Arabidopsis ECERIFERUM2 Is a Component of the Fatty Acid Elongation Machinery Required for Fatty Acid Extension to Exceptional Lengths\textsuperscript{1[W][OA]}

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Primary aerial surfaces of land plants are coated by a lipidic cuticle, which forms a barrier against transpirational water loss and protects the plant from diverse stresses. Four enzymes of a fatty acid elongase complex are required for the synthesis of very-long-chain fatty acid (VLCFA) precursors of cuticular waxes. Fatty acid elongase substrate specificity is determined by a condensing enzyme that catalyzes the first reaction carried out by the complex. In Arabidopsis (Arabidopsis thaliana), characterized condensing enzymes involved in wax synthesis can only elongate VLCFAs up to 28 carbons (C28) in length, despite the predominance of C29 to C31 monomers in Arabidopsis stem wax. This suggests additional proteins are required for elongation beyond C28. The wax-deficient mutant \textit{eceriferum2} (cer2) lacks waxes longer than C28, implying that CER2, a putative BAHD acyltransferase, is required for C28 elongation. Here, we characterize the cer2 mutant and demonstrate that green fluorescent protein-tagged CER2 localizes to the endoplasmic reticulum, the site of VLCFA biosynthesis. We use site-directed mutagenesis to show that the classification of CER2 as a BAHD acyltransferase based on sequence homology does not fit with CER2 catalytic activity. Finally, we provide evidence for the function of CER2 in C28 elongation by an assay in yeast (Saccharomyces cerevisiae).

Land plants have a lipidic cuticle that seals the outer surface of all of their primary aerial organs. Structurally, the cuticle consists of two components, cutin and cuticular waxes. Together these form a hydrophobic barrier that plays a critical role in plant survival by restricting nonstomatal water loss (Riederer and Schreiber, 2001). Cuticles also protect the plant from biotic and abiotic stresses, profoundly affect plant-insect interactions (Müller, 2006), prevent epidermal fusions (Sieber et al., 2000), and are involved in drought stress signaling (Wang et al., 2011).

Cutin is a polymer of mainly midchain- and \textit{ω}-hydroxy and -epoxy 16 carbon (C16) and C18 fatty acids, which are cross-linked in ester bonds directly or through a glycerol backbone (Pollard et al., 2008). Cuticular waxes are aliphatic monomers that are deposited within the cutin matrix as intracuticular wax, and on top of it as epicuticular wax film and crystals. Wax is a heterogeneous mixture of very-long-chain fatty acids (VLCFAs) and their alkane, aldehyde, alcohol, ketone, and ester derivatives, which typically range from C24 to C32 in length (Samuels et al., 2008). The composition of cuticular wax varies greatly among species and tissues, often providing physical and chemical properties to the plant surface that are advantageous in specific environments.

Genetic analyses have revealed that a fatty acid elongase (FAE) complex is responsible for the synthesis of VLCFA wax precursors (Millar et al., 1999; Fiebig et al., 2000; Kunst and Samuels, 2009). FAE complexes are heterotetramers of independently transcribed, monofunctional proteins localized to the endoplasmic reticulum (ER). Together, they catalyze a series of four reactions to elongate long-chain acyl-CoAs or very-long-chain acyl-CoAs by sequential addition of two carbon units. The condensing enzyme, or \textit{β}-ketoacyl-CoA synthase (KCS), catalyzes the first reaction in this sequence and is both rate limiting and specific for the chain length of acyl-CoA synthesized (Millar and Kunst, 1997). Two very dissimilar families of KCSs have been identified in Arabidopsis (Arabidopsis thaliana): a FAE1-type family homologous to the first such KCS enzyme discovered in association with seed oil biosynthesis (Kunst et al., 1992; James et al., 1995; Lassner et al., 1996), and an \textit{Elongation Defective} (ELO)-like family homologous to the yeast (\textit{Saccharomyces cerevisiae}) ELO family responsible for sphingolipid synthesis (Dunn et al., 2004). To date, no function has been ascribed to Arabidopsis ELOs. Of the
21 FAE1-type KCS enzymes in Arabidopsis (Joubès et al., 2008), 11 have been shown by microarray analysis to be up-regulated in the stem epidermis (Suh et al., 2005). Only one of these, ECRIFERUM6 (CER6/KCS6/CUT1; Millar et al., 1999; Fiebig et al., 2000; Joubès et al., 2008), has a dominant role in the elongation of VLCFAs for cuticular wax synthesis, as CER6 suppression results in a dramatic reduction of all wax monomers longer than C24 (Millar et al., 1999). Heterologous expression of CER6 in yeast has demonstrated that the CER6 condensing enzyme can produce C28 VLCFAs (O. Rowland and L. Kunst, unpublished data). However, CER6 appears to be unable to produce VLCFAs longer than C28 in yeast; this presents a problem as the bulk of Arabidopsis stem wax is made up of C29 alkanes, secondary alcohols, and ketones derived from C30 VLCFAs. Mutant screens have not revealed any other KCS enzymes necessary for VLCFA elongation past C28 in Arabidopsis. Therefore, there may be other proteins unrelated to condensing enzymes that are required for acyl chain extension beyond C28 that remain unknown.

The wax-deficient mutant cer2 shows a dramatic reduction in all stem waxes longer than C28 and increased accumulation of waxes C28 or shorter, suggesting that CER2 has a role in the final steps of VLCFA elongation. Surprisingly, the cer2 mutation has been mapped to At4g24510 (Negrúk et al., 1996; Xia et al., 1996), a gene homologous to plant BAHD acyltransferases. However, the CER2 protein was reported to localize exclusively to the nucleus (Xia et al., 1997). This does not fit with CER2 annotation as a BAHD acyltransferase, as all characterized BAHD acyltransferases are soluble cytosolic enzymes (D’Auria, 2006).

The objective of this work was to more precisely evaluate the role of CER2 in fatty acid elongation using a new CER2 allele, cer2-5 (Columbia-0 [Col-0] ecotype). We provide evidence that CER2 has a metabolic function specific to wax synthesis, and that the CER2 homolog CER2-LIKE1 has an analogous role in leaf wax synthesis. Despite the classification of CER2 as a BAHD acyltransferase based on sequence homology, we demonstrate that CER2 cannot share the catalytic mechanism that has been confirmed for other members of the BAHD family, and provide biochemical support for a function of CER2 in VLCFA elongation by an assay in yeast.

RESULTS

The cer2-5 Mutant Has a Reduced Wax Load and Does Not Accumulate Waxes Longer than C28

Previous studies have established both wax load and wax composition phenotypes for cer2 using transfer DNA (T-DNA) mutants BRL5 (cer2-3) and BRL9 (cer2-4) in Wassilewskija (McNevin et al., 1993, Jenks et al., 1995; Negrúk et al., 1996), and ethyl methanesulfonate-mutagenized lines cer2-1 and cer2-2 in Landsberg erecta (Koornneef et al., 1989; Xia et al., 1996). All of these mutant alleles have glossy green stems, and observation of cer2-1 and cer2-2 stems by scanning electron microscopy (SEM) revealed an absence of epicuticular wax crystals (Koornneef et al., 1989; Xia et al., 1996). Gas chromatography with flame ionization detection (GC-FID) analysis of cuticular wax load and composition of the cer2-4 mutant revealed a 65% decrease in total wax load, reduction of all wax monomers longer than C28 to trace amounts, and increased load of most wax monomers C28 or shorter (Jenks et al., 1995). A summary of characterized cer2 alleles is shown in Supplemental Table S1.

For our further investigations into CER2 function, we acquired a T-DNA insertional mutant line in Col-0 ecotype from the Arabidopsis Biological Resource Center, SALK 084443 (Alonso et al., 2003), hereafter referred to as cer2-5. Homozygous mutants were identified by PCR-based genotyping, and direct sequencing of genomic DNA revealed a T-DNA insertion in the second exon of the CER2 gene (Fig. 1A). Stems of cer2-5 individuals had a glossy appearance characteristic of ciceriferum mutants with decreased wax load (Fig. 1B). We further investigated this phenotype using SEM; whereas stems of wild-type Col-0 plants are densely

![Figure 1](https://example.com/f1.png)

**Figure 1.** Characterization of the cer2-5 mutant. A, T-DNA insertion site in the second exon of CER2. Primers used for sequencing and qPCR are denoted by numbered arrows and described in Supplemental Table S3. B, Stems of wild-type Col-0 (left) and cer2-5 (right). C, SEM of wild-type Col-0 (left) and cer2-5 (right) inflorescence stems. Scale bars = 20 μm. D, Total stem wax load of wild-type Col-0 and cer2-5, measured as average values for four biological replicates. Error bars represent sd. Statistical significance of the difference between the wild type and mutant was confirmed with a Student’s t test, P < 0.005. E, Wax composition of wild-type Col-0 and cer2-5 stems, measured as average values for four biological replicates. Error bars represent sd.
coated with rod-shaped, tubular, and platelet-shaped wax crystals, cer2-5 stems appear completely devoid of such structures (Fig. 1C).

We analyzed stem waxes by GC-FID to determine how wax load and composition of the cer2-5 mutant compared with that of the wild type and other previously described cer2 alleles. We detected a 60% reduction in total wax load (Fig. 1D). All wax monomers longer than C28 were reduced to trace amounts in the mutant, and relative increases were observed for several wax monomers shorter than C28 (Fig. 1E). These results suggest a block in acyl chain elongation past C28 in cer2-5. We also examined the wax profile of rosette leaves in the cer2-5 mutant; no change in rosette leaf wax composition or load compared with the wild type was detected (data not shown). These results agree with the published data for other cer2 alleles.

CER2 Localizes to the ER

Based on the wax phenotype of cer2 mutant alleles, we hypothesized that CER2 has a role in fatty acid elongation of wax precursors. Given that the four characterized enzymes of the FAE complex are localized to the ER (Zheng et al., 2005; Bach et al., 2008; Joubès et al., 2008; Beaudoin et al., 2009), we predicted that the CER2 gene product should localize to the ER as well. To determine if this is indeed the case, the genomic CER2 sequence was fused to GFP and expressed downstream of the 35S promoter in cer2-5 and wild-type Arabidopsis. Multiple cer2-5 transgenic individuals were recovered, which displayed complementation of the cer2-5 wax-deficient phenotype (i.e. had glaucous stems, and restored wax load and composition as determined by GC-FID analysis). In both genetic backgrounds, GFP fluorescence strongly labeled a reticulate structure in young, wax-producing stem epidermal cells (Fig. 2A). When plant stems were stained with the ER dye hexyl rhodamine B (Boevink et al., 1996), the stain and GFP fluorescence showed clear colocalization (Fig. 2, B and C). We also observed an inconsistent CER2:GFP signal in nuclei in some cells in addition to the strong and highly consistent signal from the ER. ER localization of CER2 supports a role for CER2 in wax biosynthesis.

CER2 Is Expressed throughout the Aerial Epidermis

Organ-specific expression patterns of CER2 were investigated by quantitative PCR (qPCR). We detected CER2 transcript in all tissues except roots (Fig. 3A), suggesting either that CER2 may contribute to wax biosynthesis in organs other than stem, or that it may be involved in other metabolic pathways that require VLCFAs.

To determine tissue- and cell type-specific expression patterns of CER2, we examined GFP fluorescence in the wild type and cer2-5 plants transformed with a CER2:GFP transgene driven by the native CER2 promoter, which complemented the cer2-5 mutant phenotype. In stems, siliques, rosette leaves, and cauline leaves, GFP was detected uniquely in epidermal cells (Fig. 3, B and D–F). These results support the notion that CER2 function is specific to cuticular lipid synthesis, which occurs exclusively in the epidermis, and is similar to previous CER2 expression patterns reported by Xia et al. (1996, 1997). We also observed that whereas GFP fluorescence was very strong at the stem apex, there was a reduction in fluorescence at a distance of only 3 cm from the apex (Fig. 3, B and C). This observation corroborates previous findings that wax biosynthesis occurs predominantly at the shoot tip (Suh et al., 2005).

CER2 and Two Paralogs Are Distantly Related to Characterized Members of the BAHD Acyltransferase Family

The cer2 mutation was previously mapped to At4g24510 (Negruk et al., 1996; Xia et al., 1996), which was annotated to encode a BAHD acyltransferase on the basis of sequence similarity with members of this gene family. The BAHD family is specific to and widely distributed among plants, and characterized members have been associated with a broad range of metabolic pathways (D’Auria, 2006). We carried out a phylogenetic analysis using the CER2 protein sequence, its three closest Arabidopsis homologs, and all of the characterized BAHD acyltransferases from Arabidopsis that we are aware of (Supplemental Fig. S1A; proteins annotated in Supplemental Table S2). We found that CER2 was distantly related to most of the other proteins included in our analysis, particularly those with demonstrated acyltransferase activity. Two hypothetical proteins that appeared the most closely related to CER2 were named CER2-LIKE1 (At4g15380) and CER2-LIKE2 (At5g23840).

BAHD acyltransferases catalyze the transfer of an acyl group from acyl-CoA to either an alcohol or an amine acceptor molecule (D’Auria, 2006). Multiple studies have demonstrated through structural analysis and site-directed mutagenesis that BAHD acyltransferase
activity, and indeed that of several other acyltransferase families, is dependent on a catalytic His residue within a conserved “HXXXD” site. The His residue is singly responsible for deprotonating the alcohol or amine of the acyl acceptor (Suzuki et al., 2003; Bayer et al., 2004; Ma et al., 2005; Unno et al., 2007); this deprotonation creates a nucleophile that attacks the carbonyl carbon of the acyl-CoA, resulting in release of the CoA group and formation of a new ester or amide bond. Whereas the conserved HXXXD site is present in the CER2 sequence, we noted that the catalytic His residue is replaced by an Asn residue in the putative Arabidopsis protein with highest homology to CER2, CER2-LIKE1 (Supplemental Fig. S1B). Because of this peculiarity, we undertook an investigation into the function of this CER2 paralog.

CER2-LIKE1 Has a Similar Role to CER2 in VLCFA Elongation Specific to Leaf

We obtained a T-DNA insertional mutant of CER2-LIKE1 from the Arabidopsis Biological Resource Center, SALK_087857, hereafter referred to as cer2-like1-1. Homozygous mutants were identified by PCR-based genotyping, and direct sequencing of genomic DNA revealed a T-DNA insertion in the single intron of CER2-LIKE1 (Fig. 4A). Publicly available microarray data on the Arabidopsis eFP browser (Winter et al., 2007) indicate that CER2-LIKE1 is highly expressed in leaves. As Arabidopsis leaves carry roughly 10-fold less wax than Arabidopsis stems, wax crystals do not form, and it is difficult to detect any change in wax load by sight or with SEM. Because there was no obvious mutant phenotype indicating that cer2-like1-1 is a loss-of-function allele, we first quantified CER2-LIKE1 expression in various wild-type tissues (Fig. 4B) and confirmed that CER2-LIKE1 is expressed in leaves, with lower levels of expression in siliques and seedlings. We then compared CER2-LIKE1 transcript levels between rosette leaves of the wild type and cer2-like1-1 using semiquantitative reverse transcription (RT)-PCR. We could not detect any transcript in the mutant, indicating that cer2-like1-1 is a loss-of-function allele suitable for further analysis (Fig. 4C).

To determine if CER2-LIKE1 plays a role in leaf wax synthesis, we analyzed wax composition of cer2-like1-1 and wild-type rosette leaves. We found that cer2-like1-1 leaves had a 56% reduction in C31 alkane, and accumulated 86% more C29 alkane than the wild type (Fig. 4E). Changes in chain length distribution of less abundant waxes such as aldehydes, alcohols, and fatty acids were less obvious. The shift in alkane chain length distribution does, however, suggest a function for CER2-LIKE1 in C30 VLCFA elongation to C32, parallel to the role of CER2 in C28 elongation to C30. The cer2-like1-1 mutant does still accumulate C32 aldehyde, C33 alkane, and a reduced amount of C31 alkane, which implies that there may be genetic redundancy for the C30 to C32 elongation step. Because CER2, CER2-LIKE1, and CER2-LIKE2 are all expressed in rosette leaves (Figs. 3A and 4B; Supplemental Fig. S2), we suspect that several or all of these genes have a role in elongating C30 to C32 VLCFAs. Thus, the remaining C31 and C33 waxes in the cer2-like1-1 mutant may be produced by either or both CER2 or CER2-LIKE2 activity. The fact that the cer2-5 mutant

Figure 3. CER2 expression patterns as determined by qPCR and CER2pro:CER2:GFP expression in wild-type Col-0. A, qPCR analysis of CER2 expression patterns in the wild type relative to root and normalized to GAPC1 expression, shown as the average of four technical replicates; error bars represent so. CER2pro:CER2:GFP expression in stem sections 1 cm from the apex (B), stem sections 3 cm from the apex (C), developing siliques (D), young rosette leaf (E), and young cauline leaf (F). Scale bars = 50 μm.
had no rosette leaf wax phenotype, despite expression of CER2 in rosette leaves, suggests that CER2 is not the only protein with a function in VLCFA elongation from C28 to C30. Thus, there may be functional overlap among CER2, CER2-LIKE1, and CER2-LIKE2 with respect to both C28 to C30 and C30 to C32 elongation in rosette leaves. Conversely, the cer2-5 phenotype is obvious in stems because it is the only gene from this group that is expressed in the stem epidermis (Figs. 3A and 4B; Supplemental Fig. S2).

To explore the possibility of functional redundancy between CER2 and CER2-LIKE1, we generated a cer2-5 cer2-like1-1 double mutant and analyzed rosette leaf wax composition. cer2-5 cer2-like1-1 rosette leaf wax showed substantial accumulation of C28 primary alcohol and C27 alkane, showed reduction in C31 alkane, and unlike the cer2-like1-1 single mutant, did not accumulate C29 alkane (Fig. 4E). Overall, we interpreted this result as an accumulation of leaf cuticular waxes C28 and shorter, and a reduction in waxes longer than C28. The fact that the reduction in leaf wax elongation occurred at C28 in the double mutant suggests that CER2-LIKE1, similar to CER2, can elongate C28 to C30. However, the double mutant produced wild-type levels of some C30, C32, and C33 wax monomers, indicating that the block in elongation in the double mutant is incomplete. We suggest that the second paralog of CER2 we identified, CER2-LIKE2, may be involved in VLCFA elongation in leaves, and that only a triple mutant will show a complete block in VLCFA elongation from C28 to C32.

Unexpectedly, we found that the average total leaf wax load of the cer2-like1-1 mutant was 18.5% greater than the wild type, although this difference was determined to be statistically insignificant using a Student’s t test. (Fig. 4D). We are unsure of the reason for the increase in wax load we observed here, and at this time our best explanation for this result is that we may be observing a compensatory effort by the plant to produce an effective cuticular barrier by producing more wax when the cuticle structure is compromised. Further investigations of the cer2-like1-1 phenotype, exploration of the cer2-like2 phenotype, as well as double and triple mutant analyses, are currently underway.
The Catalytic Acyltransferase Motif Is Not Required for CER2 Function

Our analysis of the cer2-like1-1 mutant revealed that despite having the catalytic His residue of the conserved HXXXD acyltransferase motif replaced by an Asn (Supplemental Fig. S1B), CER2-LIKE1 has a metabolic function in wax biosynthesis. This is in stark contrast to other characterized BAHD acyltransferases (with the exception of At5g67160, which also lacks this residue; Zheng et al., 2009), and suggests that CER2-LIKE1 may not be an acyltransferase. CER2 homology to CER2-LIKE1 and similar roles of these proteins in VLCFA elongation led us to question whether CER2 has acyltransferase activity. We used site-directed mutagenesis to modify the catalytic His residue of CER2 to determine if it is required for CER2 function. We replaced His with Ala, to presumably knock out any catalytic activity at this site, and Asn, to mimic CER2-LIKE1. We then transformed constructs carrying these mutant alleles of CER2, as well as a wild-type control, into cer2-5 plants. In each case, we confirmed the insertion of the transgene by PCR-based genotyping, and presence of the mutation by Sanger sequencing of the transgene. Both mutant alleles fully complemented the mutant phenotype, indicating that CER2 does not share the same catalytic mechanism as characterized members of the BAHD acyltransferase family (Fig. 5, A and B; Supplemental Fig. S3).

Together, CER2 and CER6 Elongate C28 Fatty Acids to C30 Fatty Acids in Yeast

The wax phenotypes of cer2-5 and cer2-like1-1 mutants demonstrate a role for CER2 and CER2-LIKE1 in specific VLCFA elongation steps required for wax synthesis. However, their biochemical functions are uncertain. They could serve as condensing enzymes in the elongation of VLCFAs longer than C28, or facilitate acyl chain extension past C28 catalyzed by a condensing enzyme such as CER6. To establish whether CER2 is sufficient for C28 elongation to C30, we expressed CER2 in yeast cells in combination with LfKCS45 from the crucifer Lesquerella fendleri (Moon et al., 2004). LfKCS45 elongates C26 VLCFA to C28, the proposed substrate for CER2, and also produces a lesser amount of C30 VLCFA. We detected C28 VLCFA and trace amounts of C30 VLCFA in yeast cells transformed with CER2 and LfKCS45, equivalent to what is produced when LfKCS45 is expressed alone (Supplemental Fig. S4). This result indicates that CER2 is not sufficient for C28 elongation.

The dominant role of the CER6 condensing enzyme in cuticular wax elongation led us to suspect that concerted activity of CER2 and CER6 may be required for the production of C30 VLCFA. Therefore, we expressed CER2 and CER6 individually and together in yeast. Cells transformed with CER2 alone showed no change in fatty acid profile relative to the wild type (Fig. 6). A very small amount of C28 was detected in the strain expressing CER2; however, this can also be seen, though to a lesser degree, in the wild-type strain. It has been previously reported that wild-type yeast can produce very small amounts of this VLCFA (Oh et al., 1997.) Cells expressing CER6 accumulated a much greater amount of C28 VLCFA, but no C30 VLCFA. Remarkably, in the strain expressing both CER6 and CER2, we detected a 2-fold increase in the yield of C28 VLCFA compared with the strain expressing only CER6, and an additional peak, which we identified as C30 fatty acid. Therefore, the combined activity of CER6 and CER2 is necessary and sufficient for acyl chain elongation to C30 for cuticular wax production in Arabidopsis.

DISCUSSION

Previous work has established that FAE complexes carry out VLCFA elongation for wax biosynthesis, and that the condensing enzyme CER6 is responsible for VLCFA elongation from C24 to C28 in Arabidopsis (Millar et al., 1999). However, the inability of CER6 to produce fatty acids longer than C28 in yeast suggests that this process requires one or more additional players. This is particularly puzzling given that there have been no other kcs mutants identified with deficiencies in VLCFA elongation past C28. The only known mutant defective in the conversion of C28 to C30 VLCFA is cer2. We hypothesized that CER2 is required for this elongation step, and in this study, we provide evidence that this is indeed the case.

CER2 Resides in the ER in Epidermal Cells Active in Cuticular Wax Synthesis

The enzymes of the FAE complex that generate VLCFA wax precursors, as well as all of the characterized
wax biosynthetic enzymes, reside in ER membranes (Samuels et al., 2008). We anticipated that likewise, if it were involved in fatty acid elongation, CER2 would localize to the ER. Confocal microscopy performed on transgenic lines expressing \textit{CER2}:GFP driven by the \textit{35S} promoter confirmed CER2 localization to the ER in epidermal cells at the inflorescence stem apex, which actively synthesize cuticular lipids. In these cells, we observed strong CER2-GFP fluorescence throughout the ER; the reticulate cortical ER, transvacuolar strands, and the nuclear envelope. Such localization is consistent with a metabolic function for CER2 in VLCFA biosynthesis. This result was not unexpected, because the \textit{GLASSY2} (GL2) ortholog of CER2 from maize (\textit{Zea mays}) was also localized to a membrane fraction enriched with ER and mitochondria, as well as a soluble cytoplasmic fraction, using polyclonal antibodies (Velasco et al., 2002).

However, this finding contradicts previous work by Xia et al. (1997), who reported that CER2 is a soluble protein and is localized exclusively in the nucleus. Their conclusion was based on probing cell fractions prepared from entire shoots with a polyclonal antibody raised against CER2. We do not discount the possibility that CER2 may be localized to the nucleus in some tissues or under specific conditions. We did, inconsistently, observe the CER2-GFP signal in nuclei of some cells, in addition to the strong and highly consistent signal from the ER. Certainly, this is a topic that requires further investigation.

Whereas ER localization of CER2 is compatible with a biochemical function in wax synthesis, it presents yet another peculiarity of CER2 in the context of homology to BAHD acyltransferases, as all BAHDs localized to date are soluble, cytosolic enzymes (D’Auria, 2006; Panikashvili et al., 2009; Rautengarten et al., 2012). Furthermore, localization of maize GL2 to both the ER and the cytoplasm suggests that CER2 may reside in both compartments and be both soluble and membrane bound. At present, it is not clear how CER2 associates with the ER, and further work is needed to determine if ER localization of CER2 requires interaction with the members of the FAE complex, or other wax biosynthetic enzymes.

\textbf{CER2 and a Small Cluster of Genes within the BAHD Family Do Not Have Typical BAHD Acyltransferase Activity}

Both of the genes investigated in this work, \textit{CER2} and \textit{CER2-LIKE1}, are classified as BAHD acyltransferases based on sequence homology. We were confounded as to how acyltransferases could contribute to VLCFA elongation, given what is known about the enzymology of fatty acid elongation. Analysis of CER2 and CER2-LIKE1 amino acid sequences revealed that despite overall homology, there are important differences between these proteins relative to the rest of the BAHD family. Two conserved domains have been described for BAHDs: a carboxy-terminal DFGWG, which is predicted to have a function in retaining structural stability of the enzyme but is not present in all BAHDs, and HXXXD, which has a confirmed role in catalyzing the acyltransferase reaction (Suzuki et al., 2003; Bayer et al., 2004; Ma et al., 2005; Unno et al., 2007). Both CER2 and CER2-LIKE1 lack the putatively stabilizing DFGWG domain. This may not be surprising given that BAHDs are soluble enzymes (D’Auria, 2006; Panikashvili et al., 2009; Rautengarten et al., 2012), whereas our hypothesis regarding CER2 and CER2-LIKE1 function suggests their physical association with the ER membrane-integrated FAE complex. Perhaps the DFGWG domain is not required when these proteins are in association with the complex.

The substitution of His with Asn in the HXXXD domain of CER2-LIKE1 was far more striking, particularly...
after our analyses of the *cer2-like1-1* mutant demonstrated that this protein is required for normal rosette leaf wax accumulation. In addition to numerous structural studies, site-directed mutagenesis experiments have confirmed the essential nature of the His residue for catalysis in several BAHD acyltransferases (Suzuki et al., 2003; Bayer et al., 2004). We therefore questioned whether the HXXXD domain is relevant to the function of *CER2-LIKE1*, or that of *CER2*. Site-directed mutagenesis experiments converting the His residue of *CER2* to an Ala or an Asn revealed that, just like *CER2-LIKE1*, *CER2* does not require the HXXXD sequence for activity. Therefore, *CER2*, *CER2-LIKE1*, and probably *CER2-LIKE2* represent a protein family with biochemical function distinct from the described functions of characterized BAHD family members.

**CER2 and CER6 Are Necessary and Sufficient for C30 VLCFA Synthesis**

Plant KCS enzymes expressed in yeast are able to work with other components of the native FAE complex to synthesize their specific products (Millar and Kunst, 1997). Therefore, yeast has been a useful system for the functional characterization of KCS enzymes from Arabidopsis. This approach has revealed an inconsistency in that the *CER6* enzyme, the key KCS associated with VLCFA elongation for cuticular wax synthesis, is unable to elongate VLCFAs beyond C28. Indeed, there are very few KCSs that have been reported to elongate VLCFAs to C30; Arabidopsis *CER60* (Trenkamp et al., 2004) and *L. fendleri* LfKCS45 (Moon et al., 2004) are the only plant KCSs with reported ability to elongate VLCFAs to C30. When we introduced *LfKCS45* alone to yeast cells, both C28 and a small amount of C30 VLCFA were produced, as previously reported by Moon et al. (2004). When we introduced *CER2* alone to yeast cells, we did not observe any change in the fatty acid profile relative to the wild type. The fatty acid profile of cells expressing *CER2* alongside *LfKCS45* was no different from that of cells expressing only *LfKCS45*, demonstrating that *CER2* is itself insufficient for VLCFA elongation past C28. Expression of *CER6* alone in yeast resulted in C28 accumulation, as expected. In contrast, coexpression of *CER2* and *CER6* in yeast cells not only resulted in an increase in the yield of C28 VLCFA relative to cells expressing *CER6* alone, but also a remarkably high yield of C30 VLCFA. These findings demonstrate that *CER2* and *CER6* are necessary and sufficient for C30 VLCFA synthesis, and also indicate that *CER2* enhances the elongation of shorter VLCFAs catalyzed by *CER6*.

Our results indicate that the *CER6* condensing enzyme has a property absent from *LfKCS45* that is necessary for *CER2*-associated elongation. What this property is and how unique it is to *CER6* remains unknown, and is a topic of our present research. It also remains to be determined whether *CER2* has a distinct activity that contributes to VLCFA elongation, if it plays a structural or stabilizing role necessary for the elongation of VLCFAs by *CER6*, or has a role in allosteric regulation of condensing enzymes.

As discussed above, *CER2* appears not to have acyltransferase activity, as it does not need the conserved His residue required to activate the acyl acceptor molecule for nucleophilic attack. BAHD acyltransferases are known to utilize a ternary complex mechanism of acyl transfer (Suzuki et al., 2003), which entails direct transfer of the acyl group off of CoA onto the acceptor molecule as a result of the deprotonated acceptor’s nucleophilic properties. Thus, we can draw two conclusions from the dispensability of the His residue in *CER2*. (1) *CER2* cannot transfer acyl groups, at least not by the mechanism described based on other BAHD acyltransferases, and (2) *CER2* cannot cleave the acyl group from CoA, as CoA release is a downstream effect of the initial deprotonation of the acyl acceptor. However, *CER2* may still share with other BAHD acyltransferases the capacity to bind acyl-CoA groups. It is tempting to speculate that *CER2* binding an acyl-CoA substrate, such as C28-CoA or malonyl-CoA, could induce conformational changes in the *CER2* protein structure, and that these could in turn affect *CER2* protein-protein interactions or targeting, or even the conformations and activities of components of the FAE complex. Further investigation of these putative protein-protein interactions will be fundamental to our understanding of the biochemical function of *CER2*.

VLCFAs longer than C28 are not common in nature, and we are not aware of any reports of KCSs that can produce more than trace amounts of C30 VLCFAs or longer. We therefore suspect that the canonical FAE complex-catalyzed mechanism of VLCFA elongation holds exceptions after C28 synthesis. We were able to find putative orthologs of *CER2* across the plant kingdom using a BLAST search, and previous phylogenetic analyses have confirmed pervasive distribution of the clade of BAHD acyltransferases to which *CER2* belongs among vascular plants (Tuominen et al., 2011). This suggests that the *CER2* family has a necessary, conserved role in VLCFA elongation, and that *CER2* activity is critical to cuticular wax biosynthesis.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Seed stocks of *cer2-5* (At4g24510; SALK_84443) and *cer2-like1-1* (At4g13840; SALK_087857) were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003; http://www.arabidopsis.org/). Single and double mutants were genotyped using primer 1 (all primer sequences are listed in Supplemental Table S3) specific for the T-DNA insertion, primers 2 and 3 specific for *cer2-5*, and primers 4 and 5 specific for *cer2-like1-1*. Double mutants were generated by crossing homozygous mutant lines and selecting the appropriate lines from the F2 generation by genotyping. The precise site of
the DNA insertion in the mutant alleles was determined by sequencing the PCR product amplified using the T-DNA-specific primer (primer 1) and one gene-specific primer (primer 3 or 5). Seeds were germinated on Arabidopsis (Arabidopsis thaliana; AT) medium (Somerville and Ogren, 1982) supplemented with agar and, when necessary, with appropriate antibiotics. Seven-day-old seedlings were transferred to soil (Sunshine Mix 4, SunGro) and grown under continuous light (100 μE m⁻² s⁻¹ of photosynthetically active radiation) at 20°C.

RT-PCR

RNA was extracted from 4-week-old wild-type Col-0 and cer2-like1-1 rosette leaves by phenol:chloroform:isoamyl alcohol separation, and purified by lithium chloride and sodium acetate precipitations (Wilkins and Smart, 1995). Residual genomic DNA was removed from the system by treatment with DNasel (New England Biolabs), according to the manufacturer's protocol. 1.5 μg of each RNA sample was reverse-transcribed to single-stranded complementary DNA (cDNA) using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. The gene GAPC1 was used as a control for constitutive expression, using primers 11 and 12. Primers 8 and 10 were used for CER2-LIKE1 detection.

qPCR

Seedling and root RNA was extracted from young wild-type Col-0 seedlings grown on Arabidopsis medium, and stem, flower, silique, cauline leaf, and rosette leaf RNA was extracted from mature plants by phenol:chloroform: isoamyl alcohol separation, and purified by lithium chloride and sodium acetate precipitations (Wilkins and Smart, 1995). RT and DNasel treatment were performed as described above. The gene GAPC1 was used as a control for constitutive expression for qPCR analysis. CER2 primers 6 and 7 and CER2-LIKE1 primers 8 and 9 were used to measure expression levels. cDNA was quantified using iQ SYBR green Supermix (BioRad), according to the manufacturer's protocol, in an MJ Mini Option conal Thermocycler (BioRad). Four technical replicates were analyzed for each sample.

Wax Analysis

Wax analysis was carried out using the top 10 cm of 3- to 5-week-old primary stems, or 3- to 4-week-old entire rosette leaves. Tissues were photographed to determine sample surface area. Surface area was determined by measuring two-dimensional area in Adobe Photoshop, and for stems, by multiplying the flat area by π. Tissues were submerged for 30 s in approximately 8 mL of chloroform containing 10 μg tetracosane as an internal standard. Chloroform was blown off under nitrogen gas, and the waxes silylated by heating in pyridine and N,O-bis(trimethylsilyl)-trimethylfluoracetamide with 1% trimethylchlorosilane at 80°C for 1 h. Solvent was again blown off under nitrogen gas, and the waxes resuspended in 50 μL of chloroform for GC-FID analysis. Waxes were analyzed on an Agilent 7890A, gas chromatography system with an HP1 methyl siloxane column. The program ran at 50°C for analysis. Waxes were analyzed on an Agilent 7890A gas chromatography system with an HP1 methyl siloxane column. The program ran at 50°C for analysis. Waxes were analyzed on an Agilent 7890A gas chromatography system with an HP1 methyl siloxane column. The program ran at 50°C for analysis.

Confocal Microscope Imaging

Subcellular localization of GFP-tagged CER2 was carried out on a PerkinElmer UltraView VoX Spinning Disk Confocal Microscope, using a 63× glycerol immersion objective. Stem tissue was dyed with hexyl rhodamine B to counterstain the ER. GFP was excited with a 488-nm laser, and hexyl rhodamine B was excited with a 561-nm laser. Cell-specific expression patterns were observed on a Nikon Eclipse 80i Scanning Laser Confocal Microscope using a 20× objective. GFP was excited with a 488-nm laser, and chlorophyll autofluorescence was detected for contrast.

Site-Directed Mutagenesis

The CER2pro:CER2::GFP construct was used as a template for overlap extension mutagenesis (Ho et al., 1989). The genomic CER2 sequence was split in two fragments for amplification of altered sequences. For the His to Ala mutation, primers 14 and 17 were used to amplify the 5′ fragment, primers 18 and 16 were used for amplifying the 3′ fragment, and primers 14 and 16 were used for the final overlap extension PCR. For the His to Asn mutation, primers 14 and 19 were used for amplifying the 5′ fragment, primers 20 and 16 were used for amplifying the 3′ fragment, and primers 14 and 16 were used for the final overlap extension PCR. Following amplification of the split CER2 fragments, PCR products were purified on a DNA-binding column (Epoch Life Science) prior to overlap extension. A wild-type control was amplified from the CER2pro:CER2::GFP construct using primers 14 and 16 directly. CER2 alleles were cloned into the pDONR221 (Invitrogen) and pGWB4 (Nakagawa et al., 2007) vectors, and transformed into cer2-5 Arabidopsis plants following the procedure outlined under “plasmid construction and plant transformation.” Insertion of the T-DNA and presence of the correct mutation were confirmed by PCR-based genotyping and sequencing.

Yeast Assay

cDNA was prepared as described above under RT-PCR from Col-0 stem tissue. CER6 and CER2 cDNA sequences were amplified using primers 23 and 24, and 21 and 22, respectively. CER6 was cloned into the pESC-URA yeast (Saccharomyces cerevisiae) expression vector (Agilent) downstream of the GAL10 inducible promoter, and CER2 was cloned into the p423 yeast expression vector with His selection, downstream of the strong constitutive GPD (glycereraldehyde-3-P-dehydrogenase) promoter (Mumberg et al., 1995). Correct plasmid construction was confirmed by colony PCR, restriction digestion, and sequencing. Yeast strain W3031a was transformed using the protocol described by Gietz and Woods (2002). The LKCS45 yeast expression construct generated and described by Moom et al. (2004) was used. Transgenic yeast cells were grown on synthetic complete (SC) selection medium (Sherman, 2002) lacking the appropriate amino acids. Successful transformation was confirmed by colony PCR. Three individual cell lines were selected from each transgenic strain for induction of transgene expression and lipid analysis. Cultures were grown in 5-mL SC medium with 2% Gal overnight, then plated on solid SC.
medium with 2% Gal and incubated for 2 to 4 d (strains harboring plasmids grew slower than the wild type). Cultures were scraped from the plates and transmethylated to their fatty acid methyl esters in methanolic HCl for 2 h at 80°C, and the lipids extracted in hexane. Fatty acid methyl esters were analyzed by GC-FID, and peaks identified based on comparison of retention times with known standards.

Phylogenetic Analysis

A BLAST search of the deduced CER2 protein sequence was used to select the closest homologs, and characterized Arabidopsis BAHDs were selected based on a literature review. The selected sequences were aligned in MUSCLE (Edgar, 2004), and viewed and edited in the Mesquite program (Maddison and Maddison, 2007). A basic tree was derived from ProtTest (Abascal et al., 2005), which was used to select the WAG+G+F model for generating the final tree (Whelan and Goldman, 2001). The tree was used to select the WAG+G+F model for generating the final tree (Whelan and Goldman, 2001).

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Supplemental Data

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

Supplemental Figure S1. Phylogenetic and sequence analysis of CER2 and the BAHD acyltransferase family.

Supplemental Figure S2. qPCR analysis of CER2-LIKE2 expression.

Supplemental Figure S3. Wax composition of cer-2-5 complemented with site-directed mutant CER2 alleles.

Supplemental Figure S4. Total lipid profiles of yeast expressing Lyc/CS45 and CER2.

Supplemental Table S1. Summary of characterized cer2 alleles.

Supplemental Table S2. Annotation of protein sequences used for phylogenetic analysis.

Supplemental Table S3. Primer sequences used for this study.

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