ECERIFERUM2-LIKE Proteins Have Unique Biochemical and Physiological Functions in Very-Long-Chain Fatty Acid Elongation

Tegan M. Haslam, Richard Haslam, Didier Thoraval, Stéphanie Pascal, Camille Delude, Frédéric Domergue, Aurora Mañas Fernández, Frédéric Beaudoin, Johnathan A. Napier, Ljerka Kunst*, and Jérôme Joubès

Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4 (T.M.H., A.M.F., L.K.); Department of Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom (R.H., F.B., J.A.N.); Université de Bordeaux, Laboratoire de Biogenèse Membranaire, Unité Mixte de Recherche 5200, F–33000 Bordeaux, France (D.T., S.P., C.D., F.D., J.J.); and Centre National de la Recherche Scientifique, Laboratoire de Biogenèse Membranaire, Unité Mixte de Recherche 5200, F–33000 Bordeaux, France (D.T., S.P., C.D., F.D., J.J.)

The extension of very-long-chain fatty acids (VLCFAs) for the synthesis of specialized apoplastic lipids requires unique biochemical machinery. Condensing enzymes catalyze the first reaction in fatty acid elongation and determine the chain length of fatty acids accepted and produced by the fatty acid elongation complex. Although necessary for the elongation of all VLCFAs, known condensing enzymes cannot efficiently synthesize VLCFAs longer than 28 carbons, despite the prevalence of C28 to C34 acyl lipids in cuticular wax and the pollen coat. The eceriferum2 (cer2) mutant of Arabidopsis (Arabidopsis thaliana) was previously shown to have a specific deficiency in cuticular waxes longer than 28 carbons, and heterologous expression of CER2 in yeast (Saccharomyces cerevisiae) demonstrated that it can modify the acyl chain length produced by a condensing enzyme from 28 to 30 carbon atoms. Here, we report the physiological functions and biochemical specificities of the CER2 homologs CER2-LIKE1 and CER2-LIKE2 by mutant analysis and heterologous expression in yeast. We demonstrate that all three CER2-LIKEs function with the same small subset of condensing enzymes, and that they have different effects on the substrate specificity of the same condensing enzyme. Finally, we show that the changes in acyl chain length caused by each CER2-LIKE protein are of substantial importance for cuticle formation and pollen coat function.

The extension of fatty acids to lengths greater than 28 carbons (C28) is an exceptional process in plant metabolism in that it requires unique biochemical machinery, and the elongation products are used for the synthesis of specialized plant metabolites. Derivatives of C30 to C34 fatty acids make up the bulk of plant cuticular wax, which coats all of a plant’s primary aerial surfaces. Cuticular wax serves as a barrier against transpirational water loss (Riederer and Schreiber, 2001) and protects the plant from both biotic (Eigenbrode, 1996) and abiotic (Grace and van Gardingen, 1996) stresses. C30 to C34 fatty acid-derived lipids are also components of the pollen coat, where they function in pollen hydration and germination on dry stigma (Elleman et al., 1992; Preuss et al., 1993).

The core complex that elongates long-chain fatty acids (C16–C18) to very-long-chain fatty acids (VLCFAs; C20–C34) consists of four interacting proteins localized to the endoplasmic reticulum (ER). β-Keto-acyl-CoA synthases (KCSs), also known as condensing enzymes, catalyze the first reaction required for VLCFA elongation, condensing malonyl-CoA with an acyl-CoA (n) to produce a β-keto-acyl-CoA (n + 2). Condensation is both a specific and rate-limiting step in elongation (Millar and Kunst, 1997). Chain length specificity of KCSs is of particular importance because VLCFA length determines the downstream use of the fatty acid (for review, see Joubès et al., 2008; Haslam and Kunst, 2013a). There are two families of condensing enzymes in Arabidopsis (Arabidopsis thaliana). The ELONGATION-DEFECTIVE (ELO)-LIKE family is homologous to yeast (Saccharomyces cerevisiae) ELOs, and has putative functions in sphingolipid biosynthesis (Quist et al., 2009). Although our
current understanding of plant ELO-LIKE physiological function and biochemical activity is limited, the mechanism of yeast Elo protein activity has been thoroughly investigated (Denic and Weissman, 2007). The FATTY ACID ELONGATION1 (FAE1)-type family is homologous to the first condensing enzyme identified in Arabidopsis, which is required for the synthesis of C20 to C22 VLCFAs in Arabidopsis oilseeds. Many of the 21 FAE1-type condensing enzymes of Arabidopsis have been characterized using reverse genetics and heterologous expression in yeast (Trenkamp et al., 2004; Blacklock and Jaworski, 2006; Paul et al., 2006; Tresch et al., 2012). This work has revealed the intriguing caveat that, although FAE1-type KCs are involved in the synthesis of diverse downstream metabolites and use a broad range of acyl chain lengths, none are able to efficiently elongate VLCFAs beyond C28 (for review, see Haslam and Kunst, 2013a), which is essential for the production of cuticular wax components.

Eceriferum2 (cer2) and glossy2 (gl2) mutants of Arabidopsis and Zea mays, respectively, are deficient in specific VLCFA-derived waxes longer than C28 (Bianchi et al., 1975; McNevin et al., 1993; Jenks et al., 1995). Both mutations were mapped to genes that do not resemble any component of the elongase complex (Tacke et al., 1995; Xia et al., 1996), but are homologous to the BAHD family of acyltransferases (St-Pierre et al., 1998). However, site-directed mutagenesis of conserved acyltransferase catalytic site amino acids in CER2 revealed that this motif is not required for CER2 function in cuticular wax synthesis (Haslam et al., 2012).

CER6 is a condensing enzyme necessary for the accumulation of stem cuticular waxes in Arabidopsis, but when expressed in yeast, CER6 can only elongate VLCFAs to C28. When CER2 is expressed in yeast, it has no elongation activity. However, coexpression of CER2 and CER6 results in efficient production of C30 VLCFAs. Coexpression of CER2 with LfKCS45, a condensing enzyme from the crucifer Lesquerella fendleri that generates C28 and a small amount of C30 VLCFAs (Moon et al., 2004), does not alter product chain length (Haslam et al., 2012). Based on these observations, it was hypothesized that CER2 modifies the chain length specificity of the core elongase complex by interaction with specific KCS enzymes (Haslam et al., 2012).

CER2 homologs are found in diverse flowering plant lineages, and many species have multiple CER2 homologs (Tuomainen et al., 2011). A BLAST search of proteins from Arabidopsis identified two sequences with substantial similarity to CER2. NP_193120 is 36% identical to CER2, and is encoded by the gene At4g13840. We named this gene CER2-LIKE1 (also known as CER26) (Pascal et al., 2013). NP_566741 is 38% identical to CER2, and is encoded by the gene At3g23840. We named this gene CER2-LIKE2 (also named CER26-LIKE) (Pascal et al., 2013). Characterization of a cer2-like1 null mutant revealed a role for the CER2-LIKE1 protein in the elongation of leaf wax precursors beyond C30, analogous to the role of CER2 in C28 elongation in stems (Haslam et al., 2012; Pascal et al., 2013). cer2 cer2-like1 double mutants are deficient in the formation of wax components longer than C28 in both stems and leaves. As the cer2 single mutant has no leaf wax phenotype, the additive effect of these two mutations on leaf wax composition indicates that there is partial functional redundancy between the two genes.

A comprehensive investigation of the biochemical and physiological functions of CER2-LIKE proteins is necessary. Beyond the value of knowing the specific roles of each homolog, such an investigation has potential to elucidate the nature of CER2-LIKE protein function. With this objective, we used our data to address the following questions: (1) Do CER2-LIKE proteins function with CER6 alone, or can they modify the activity of other FAE1-type condensing enzymes? (2) Do CER2-LIKE proteins have different effects on the substrate specificity of the same condensing enzyme, or is substrate specificity determined exclusively by the condensing enzyme? (3) What is the physiological relevance of the subtle changes in acyl lipid chain length that CER2-LIKE proteins induce?

RESULTS

CER2-LIKE Proteins Impart Different Substrate Specificities to the Same Condensing Enzyme

Mutant phenotypes described in Haslam et al. (2012) and Pascal et al. (2013) suggest that CER2 and its homolog CER2-LIKE1 have different substrate specificities. We reasoned that this difference in specificity could be due to either CER2 and CER2-LIKE1 each functioning with different condensing enzymes, or CER2 and CER2-LIKE1 imparting their own unique substrate specificity to the same condensing enzyme. To differentiate between these two possibilities, we coexpressed the three Arabidopsis CER2-LIKE proteins with the CER6 condensing enzyme in yeast, and analyzed yeast fatty acyl chains by gas chromatography (GC)-mass spectrometry (MS).

None of the CER2-LIKE proteins had any detectable activity when expressed alone, but elongation activity was detected when each of the three CER2-LIKEs were expressed alongside CER6. As reported, CER2 and CER6 coexpression in yeast produced more C28 and a large amount of C30 VLCFAs, whereas yeast coexpressing CER2-LIKE1 with CER6 accumulated C28, C30, C32, and C34 VLCFAs (Fig. 1A). Yeast cells coexpressing CER6 and CER2-LIKE2 synthesized C28, C30, and traces of C32 VLCFAs. Coexpression of CER2, CER2-LIKE1, and CER6, or CER2-LIKE1, CER2-LIKE2, and CER6, produced VLCFA profiles similar to CER2-LIKE1 and CER6 alone, indicating that there is no synergistic effect of having multiple CER2-LIKE proteins. These results demonstrate that CER2-LIKE proteins have unique substrate specificities in the presence of the same condensing enzyme. The specificity of CER2-LIKE1 observed here is in agreement with the fact that the cer2-like1-1 mutant rosette leaf wax has reduced amounts of C31, C32, and C33 waxes, which are abundant in wild-type leaf cuticular wax (Haslam et al., 2012; Pascal et al., 2013).
CER2-LIKEs Can Modify the Activity of Select Condensing Enzymes Involved in Cuticular Wax Biosynthesis

We demonstrated previously that CER2 can alter chain length specificity of the elongation complex when it is coexpressed with CER6, but not when it is coexpressed with the LfKCS45 condensing enzyme from the crucifer *L. fendleri* (Haslam et al., 2012). We were curious whether CER2-LIKE activity could be observed strictly with CER6, or if any of the CER2-LIKEs have multiple condensing enzyme partners. We therefore coexpressed each of the CER2-LIKE proteins with the following condensing enzymes from Arabidopsis: ECERIFERUM60 (CER60/KCS5; Fiebig et al., 2000), FIDDLEHEAD (KCS10; Pruitt et al., 2000), KCS1 (Todd et al., 1999), KCS9 (Lü et al., 2012), and KCS20 (Lee et al., 2009). These condensing enzymes were selected because their null mutants have cuticle defects, they belong to different subclasses of the FAE1-type KCS family (Joubès et al., 2008), and because they have demonstrated activity in yeast (Tresch et al., 2012). CER2-LIKE activity was not tested alongside ELO-LIKE proteins. Beyond CER6, the three CER2-LIKEs only had an effect on the specificity of CER60. Although the activities of CER6 and CER60 in yeast are different in that CER60 produces more C28 than CER6, all three CER2-LIKEs modified the activity of CER60 in a fashion similar to when partnered with CER6 (Fig. 1B; data for CER2-LIKEs paired with KCSs which were not altered by coexpression are not shown).

Together, the yeast expression experiments described here reveal that the effect of CER2-LIKE proteins on fatty acid elongation is limited to specific condensing enzyme partners, and that CER2-LIKE proteins either have their own unique substrate specificity, or a unique effect on the substrate specificity of the condensing enzyme with which they function. Although the identical effects of CER2-LIKEs on CER6 and CER60 activity suggest that the specificity of CER2-LIKEs does not vary with different condensing enzyme partners, it remains a possibility that CER2-LIKE activity may be different with other condensing enzymes which were not investigated here.

CER2 and CER2-LIKE2 Have Different Expression Patterns in Reproductive Organs

We previously reported the expression patterns of CER2, CER2-LIKE1, and CER2-LIKE2. CER2 is expressed in the epidermis of stems, leaves, flowers, and siliques; CER2-LIKE1 is expressed in rosette and cauline leaves; and CER2-LIKE2 is largely expressed in flowers (Haslam et al., 2012; Pascal et al., 2013). Although the functions of CER2 and CER2-LIKE1 were described in these publications, the role of CER2-LIKE2 was not investigated.
High expression levels of CER2-LIKE2 in flowers suggest that it could be required for the production of the unique cuticular lipids on floral organs, or the synthesis of lipidic components of the pollen coat or sporopollenin. To explore the function of CER2-LIKE2, we performed quantitative PCR on flowers throughout development of Columbia-0 (Col-0) plants, following the staging described by Smyth et al. (1990) for early floral development. We compared temporal CER2-LIKE2 expression patterns to those of CER2 and CER1 (Fig. 2A). CER1 encodes an alkane-forming enzyme required for cuticular wax biosynthesis (Bernard et al., 2012). CER2 and CER1 are both expressed in different floral organs throughout development and contribute to cuticle formation (Bourdenx et al., 2011; Pascal et al., 2013). Further, specific mutant alleles of both of these genes cause conditional male sterility at low humidity (Koornneef et al., 1989; Preuss et al., 1993), suggesting that they are required for the synthesis of pollen coat lipids. We also compared CER2-LIKE2 expression to that of FATTY ACID REDUCTASE2 (FAR2), which has a role in the synthesis of fatty alcohol monomers of sporopollenin (Aarts et al., 1997; Kapoor et al., 2002; Choi et al., 2014). In our case and perhaps in those cited, secretion of either the GUS protein or its CIβ-indigo product, or their release upon degeneration of tapetal cells, may have caused the observed staining of microspores. In stage nine flowers, GUS staining was observed exclusively in tapetal cells, and no staining was observed in stage 11 flowers. Together, the spatial and temporal expression patterns of CER2-LIKE2 suggest that its gene product is required for the pollen coat or sporopollenin biosynthesis.

CER2-LIKE Proteins Are Required for Male Fertility

The metabolic function of CER2-LIKE2 in planta has not been investigated. Further, previous reports on CER2 and CER2-LIKE1 functions may be incomplete due to functional redundancy with CER2-LIKE2. We obtained transfer DNA (T-DNA) insertional mutant lines for all three CER2-LIKE genes, and confirmed loss of full-length transcript for each by reverse transcription-PCR (Haslam et al., 2012; Pascal et al., 2013; Supplemental Fig. S1). All combinations of double mutants and a triple mutant were generated to observe possible redundant functions of CER2, CER2-LIKE1, and CER2-LIKE2. Homozygous mutant lines were selected from the F2 generation of each cross by PCR-based genotyping.

Figure 2. Tissue-specific expression of CER2-LIKE2. A, Differential expression analysis of CER2, CER2-LIKE2, CER1, and FAR2 genes in Arabidopsis flowers. The gene expression level was determined by real-time PCR analysis. The relative transcript abundance of ACTIN2 and EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1 in each sample was determined and used to normalize for differences of total RNA amount. Results are presented as transcript abundance for each gene compared with expression levels at stage less than 9. The data represent the means ± so of three replicates. B, Anther-specific staining of GUS in flowers in transgenic Arabidopsis plants harboring the CER2-LIKE2 promoter fused to the GUS reporter gene. Promoter activity was visualized through histochemical GUS staining of a flower bud (a) and on semithin sections of anthers at stages 8 (a), 9 (b), and 11 (c) of flower development. Bars = 0.5 mm.
We immediately noticed that cer2-5 cer2-like2-1 double mutant and cer2-5 cer2-like1-1 cer2-like2-1 triple mutant had stunted siliques, which were largely devoid of viable seeds at maturity (Fig. 3A). This phenotype was rescued by growing the mutants at high humidity, suggesting that they may be male sterile due to a pollen coat defect. To confirm this, we performed reciprocal crosses between the two mutant lines and the wild type. Normal seed and silique development were observed when wild-type pollen was used to pollinate the triple mutant (Fig. 3B), but few or no seeds could be recovered when pollen from the mutant was used to pollinate wild-type stigmas (Fig. 3C). These results indicate that CER2 and CER2-LIKE2 have semiredundant functions required for male fertility. No obvious structural abnormalities were observed when cer2-5 cer2-like1-1 cer2-like2-1 pollen was inspected by transmission electron microscopy (Supplemental Fig. S2).

CER2-LIKE Proteins Have Semiredundant Functions in the Elongation of Cuticular Wax Precursors

As CER2, CER2-LIKE1, and CER2-LIKE2 are all expressed in rosette leaves, we analyzed rosette leaf wax and the acyl-CoA content of the single, double, and triple mutants to gain a more complete understanding of their substrate specificities in planta.

The rosette leaf cuticular wax profiles of the cer2-5 and cer2-like2-1 single mutants were indistinguishable from the wild type. cer2-like1-1 alone was different, with increased levels of C30-derived waxes and less C32-derived C31 alkane (Fig. 4A). Among the double mutants, the wax profile of cer2-5 cer2-like2-1 appeared to be identical to the wild type, whereas the profile of cer2-like1-1 cer2-like2-1 was very similar to that of the cer2-like1-1 single mutant (Fig. 4B). These two phenotypes suggest that CER2-LIKE2 has either no function or a redundant function in wax precursor elongation in leaf.

The cer2-5 cer2-like1-1 double mutant, like the cer2-like1-1 mutant, had reduced levels of C32-derived waxes; however, cer2-5 cer2-like1-1 was different in that it did not accumulate substantially more C30-derived waxes than the wild type and accumulated more C28-derived waxes. This suggests that CER2 and CER2-LIKE1 have redundant functions in elongation past C28 in leaves. The wax profile of the cer2-5 cer2-like1-1 cer2-like2-1 triple mutant was nearly indistinguishable from that of the cer2-5 cer2-like1-1 double mutant, with a slightly greater reduction in the load of waxes longer than C30. It is important to note that none of the mutants, including the triple, were completely lacking leaf waxes of any given length. Therefore, there must be other pathway components in leaf epidermal cells, absent from stem epidermal cells, which contribute to VLCFA elongation to exceptional lengths. Alternatively, truncated proteins could be produced in cer2-like1-1 or cer2-like2-1 mutants (cer2-5 is clearly a null based on its stem wax phenotype), which may retain partial activity.

VLCFA metabolism in cer2-like mutant lines was further investigated by profiling the acyl-CoA pools of young rosette leaves (Fig. 4C). The acyl-CoA pools of cer2-5, cer2-like1-1, and the cer2-5 cer2-like2-1 double mutants were not different from the wild type. cer2-like1-1 and the cer2-like1-1 cer2-like2-1 double mutant showed a 3-fold increase in C30 acyl-CoA, a 50% reduction in the amount of C32 acyl-CoA, and only trace amounts of C34 acyl-CoA. cer2-5 cer2-like1-1 and the triple mutant had a 2-fold increase in the amount of C28 acyl-CoA, no difference in the amount of C30 acyl-CoA, and reduced amounts of both C32 and C34 acyl-CoA. Both had only trace amounts of C34; however, the cer2-5 cer2-like1-1 double mutant had 50% of the wild-type amount of C32 acyl-CoA, whereas C32 was reduced to trace levels in the triple mutant. These results support our hypotheses based on the leaf cuticular wax phenotypes of the mutants: CER2 and CER2-LIKE1 both contribute to the elongation of C28 acyl-CoAs in leaf, CER2-LIKE1 contributes to elongation past C30, and CER2-LIKE2 activity makes a very small contribution to the elongation process.

CER2-LIKE1 Partially Rescues the Stem Wax-Deficient Phenotype of cer2-5 and Localizes to the ER

To determine whether different CER2-LIKE proteins can substitute for each other’s functions, we expressed a CER2-LIKE1-GFP fusion ectopically in the stem epidermis of cer2-5 mutants. We used the promoter of the condensing enzyme CER6, which was thoroughly characterized.

Figure 3. Sterility phenotypes of cer2-like mutants. A, Mature siliques of single, double, and triple mutants. Siliques of cer2-5 cer2-like2-1 and cer2-5 cer2-like1-1 cer2-like2-1 are stunted and contain few or no seeds at maturity. B, Siliques of cer2-5 cer2-like1-1 cer2-like2-1 triple mutant manually pollinated with wild-type pollen. C, Siliques of wild-type plants manually pollinated with cer2-5 cer2-like1-1 cer2-like2-1 triple mutant pollen.
by Hooker et al. (2002), to drive CER2-LIKE1 expression in both the wild-type and cer2-5 mutant background.

GC with flame ionization detection analysis of stem wax revealed that there was little or no reduction in the total wax load of transgenics in wild-type background (Fig. 5A), and transgene expression in the cer2-5 mutant background recovered more than one-half the wild-type wax load (Fig. 5B). That the total stem wax load of the cer2-5 mutant was partially recovered demonstrates that different CER2-LIKE proteins are able to partially substitute for each other in planta. Further, as expected based on the results of our yeast expression experiment, CER2-LIKE1 conferred its own unique substrate specificity, causing a shift in the stem cuticular wax profile to longer chain lengths; for transgenic plants in either a Col-0 or cer2-5 background, the ratio of C31:C29 wax components is greater than 1, whereas it is 0.03 in Col-0 wild type.

Plants carrying the CER2-LIKE1-GFP transgene in either a Col-0 or cer2-5 background appeared glossy, despite the fact that they had wild-type or partially recovered stem wax loads, respectively. A similar observation has been made for the cer2-like1-1 mutant complemented with a 35Spro::CER2-LIKE1 construct (Pascal et al., 2013). Examination of the progeny of three independent transgenic lines in each background by scanning electron microscopy (SEM) revealed that stems had a greatly reduced density of wax crystals, and that these consisted of irregularly shaped, thin flakes on the stem surface (Fig. 5C). This demonstrates that modification of relative amounts of different wax component chain lengths is sufficient to modify wax crystal organization.

The CER2 gene has been localized by several research groups, with conflicting results (Xia et al., 1997; Haslam et al., 2012). The CER2-LIKE1-GFP protein localized to
the ER when ectopically expressed in stem epidermis under control of the CER6 promoter (Fig. 6A). We generated a similar construct, CER2-LIKE1::CER2-LIKE1-GFP, which we transformed into the cer2-like1-1 mutant, and which complemented the cer2-like1-1 wax-deficient phenotype. CER2-LIKE1-GFP also localized to the ER in the leaves of transgenic plants (Fig. 6B). As the CER2-LIKE1-GFP fusions clearly localized to the ER in both of these lines, in cells where the protein was demonstrated to complement background mutations, we confirm that the ER is the site of CER2-LIKE protein activity during VLCFA elongation.

DISCUSSION

CER2-LIKE Proteins Impart Unique Substrate Specificity to Fatty Acid Elongation

Previous work demonstrated that the cer2-like1 mutant, similar to the cer2 mutant, is deficient in cuticular wax precursor elongation. Cuticular wax and acyl-CoA profiles of the two mutants showed that each gene product affects elongation at a different chain length. These observations suggested two hypotheses: (1) different CER2-LIKE proteins could associate with and thereby alter the specificity of different condensing enzymes; and (2) different CER2-LIKE proteins could have unique effects on the specificity of the same condensing enzyme. These possibilities are not mutually exclusive, but at least one of them had to be correct to account for the different phenotypes that were observed in the cer2 and cer2-like1 mutants.

We tested these two hypotheses by coexpressing CER2, CER2-LIKE1, and CER2-LIKE2 with the condensing enzyme CER6, and with several other condensing enzymes known to contribute to the synthesis of cuticular lipid precursors. The three CER2-LIKE proteins had activity with only two condensing enzymes, CER6 and CER60/KCS5. The chain length specificities of CER2-LIKEs were different, but did not change when they were coexpressed with either CER6 or CER60.
Coexpression of multiple CER2-LIKEs with CER6 demonstrated that CER2-LIKEs do not have synergistic activity. When CER6, CER2, and CER2-LIKE1 are coexpressed, the yeast fatty acid methyl esters (FAMEs) profile is similar to that of only CER2-LIKE1 and CER6 together, but with slightly more C30 accumulation, as is observed with CER2 and CER6. The same pattern is also observed with CER6, CER2-LIKE1, and CER2-LIKE2 coexpression. These results suggest that there could be competition among CER2-LIKEs for activity alongside the core elongation machinery. This idea is further supported by the dramatic accumulation of C30 acyl-CoAs or C30-derived waxes in the cer2-like1 single mutant, which easily exceeds the loss of C32 and C34 acyl-CoAs or C32- and C34-derived waxes.

That the CER2-LIKEs functioned with both CER6 and CER60 was not unexpected, as the amino acid sequences of these two condensing enzymes are 88.6% identical and they belong to the same subclass of the FAE1-type KCS family (Joubiès et al., 2008). We conclude that CER2-LIKE proteins have their own unique acyl chain length specificity, which is observed only when they are coexpressed with an appropriate condensing enzyme partner. The CER2-LIKE1 and CER2-LIKE2 amino acid sequences are 66% identical, but are only 36% and 38% identical to CER2, respectively (Pascal et al., 2013).

As such, we were surprised to find that CER2 and CER2-LIKE2 both contribute to VLCFA elongation to C30, whereas CER2-LIKE1 uniquely supports elongation up to C34. This observation will be valuable in directing future work dissecting the basis of substrate specificity within the CER2-LIKE gene family.

**Modification of FAE1-Type KCS Specificity by CER2-LIKEs Is of Physiological Importance**

Heterologous expression in yeast demonstrated that CER2-LIKE proteins have the potential to alter the substrate specificity of different condensing enzymes, but did not address whether CER2-LIKE proteins function alongside the same condensing enzymes in planta, and whether the activity of a CER2-LIKE protein is sufficient to determine the chain length profile of downstream acyl-lipid products. Work by Pascal et al. (2013) demonstrated that ectopic expression of CER2-LIKE1 using the 35S promoter was sufficient to increase the amount of C31, C32, and C33 waxes relative to the total wax load in the stem cuticle. Here, we have repeated that experiment using the strong, epidermis-specific CER6 promoter, with similar results. This construct was able to partially complement the reduced wax load of the cer2-5 mutant, demonstrating that CER2-LIKE proteins can substitute for one another’s in vivo function. We observed that the CER2-LIKE1 protein localized to the ER when ectopically expressed in stem epidermal cells and when expressed by its native promoter in leaf epidermal cells. We conclude that CER2-LIKE proteins localize to the ER for function paired with FAE1-type condensing enzymes.

We observed altered wax chain length profiles when the CER6pro::CER2-LIKE1-GFP construct was expressed in wild-type and cer2-5 backgrounds, with both showing a substantial increase in C31 and C33 waxes and a decrease in C29 waxes relative to the untransformed background. The total wax load of the transgenics in the wild-type background did not differ substantially from the nontransformed controls. This was somewhat counterintuitive, as the stems of the transgenics of both backgrounds were glossy, and we predicted that we would observe a total wax load deficiency. As the changes in relative amounts of different types of acyl lipids (e.g., ketones, alkanes, secondary alcohols, etc.) were minor compared to the relative changes in amount of acyl chain lengths, we interpret this result as an indication that wax component chain length affects the formation of wax crystals. This hypothesis is in agreement with the fact that Arabidopsis leaf cuticular waxes, which are predominantly composed of C32-derived hydrocarbons, do not produce a glaucous, waxy appearance, whereas inflorescence stem cuticular waxes are largely composed of aliphatics derived from C30 VLCFAs and have a white, glaucous appearance. Therefore, the effects of CER2-LIKE proteins on the final steps of VLCFA elongation have an important effect on the structure and physical properties of the cuticle.
Male sterility dependent on humidity of the environment is a phenotype associated with pollen coat deficiencies, and has been observed in several Arabidopsis mutants with deficiencies in cuticular wax metabolism (Koornneef et al., 1989; Preuss et al., 1993) including one cer2 allele (Preuss et al., 1993; Eijsner et al., 1998). The most abundant acyl lipids in the pollen coat are C29 alkane, C29 ketone, and C31 alkene (Jessen et al., 2011), and so it is not surprising that the biosynthesis of cuticular waxes and pollen coat could require the same or similar enzymes. We have not observed any reduction in seed set in the single cer2-5 mutant relative to the wild type, and we suggest that this discrepancy with previous work could be due to subtle differences in growth conditions, or the different alleles selected for study. Here, we showed that coinactivation of CER2 and CER2-LIKE2 causes male sterility in low-humidity environments. These results suggest that the chain length of acyl lipid pollen coat components affects their function, or that chain length of VLCFA precursors is important for their conversion to alkanes and other aliphatics found in the pollen coat. The precise role of VLC acyl lipids in the pollen coat remains unknown, but it has been suggested that they could be required for physical contact between the pollen grain and stigmatic papillae, allowing for diffusion of signaling molecules; they could provide a matrix that could stabilize other trypine components; or that VLC acyl lipids could themselves function as signaling molecules (Preuss et al., 1993). Regardless of which of these functions VLCFA-derived lipids fulfill in the pollen coat, our data show that CER2-LIKEs paired with condensing enzymes are required for fertilization.

MATERIALS AND METHODS

Plant Material

Seed stocks of cer2-5 (At4g24510; SALK_084443), cer2-like1-1 (At4g13840; SALK_087857), and cer2-like2-1 (At3g23840; GK-180G04-013602) were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003; http://www.arabidopsis.org/). Homozygous cer2-like2-1 mutants were selected by PCR-based genotyping, and precise T-DNA insertion sites were determined by sequencing the PCR product generated by genotyping homozygous mutant PCR-based genotyping, and precise T-DNA insertion sites were determined by standard Gateway cloning procedures. (Primer sequences are listed in Supplemental Table S1.) Constructs were confirmed by Sanger sequencing, and transformed into competent GV3101 Agrobacterium tumefaciens cells. Arabidopsis plants were transformed by the floral spray method. Transformed lines were selected by resistance to antibiotics, and genotypes were confirmed by PCR in lines selected for phenotype analysis.

The CER-LIKE2 promoter sequence was amplified from Arabidopsis genomic DNA using primers listed in Supplemental Table S1. The PCR product was cloned into the pDONR221 ENTRY vector using Gateway recombination technology, and subsequently transferred into the pGWFS7 destination vector (Karimi et al., 2002). The construct was transferred into the A. tumefaciens CS9C1Rif strain harboring the plasmid pMP90, and was transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998). Histochemical GUS analyses were performed as described in Joubs et al. (2008).

The CER-LIKE and KCS open reading frames were cloned into the pDONR221 ENTRY vector using the primers described in Supplemental Table S1, and were transferred into yeast (Saccharomyces cerevisiae) expression vectors as detailed in Supplemental Table S2. Yeast strain INVSc1 (MATa/MATa his3D1/ his3D1 leu2/leu2 trpl-289/trpl-289 ura3-52/ura3-52) cells were transformed with different combinations of constructs by a polyethylene glycol/lithium acetate protocol (Ausubel et al., 1995) and grown on minimal medium lacking appropriate amino acids as indicated in Supplemental Table S2.

Reverse Transcription-PCR and Quantitative PCR

RNA was extracted from wild-type and mutant leaves by phenolchloroform:isoamyl alcohol separation and purified by lithium chloride and sodium acetate precipitations (Wilkins and Smart, 1995). Genomic DNA was removed by treatment with DNase I (New England Biolabs) according to the manufacturer’s protocol. Single-stranded complementary DNA was synthesized from RNA using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s protocol. CER2-LIKE expression in the cer2-like2-1 mutant was examined using the primers described in Supplemental Table S1.

RNA from Arabidopsis flowers was extracted with the RNeasy Plant mini kit (Qiagen). Purified RNA was treated with DNase I using the DNA-free kit (Ambion). First-strand complementary DNA was prepared as described in Pascal et al. (2013). The PCR amplification was performed with the gene-specific primers listed in Supplemental Table S1 as described in Pascal et al. (2013).

Lipid Analyses

Yeast FAMES Analysis

To analyze fatty acyl composition of individual transformed yeast cell lines, cells were grown in 5 mL of appropriate liquid minimal medium supplemented with 2% (w/v) Gal to induce expression of Arabidopsis transgenes. Starting from precultures in 2% (w/v) raffinose medium, expression cultures were grown for 4 to 6 d at 30°C. Cultures were pelleted and washed in 2 mL of 2.5% (w/v) Salt before fatty acyl analysis. FAMES were synthesized by formylation of yeast cell sediments at 85°C for 1 h and 20 min with 1 mL of 0.5 M sulfuric acid in methanol containing 2% (v/v) dimethoxypropane and 50 μg of heptadecanoic acid (C17:0) as an internal standard. After cooling, 1 mL of 2.5% (w/v) NaCl was added, and fatty acyl chains were extracted in 1 mL of hexane and analyzed by GC-MS as previously described (Domenge et al., 2010).

Wax Analysis

Wax analysis of rosette leaves was carried out as described in Haslam and Kunst (2013b).

Acyl-CoA Analysis

Freshly harvested plant material was frozen in liquid nitrogen, and acyl CoAs was extracted as described by Larson and Graham (2001) and analyzed by
using electrospray ionization-tandem mass spectrometry (MS/MS) MS/MS/MS + multiply reaction monitoring (MRM) or liquid chromatography (LC)-MS/MS/MS + MRM in positive ion mode. The LC-MS/MS + MRM analysis (using an ABISciex 4000 QTRAP) was performed as described by Haynes et al. (2008) Agilent 1200 LC system; Gemini C18 column [Phenomenex, 2-mm i.d., 150-mm length, 5-μm particle size]. For the purpose of identification and calibration, standard acyl-CoA esters with acyl chain lengths from C14 to C20 were purchased from Sigma as free acids or lithium salts.

Microscopy

Confocal Microscopy

Subcellular localization of GFP-tagged CER2-LIKE1 was determined using a PerkinElmer Ultraview VoX Spinning Disk Confocal Microscope. Images were captured using a 63× glycerol-immersion lens, with a 488-nm laser for GFP excitation. Leaves were counterstained with hexyl rhodamine B to label the ER (Grabski et al., 1993).

SEM

One-centimeter-thick stem segments approximately 10 cm from the apex were cut, mounted, air dried, and sputter coated with gold particles to a thickness of 10 nm using a Leica EM ACE200 Low Vacuum Coater. Coated samples were viewed with an Hitachi S4700 field emission SEM using an accelerating voltage of 5 kV and a working distance of 12 mm.

Transmission Electron Microscopy

Arabidopsis wild-type and triple mutant flowers at stage 13 of floral development were fixed in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C, postfixed in osmium tetroxide for 2 h at room temperature (22°C–25°C), and poststained with uranyl acetate overnight at 4°C. After dehydration and embedding in Spurr’s resin, ultrathin sections (80–90 nm) were obtained on a Reichert 2010 microtome and mounted on copper grids. Ultrathin sections were observed with a Tecnai G2 Spirit TWIN transmission electron microscope at 120 kV.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CER2, At4g24510; CER2-LIKE1, At4g13840; CER2-LIKE2, At3g3840; CER1, At1g02205; FAR2, At3g19420; elF4A-1, At4g13920, GAPC.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Reverse transcription-PCR of CER2-LIKE2 in wild-type and cer2-like2-1 mutants.

Supplemental Figure S2. Pollen coat formation of cer2-5 cer2-like1-1 cer2-like2-1 plants.

Supplemental Table S1. Primers used for cloning, reverse transcription-PCR, and quantitative PCR.

Supplemental Table S2. Transgenic yeast cell lines.

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