Arabidopsis ECERIFERUM9 Involvement in Cuticle Formation and Maintenance of Plant Water Status

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Mutation of the ECERIFERUM9 (CER9) gene in Arabidopsis (Arabidopsis thaliana) 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 tetracosanoic acid (C24) and hexacosanoic acid (C26). Relative to the 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 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 that it encodes an E3 ubiquitin ligase homologous to yeast Doa10 (previously shown to target endoplasmic reticulum 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 analyses. For both wax and cutin, cer9 showed mostly additive effects with cer6, long-chain acyl-CoA synthetase1 (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. The discovery of CER9 lays new groundwork for developing novel cuticle-based strategies for improving the drought tolerance and water use efficiency of crop plants.

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 (Schröder et al., 2001; Chaerle et al., 2005; Nilson and Assmann, 2007; Sirichandra et al., 2009; 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 nonstomatal 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 the substomatal chamber (Xiao et al., 2004; Kerstiens, 2006; Kosma et al., 2009; Lü et al., 2009). The cuticle is composed primarily of two lipid classes, the nonpolymerized cuticular waxes and the cutin polyester. Most waxes are very-long-chain (C25–C34) saturated lipids that occur as epicuticular and intracuticular lipids, whereas the more hydrophilic cutin polyester consists of C16 and C18 fatty acid derivatives (e.g. hydroxy fatty acids and dicarboxylic acids) linked primarily by ester bonds.

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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 (*Solanum lycopersicum*) eceriferum6 (*cer6*) and positional sterile mutants (Leide et al., 2007, 2011), cutin monomer mutants having wild-type waxes such as *att1*, *hothead*, and gpat4/*gpat8* (Xiao et al., 2004; Kurdyukov et al., 2006b; Li et al., 2007), and mutants conferring alterations in both waxes and cutin monomers, such as *lacs1*, *lacs2*, bodyguard, glosshead1 (Chen et al., 2003; Kurdyukov et al., 2006a; Lü et al., 2009, 2011), and *wax2* (E.P. Parsons, unpublished data); all were shown to exhibit elevated cuticle permeability. Additionally, Arabidopsis (*Arabidopsis thaliana*) plants exposed to water deficit conditions induce the synthesis of cuticular waxes and cutin monomers, especially waxes, which is often associated with improved plant tolerance to water deficiency (Cameron et al., 2006; Kosma et al., 2009; Seo et al., 2011). When expressed ectopically, the transcription factors MYB96 and WAX INDUCER1/SHINE1 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 (Aharoni et al., 2004; Zhang et al., 2005, 2007; Seo et al., 2011). 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 the 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 *cer9* was shown to have a semiglossy 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) tetracosanoic acid (*C_{24}*), and hexacosanoic acid (*C_{26}*), which are constituents at near trace amounts on the wild type (Jenks et al., 1995; Goodwin et al., 2005). The effect of the *cer9* mutation on cutin monomer composition is reported here, to our knowledge for the first time, revealing a major increase in the amount of this lipid class. We describe the 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 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 118.2 centimorgan on chromosome 4 using the recessive ethyl methanesulfonate-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 polymorphism (SSLP) markers F015.9M and F016.5M 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 derived CAPS, and narrowed the location of the *cer9*-1 mutation to a 63-kb interval between markers F016.296M and F016.359M (Fig. 1B). DNA sequencing of this 63-kb region revealed a G-to-A single nucleotide polymorphism in *cer9*-1 that causes the conversion of a highly conserved Cys to Tyr 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, that 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). Semiquantitative reverse transcription (RT)-PCR using RNA isolated from leaves showed that the total abundance of the At4g34100 transcript in *cer9*-1 was similar to the wild type, suggesting that the point mutation in *cer9*-1 does not affect 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). This 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 the translation of a full protein. *cer9*-2 also exhibited a semiglossy stem as well as stem and leaf wax and cutin monomer composition essentially identical to that of the *cer9*-1 allelic mutant (see Figs. 3 and 5 below; Supplemental Figs. S3 and 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 the wild type, further verifying CER9 (At4g34100) control over the associated phenotypes (Supplemental Fig. S1, A–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 CER9 transcript levels in leaves of cer9 mutants compared with the corresponding wild type using primer pairs as shown in C.

Organ- and Tissue-Specific Expression of CER9

We constructed a CER9pro::GUS construct for the 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-d-old seedlings (Fig. 2A). Relatively strong signals were detected in roots, cotyledons, and true leaves of 5- and 10-d-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, inflorescences, and siliques (Fig. 2, D and F–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(45)-C-x(1)-C-x(10-25)-C-x(2)-C, which is different from that of the 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...

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**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 2,000 F2 plants. Numbers above the horizontal lines in A and B are the number of recombinants between the indicated marker and cer9-1. C, Sequenced region of the 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. Gray arrows represent genes in the sequenced region. The dashed line shows the mutated site of cer9-1. Start, Start codon; Stop, stop codon. The black bar represents the untranslated region, and the gray bar represents the 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 primer pairs as shown in C.
Cuticular Waxes of the TD domain (transmembranes 5 TD (for TEB4-Doa10; Kreft and Hochstrasser, 2011). The conserved segment of approximately 130 residues called the RING-CH domain (as noted above) and an internal high similarity with that of Doa10 and TEB4, with 49% identity, respectively (Supplemental Fig. S2).

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

the 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 with that of Doa10 and TEB4, with 49% and 57% identity, respectively (Supplemental Fig. S2). All Doa10 orthologs are characterized by an N-terminal and 57% identity, respectively (Supplemental Fig. S2).

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 C18:2 dioic acid, was increased to 187% (P < 0.0001) above wild-type levels, whereas 18-OH-C18:1 acid was increased by 124% (Fig. 5A). There was no significant difference observed in 16-OH-C16:0 monoesters having over 120% more total esters than the wild type. 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. 4).
monomers on cer9-1 stems compared with 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 the wild type in both leaves (both adaxial and abaxial surfaces) and stems (Fig. 6, A–F). The cuticle membrane that forms the cuticular ridge over the outside of the stomatal pore (forming the antechamber) was larger in both leaf and stem of cer9-1 (Fig. 6, G–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 the 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 increases 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%

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**Table 1. Cuticular wax composition of inflorescence stems of Arabidopsis Ler-0 and cer9-1 and of leaves of Ler-0 and cer9-1**

Values shown are means ± SD (µg dm⁻²) total wax amounts and coverage of individual compound classes (n = 3–4). –, Undetectable.

| Sample       | Total Load | Fatty Acids | Aldehydes | 1-Alcohols | Alkanes | 2-Alcohols | Ketones | Esters |
|--------------|------------|-------------|-----------|------------|---------|------------|---------|--------|
| Inflorescence stems |
| Ler-0        | 2,175.0 ± 159.1 | 29.9 ± 3.2 | 105.4 ± 15.1 | 137.4 ± 9.2 | 1,196.0 ± 90.4 | 85.5 ± 9.9 | 589.1 ± 42.4 |       |
| cer9-1       | 947.6 ± 27.7  | 65.9 ± 2.7  | 53.3 ± 4.6   | 194.9 ± 7.3  | 310.1 ± 8.9  | 31.3 ± 4.4  | 222.3 ± 8.5  | 69.7 ± 6.2 |
| Rosette leaves |
| Ler-0        | 128.9 ± 11.1  | 11.2 ± 1.6  | 4.6 ± 0.2    | 23.4 ± 3.7   | 89.1 ± 6.3  | –           | 0.65 ± 0.3   | –      |
| cer9-1       | 164.3 ± 16.7  | 150.7 ± 15.2 | 1.4 ± 0.2    | 3.9 ± 0.4    | 7.8 ± 1.5   | –           | 0.16 ± 0.06  | –      |
reduction in C_{28} 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 by Lü et al. (2009), the lacs2-3 mutant has lower C_{28}-C_{30} constituents for each wax class examined (except acids, for which only C_{30} 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 that 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 C_{32}, C_{34}, and C_{26} fatty acids increased by 292-, 217-, and 20-fold, respectively, relative to the 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 in 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 a 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 (Millar et al., 1999), the lack of 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 the 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 C_{18:0} dioic acid (P < 0.01), a 45% reduction in C_{18:1} dioic acid (P < 0.001), an 83% reduction in 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 monomer profile detected in the lacs2-3 cer9-2 double mutant is similar to that of single mutant lacs2-3, indicating that the 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). Furthermore, the differences in transpiration rate were much greater in light than in dark conditions, implicating cer9 effects in stomatal water loss (Fig. 8A and B). Interestingly, the
lower transpiration rate of the cer9-2 mutant was overcome when cer9-2 was 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 the stomata density, pavement cell density, and stomatal index of cer9-2, lacs2-3, and lacs2-3 cer9-2 mutants are unchanged, being similar to those of wild-type Col-0 (Fig. 8, C–E).

We also estimated the effect of several cuticle mutants on integrated WUE. WUE is the ratio of carbon gained per unit of water lost through transpiration. WUE 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 CO2 inside the leaf and can be generated by variation in either stomatal constraint on the diffusion of CO2 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 (Triticum aestivum; 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, att1-2, and wax2. In the Col-0 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 posthoc Tukey honestly significant difference test determined that the cer9-2 mutant showed significantly less-negative \( \delta^{13}C \) (associated with higher WUE) than the isogenic parent Col-0, while the other cutin mutants, lacs1-1, lacs2-3, att1-2, and wax2, were all indistinguishable from the 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/Eidgenössische Technische Hochschule) 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

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

| With water | water withdrawal |
|------------|-----------------|
| **Ler-0**  | 88.7±6.5        | 33.4±2.8        |
| **cer9-1** | 87.5±5.9        | 58.3±3.9        |
| **Col-0**  | 89.1±6.9        | 38.3±3.8        |
| **cer9-2** | 90.1±5.4        | 69.5±3.2        |
types, while the C_{22:0} acids decreased to 57% of wild-type levels (but this is only significant in cer9-1). Other acid monomers (C_{16:0}, C_{18:0}, and C_{24:0}) were not changed significantly. Dioic acids C_{18:0} and C_{20:0} were increased to 181% and 146% in cer9-1 roots, respectively, and increased by 77% and 56% in cer9-2, respectively. Other dioic acid monomers were unchanged relative to the wild type. All ω-OH acids (C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2}, and C_{20:0}) in cer9-1 and cer9-2 were increased, except that C_{22:0} was 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 d) 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 with the Col-0...
wild-type parent are shown in Supplemental Tables S2 (up-regulated in cer9-2) and 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 associated with lipid biosynthetic pathways were up-regulated 2-fold or more in cer9-2: genes encoding cinnamoyl-CoA reductase-related protein (AT5G14700; 5.36-fold), hydrolase α/β-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 three lipid genes were down-regulated: genes encoding two lipid transfer proteins (AT1G62510 and AT2G37870; 2.64- and 3.72-fold, respectively) and one lipid-binding protein (AT4G33550; 2.47-fold; Fig. 11; Supplemental Tables S2 and 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). 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 seven 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 (ER) stress inducer (Iwata et al., 2008). Most other genes whose expression was modified 2-fold or more resembled fundamental metabolic (housekeeping) 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 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 (in the light) or closed (in the dark). Moreover, the cer9 mutant had higher WUE (less-negative δ13C), 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 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 the wild type, and the stem cuticle membrane thickness was comparably increased by 2.1-fold. The cer9 leaf cuticles were altered in a similar way. 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, so the elevated cutin on cer9 should likewise not be expected to improve cer9’s cuticular diffusion barrier. Notwithstanding, the physicochemical 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 nonpermeable 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 the 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, although 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 a more impermeable crystalline-like cuticle barrier. Further studies with cer9 (and other cuticle mutants) using NMR, atomic force microscopy, or other nanotechnology tools may shed light on these questions.

The cer9 mutant displayed another unique transpiration phenotype. Relative to the 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 to the 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 the 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 the 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 physicochemical
properties of cer9’s mesophyll cuticle may be altered in such a way 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), 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 WUE of cer9. The findings presented here open up new avenues for exploring the role of the 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/Eidgenössisch Technische Hochschule), 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 the 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 the 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 C₄₆ and C₅₀ VLCFAs, especially on leaves, and inhibited the synthesis of all other wax compounds, causing an 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 the normal synthesis of 24- and 26-carbon length VLCFAs and, in the case of cutin, elevates the 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 human), 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 Cys of the RING-CH domain, which has been mutated to a Tyr (C114Y). CER9’s RING-CH domain likely plays an important function, since the cer9-1 mutant expressing a full-length transcript (with a 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-associated degradation (ERAD) of misfolded 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/EB5 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, led 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 and Samuels, 2009); 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, remain to be determined.

The interaction of cer9 with other genes was examined using double mutants 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) and Lü et al. (2009) revealed comparable results as in leaves and a similar interpretation that CER9 functions in the 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 the wild type, whereas 91 genes were down-regulated 2-fold or higher transcript abundance in the wild type, whereas 91 genes were down-regulated 2-fold or higher transcript abundance. Although cuticle lipids are greatly altered in cer9, the expression of a few lipid-associated genes was affected by the cer9 mutation, and none of these were 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 the 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 the synthesis of cuticle lipids that serve as regulators of the 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 degredation 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. The 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.

This study of the cer9 mutant reveals, to our knowledge, the first described cuticle biosynthesis gene whose deficiency increases cuticle lipid deposition, 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 to Doa10 in yeast. Due to its novel impact on plant water status, elucidation of CER9 function in ER-associated protein degradation and WUE of crop plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seed stocks of Arabidopsis (Arabidopsis thaliana) cer8-1, cer9-1, cer6-1, and cer9-2 (GABI_388A06) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). cer8-1 cer9-1 and cer9-1 cer8-1 were as reported by Goodwin et al. (2005). lac2-3 (GABI_368C02) was kindly provided by Dr. Christiane Nawrath (Besore et al., 2007). The cer9-2 line was backcrossed to the wild-type Col-0 and advanced to the F2 generation for genetic analysis. The ratio of the wild type to the glossy mutant was approximately 3:1 (453:153 = 2.9524:1, \(P < 0.05\)). In total, 310 plants were genotyped for 24 h at 70°C. Two milligrams of DNA from each plant were used for the PCR test on 310 plants, which were grown in soil and placed on one of 20 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) at 5-min intervals for 1 d and two 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 digitalizing images of all leaves and using ImageJ software to determine leaf area.

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 h of photosynthetically active light (100–120 mmol m\(^{-2}\) s\(^{-1}\)), with mean day and night temperatures of 22°C 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 20 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) at 5-min intervals for 1 d and two 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 digitalizing 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

Ten individuals of Col-0, Ler-0, lac2-1, lac2-3, 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 (10 h of light/14 h of 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 h at 70°C. Two milligrams of finely ground dry powder were placed in capsules and then analyzed at the University of California Davis Stable Isotope Facility (http://stableisotopofacility.ucdavis.edu). Carbon isotope composition of samples is presented as values relative to the PeeDee Belemnite standard.
We performed factorial ANOVAs to test the hypothesis that mutations in cuticle biosynthesis affect δ13C as compared with the wild type. For mutants in the Col-0 background, we performed an ANOVA with genotype as a fixed effect and experimental block as a random effect. We performed a separate ANOVA to test for a difference in WUE between Ler-0 and the cer9-1 mutant. All analyses were performed in JMP 8.0.

Wiltting Assays

Water deficit stress was imposed by withholding water from containers of soilless medium containing nine plants (3 weeks old; 10 replicates for each genotype). Containers were irrigated with water to saturation. Photographs were taken after 10 d without water. In the whole process, two replicates were used to measure the leaf relative water content of fully expanded leaves of 3-week-old plants after the containers were saturated with water. Two replicates were used to measure the leaf relative water content of leaves, after which watering was stopped for 10 d. Leaves were removed and immediately weighed to obtain leaf fresh weight (FW). Leaves were then placed into vials filled with distilled water for 24 h, blotted to remove excess water, and then weighed to obtain leaf turgid weight (TW). Leaves were then dried to a constant weight at 65°C and reweighed to obtain leaf dry weight (DW). Leaf relative water content was calculated as (FW – DW)/(TW – DW) × 100.

Grafted seedlings were grown in soil as described previously by Rus et al. (2000). To measure wilting resistance, grafted plants were transferred to soil and grown for 3 weeks, after which watering was stopped and plants were observed for symptoms of wilting for 11 d.

RT-PCR and Quantitative RT-PCR

RT-PCR was used to analyze the expression levels of CER9 in the different cer9 alleles and their corresponding wild-type ecotypes together with cer9-2 transgenic lines harboring the 35S::CER9 construct. Total RNA was extracted from 4- to 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 (At5g18780) 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 analysis, total RNA was extracted from tissues of plants grown under the same conditions as those used for DNA microarray analysis. Quantitative RT-PCR was 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 the gene-specific primers listed in Supplemental Table S1. Data were analyzed using SDS software (Applied Biosystems version 1.0). Cycle threshold (CT) values were determined based on the efficiency of amplification. The mean CT values were normalized against the corresponding ACTIN2 gene. ΔCT values were calculated as ΔCT = CT gene of interest – CT ACTIN2, and ∆∆CT values was calculated as (∆∆CT = CT cer9-2 – CT wt [where wt indicates the wild type]). The relative expression level of selected genes was calculated using the 2−ΔΔCT method. A 2−ΔΔCT 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 an RNeasy plant mini kit (Qiagen) following the manufacturer’s instructions. Total RNA was quantified and quality checked using a Nanodrop-ND 8000 spectrophotometer (Thermo Fisher Scientific) and the 2100 Bioanalyzer (Agilent Technologies). Replicated samples with a RNA integrity number of 7.5 and above were chosen for further analysis. DNA microarray analysis was performed as described by Lu et al. (2011).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. SEM results showing stem epicuticular wax crystals in complemented transgenic lines.

Supplemental Figure S2. Sequence alignment (ClustalW) of Arabidopsis CER9 (At4g34180) with five related proteins.

Supplemental Figure S3. Cuticular wax composition on leaves and stems of Arabidopsis.

Supplemental Figure S4. Cutin monomer composition on leaves and stems of Arabidopsis.

Supplemental Figure S5. Quantitative RT-PCR analysis of selected stress-related genes that are differentially modulated between cer9-2 and wild-type (Col-0) plants.

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.

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