The Acyl Desaturase CER17 Is Involved in Producing Wax Unsaturated Primary Alcohols and Cutin Monomers

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We report n-6 monounsaturated primary alcohols (C_{26:1}, C_{28:1}, and C_{30:1} homologs) in the cuticular waxes of Arabidopsis (Arabidopsis thaliana) inflorescence stem, a class of wax not previously reported in Arabidopsis. The Arabidopsis cer17 mutant was completely deficient in these monounsaturated alcohols, and CER17 was found to encode a predicted ACYL-COENZYME A DESATURASE LIKE4 (ADS4). Studies of the Arabidopsis cer4 mutant and yeast variously expressing CER4 (a predicted fatty acyl-CoA reductase) with CER17/ADS4, demonstrated CER4’s principal role in synthesis of these monounsaturated alcohols. Besides unsaturated alcohol deficiency, cer17 mutants exhibited a thickened and irregular cuticle ultrastructure and increased amounts of cutin monomers. Although unsaturated alcohols were absent throughout the cer17 stem, the mutation’s effects on cutin monomers and cuticle ultrastructure were much more severe in distal than basal stems, consistent with observations that the CER17/ADS4 transcript was much more abundant in distal than basal stems. Furthermore, distal but not basal stems of a double mutant deficient for both CER17/ADS4 and LONG-CHAIN ACYL-COA SYNTHETASE1 produced even more cutin monomers and a thicker and more disorganized cuticle ultrastructure and higher cuticle permeability than observed for wild type or either mutant parent, indicating a dramatic genetic interaction on conversion of very long chain acyl-CoA precursors. These results provide evidence that CER17/ADS4 performs n-6 desaturation of very long chain acyl-CoAs in both distal and basal stems and has a major function associated with governing cutin monomer amounts primarily in the distal segments of the inflorescence stem.

Plant cuticle coats most aerial surfaces of vascular plants and plays a major role in coordinating interactions between the plant and its environment (Rensing et al., 2008; Yeats and Rose, 2013). The cuticle is primarily composed of two lipid classes, the non-polymerized (free) cuticular waxes and the cutin polyester, both of which are synthesized by epidermal cells. Common plant wax compounds are the very long chain fatty acids (VLCFAs) and their derivatives including aldehydes, primary alcohols, alkanes, secondary alcohols, ketones, and esters (Samuels et al., 2008). As much as 4.0% of total waxes on Arabidopsis (Arabidopsis thaliana) inflorescence stems are composed of numerous yet unidentified wax compounds (Jenks et al., 1995). Cutin consists primarily of C_{16} and C_{18} fatty acid derivatives (e.g. hydroxy fatty acids and dicarboxylic acids), which are linked by ester bonds; however, glycerol and small amounts of longer chain cutin monomers have also been reported (Pollard et al., 2008).

In the past decade, there has been significant progress toward understanding the molecular mechanisms controlling the cuticular wax biosynthetic pathway based primarily on studies of wax-deficient mutants (Bernard and Joubès, 2013; Yeats and Rose, 2013). Synthesis of wax occurs in epidermal cells and begins with C_{16} and C_{18} long-chain acyl-coenzyme A (CoA) precursors, which are synthesized by long-chain acyl-CoA

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synthetases (LACSs, mainly by LACS1 and LACS2) using plastid-derived C_{16} and C_{18} long-chain fatty acids as substrates (Lü et al., 2009). These are elongated further in the endoplasmic reticulum (ER) by fatty acid elongase complexes (Yeats and Rose, 2013) to very long-chain fatty acyl-CoAs (VLCFA-CoAs; 18 carbons). The elongated VLCFA-CoAs can either be converted to free VLCFAs by yet-unknown thioesterases, or enter one of two ER pathways, an acyl reduction pathway that produces primary alcohols and wax esters involving CER4 and WSD1 (Rowland et al., 2006; Li et al., 2008), or a pathway that produces aldehydes, alkanes, secondary alcohols, and ketones involving CER1, CER3/WAX2, and MAH1 as well as other metabolic and regulatory genes (Aarts et al., 1995; Chen et al., 2003; Bernard et al., 2012; Greer et al., 2007; Yeats and Rose, 2013). The most commonly reported cutin monomers in the Arabidopsis stem are C_{16} and C_{18} monomers (Lü et al., 2009). The predominant C_{18} monomers are the 18-OH C_{18:0} fatty acids, the C_{16:0} dioic acids and 10,16-diOH C_{16:0} fatty acids, while the principal C_{18} monomers are the 18-OH C_{18:0} fatty acids and the C_{18:1} and C_{18:2} dioic acids. In recent years, the genes CYP86A4, CYP77A6, and GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE6 have been directly associated with biosynthesis of the C_{16} monomer and their assembly into a polymeric structure (Li-Beisson et al., 2009; Lü et al., 2009). Biosynthesis of C_{18} monomers has been understood primarily through study of ATT1, GPAT4/8, LACS2, and DCR. Mutants deficient in the expression of these genes showed specific reductions of the C_{18:2} dioic acids, suggesting that these genes have primary effects on C_{18} monomer biosynthesis (Bonaventure et al., 2004; Schnurr et al., 2004; Xiao et al., 2004; Li et al., 2007; Panikashvili et al., 2009).

In Arabidopsis, unsaturated aliphatic components accounted for about 60% of total stem cutin load, with C_{18:2} dioic acids comprising the dominant unsaturated component and C_{16:0} dioic acids being the second predominant saturated monomer (Bonaventure et al., 2004; Lü et al., 2009). A previous report showed that FATTY ACID DESATURASE2 (FAD2) affected cutin monomer composition, since a transcriptional knock-out of this gene caused a 2-fold reduction in the levels of C_{18:2} dioic acids and a 3-fold increase in the levels of C_{18:1} dioic acids (Bonaventure et al., 2004). FAD2 catalyzes the desaturation of 18:1 fatty acyls to form cis-delta12 double bonds and 18:2 fatty acyls. As a result, the fad2 mutant accumulates high levels of C_{18:1} and has reduced C_{18:2} content, which consequently impacts the contents of cutin monomers C_{18:1} and C_{18:2} dioic acid. Clearly, fatty acid desaturation reactions are fundamental to cutin biosynthesis. In cuticular wax biosynthesis, mono- and polyunsaturated waxes have been described. Bauer et al. (2004) described alkadien-1-ols (n-6,9) and alkatrien-1-ols (n-3,6,9) and alkadienes (n-6,9).
as components of tomato (Solanum species) fruit wax. Similarly, monounsaturates have been reported as trace components in the waxes of naval orange (Citrus sinensis) fruit (Liu et al., 2015). Alkenes have been reported in the waxes of rice and maize silks (Perera et al., 2010; Qin et al., 2013). In the aforementioned case of maize silks, double-bond positions were reported as carbon numbers 4, 6, and 9. Monounsaturated alcohols (C_{26:1} and C_{28:1}) have also been reported in three species of microalgae (Volkman et al., 1999). The biosynthesis of unsaturated wax compounds remains largely unknown, and no genes or gene products involved in their synthesis have been identified.

Candidates for these putative plant wax desaturases include members of the acyl-CoA desaturase-like (ADS) gene family found in dicots. In Arabidopsis, the ADS family has nine members showing homology to the Δ9 acyl-CoA desaturases of mammals and yeast (Smith et al., 2013). The Arabidopsis AtADS2 gene encoding an extraplastidial enzyme that desaturates VLCFA-CoA substrates was crucial for synthesis of the C_{24:1} (n-9) and C_{26:1} (n-9) components of seed lipids, sphingolipids, and the membrane phospholipids phosphatidyl-Ser and phosphatidylethanolamine (Smith et al., 2013). Six other Arabidopsis extraplastidial ADS gene products were shown to have VLCFA-CoA desaturase activity but exhibited diverse regiospecificity, with introduction of double bonds into different positions relative to the methyl end (n-x): AtADS1 (n-9), AtADS2 (n-9), AtADS1.3 (n-7), AtADS4 (n-6), AtADS4.2 (n-7), AtADS1.2 (Δ9), and AtADS1.4 (Δ9; Smith et al., 2013). Whether any of these Arabidopsis ADS genes function in synthesis of unsaturated plant wax or cutin monomer compounds has yet to be established.

In this study, we performed chromosome fine mapping of the previously described wax-deficient mutant ceriferum17 (cer17-1) locus (Rashotte et al., 2001, 2004). A candidate gene approach was used to identify CER17 as At1g06350, a gene previously described as ADS4, which has VLCFA-CoA desaturase activity when expressed in yeast cells (Smith et al., 2013). We confirmed CER17/ADS4 involvement in wax synthesis by identifying two independent mutants, designated cer17-2 and cer17-3, from the SALK collection (Salk Institute Genomic Analysis Laboratory) that disrupt expression of the ADS4 gene. Here, we report monounsaturated primary alcohols as constituents of Arabidopsis cuticular waxes and then demonstrate that these specific compounds are deficient in the cer17 mutants. Furthermore, we demonstrate that CER17/ADS4 plays a principal role in synthesis of these unsaturated primary alcohols, likely by acting on VLCFA-CoA substrates that are subsequently converted to free monounsaturated alcohols through

Figure 2. The expression pattern of CER17 in different tissues. A, Three-day-old seedling. B, Five-day-old seedling. Arrow indicates lateral root. C, Seven-day-old seedling. D, Roots of 2-week-old seedling. E, Two-week-old seedling. F, Inflorescence. G, Three-week-old plant growing in soil. H, One rosette leaf of 3-week-old plant growing in soil. I, Transverse section of stem. J, Flower. K, Quantitative RT-PCR analysis of CER17 in different organs of wild-type Col-0. RNA was extracted from different organs. ACTIN2 was used as internal control. Data are mean ± SD (n = 3) from one representative experiment. Three independent experiments were performed; the results from each experiment exhibit similar relative trends.
the fatty acyl-CoA reductase activity of CER4. Besides a role in unsaturated wax synthesis, our findings show that CER17/ADS4 also plays a role in cutin monomer deposition principally on distal (upper) stem segments in the fully expanded inflorescence. A mutation in the LACS1 gene was shown to genetically interact with the CER17 mutation in the synthesis of both unsaturated alcohols and cutin monomers, revealing new insights as well as new questions about the associated wax and cutin metabolic pathways.

RESULTS

Isolation of CER17/ADS4

The Arabidopsis ceciferum17 (cer17) mutant was generated using ethyl methanesulfonate, and first identified as a wax-deficient mutant based on its visible semiglossy stem phenotype (Koornneef et al., 1989). The mutation of CER17 was previously rough-mapped to 73.6 cM on chromosome 1 using the recessive cer17-1 allele (Rashotte et al., 2004). We fine mapped the causal locus of cer17-1 using a 96-plant F2 mapping population to a region of 500 kb on chromosome 1 between the simple sequence length polymorphic markers Chr1-1.837M and Chr1-2.354M. ADS4 (AT1G06350) is in the mapping interval and was previously shown to have extremely high epidermal expression within the upper inflorescence stem (Suh et al., 2005). The entire open reading frame of ADS4 was subsequently sequenced to reveal a G-to-A nucleotide change in the cer17-1 mutant (Fig. 1C). Although this point mutation in cer17-1 did not influence the expression CER17/ADS4 at the mRNA abundance level (Fig. 1B), it is predicted to cause a single amino acid substitution from Pro to Leu (P to L), which is a highly conserved position among ADS family proteins (Fig. 1C) and thus possibly affects enzyme activity. The VLCFA-CoA desaturase activity of CER17/ADS4 and its mutated form mCER17 from cer17-1 was further analyzed using a yeast expression system, as previously described (Smith et al., 2013). As shown in Figure 1D, CER17/ADS4 had VLCFA-CoA desaturase activity and generated C\textsubscript{24:1} and C\textsubscript{26:1} fatty acids as expected, while the mutated form lost this activity. To further confirm that the mutation of At1g06350 caused the wax-deficient phenotype observed in cer17-1, we obtained two T-DNA insertion alleles of At1g06350, Salk_043674, and SM_3_20206, which were then designated as cer17-2 and cer17-3, respectively. The insertion site of cer17-2 (Salk_043674) was in the 132-bp upstream region of the translational start site (ATG) of At1g06350, while the insertion site of cer17-3 (SM_3_20206) occurred in the second exon, which was located 452 bp downstream from the translation start site (ATG; Fig. 1A). Reverse-transcription PCR (RT-PCR) analysis showed that the At1g06350 transcript abundance in cer17-2 was dramatically reduced, whereas the At1g06350 transcript in cer17-3 was absent (Fig. 1B). Similar to cer17-1, both cer17-2 and cer17-3 exhibited glossy stems altered mostly in the distal stem segments of the inflorescence. We

![Figure 3.](image-url) CER17 localizes to the ER. A, The expression of 35S-CER17::YFP protein with ER-mCherry (CD3-959) in Arabidopsis protoplast. B, Transient colocalization of 35S-CER17::YFP protein with ER-mCherry (CD3-959) in tobacco epidermal cells.

![Figure 4.](image-url) Scanning electron micrographs of epicuticular wax crystals on distal and basal stem regions of wild type, cer17-1, and cer17-2 mutants. Scale bar = 5 μm.
hybridized cer17-1 and cer17-2 and observed that the stem of the cer17-1 cer17-2 F1 hybrid had similar glossiness and flattened wax crystals as the single mutant parent (Supplemental Fig. S1). These allelism tests confirmed that cer17-2 did not complement cer17-1, indicating the visible and microscopic wax deficient phenotype is caused by defects of the same gene (Supplemental Fig. S1).

CER17/ADS4 Exhibits Highest Expression in the Upper Inflorescence Stem

To examine the organ-specific expression pattern of the ADS4 gene, we cloned a 2-kb upstream region of CER17/ADS4 and fused it to a GUS reporter gene. The fusion construct was transformed into Arabidopsis, and the CER17/ADS4 expression pattern was examined in GUS-positive plants. Strong GUS-related staining was detected in newly emerged leaves and near the junction region between hypocotyl and root, in 3-d-old seedlings (Fig. 2A). In 5-, 7-, or 14-d-old seedlings, GUS was still highly expressed in the new leaves and hypocotyl, though the signal spread throughout the entire hypocotyl later (Fig. 2, B, C, and E). GUS expression was also detected in the lateral roots (Fig. 2, B-D). In 3-week-old plants, GUS expression was detected in trichomes, and in regions undergoing active cell division and elongation (Fig. 2, G and H). Along the length of the fully expanded and more mature inflorescence stem, GUS expression was significantly enhanced in the distal (upper) regions of the stem (Fig. 2F). In the transverse section of the stems, GUS was exclusively detected in epidermal cells (Fig. 2I). Within the flower, GUS expression was detected in both stigma and pedicel (Fig. 2J). The transcripts of CER17/ADS4 were also quantified in different organs using quantitative RT-PCR (Fig. 2K). The results showed that this gene was highly expressed in flower, distal stem segments, and the silique, and moderately expressed in cauline leaves, basal segments of the stem, and in young seedlings. Very low CER17/ADS4 expression levels were detected in the root and rosette leaves, consistent with our GUS staining results.

The CER17/ADS4 Protein Localizes to the ER

The substrate specificity and regiospecificity of ADS enzymes greatly depends on their localization in subcellular compartments (Heilmann et al., 2004). To experimentally identify the subcellular location of CER17/ADS4, the fluorescent-tagged fusion protein CER17-YFP and ER membrane marker CD3-959 were cotransformed into Arabidopsis protoplasts. Our results showed that CER17/ADS4 was colocalized with the ER membrane marker CD3-959 (Nelson et al., 2007; Fig. 3A), indicating it was localized to the ER membrane. Its ER colocalization was also confirmed in tobacco cells using infiltrated Nicotiana benthamiana leaves (Fig. 3B), suggesting that CER17/ADS4 is a component of the ER membrane system, which is similar to ADS1 and ADS2 (Smith et al., 2013) and most cuticle-metabolism-related proteins (Yeats and Rose, 2013).

Cuticular Waxes on Distal and Basal Segments of the cer17 Inflorescence Stem

We examined the cuticle ultrastructure using scanning electron microscopy, and wax chemical composition using gas chromatography-mass spectrometry (GC-MS), of the distal and basal segments of the inflorescence stem. Scanning electron microscopy was used to show that the wax crystals of the distal stem were slightly different from those of the basal stem (Fig. 4). Even though total wax loads on distal stems of wild-type plants were similar to that of the basal stems, some wax constituents differed significantly (Table I). Total aldehydes on the distal stem were 30% lower than on

Table 1. Cuticular wax composition of both distal and basal inflorescence stems of Arabidopsis Col-0, cer17-2, cer17-3, lacs1-1, and cer17-2 lacs1-1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Load</th>
<th>Fatty Acids</th>
<th>Aldehydes</th>
<th>SA-1-Alcohols</th>
<th>UNSA-1-Alcohols</th>
<th>Alkanes</th>
<th>2-Alcohol</th>
<th>Ketone</th>
<th>Esters</th>
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<tbody>
<tr>
<td>Distal stem</td>
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<tr>
<td>Col-0</td>
<td>2167 ± 102</td>
<td>36.6 ± 2.0</td>
<td>70.5 ± 9.0</td>
<td>232.9 ± 17</td>
<td>50.2 ± 3.0</td>
<td>1028 ± 17</td>
<td>119.0 ± 10</td>
<td>607 ± 13</td>
<td>23.3 ± 4.4</td>
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<tr>
<td>cer17-2</td>
<td>2543 ± 115</td>
<td>23.9 ± 2.0</td>
<td>83.5 ± 6.0</td>
<td>308.0 ± 12</td>
<td>–</td>
<td>1197 ± 80</td>
<td>189.0 ± 24</td>
<td>665 ± 49</td>
<td>26.9 ± 2.0</td>
</tr>
<tr>
<td>cer17-3</td>
<td>2532 ± 178</td>
<td>23.0 ± 2.0</td>
<td>82.8 ± 10.0</td>
<td>302.5 ± 17</td>
<td>–</td>
<td>1165 ± 77</td>
<td>196.0 ± 37</td>
<td>684 ± 40</td>
<td>30.0 ± 2.6</td>
</tr>
<tr>
<td>lacs1-1</td>
<td>1605 ± 242</td>
<td>314 ± 107</td>
<td>86.8 ± 13</td>
<td>218.2 ± 20</td>
<td>76.7 ± 10.5</td>
<td>477 ± 37</td>
<td>67.7 ± 9</td>
<td>324 ± 41</td>
<td>40.2 ± 7.5</td>
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<tr>
<td>cer17-2</td>
<td>1511 ± 275</td>
<td>378.8 ± 90</td>
<td>93.4 ± 18</td>
<td>230.6 ± 40</td>
<td>24.3 ± 0.87</td>
<td>435 ± 67</td>
<td>58.9 ± 16</td>
<td>260 ± 49</td>
<td>30.9 ± 8.3</td>
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<tr>
<td>lacs1-1</td>
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<tr>
<td>Basal stem</td>
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</tr>
<tr>
<td>Col-0</td>
<td>2201 ± 97</td>
<td>23.8 ± 2.3</td>
<td>235 ± 15</td>
<td>191.7 ± 16</td>
<td>13.1 ± 0.83</td>
<td>1092 ± 43</td>
<td>68.9 ± 7.5</td>
<td>564 ± 30</td>
<td>13.1 ± 1.3</td>
</tr>
<tr>
<td>cer17-2</td>
<td>2278 ± 163</td>
<td>20.8 ± 1.7</td>
<td>237 ± 15</td>
<td>219.4 ± 6.8</td>
<td>–</td>
<td>1158 ± 100</td>
<td>67.0 ± 4.5</td>
<td>555 ± 42</td>
<td>21.0 ± 3.8</td>
</tr>
<tr>
<td>cer17-3</td>
<td>2280 ± 53</td>
<td>22.1 ± 1.3</td>
<td>223 ± 17</td>
<td>217.8 ± 17</td>
<td>–</td>
<td>1148 ± 24</td>
<td>86.7 ± 9.7</td>
<td>562 ± 23</td>
<td>20.6 ± 3.8</td>
</tr>
<tr>
<td>lacs1-1</td>
<td>1802 ± 48</td>
<td>306 ± 74</td>
<td>214 ± 11</td>
<td>249.7 ± 9.0</td>
<td>30.3 ± 3.3</td>
<td>632 ± 18</td>
<td>53.1 ± 4.0</td>
<td>303 ± 11</td>
<td>13.0 ± 1.8</td>
</tr>
<tr>
<td>cer17-2</td>
<td>1835 ± 84</td>
<td>384 ± 24</td>
<td>227 ± 12</td>
<td>251.4 ± 4.8</td>
<td>13.5 ± 2.1</td>
<td>596 ± 46</td>
<td>52.3 ± 4.3</td>
<td>306 ± 30</td>
<td>17.2 ± 1.0</td>
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<td>lacs1-1</td>
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Figure 5. Cuticular wax profiles of Arabidopsis derived from both distal and basal inflorescence stems of wild-type Col-0, cer17-2, and cer17-3 mutant. Wax coverage is expressed as µg dm$^{-2}$ stem surface area. Each wax constituent is designated by carbon chain length and is labeled by chemical class along the x axis. Values shown are means ± SD ($n = 4$–5).
the basal stem segments, whereas the total fatty acids, saturated 1-alcohols, 2-alcohols, and esters were 154, 121, 173, and 178% higher on the distal stem, respectively (Table I; Fig. 5). Scanning electron microscopy analysis showed that the wax crystals on distal and basal stem surfaces of cer17-1 and cer17-2 were generally less dense and flatter than wild type (Fig. 4; Supplemental Fig. S1). The more significant change in wax crystallization patterns at the top of the stems likely explains the increased glossiness (Fig. 4; Supplemental Fig. S1). The total stem wax deposition per area on the distal segments of the stems of cer17-2 and cer17-3 were 117 and 116.8% of wild-type levels (including newly identified monounsaturated primary alcohols, see below; Table I). This was due to a general increase in all major wax constituents, including the most abundant C29 alkanes, C29 secondary alcohols, and C29 ketones (Table I). The amounts of saturated primary alcohols in the cer17-2 and cer17-3 stems increased to 119 and 148% of wild-type levels, respectively (Table I). The C40 and C42 esters on the distal stem segments were 203 and 242%, and 172 and 195%, of wild-type levels for cer17-2 and cer17-3, respectively (Fig. 5 and Table I). Similar to distal stems, the total wax amounts on basal stem segments of cer17-2 and cer17-3 were likewise much higher than observed for the wild-type basal stems, though the effect was slightly less than for distal stems (Table I; Fig. 5). The amounts of total saturated primary alcohols and total esters on cer17-2 and cer17-3 were 113 and 115%, and 157 and 160%, of wild-type levels, respectively (Table I; Fig. 5).

Monounsaturated Primary Alcohol Deficiency of the cer17 Mutants

Upon close examination, we observed that three chromatographic peaks disappeared in the GC-MS profiles of cer17 mutant stems, which were adjacent to the primary alcohols in the chromatograms of wild-type stems. Structural analysis of these compounds, including double-bond positions, was evaluated using thin-layer chromatography (TLC) and GC-MS via the dimethyl disulfide (DMDS)-alcohol adducts (Fig. 6; Supplemental Figs. S2 and S3). These compounds were identified as monounsaturated primary alcohols, including C26:1 (n-6), C28:1 (n-6), and C30:1 (n-6) primary alcohols (double-bond nomenclature is given as (n-x) relative to the methyl end of the molecule; Supplemental Fig. S3, C, F, and I). The total coverage of detected monounsaturated primary alcohols on the distal stem segments of wild type was 50.2 µg dm⁻², which is much higher than that of total monounsaturated primary alcohols observed at the basal part of the wild-type stem at 13.1 µg dm⁻² (Table I). The amounts of the C26:1, C28:1, and C30:1 primary alcohols deposited on the distal stem segments of wild type was 3.8-, 3.5-, and 4.3-fold higher than that recorded for the lower stem of the wild type, respectively (Table II), which is consistent with the higher expression pattern of CER17/ADS4 in upper stem regions (Fig. 2, F and K). We observed that these three unsaturated primary alcohols, the monounsaturated C26:1, C28:1, and C30:1 primary alcohols, disappeared completely from the wax profiles of CER17/ADS4 mutant stems, from both upper and lower stem segments (Fig. 6; Supplemental Fig. S2). The cer17-2 transgenic complementation lines harboring the 35S-CER17::YFP construct exhibited monounsaturated primary alcohols similar to the wild type on distal stems.

Figure 6. Gas chromatographs showing monounsaturated and saturated primary alcohols. Total wax was first extracted from distal stems of wild-type (WT), cer17-2, cer17-3, and cer17-2 transgenic complementation line, as well as basal stem from WT and CER17 over-expression line. Subsequently, TLC was used to separate the primary alcohols prior to GC analysis.
stem. Furthermore, CER17/ADS4 transgenic lines in both Col-0 and cer17-2 background (harboring the 35S-CER17::YFP construct) had much higher amounts of monounsaturated primary alcohols in basal stems than that of wild-type basal stems (Fig. 6; Supplemental Table S1), which is consistent with the low levels of the native CER17/ADS4 transcript observed in basal stems (Fig. 2, F and K). The amounts of C_{30:1} primary alcohol in CER17/ADS4 overexpression line was even higher than that of C_{30:0} primary alcohol (Fig. 6).

### CER4 Has a Principal Function in the Synthesis of Monounsaturated Primary Alcohols

To test if CER17/ADS4 could directly desaturate primary alcohols, we expressed CER17/ADS4 in yeast cells fed with C_{20:0} saturated primary alcohols; however, no unsaturated C_{20} primary alcohols were detected (Supplemental Fig. S4). This indicated that saturated primary alcohols are likely not the direct substrates for CER17/ADS4 during synthesis of the monounsaturated primary alcohols. Then we hypothesized that CER4, an alcohol-forming fatty acyl-CoA reductase, which is responsible for the synthesis of very long-chain saturated fatty primary alcohols in Arabidopsis (Rowland et al., 2006), might be also involved in the synthesis of the newly identified monounsaturated alcohols. To test this hypothesis, we analyzed the wax composition of the distal stem of cer4-4 (SALK_000575). The total amounts of monounsaturated primary alcohols were dramatically decreased in the cer4-4 mutant compared to that of wild type (Fig. 7, A and B). This suggested that monounsaturated VLCFA-CoAs generated by CER17/ADS4 were substrates of CER4 in the synthesis of monounsaturated primary alcohols in Arabidopsis stems. To test this, we first coexpressed CER4 and CER17/ADS4 in the yeast line Fat1Δ. A mutant strain has high amounts of saturated VLCFAs, which was more effectively to characterize the activity of the ADS proteins (Smith et al., 2013). However, we could not detect the monounsaturated primary alcohols in this background (Supplemental Fig. S5), presumably because the deletion of the FAT1 gene affected the production of primary alcohols, since the primary alcohol content was greatly reduced in the Fat1Δ line compared to that of a wild-type yeast expressing CER4 (Supplemental Fig. S6A). We then used wild-type yeast line BY4741 to test our hypothesis, since expressing CER17 in this line could still produce 30% of C_{24:1} and almost equal amount of C_{26:1} fatty acids compared to that generated in Fat1Δ line. (Supplemental Fig. S6, B and C). The wild-type yeast line coexpressing CER4 with CER17/ADS4 produced n-6 monounsaturated primary alcohols C_{24:1}-OH and C_{26:2}-OH, which was confirmed by GC-MS (Fig. 7, C–F). These results provide further support to our hypothesis that CER4 and CER17 function together in the synthesis of very long-chain monounsaturated alcohols.

### CER17/ADS4 Deficiency Caused an Increase in Total Cutin Monomer Amounts and Alteration in Stem Cuticle Membrane Ultrastructure

We determined the chemical composition of cutin monomers on both the distal and basal segments of stems for wild type and both cer17 allelic mutants. The total cutin monomer amount per dry weight of the distal stem is about 1.5-fold higher than at the base of the stem (Fig. 8A), which is consistent with a previous report (Suh et al., 2005). All detected cutin monomers were more abundant on the distal than basal stem (Fig. 9), except for C_{16:0} and C_{18:0} dioic acids, which were similar on both distal and basal stem segments. Although total cutin is higher on distal stems, transmission electron microscopy (TEM) revealed that the cuticle membrane on the distal stem is slightly thinner than on the basal stem. However, the distal stem cuticle

<table>
<thead>
<tr>
<th>Sample</th>
<th>Monounsaturated 1-Alcohols</th>
<th>Saturated 1-Alcohols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26:1 28:1 30:1</td>
<td>24:0 26:0 28:0 30:0</td>
</tr>
<tr>
<td>Distal stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>3.26 ± 0.38 23.8 ± 1.57 23.12 ± 1.54</td>
<td>6.06 ± 0.59 58.66 ± 5.92 112.19 ± 9.55 56.02 ± 0.98</td>
</tr>
<tr>
<td>cer17-2</td>
<td>– – –</td>
<td>5.40 ± 0.20 69.36 ± 3.64 150.06 ± 6.73 83.09 ± 1.19</td>
</tr>
<tr>
<td>cer17-3</td>
<td>– – –</td>
<td>5.65 ± 0.29 68.38 ± 3.59 148.70 ± 7.59 79.76 ± 5.23</td>
</tr>
<tr>
<td>lac5-1</td>
<td>7.26 ± 0.94 44.11 ± 5.45 23.31 ± 4.40</td>
<td>7.86 ± 0.72 77.14 ± 7.92 112.99 ± 10.71 20.20 ± 1.20</td>
</tr>
<tr>
<td>cer17-2 lac5-1</td>
<td>2.07 ± 0.06 12.81 ± 0.19 9.85 ± 1.00</td>
<td>6.26 ± 1.37 84.21 ± 15.47 120.75 ± 20.62 19.35 ± 2.18</td>
</tr>
<tr>
<td>Basal stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>0.85 ± 0.10 6.87 ± 0.55 5.35 ± 0.26</td>
<td>4.40 ± 0.38 52.72 ± 3.86 95.62 ± 7.76 38.91 ± 3.68</td>
</tr>
<tr>
<td>cer17-2</td>
<td>– – –</td>
<td>4.21 ± 0.25 59.01 ± 1.62 111.67 ± 3.84 44.55 ± 1.07</td>
</tr>
<tr>
<td>cer17-3</td>
<td>– – –</td>
<td>4.34 ± 0.16 58.19 ± 1.64 110.56 ± 3.49 44.73 ± 1.81</td>
</tr>
<tr>
<td>lac5-1</td>
<td>2.17 ± 0.05 13.95 ± 0.9 14.21 ± 3.07</td>
<td>6.65 ± 0.24 88.74 ± 2.94 129.88 ± 4.85 24.41 ± 1.13</td>
</tr>
<tr>
<td>cer17-2 lac5-1</td>
<td>0.72 ± 0.05 3.13 ± 1.41 9.69 ± 0.62</td>
<td>6.49 ± 0.93 93.57 ± 8.92 139.04 ± 11.54 23.68 ± 1.95</td>
</tr>
</tbody>
</table>

Values shown are means ± so (μg dm⁻²) coverage of individual monounsaturated alcohols, and saturated 1-alcohols (n = 4–5). –, Undetectable.
membrane does appear darker in the micrographs than basal stem, revealing heavier osmium staining/deposition (Fig. 8B). Compared to the wild type, the total cutin loads on distal stems from both cer17-2 and cer17-3 were 131 and 125% of wild-type levels, respectively, whereas the basal stem segments of cer17-2 and cer17-3 were 122% and 114% of wild-type levels, respectively (Fig. 8A). The major cutin monomer C18:2 dioic acids on the distal stem segments of cer17-2 and cer17-3 was 140 and 132% of wild type, respectively, whereas these compounds on basal stem of cer17-2 and cer17-3 were 125 and 117% of wild-type levels, respectively (Fig. 9, A and B). Other cutin monomers, on both the distal and basal stem segments, changed little in cer17 mutants.

TEM was used to show that the cuticle membrane ultrastructure of cer17-2 was somewhat disorganized and structurally irregular relative to the wild type, on stems at both distal and basal segments of the inflorescence (Fig. 8B). These results indicated that CER17/ADS4 deficiency affects stem cutin biosynthesis, with stronger effects on cutin deposition on the distal than basal stem segments of the inflorescence.

CER17/ADS4 Interacts Genetically with LACS1/CER8 in Cuticle Synthesis

While CER17/ADS4 shows higher transcript level (8-fold) in the distal compared to basal segments of the wild-type inflorescence stem, previous reports showed that LACS1 also has higher transcript abundance (3.4-fold) in the distal compared to basal stems (Suh et al., 2005). It was also previously shown that very long-chain acyl-CoAs synthesized by LACS1 are precursors for both wax and cutin biosynthesis (Lü et al., 2009), making the acyl-CoA products of LACS1 potential substrates of ADS4 (Smith et al., 2013). To investigate the potential for LACS1 genetic interaction with CER7/ADS4, a cer17-2 lacs1-1 double mutant was created. On the upper stem of lacs1-1, the C16:0 dioic acids and 10,16-diOH C16:0 fatty acids were reduced by 27 and 48%, whereas the C18:2 dioic acids and 18-OH C18:0 acids were elevated by 25 and 11%, respectively, relative to wild type (Fig. 9A). On the top stems of the cer17-2 lacs1-1 double mutant, the cutin monomers 16-OH C16:0, C18:1, and C18:2 dioic acids, and 18-OH C18:0 acids were 154, 177, 266, and 171%, of wild-type
crease of the major monomer C18:2 dioic acids, the levels, respectively (Fig. 9A). Owing mainly to an increase in the major monomer C18:2 dioic acids, the total amount of cutin monomers in the cer17-2 lacs1-1 double mutant was 229, 183, and 198% of wild type, cer17-2, and lacs1-1, respectively. Compared to the cuticle membrane ultrastructure at the top of the stem of wild type, both cer17-2 and lacs1-1 have a slightly thicker and more disorganized cuticle membrane as visible in TEM micrographs (Fig. 10, A–C). By comparison, the cuticle membrane on the upper stem sections of the cer17-2 lacs1-1 mutant was much thicker than that observed in the wild type or either of the mutant parents, and the membrane ultrastructure was even more severely affected (Fig. 10, D–F). Also, osmophilic material that was visually identical to cuticle membrane material was observed deposited within the cell wall and disconnected from the normal cuticle (Fig. 10F). The cuticle membrane of the double mutant appeared to have a higher level of discontinuity and had a more crystalline-type structure than either mutant parent (Fig. 10F). Further, the cuticle deposition that occurs over the junction above the anticlinal walls between two epidermal cells was thicker in the cer17-2 lacs1-1 double mutant (Fig. 10E). Unlike the top segments of the stem, the total cutin monomer compositional profile of the basal stem of cer17-2 lacs1-1 shows very little difference from the lacs1-1 parent (Fig. 9B). The cuticle membrane ultrastructure of the basal stem of the double mutant is likewise very similar to that of the single lacs1-1 mutant parent (Supplemental Fig. S7), revealing that lacs1 is epistatic in the basal inflorescence stem to cer17 for cutin chemical composition and cuticle membrane ultrastructure.

We further checked the cuticle permeability of distal and basal stem segments of wild type, and single and double mutants, using a toluidine blue (TB) staining assay. Distal stem segments of the cer17-2 lacs1-1 double mutant had much higher cuticle permeability than distal stems of wild type or either parent (Fig. 11A). Furthermore, water loss analysis showed that the distal stem of the cer17-2 lacs1-1 double mutant lost water at a much faster rate than wild type or either of the single mutant parents (Fig. 11B), consistent with the TB staining results. However, cuticle permeability of the basal stem was found to be similar among all lines on both TB staining and water loss assay (Fig. 11, C and D). The more severely disrupted cuticle membrane of the distal stem segments (but not basal stem) of the cer17-2 lacs1-1 double mutant is thus closely linked with this higher permeability.

As previously reported, LACS2 plays an important role in cutin monomer biosynthesis and has overlapping function with LACS1 in both wax and cutin synthesis (Schnurr et al., 2004; Lü et al., 2009). To determine if LACS2 could also coordinate with ADS4 in wax or cutin biosynthesis, the lacs2 mutation was introduced into the cer17-2 mutant background by hybridization and selection in the F2. Biochemical analysis showed that the cutin monomer composition of the upper and lower stem segments of the cer17-2 lacs2-3 double mutant closely resembled lacs2-3 (Supplemental Fig. S8, A and B), indicating lacs2 is epistatic to cer17 for these cuticle traits throughout the length of the stem. The cuticle membrane ultrastructure of both distal and basal stem segments of the cer17-2 lacs2-3 double mutant was also similar to that of lacs2-3, likewise revealing lacs2 epistasis (Supplemental Fig. S8, C and D).

Our wax chemical analysis showed that the total wax load at the distal and basal segments of the stems of cer17-2 lacs1-1 was nearly identical to that of lacs1-1 (Table I). This indicated that LACS1 functions upstream of CER17/ADS4 in wax biosynthesis, which is different than we describe above for CER17/ADS4 and LACS1 genetic interaction in cutin biosynthesis. Although the wax chemical composition of the double mutant appeared very similar to lacs1, we did observe that the wax crystals on the distal stem of the cer17-2 lacs1-1 double mutant were slightly less dense and slightly more patchy in their distribution when compared with wild type or either single parent, whereas the wax crystallization patterns on the basal stem segments were essentially identical to those on lower stem segments of lacs1-1 (Fig. 12). Not previously reported, the lacs1-1 mutation alone caused a slight elevation in the amounts of monounsaturated C26:1 and C28:1 primary alcohols on upper stems and an increase in all three monounsaturated C26:1, C28:1, and C30:1 primary alcohols on lower stems (Table II). Unexpectedly, the
monounsaturated C_{26:1}, C_{28:1}, and C_{30:1} primary alcohols detected on the double mutant (even though these were not detectable in the cer17 single mutants), with amounts being increased even above wild-type levels for the monounsaturated C_{30:1} primary alcohols in the lower stem but remaining lower than lacs1-l for all monounsaturated primary alcohols elsewhere (Table II). The double bond position of these monounsaturated primary alcohols was also n-6 relative to the methyl end of the molecule based on DMDS analysis (Supplemental Fig. S9).

**DISCUSSION**

Based on the very high transcript abundance of ADS4 in the epidermis of expanding inflorescence stems of Arabidopsis (Suh et al., 2005) and chromosome mapping of the previously reported Arabidopsis cer17 wax locus by Rashotte et al. (2004) and herein, we employed a candidate gene approach to show that ADS4 is the causal gene whose mutation is responsible for the visible semiglossy stem phenotype of cer17-1 as well as two allelic cer17 mutants. The CER17/ADS4 protein belongs to a nine-member family of predicted desaturases in Arabidopsis, and preliminary reports indicated that these would have different functions depending on their subcellular localization and substrate availability (Heilmann et al., 2004). We report the presence of unsaturated aliphatic compounds in Arabidopsis cuticular wax and the monounsaturated primary alcohols (alken-1-ols), and we then demonstrate using mutation and transgenic complementation analysis a direct role for CER17/ADS4 in synthesis of these unsaturated waxes. The CER1 and WAX2/CER3 genes encode proteins with homology to desaturases and their association with wax synthesis was previously reported (Aarts et al., 1995; Chen et al., 2003; Rowland et al., 2007). However, the polypeptide sequences of CER1 and WAX2/CER3 are highly divergent relative to typical desaturases, and a newer report indicates these enzymes function as part of an alkane decarbonylase complex, rather than as typical desaturases (Bernard et al., 2012). As such, ADS4 is to our knowledge the first gene reported as being directly associated with the desaturation of plant wax compounds.

We report the presence of monounsaturated C_{26:1}, C_{28:1}, and C_{30:1} primary alcohols having double bonds at the n-6 position on inflorescence stems of wild-type Arabidopsis and that Arabidopsis mutants in the cer17 allelic group are completely deficient in these compounds. The exact substrates modified by the
ER-localized CER17/ADS4 enzyme are uncertain; however, previous reports indicate that the substrates are VLCFA-CoAs since the CER17/ADS4 protein could generate C24:1 \( \Delta 18 \) (n-6) and C26:1 \( \Delta 20 \) (n-6) fatty acids when expressed in the VLCFA-accumulating yeast line Fat1Δ (Smith et al., 2013), and this was confirmed in this study (Fig. 1D). The position of the double bond at n-6 (relative to the methyl end of the molecule) in all of these monounsaturated primary alcohols argues against a mode of biosynthesis in which elongation to C26 and longer chain lengths is preceded by the activities of previously reported plastidial STEAROYL-ACP DESATURASE or ER-localized FAD2 or FAD3 (Shanklin and Cahoon, 1998). Instead, our findings indicate that CER17/ADS4 catalyzes the direct desaturation of C26-C30 VLCFA-CoAs, which is consistent with Smith et al. (2013). Besides CER17/ADS4 involvement, the predicted FATTY ACYL-COA REDUCTASE, CER4 is also involved in the synthesis of these monounsaturated primary alcohols, since these compounds were greatly reduced in cer4-4 mutant stems, and wild-type yeast line BY4741 expressing CER4 with CER17/ADS4 was newly capable of generating very long-chain monounsaturated (n-6) primary alcohols. Evidence indicates that CER17/ADS4 utilizes very long-chain saturated acyl-CoAs as substrate, rather than saturated primary alcohols. CER4 function in monounsaturated primary alcohol synthesis thus occurs downstream of CER17/ADS4, with CER4 being capable of utilizing the monounsaturated acyl-CoAs generated by CER17/ADS4 as substrate.

Consistent with CER17/ADS4 having its highest transcript expression in the distal (upper) segments of the stem, the cer17 mutants have more severely altered cuticle membrane ultrastructure and cutin monomer composition in the distal than the basal stem. CER17/ADS4

Figure 10. Transmission electron micrographs of the cuticle layer of epidermal cells. The cuticle is indicated by an arrow. Scale bars = 500 nm in A-D and F and 2 \( \mu \text{m} \) in E.

Figure 11. Cuticle permeability of cer17-2, lacs1-1, and corresponding double mutants. Distal stems (A) and basal stems (C) of 6-week-old plants were immersed for 3 min in 0.05% toluidine blue-O and then rinsed with water. Excised stem from distal (B) and basal (D) water loss rates recorded over 180 min, measured as a percentage of the initial weight of fully hydrated stems. Values are mean of five replicate assays. Error bar = SD. The experiment was repeated three times with similar results.
than the lower stem, and it now appears likely that more distal in principal role in synthesizing cuticle lipids in the upper, from this, we postulated that CER17/ADS4 has a principal role in synthesizing cuticle lipids in the upper, more distal inflorescence stem. The distal stem of wild-type Arabidopsis produces more total cutin monomers than the lower stem, and it now appears likely that CER17/ADS4 plays a role in the distal stems to generate the normally higher cutin loads. Since our analysis was performed on the younger distal stems that had more recently undergone elongation, future studies may also show that CER17/ADS4 plays a role in maintaining the more rapid rates of cuticle synthesis as would be necessary to maintain continuous surface coverage by a functional cuticle barrier during the rapid elongation phases.

Besides the absence of the unsaturated primary alcohols in the cer17 mutants, we also observed that the amounts of cutin monomers were elevated relative to wild-type levels. Potentially, this could be due to shunting of VLCFA precursors away from the unsaturated wax pathway and into the cutin pathways. However, the increase of these cutin monomers in cer17 is much larger than the reduction in monounsaturated primary alcohols, suggesting that CER17/ADS4 is impacting yet-unknown metabolic or regulatory mechanisms controlling cutin amounts and cuticle ultrastructure. Although saturated waxes were also elevated in cer17, these changes were small compared to the effect on cutin and probably due to minor shunting within the pathway. CER17/ADS4 by comparison appears to have a more direct metabolic or regulatory function in the cutin synthetic pathways. Since the desaturated cutin monomers are more abundant on the cer17 mutant, we concluded that CER17/ADS4 is not necessary for the desaturation of cutin monomers. Although the results presented here indicate that CER17/ADS4 is acting as a very long-chain fatty acyl-CoA desaturase in the wax pathway, the exact function of CER17/ADS4 in cutin synthesis is still uncertain.

As the LACS1 protein is required for CoA activation of early wax and cutin metabolic precursors (Lü et al., 2009), and since LACS1 transcripts showed relatively high abundance in distal compared to basal stems like CER17/ADS4 (Suh et al., 2005), we speculated that the LACS1 protein product might interact with ADS4 in a way that sheds light on CER17/ADS4 function. The total cutin monomer amounts on the distal stem of the cer17-2 lacs1-1 double mutant were much higher than on wild type and both single mutation parents, whereas cutin monomer loads on the double's basal stem were only slightly elevated. It was also observed that cuticle permeability of the double mutant stem was much higher than for the wild-type and parental lines. A double mutant of cer17-2 lacs2-3 was also examined (even though the LACS2 gene had much lower expression than LACS1 in upper stems [Suh et al., 2005]), but this double mutant produced cutin monomers that were essentially identical to that of lacs2-3 on both distal and basal stems, indicating lacs2 epistasis over cer17 throughout the stem. From these results, it is clear that a particularly unique genetic interaction occurs between CER17/ADS4 and LACS1 in cutin monomer synthesis by the upper stems of the inflorescence. As discussed above, the CER17/ADS4 desaturase likely targets VLCFA-CoA substrates for insertion of a double bond. If we assume that LACS1 generates these VLCFA-CoA precursors for cutin synthesis in Arabidopsis as suggested by Lü et al. (2009), then loss of LACS1 in the double mutant should result in a dramatic reduction in total cutin monomers. In contrast, however, we observed a dramatic increase in cutin monomers on the double mutant that was far above wild-type and mutant parent levels. Possibly, the VLCFA-CoAs synthesized by LACS1 are not the main substrates for CER17/ADS4, and the normal acyl and CoA substrates of LACS1 are thus being shunted toward an alternative LACS pathway toward cutin synthesis. It is notable that the cer17 mutant makes more stem cutin monomers than wild type, strongly suggesting that the cer17 and lacs1 defects together generate a synergistic up-regulation of cutin pathways. Not previously reported, we show here that

Figure 12. Scanning electron micrographs of stem epicuticular wax crystals of distal and basal stem regions of Arabidopsis. Scale bar = 5 μm.
the C_{26:1}, C_{28:1} and C_{30:1} homologs of the monounsaturated primary alcohols are also significantly elevated above wild-type levels in the lacs1-1 mutant. We also demonstrated that the free C_{26}, C_{28} and C_{30} VLCFAs are also dramatically elevated on cer17-2 lacs1-1 (Table I), and this above wild-type and parental levels. These results provide further evidence that alternative cuticle lipid pathways (perhaps involving other members of the LACS and ADS families) are being highly activated in the genetic background of this double mutant.

Finally, we observed that large amounts of the C_{26:1}, C_{28:1}, and C_{30:1} monounsaturated primary alcohols were produced in the cer17-2 lacs1-1 double mutant even though the single cer17 mutation apparently blocked all monounsaturated primary alcohol production, since these compounds could not be detected using GC-MS in the cer17 mutant. And the C_{26:1} and C_{30:1} monounsaturated primary alcohols were actually synthesized at higher levels by the double mutant than by the wild type. The double bond position of these monounsaturated primary alcohols were identical to that in wild type, suggesting these products were generated by CER17/ADS4 (n-6) since other ER-localized ADS proteins exhibited different regiospecificity (Smith et al., 2013). One possible explanation is that the trace amounts of the CER17/ADS4 transcripts produced by the cer17-2 allele are still functional in the double mutant and somehow induced or otherwise more effective. The expression levels of the other ADS genes were not changed in distal stem of either single mutant or double mutant compared to wild type (Supplemental Fig. S10), further excludes the possibility of other members of the ADS family participating in the synthesis of these monounsaturated primary alcohols in the cer17-2 lacs1-1 double mutant.

CONCLUSION

Consistent with its predicted function as an acyl-CoA desaturase, disruption of the CER17/ADS4 gene prevents the synthesis of an entire chain-length series of monounsaturated primary alcohols, a wax constituent, to our knowledge, not previously reported in Arabidopsis, suggesting that CER17/ADS4 plays a principal role in synthesis of these unsaturated waxes, most likely by acting on VLCFA-CoA substrates. The unsaturated VLCFA-CoAs are subsequently converted to free monounsaturated alcohols through the fatty acyl-CoA reductase activity of CER4. Besides waxes, the CER17/ADS4 disruption also caused an increase in the amount of cutin monomers, especially desaturated monomers, and double mutant analysis revealed that CER17/ADS4 interacted genetically with LACS1 in determining cutin deposition, especially by the distal inflorescence stem. The initiation of further studies to elucidate genes involvement in these novel wax and cutin metabolic pathways, as well as the ecological significance of monounsaturated primary alcohols in plants, is an important subject for future studies.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds stocks of cer17-1 (C599), cer17-2 (Salk_043674), cer17-3 (SM_3_2006), cer4-4 (SALK_000578), and lacs1-1 (Salk_127191) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). lacs2-3 (GABI-Kat line 368C02) was kindly provided by Dr. Christiane Nawrath (University of Lausanne, Switzerland; Bessire et al., 2007). The cer17-2 mutant was crossed with lacs1-1 and lacs2-3 to generate double mutants cer17-2 lacs1-1 and cer17-2 lacs2-3, respectively. Arabidopsis (Arabidopsis thaliana) wild-type and mutant seeds were stratified for 3 to 4 d at 4°C, and plants were grown in soil at 21°C to 22°C in a growth room with 30 to 60% humidity, a 16-h/8-h light/dark cycle, and a light intensity of 90 to 110 mol m^-2 s^-1.

RT-PCR and qRT-PCR

RT-PCR and qRT-PCR was used to analyze the expression levels of CER17/ADS4, ADS1, ADS1.2, ADS1.4, ADS2, and ADS4.2 in the different cer17 allelic mutants and double mutants as well as wild type. Total RNA was extracted from 6-week-old inflorescence stem using the TRIZOL reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol. First-strand cDNA was synthesized using reverse transcriptase (Promega) 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. qRT-PCR was carried out as described by Liu et al. (2011). The primers used in this study are listed in Supplemental Table S2.

Generation of Gene Constructs and Transgenic Plants

To make the 35S-CER17::YFP construct, the full-length coding sequence of CER17/ADS4 was amplified from the cDNA of wild-type Col-0 using the At1g06350-F and At1g06350-R primers. The PCR products were first subcloned into pENTRTM/D-TOPO vector then into destination vector pEarleygate101, by the LR reaction. Transgenic plants were generated using Agrobacterium tumefaciens-mediated transformation using the floral-dipping method (Clough and Bent, 1998). T2 generation transgenic lines were used for further study.

Promoter-GUS Reporter Gene Fusions and GUS Histochemical Staining

To generate the CER17::GUS fusion construct, a 2,032 bp promoter fragment was PCR-amplified from genomic DNA using the At1g06350-F and At1g06350-R primers. The PCR products were digested with Sall and Smal and cloned into the same sites of pBI101.2. The constructs were fully sequenced to ensure no PCR errors were introduced. The CER17::GUS construct was introduced into Arabidopsis Col-0 by A. tumefaciens-mediated transformation, and T2 generation lines were used for GUS histochemical assays. Plant materials were incubated in ice-cold 80% acetone for 30 min, rinsed with 100 mx sodium phosphate buffer twice, and then incubated in a 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide solution at 37°C for 1 h to overnight. The stained materials were cleared overnight in 90% ethanol. Samples were examined and photographed under a stereomicroscope. For stem cross sections, stained stems were fixed and embedded in paraffin according to Liu et al. (2009), and a microtome was used to prepare 12-μm-thin sections for photograph under the same microscope.

Subcellular Localization by Transient Expression in Arabidopsis Protoplasts and Nicotiana benthamiana

The 35S-CER17::YFP construct was used for CER17/ADS4 subcellular localization assays, since public to complement the glossy phenotype of the cer17-2 mutant. mCherry fluorescent protein-tagged ER marker CD3-959 was used for protein colocalization. For protoplast transformation, fully expanded healthy leaves of 3- to 4-week-old plants were used. Arabidopsis protoplast transformation was performed as described (Yoo et al., 2007). For tobacco infiltration experiments, overnight bacterial culture was centrifuged and then suspended in liquid Murashige and Skoog medium. The solution was infiltrated into N. benthamiana leaves using a plastic 1-ml syringe. After infiltration, the infiltrated leaves were marked and the plants were moved to the growth chamber.
before imaging. Photographs were imaged by confocal laser-scanning microscopy (LSM710 Meta; Carl Zeiss).

Cuticular Wax and Polyester Analysis

The cuticular wax and cutin monomer composition from distal and basal inflorescence stems of 6-week-old plants were determined as described by Lü et al. (2011). For identification of monounsaturated primary alcohols, TLC analysis was used to purify the primary alcohols. The total wax mixtures were separated from heated for 2 h. After cooling down, 5 mL 0.9% NaCl and 5 mL hexane were added. Fatty acid methyl esters and fatty alcohol were recovered by collecting hexane fraction. The hexane fraction was dried under nitrogen and derivatized with acetic anhydride, then analyzed by gas chromatography-

PCR and subcultured in liquid synthetic complete medium containing 2% GAL and 5 mL hexane were added. Fatty acid methyl esters and fatty alcohol were separated on silica gel plates (20:80:1 [v/v/v]) as the mobile phase and visualized under UV light after spraying with primuline (Sigma-Aldrich). The alcohol fraction was scraped off and extracted in chloroform, dried under nitrogen, and derivatized with BSTFA, then analyzed on gas chromatography-

Yeast Assay

PCR-based gene deletion strategy was used in the construct of yeast Fat1Δ lines (Batudin et al., 1995). The KanMX4 were cloned from the plasmids pFLKanMXV, then transformed to the yeast line BY4741 to generate Fat1Δ lines. CER17 and CER4 were amplified using cDNA from Col-0 by the corresponding primers. The CER17 and CER4 were then cloned into the downstream of GPD promoter in p426 vector and downstream of GAL promoter in pESC-Leu vector, respectively, to generate p426-CER17 and pESC-CER4. p426-CER17 and pESC-CER4 were transformed into the yeast. The transformants were selected on the synthetic complete selection medium. Successful clones were confirmed by PCR and subcultured in liquid synthetic complete medium containing 2% GAL, for 3 d for lipid analysis. Cells were pelleted and suspended in 2 mL 5% sulfuric acid in methanol, which was heated for 2 h. After cooling down, 5 mL 0.09% NaCl and 5 mL hexane were added. Fatty acid methyl esters and fatty alcohol were recovered by collecting hexane fraction. The hexane fraction was dried under nitrogen and derivatized with acetic anhydride, then analyzed by gas chromatography-flame ionization detection and GC-MS.

Assessment of Cuticle Permeability

The toluidine blue staining protocol was followed as described previously (Tanaka et al., 2004). Briefly, both distal and basal stem sections of 6-week-old plants were immersed for 3 min in 0.05% toluidine blue then rinsed with water. For water loss measurements, both distal and basal stems of fully watered 6-week-old plants were detached and weighed every 20 min using a micro-balance in complete darkness. Water loss rates were recorded over 180 min and measured as a percentage of the initial weight.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Scanning electron micrographs of epicuticular wax crystals.

Supplemental Figure S2. Gas chromatographs showing monounsaturated and saturated primary alcohols.

Supplemental Figure S3. Mass spectra of monounsaturated and saturated primary alcohols as well as DMDs-alcohol adducts.

Supplemental Figure S4. Heterologous expression of CER17 in yeast line Fat1Δ fed with C28:0 alcohol.

Supplemental Figure S5. Heterologous coexpression of CER4 and CER17 in yeast line Fat1Δ.

Supplemental Figure S6. Heterologous expression of CER17 or CER4 in yeast lines BY4741 and Fat1Δ.

Supplemental Figure S7. Transmission electron micrographs of the basal stem cuticle layer of epidermal cells.

Supplemental Figure S8. Total cutin monomer amount and cuticle membrane ultrastructure of stems of wild type, cer17-2, lac2-3, and cer17-2 lac2-3 mutants.

Supplemental Figure S9. Double-bond position analysis monounsaturated primary alcohols isolated from stems of cer17-2 lac2-1.

Supplemental Figure S10. Transcript levels of genes of ADS family in distal stem of wild type, cer17-2, cer17-2 lac2-1, and cer17-2 lac2-3 double mutants.

Supplemental Table S1. Quantification of monounsaturated 1-alcohols in basal inflorescence stems of Arabidopsis wild type and cer17 mutants, transgenic complementation, and overexpression lines.

Supplemental Table S2. Primers used in this study.

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