild-type Col-0 (Fig. 8, C, D and E).

We also estimated the effect of several cuticle mutants on integrated water use efficiency (WUE). WUE is the ratio of carbon gained per unit water lost through transpiration. Water use efficiency can be estimated by calculating the stable carbon isotope ratio \( \delta^{13}C \) of leaves when applied to C3 plants (Farquhar et al., 1989; Dawson et al., 2002). Variation in \( \delta^{13}C \) reflects differences in the partial pressures of CO\(_2\) inside the leaf and can be generated by variation in either stomatal constraint on the diffusion of CO\(_2\) or through photosynthetic biochemistry (Farquhar et al., 1989).
Considerable natural variation in \( \delta^{13}C \) has been documented in Arabidopsis (McKay et al., 2003; Hausmann et al., 2005; Juenger et al., 2005) and artificial selection for low \( \delta^{13}C \) has improved yield in wheat (Rebetzke et al., 2002). \( \delta^{13}C \) was determined for cer9-1, cer9-2, and the corresponding wild types, as well as for the other known cutin mutants lacs1-1, lacs2-3, attl-2, and wax2. In the Columbia background, we found a significant effect on \( \delta^{13}C \) in comparisons between these cuticle associated genotypes (ANOVA: \( F_{5,39} = 22.4577, p<0.0001 \)). A post-hoc Tukey HSD test determined that the cer9-2 mutant showed significantly less-negative \( \delta^{13}C \) (associated with higher water use efficiency) than the isogenic parent Columbia, while the other cutin mutants, lacs1-1, lacs2-3, att1-2, and wax2, were all indistinguishable from wild-type using this test at \( \alpha = 0.05 \) (Fig. 8F). A separate ANOVA confirmed that the cer9-1 mutant exhibits significantly less-negative \( \delta^{13}C \) than its isogenic parent Ler-0 (\( F_{1,19} = 22.5544, p=0.0003 \)) (Fig. 8F).

Suberin content in cer9 roots

Genevestigator (©NEBION/ETH, Zurich, 2008) analysis of CER9 transcript expression indicated a possible function of CER9 in roots. Since suberin is chemically similar to cutin (raising the potential biochemical-genetic connection), we quantified the aliphatic suberin monomer content in roots of cer9-1 and cer9-2 and the corresponding wild-type ecotypes Ler-0 and Col-0, respectively (Fig. 9). The cer9 mutants exhibited small but significant increases in overall suberin content of 12% and 23% in the cer9-1 and cer9-2 mutants relative to the near isogenic wild types, respectively. For specific suberin monomers, the C20:0 acids were increased to 208% and 181% in cer9-1 and cer9-2, respectively, relative to the wild-types, while the C22:0 acids decreased to 57% of wild-type levels (but this is only significant in cer9-1). Other acids monomers (C16:0, C18:0, and C24:0) were not changed significantly. Dioic acids C18:0 and C20:0 were increased to 181% and 146% in cer9-1 roots, respectively, and increased by 77% and 56% in cer9-2, respectively. Other dioic acids monomers were unchanged relative to wild type. All \( \omega \)-OH acids (C16:0, C18:0, C18:1, C18:2, C20:0)
in cer9-1 and cer9-2 are increased, except that C_{22:0} is decreased slightly in cer9-1. To test whether the delayed wilting phenotype observed in cer9 mutants is related to elevated root suberin and reduced daytime transpiration rates in cer9, reciprocal grafting experiments were carried out. Plants with cer9-2 shoot grafted onto wild-type or cer9-2 roots showed reduced wilting and higher leaf relative water content, similar to cer9-2 mutant plants; whereas plants with wild-type shoots all wilted earlier (within 11 days) regardless of root system, and had lower leaf relative water content. This reveals that delayed wilting in the cer9 mutant is a shoot-dependent phenomenon and that the changes in cer9 root suberin had no significant effect on transpiration (Fig. 10).

**Transcriptome analysis of the cer9 mutant**

We conducted microarray analysis to estimate the impact of the cer9 mutation on the transcriptome. All genes showing at least 2-fold or greater change in transcript abundance in cer9-2 compared to the Col-0 wild-type parent are shown in Supplemental Table S2 (up-regulated in cer9-2) and Supplemental Table S3 (down-regulated in cer9-2). In cer9-2, 591 genes were up-regulated by 2-fold or greater (Fig. 11A), whereas 91 genes were down-regulated 2-fold or greater relative to the wild-type control (Fig. 11B). Because cer9 has a major impact on cuticle lipids, we expected that genes related to wax and cutin biosynthesis would show altered expression. However, none of the previously reported wax and cutin biosynthesis-associated genes showed altered expression. Only five genes previously association with lipid biosynthetic pathways were up-regulated 2-fold or more in cer9-2 including genes encoding cinnamoyl-CoA reductase-related protein (AT5G14700; 5.36 fold), hydrolase alpha/beta fold family protein (AT4G24160; 2.31 fold), lipase class 3 family protein (AT1G30370; 16.1 fold), pectin esterase (ATPMEPCRB; 3.98 fold) and triacylglycerol lipase (AT5G24200; 2.43 fold), while only 3 lipid genes were down-regulated including ones encoding two lipid transfer proteins (AT1G62510; 2.64 fold and AT2G37870; 3.72 fold) and one lipid binding
protein (AT4G33550; 2.47 fold) (Fig. 11, A and B; Supplemental Table S2 and Table S3). The major class of genes impacted by the cer9 mutation was associated with stress tolerance and response (both abiotic and biotic), with cer9 exhibiting 95 up-regulated stress-associated genes and 10 down-regulated stress-associated genes (Fig. 11, A and B). Twenty-one genes highly induced in cer9-2 are involved in protein ubiquitination and degradation, while only two such genes are decreased (Fig. 11). Many hormone response-associated genes showed altered regulation in cer9-2, with 30 such genes showing up-regulation and 7 showing down-regulation (Fig. 11). Four genes related to the unfolded protein response -- which is potentially linked to a predicted CER9 E3 ubiquitin ligase function -- are induced in cer9-2, including AtbZIP60 (2.64 fold), BIP3 (2.81 fold), CYP71A12 (7.78 fold) and a C2H2 type zinc finger protein (AT3G46080; 4.18 fold). These four genes are also highly induced by tunicamycin, which is an endoplasmic reticulum stress inducer (Iwata et al., 2008). Most other genes whose expression was modified 2-fold or more resembled fundamental metabolic (house-keeping) genes (Fig. 11). Quantitative RT-PCR analysis of selected stress related genes (those whose expression was altered on the array) was used to confirm the high reliability of the microarray data (Supplemental Fig. S5).

DISCUSSION
In plants, many cuticle-associated mutants have been reported (Kosma and Jenks, 2007), but none of these exhibited reduced transpiration rates, improved water use efficiency (WUE), and elevated tolerance to drought-like conditions. The cer9 mutant reported here shows delayed leaf wilting when exposed to increasing water deprivation, which was associated with reduced whole plant transpiration rates regardless of whether stomata were open (under light) or closed (in dark). Moreover, the cer9 mutant had higher WUE (less negative $\delta^{13}$C), while no other cuticle mutants we examined here (even those with known cuticle permeability defects) showed any change in WUE. Besides a dramatic elevation in the very-long chain fatty acid
(VLCFA) pool of the cuticular waxes of the cer9 mutant, cer9 also possessed major changes in the cutin monomer composition, and a highly modified cuticle membrane ultrastructure. The total cutin monomer amount on the stem of cer9 was 1.6-fold greater than on wild type, and the stem cuticle membrane thickness was comparably increased by 2.1-fold. The cer9 leaf cuticles were in a similar way altered. Previous reports indicated that wax VLCFAs create poor hydrophobic barriers to water diffusion through natural or artificial cuticle membranes (Grncarevic and Radler, 1967), probably due to their charged end groups that interact closely with water molecules (Kosma et al., 2009). These reports suggested that alkanes create the most effective barriers (Grncarevic and Radler, 1967), and show that alkanes are the major constituents increased on plants under osmotic stress (Kosma et al., 2009), and are also increased more than any other wax class in the Arabidopsis drought-tolerant myb96-1D activation-tag mutant (Seo et al., 2011). These findings indicate that the elevated VLCFAs on cer9 are unlikely to explain the observed reduction in cer9’s cuticular permeability. Cutin is relatively hydrophilic, and so the elevated cutin on cer9 should likewise not be expected to improve cer9’s cuticular diffusion barrier. Notwithstanding, the physico-chemical basis for lipid involvement in cuticle permeability remains poorly understood (Kosma and Jenks, 2007). Previous reports suggest that cutin may provide a matrix in which hydrophobic waxes are packed into non-permeable regions of the cuticular membrane defining amorphous and/or polar diffusion pathways (Casado and Heredia, 2001; Riederer and Schreiber, 2001; Kerstiens, 2006; Kosma and Jenks, 2007). Our observation that cer9 has an increased thickness of the cutin matrix (via cuticle membrane) could indicate that cer9 deposits more total waxes inside the larger cutin layer, and thus possesses a longer and more circuitous pathway for water movement through the cuticle. Whether cer9’s elevated wax VLCFAs contribute directly to this barrier is unknown, though it is possible that the VLCFAs orient their charged ends to one side of the cuticle membrane in a way that allows their long saturated tails to pack closely and create to a more impermeable crystalline-like cuticle barrier. Further studies with cer9 (and
other cuticle mutants) using NMR, atomic force microscopy, or other nano-technology tools may shed light on these questions.

The cer9 mutant displayed another unique transpiration phenotype. Relative to wild type, the cer9 transpiration rate was suppressed more when exposed to light than darkness, indicating that cer9 was also having a direct effect on transpirational flux through stomatal pores. The cer9 mutant had similar stomatal density and stomatal index as wild type, so these aspects of epidermal architecture were not factors. We found using TEM that the cer9 stomatal ledges (or lips) lining the outer rim of the stomatal pore were larger than in wild type. The larger cer9 ledges occlude the pore, and may thus be expected to inhibit water vapor movement out of the stomata, to a greater extent than in wild type. Furthermore, cuticle is also deposited as a lining that coats the substomatal chamber in Arabidopsis (Xiao et al., 2004), and this mesophyll cuticle likely has a significant effect on the diffusion of CO₂ and water vapor from within mesophyll cells and into the substomatal chamber. Although we were unable to establish a statistically significant change in cer9’s substomatal cuticle thickness or ultrastructure, it should still be considered that the physico-chemical properties of cer9’s mesophyll cuticle may be altered in such a way as to explain cer9’s altered gas exchange. Previous studies demonstrate that CO₂ conductance through the cuticle is only 5.7% of that for water vapor (Boyer et al., 1997), and so even small changes in the mesophyll cuticle of the substomatal chamber of cer9 could have a large impact on the diffusion of these gases, especially CO₂, and thereby contribute to the improved water use efficiency of cer9. Findings here open up new avenues for exploring the role of stomatal cuticle as a target to improve plant drought tolerance and WUE.

The CER9 reporter gene studies reported here, as well as transcript accumulation based on Genevestigator (©NEBION/ETH, Zurich, 2008), showed that CER9 is expressed in roots. As suberin is chemically very similar to cutin, we examined suberin in cer9 roots, revealing that suberin levels were, in fact, higher in the cer9 mutant. These findings led us to speculate that a restriction in root uptake of water
may have contributed to *cer9*’s altered water relations. A recent report showed that the elevated root suberin in the *esb1* mutant lowered transpiration rates, observations that supported the standard model that suberin acts as an extracellular transport barrier limiting apoplastic radial transport of water and solutes (Baxter et al., 2009). Notwithstanding, the shoot-root grafting experiments presented here revealed that the higher suberin content of *cer9* roots played no apparent role in *cer9*’s lower transpiration and delayed leaf wilting phenotype, and that cuticle-associated changes in *cer9*’s aerial organs were likely responsible.

Changes in *cer9* cuticle lipid composition, combined with identification of the *CER9* gene sequence and its predicted protein, sheds new light on the function of *CER9*. The *cer9* mutation caused a massive and specific increase in C24 and C26 VLCFAs, especially on leaves, and inhibited synthesis of all other wax compounds, causing over 27% increase in total leaf wax amount, due overwhelmingly to a 1,245% increase in the VLCFAs. The VLCFAs increased similarly on *cer9* stems, but much less, being elevated by 120% (Fig. 3B). Total cutin monomers were elevated 59% on *cer9-1* leaves, and this was associated with a 179% increase in total C18 cutin monomers, but with reductions in C16 cutin monomers. Cutin monomer changes on *cer9* stems were nearly the same. From these results, it appears that the protein encoded by *CER9* acts as a negative regulator of cuticle wax and cutin monomer synthesis, that when deficient elevates normal synthesis of 24 and 26 carbon length VLCFA, and in the case of cutin elevates synthesis of 18 carbon length cutin monomers.

The coding sequence of *CER9* was found to encode a protein highly similar to the yeast Doa10 protein (as well as its ortholog TEB4 from humans), with especially high conservation in the RING-CH-type zinc finger and TD domains (Supplemental Fig. S2) (Swanson et al., 2001; Kreft and Hochstrasser, 2011). The causative mutation of the *cer9-1* allele is in the last cysteine of the RING-CH domain, which has been mutated to a tyrosine (C114Y). CER9’s RING-CH domain likely plays an important function since the *cer9-1* mutant expressing a full-length transcript (with RING-CH
domain point mutation) exhibits essentially the same phenotype as the cer9-2 mutant expressing a highly truncated CER9 transcript. Studies have shown that the yeast Doa10 protein functions as an E3 ubiquitin ligase involved in Endoplasmic Reticulum (ER)-associated Degradation (ERAD) of mis-folded or unassembled proteins (Swanson et al., 2001; Hassink et al., 2005). Even though studies of the Arabidopsis ERAD system are limited, Arabidopsis possesses many conserved components of the yeast ERAD system, such as HRD3A/EBS5 and HRD1 of the HRD1/HRD3 complex (Liu et al., 2011; Su et al., 2011). High CER9 similarity to Doa10, and the presence of the necessary cognates for ERAD in plants, leads us to speculate that CER9 could have a similar function to Doa10. Most enzymes in wax and cutin biosynthesis are reported as ER localized (Kunst et al, 2009), and thus it is possible that CER9 works to process defective proteins that arise during cuticle metabolism. Whether CER9 actually functions as an E3 ligase, and how this might produce the observed wax and cutin metabolic alterations remains to be determined.

The interaction of cer9 with other genes was examined using double mutant to shed further light on CER9 function. The cer9 mutation generally showed additive effects for both wax and cutin synthesis by leaves when combined with the mutations cer6, cer8, and lacs2 (except cer8 cer9 cutin synthesis wherein cer9 was epistatic, and lacs2 cer9 cutin synthesis wherein lacs2 was epistatic). As such, an exact location for CER9 function in these cuticle lipid biosynthetic pathways is not apparent. Notwithstanding, these results are useful in that they indicate a role for CER9 in early steps of both the wax and cutin biosynthetic pathways. Analysis of inflorescence stem cuticle lipids on these double mutants by Goodwin et al. (2005), Lü et al. (2009; and unpublished) revealed comparable results as in leaves, and a similar interpretation that CER9 functions in early steps of cuticle lipid synthesis.

The interaction of cer9 with other genes was also examined using transcriptome analysis. A total of 591 genes representing diverse cellular processes exhibited 2-fold or higher transcript abundance in cer9 than in wild type, whereas 91 genes were down regulated 2-fold or more in cer9. Although cuticle lipids are greatly altered in cer9,
the expression of few lipid-associated genes was affected by the cer9 mutation, and none of these predicted to have direct involvement in wax or cutin biosynthesis. Somewhat surprisingly, the gene expression network most impacted by the cer9 mutation was associated with stress response. Stress-associated genes in cer9 showing altered translation included 95 that were up-regulated and 10 that were down-regulated. All the plants examined in our studies were replicated in randomized blocks in the growth chamber, well watered, and otherwise did not experience environmental stress. One possible explanation for cer9’s impact on so many stress transcripts is that CER9 impacts synthesis of cuticle lipids that serve as regulators of environmental stress response, as proposed by Wang et al. (2011). Alternatively, the cer9 mutant could be experiencing an “ER stress” due to changes brought on by the absence of CER9 and its possible E3 ubiquitin ligase protein degradation function. That ER stress related transcripts of bZIP60 and BIP3 (Iwata and Koizumi, 2005; Iwata et al., 2008), and C2H2-type zinc-finger protein and CYP71A12 are also elevated in cer9 supports this postulate. Further observation that 21 genes encoding proteins specifically involved in protein ubiquitination and degradation were also activated in the cer9 mutant provides additional evidence that CER9 may have a function in ER-associated-protein degradation.

Study of the cer9 mutant reveals the first described cuticle biosynthesis gene whose deficiency increases cuticle lipid deposition, and improves plant tolerance to water deficits, and improves plant WUE. These studies provide evidence that the CER9 protein is a negative regulator of cuticle lipid synthesis via its putative role as an E3 ubiquitin ligase, similar as Doa10 in yeast. Due to its novel impact on plant water status, elucidation of CER9’s cellular function may reveal new molecular breeding and transgenic strategies to improve the drought tolerance and WUE of crop plants.

MATERIALS AND METHODS

Plant materials and growth conditions
Seeds stocks of *cer8-1*, *cer9-1*, *cer6-1* and *cer9-2* (GABI_588A06) were obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org/). *cer8-1 cer9-1* and *cer6-1 cer9-1* were as reported in Goodwin et al. (2005), *lacs2-3* (GABI_368C02) was kindly provided by Dr. Christiane Nawrath (University of Lausanne, Switzerland) (Bessire et al., 2007). *cer9-2* line was backcrossed to the wild-type Col-0, and advanced to the F2 generation for genetic analysis. The ratio of wild type:glossy mutant was approximately 3:1 (453:153= 2.9524:1, chi-squared test on 310 plants, P <0.05) indicating recessive inheritance. *Arabidopsis thaliana* wild type and mutant seeds were stratified for 3–4 days at 4°C, and plants were grown in soil at 21–22°C in a growth room with 30–60% humidity, a 16-h/8-h light/dark cycle, and a light intensity of 90–110 mol m⁻² sec⁻¹.

**Mapping and cloning of CER9**

*cer9* was rough mapped to chromosome 4 after which PCR-based genotyping was used to further map the *cer9-1* mutation. We were able to map the gene to within a 400 kb genomic region using four SSLP markers and a population of 96 F2 plants from the Col-0 x *cer9-1* cross. Other SSLP and CAPS markers were developed within this 400 kb mapping interval and used to screen 2000 additional Col-0 x *cer9-1* F2 plants to identify informative recombinants to further narrow the mapping interval to a 63 kb region between CAPS markers Fo16.296M and Fo16.359M. Overlapping fragments of approximately 0.7~1 kb each, covering this 63 kb candidate region, were amplified from the *cer9-1* genome and sequenced. The sequence of these fragments was aligned with the wild type sequence using DNASTAR (DNASTAR, Inc., USA). All primers for molecular markers are listed in Supplemental Table S1.

**Generation of gene constructs and transgenic plants**

The whole coding region of *At4g34100* was amplified from the cDNA of wild type Col-0 using the CER9-SpeI-F and CER9-Ascl-R primers. The PCR products were cut with SpeI and Ascl and cloned into the same sites of to make the 35S-CER9 construct.
that was fully sequenced to ensure no PCR error. The \textit{35S-CER9} construct was introduced into \textit{cer9-2} by \textit{A. tumifaciens} mediated transformation using the floral dipping method. Transgenic lines were identified in the T2 generation for further analyses.

**Promoter–GUS reporter gene fusions and GUS histochemical assay**

The 2000bp promoter region of \textit{CER9} was amplified by PCR from Col-0 genomic DNA using the CER9pro-F and CER9pro-R primers (Supplemental Table S1). The amplified \textit{CER9} promoter was first subcoloned into pBluescript II KS vector and sequenced. The sequenced fragment was cut with \textit{KpnI} and \textit{BglII} and cloned into the same sites of pCAMBIA1303. The CER9pro::GUS construct was introduced into Arabidopsis Col-0 by \textit{A. tumefaciens}-mediated transformation using the floral dipping method. Single loci, homozygous T-DNA lines were identified in the T3 generation for histochemical analyses. Histochemical GUS staining was performed on different tissues during different development phases as described in Lü et al. (2009).

**Cuticular wax and polyester analysis**

The cuticular wax composition of leaves and stems of 6-week-old plants was determined as described by Chen et al. (2003) with slight modifications described by Lü et al. (2009). Stem and leaf cutin monomer content, together with root suberin monomer composition, of six-week-old plants were analyzed based on methods described by Franke et al. (2005) and Bonaventure et al. (2004) with slight modifications described by Lü et al. (2009).

**Scanning and transmission electron microscopy**

Cryogenic scanning electron microscopy (SEM) was used to view epicuticular wax crystallization patterns. Stem (second internode above the rosette) samples were collected from plants after 6 weeks of growth. Samples were prepared and viewed by cryo-SEM as described by Lü et al. (2009). The cuticle and outer cell wall...
ultra-structures of leaves and inflorescence stems of 6-week-old plants were viewed by TEM as reported by Chen et al. (2003).

**Analysis of transpiration rates**
Transpiration rates were done following the method reported by Baxter et al. (2009) with slight modifications. In brief, plants were grown for 5 weeks in 2 inch pots with 12 hr of photosynthetically active light (100–120 mmol/m²/s), with mean day and night temperatures of 22 and 19°C, respectively. For analysis of transpiration rates, pots were covered with plastic wrap to avoid water loss from the soil, and placed on one of twenty balances (EK-410i, A&D) to monitor changes in weight. Weights of pots were automatically recorded using balances connected to computers through WinWedge software (TAL technologies Inc.) at 5 min intervals for 1 day and 2 nights. A total of six to seven plants per genotype were analyzed. At the end of the experiment, total leaf area for each plant was determined by digitally recording images of all leaves and using ImageJ software to determine leaf area.

**Analysis of stomatal density and index as well as pavement cell density**
Five week old plants grown under the conditions described above for measurement of transpiration were used for measuring stomatal density and index as well as pavement cell density as described by Chen et al. (2003)

**Carbon isotope ratio analysis**
10 individuals of Col-0, Ler-0, lacs1-1, lacs2-3, att1-2, wax2, cer9-1 and cer9-2 in three randomized blocks were grown in a Percival Model ARR-66 growth chamber under short days (10hr light; 14 hr dark). Plants were well-watered throughout the growing period. At 4 weeks post-germination, all aerial structures were harvested, cleaned of any contaminating soil, and dried for 24 hours at 70°C. Two milligrams of finely ground dry powder were placed in capsules and then analyzed at the UC Davis Stable Isotope Facility (http://stableisotop facility.ucdavis.edu). Carbon isotope
compositions of samples are presented as values relative to the PeeDee Belemnite standard.

We performed factorial ANOVAs to test the hypothesis that mutants in cuticle biosynthesis affect $\delta^{13}C$ as compared to wild type. For mutants in the Columbia background, we performed an ANOVA with genotype as a fixed effect and experimental block as random effect. We performed a separate ANOVA to test for a difference in water use efficiency between Ler-0 and the cer9-1 mutant. All analyses were performed in JMP 8.0.

**Wilting assays**

Water deficit stress was imposed by withholding water from containers of soilless media containing 9 plants (3 weeks old) (10 replicates each genotype). Containers were irrigated with water to saturation. Pictures were taken after 10 days without water. In the whole process, 2 replicates were used to measure the leaf RWC of fully expanded leaves of 3-Weeks old plant after the containers were saturated with water. And 2 replicates were used to measure the leaf RWC of leaves after which watering was stopped for 10 days. Leaves were removed and immediately weighed to obtain leaf FW. Leaves were then placed into vials filled with distilled water for 24 h, blotted to remove excess water, and then weighed to obtained leaf turgid weight (TW). Leaves were then dried to a constant weight at 65°C and reweighed to obtain leaf dry weight (DW). Leaf RWC was calculated as $(FW - DW)/(TW - DW) \times 100$.

Grafted seedlings were grown in soil as previously described by Rus et al. (2006). To measure wilting resistance, grafted plants were transferred to soil and grown for three weeks, after which watering was stopped and plants observed for symptoms of wilting for 11 days.

**RT-PCR and quantitative RT-PCR**

Reverse transcriptase-PCR was used to analyze the expression levels of CER9 in the different cer9 allelic mutants and their corresponding wild-type ecotypes together
with cer9-2 transgenic lines harboring 35S-CER9 construct. Total RNA was extracted from 4-5 week-old rosette leaves using TRIZOL reagent (Invitrogen Life Technologies, http://www.invitrogen.com/) according to the manufacturer’s protocol. First-strand cDNA was synthesized using reverse transcriptase (Promega, http://www.promega.com/) according to the manufacturer’s protocol. For RT-PCR, the cycle number and amount of template were optimized for all fragments amplified to yield products in the linear range of the reaction. The actin gene ACTIN2 (At3g18780) was used as a constitutive control using primers ACTIN2-F and ACTIN2-R. Primers used for CER9 were RT-F1 and RT-F2 combined with RT-R1 or RT-R2, respectively. The sequences of the above primers are listed in Supplemental Table S1.

For quantitative RT-PCR (qRT-PCR) analysis, total RNA was extracted from tissues of plants grown under the same conditions as that used for DNA microarray analysis. qRT-PCR reactions were performed in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR system using the SYBR Green I master mix in a volume of 20 µL. The reactions were performed in biological triplicates using RNA samples extracted from three independent plant materials and gene-specific primers listed in Supplemental Table S1. Data was analyzed using the SDS software (Applied Biosystems version 1.0). CT values were determined based on efficiency of amplification. The mean CT values were normalized against the corresponding ACTIN2 gene. ΔCT values calculated as (CT. gene of interest - CT. ACTIN2) and ΔΔCT values was calculated as (ΔCT.cer9-2 - ΔCT.wt). The relative expression level of selected genes was calculated using the $2^{-\Delta\Delta CT}$ method. A $2^{-\Delta\Delta CT}$ value for the wild type was normalized to 1.

**DNA microarray analysis**

Total RNA was extracted from leaves of 5 week-old soil-grown wild-type plants and cer9-2 mutants using a RNeasy® plant mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Total RNA was quantified and quality checked using
a Nanodrop-ND 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Replicated samples with a RNA integrity number (RIN) of 7.5 and above were chosen for further analysis. DNA microarray analysis was performed as described in Lü et al. (2011).

Supplemental material

Supplemental Figure S1. SEM results showing stem epicuticular wax crystals in complemented transgenic lines. (A) wild type Col-0; (B) cer9-2; (C) transgenic line 9 of cer9-2 harboring 35S-CER9; (D) transgenic line 11 of cer9-2 harboring 35S-CER9; (E) Diagnostic PCR of the T-DNA inserted in cer9-2 and transgenic lines (9 and 11) of cer9-2 harboring 35S-CER9. Primers used for PCR are indicated above each lane. (F) RT-PCR analysis of the CER9 transcripts in leaves of Col-0 (wild-type), cer9-2 and transgenic lines (9 and 11) of cer9-2 harboring 35S-CER9 construct. The primer pairs used for RT-PCR are shown on the left. ACTIN2 was used as an internal control.

Supplemental Figure S2. Sequence alignment (CLUSTALW) of Arabidopsis CER9 (At4g34100) with five related proteins. The fourteen predicted TM segments are highlighted by horizontal bars. The conserved cysteines and histidine in the RING-CH domain are marked by an asterisk. The φPφxxG motif (where φ is a hydrophobic residue) in TM5, which is important for DOA10 function is indicated. The (C to Y) point mutation in cer9-1 is indicated by arrow. CER9 (At4g34100; Arabidopsis thaliana); Pt, Populus trichocarpa; Os, Oryza sativa; Hv, Hordeum vulgare; DOA10 (Saccharomyces cerevisiae); TEB4 (Homo sapiens).

Supplemental Figure S3. Cuticular wax composition on leaves and stems of Arabidopsis. Wax coverage is expressed as µg dm⁻² of leaf surface area. The genotype of each sample is shown in the upper right of the charts. Each wax constituent is designated by carbon chain length and is labeled by chemical class along the x-axis. (A) Wild type Ler-0 and mutants in this background. (B) Wild type Col-0 and mutants in this background. Each value represents the mean of four replicates. Error bars = SD.
**Supplemental Figure S4.** Cutin monomer composition on leaves and stems of Arabidopsis. Leaves were analyzed for aliphatic cutin monomer composition. Monomer amounts are expressed as μg dm⁻² of stem surface. (A) Wild type Ler-0 and mutants in this background. (B) Wild type Col-0 and mutants in this background. Values represent the mean of 4 replicates. Error bars = SD.

**Supplemental Figure S5.** qRT-PCR analysis of selected stress related genes that are differentially modulated between cer9-2 and wild-type (Col-0) plants. Data are mean ± SD (n = 3) from 1 representative experiment. Three independent experiments were performed; the results from each experiment exhibit similar relative trends.

**Supplemental Table S1.** Primers used in this study.

**Supplemental Table S2.** Genes whose transcript abundance is up-regulated at least 2-fold in cer9-2 leaves.

**Supplemental Table S3.** Genes whose transcript abundance is down-regulated at least 2-fold in cer9-2 leaves.
ACKNOWLEDGEMENTS

We are grateful to Dr Christiane Nawrath (University of Lausanne, Switzerland) for providing *lacs2-3* seeds. We also thank Debra Sherman and Chia-Ping Huang of the Purdue University Electron Microscopy Center for their support.
LITERATURE CITED


Lü S, Song T, Kosma DK, Parsons EP, Rowland O, Jenks MA (2009) Arabidopsis CER8 encodes a long-chain acyl CoA synthetase 1 (LACS1) and has overlapping functions with LACS2 in plant wax and cutin synthesis. Plant J 59: 553-564


Figure Legends,

Figure 1. Map-based cloning of CER9.
(A) The mutated gene was mapped between SSLP markers Fo12.3MB and Fo21.2MB using 96 F2 plants. (B) Fine mapping narrowed cer9-1 down to a 63 kb region between markers Fo16.296MB and Fo16.359MB using 2000 F2 plants. Numbers above the horizontal line in (A) and (B) are the number of recombinants between the indicated marker and cer9-1. (C) Sequenced region of cer9-1 mutant and the T-DNA insertion allele cer9-2. The white box represents the sequenced region between markers Fo16.296MB and Fo16.359MB. Grey arrows represent genes in the sequenced region. Dashed line shows the mutated site of cer9-1. Start, start codon; Stop, stop codon. Black bar represents untranslated region and gray bar represents coding region. The black triangle indicates the T-DNA insertion site of the cer9-2 allele. (D) RT-PCR analysis of CER9 transcript levels in leaves of cer9 mutants compared with the corresponding wild type using primers pairs as shown in (C).

Figure 2. Gene expression pattern of CER9 using promoter::GUS transgenic line.
Stained tissues expressing the CER9pro::GUS reporter gene fusion: (A) 2-day-old seedling after germination; (B) 5-day-old seedling; (C) 10-day-old seedling; (D) inflorescence stem of 6-week-old plant showing high expression in upper stem; (E) cross section from top portion of stem showing expression throughout epidermis and mesophyll of five-week-old plants; (F-H) GUS staining on leaf, sique and flowers of a 6-week-old plant, respectively.

Figure 3. Cuticular wax composition of inflorescence stems and leaves of wild-type Ler-0 and the cer9-1 mutant.
Wax coverage is expressed as µg dm^{-2} leaf (A) and stem (B) surface area. Each wax constituent is designated by carbon chain length and is labeled by chemical class along the x axis. Mean ± SD; n = 4.

Figure 4. SEM results showing stem epicuticular wax crystals.
(A) wild type Ler-0; (B) cer9-1; (C) wild type Col-0; (D) cer9-2. Bars = 5 µm.

Figure 5. Cutin monomer composition of inflorescence stems and rosette leaves of
wild-type Ler-0 and the cer9-1 mutant.
The C16 and C18 labels on the x axis represent the 16- and 18-carbon acid chains, respectively, whereas the number preceding the “OH” indicates chain insertion point(s). Dioic represents dioic acid. The number of double bonds is indicated after the colon. Monomer amounts are expressed as µg dm⁻² leaf (A) and stem (B). Mean ± SD; n = 4. ** P ≤ 0.01.

Figure 6. Transmission electron micrographs of the cuticle layer of epidermal cells. (A) Ler-0 leaf adaxial layer; (B) cer9-1 leaf adaxial layer; (C) Ler-0 leaf abaxial layer; (D) cer9-1 leaf abaxial layer; (E) Ler-0 stem; (F) cer9-1 stem; Scale bars: 0.2µm. The cuticular layer is indicated by the black arrow. (G) Ler-0 leaf stomata; (H) cer9-1 leaf stomata; (I) Ler-0 stem stomata; (J) cer9-1 stem stomata; Scale bars: 1µm. The cuticular ridges are indicated by the black arrow.

Figure 7. Both cer9-1 and cer9-2 are more resistant to drought stress compared to the corresponding wild type Ler-0 and Col-0, respectively. The percentage of relative water content (RWC) shown under each representative plant is the average of 10 plants.

Figure 8. cer9 mutants have reduced transpiration rate and improved water use efficiency. (A-B) Transpiration rates of five-week-old wild-type and mutant plants. Five-week-old plants of Ler-0, cer9-1, Col-0, cer9-2, lacs2-3 and lacs2-3 cer9-2 double mutant grown under 12 hr/12 hr day/night were used for the transpiration experiment. Water loss from each plant was measured as weight change at 5 minute intervals over 36 hr. At the end of the experiment, leaf area was measured and transpiration rate calculated. Data represents the mean ± standard error of n = 5 replicate plants for each genotype. Black horizontal bars represent nighttime period, white bars represent daytime period. Transpiration rates of five week old wild-type and mutant plants. (C-E), Stomatal density (the number of stomata per area), pavement cell density and stomatal index (the number of stomata per total epidermal cells), respectively, were analyzed in the leaf abaxial epidermal layers from wild-type,
cer9-2, lacs2-3 and lacs2-3 cer9-2 double mutant plants. Data are the mean of seven individual plants (mean ± SE, n = 7). (F) Carbon isotope analysis of cutin mutants of att1, cer9, lacs1, lacs2 and wax2 compared to wild type. Values are carbon isotope discrimination relative to the Pee Dee Belemnite standard. More negative values correspond to less water use efficient plants. Bars are one standard error, * P ≤ 0.05.

Figure 9. Suberin monomer composition in roots of wild-type (Col-0 and Ler-0) and cer9 mutants. The cer9 mutants exhibit higher aliphatic suberin monomer amounts than wild type. Means ± SD, n=4, * P ≤ 0.05.

Figure 10. Wilting test of grafted wild-type Col-0 and mutant plants. After grafting, plants were grown for 4 weeks in soil with regular watering, after which time watering was stopped and the plants’ wilting status recorded at 11 days after water withdrawal. The percentage of relative water content (RWC) shown under each representative plant is the average of 10 plants. (A) Wild-type Col-0 without water withdrawal. (B) cer9-2 without water withdrawal. (C) Wild-type Col-0 with water withdrawal. (D) cer9-2 with water withdrawal. (E) Self grafted wild-type Col-0 with water withdrawal. (F) Self grafted cer9-2 with water withdrawal. (G) Wild-type Col-0 shoot/cer9-2 root grafted with water withdrawal. (H) cer9-2 shoot/wild-type Col-0 root grafted.

Figure 11. DNA microarray analysis reveals altered gene expression in cer9-2 leaves relative to wild type. Functional categorization of mRNAs that differentially accumulate in cer9-2 and wild-type leaf tissues. mRNAs identified as having at least 2-fold change relative to wild type are placed in separate categories based on predicted function (the number identified in parentheses). The first figure (A) shows genes that are up-regulated in cer9-2 leaves relative to wild-type, whereas (B) shows genes that are down-regulated in cer9-2 relative to wild-type. See Supplemental Tables S2 and S3 for additional information about these genes.
Table I. Cuticular wax composition of inflorescence stems of Arabidopsis Landsberg *erecta*-0 (*Ler*-0) and *cer9*-1 and of leaves of *Ler*-0 and *cer9*-1. Mean (µg dm⁻² ± SD) total wax amounts and coverage of individual compound classes (n=3-4).

### Inflorescence Stems

<table>
<thead>
<tr>
<th>Compound Class</th>
<th><em>Ler</em>-0</th>
<th><em>cer9</em>-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total load</td>
<td>2175.0±159.1</td>
<td>947.6±27.7</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>29.9±3.2</td>
<td>65.9±2.7</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>105.4±15.1</td>
<td>53.3±4.6</td>
</tr>
<tr>
<td>1-Alcohols</td>
<td>137.4±9.2</td>
<td>194.9±7.3</td>
</tr>
<tr>
<td>Alkanes</td>
<td>1196.0±90.4</td>
<td>310.1±8.9</td>
</tr>
<tr>
<td>2-Alcohols</td>
<td>85.5±9.9</td>
<td>31.3±4.4</td>
</tr>
<tr>
<td>Ketones</td>
<td>589.1±42.4</td>
<td>222.3±8.5</td>
</tr>
<tr>
<td>Esters</td>
<td>31.7±8.2</td>
<td>69.7±6.2</td>
</tr>
</tbody>
</table>

### Rosette Leaves

<table>
<thead>
<tr>
<th>Compound Class</th>
<th><em>Ler</em>-0</th>
<th><em>cer9</em>-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total load</td>
<td>128.9±11.1</td>
<td>164.3±16.7</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>11.2±1.6</td>
<td>150.7±15.2</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>4.6±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>1-Alcohols</td>
<td>23.4±3.7</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>Alkanes</td>
<td>89.1±6.3</td>
<td>7.8±1.5</td>
</tr>
<tr>
<td>2-Alcohols</td>
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<td>-</td>
</tr>
<tr>
<td>Ketones</td>
<td>0.65±0.3</td>
<td>0.16±0.06</td>
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<tr>
<td>Esters</td>
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<td>-</td>
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<tr>
<td></td>
<td>With water</td>
<td>RWC%</td>
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<tr>
<td>Ler-0</td>
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<td>88.7±6.5</td>
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<tr>
<td>cer9-1</td>
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<td>87.5±5.9</td>
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<tr>
<td>Col-0</td>
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<tr>
<td>cer9-2</td>
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<td>90.1±5.4</td>
</tr>
</tbody>
</table>
A. Genes up-regulated >2-fold in *cer9-2* leaves

- Lipid metabolism (5)
- Response to hormones (30)
- Stress related (95)
- Protein ubiquitination and degradation (21)
- Unfolded protein response (4)
- Metabolic process (31)
- Protein phosphorylation and dephosphorylation (67)
- Growth and development (31)
- Regulation of membrane transport (36)
- Regulation of transcription and translation (83)

B. Genes down-regulated >2-fold in *cer9-2* leaves

- Lipid metabolism (3)
- Response to hormones (7)
- Stress related (10)
- Protein ubiquitination and degradation (2)
- Metabolic process (8)
- Protein phosphorylation and dephosphorylation (6)
- Growth and development (6)
- Regulation of membrane transport (6)
- Regulation of transcription and translation (6)