Molecular Characterization of the CER1 Gene of Arabidopsis Involved in Epicuticular Wax Biosynthesis and Pollen Fertility

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The aerial parts of plants are coated with an epicuticular wax layer, which is important as a first line of defense against external influences. In Arabidopsis, the ECERIFERUM (CER) genes affect different steps of the wax biosynthesis pathway. In this article, we describe the isolation of the CER1 gene, which encodes a novel protein involved in the conversion of long chain aldehydes to alkanes, a key step in wax biosynthesis. CER1 was cloned after gene tagging with the heterologous maize transposable element system Enhancer-Inhibitor, also known as Suppressor-mutator. cer1 mutants display glossy green stems and fruits and are conditionally male sterile. The similarity of the CER1 protein with a group of integral membrane enzymes, which process highly hydrophobic molecules, points to a function of the CER1 protein as a decarboxylase.

INTRODUCTION

Waxes are found in a wide variety of living organisms as a mixture of long chain fatty acid-derived substances (Kolattukudy, 1976). In plants, these components are specifically found as an epicuticular layer that covers leaves and young stems and is often visualized by a characteristic glaucous appearance (Kolattukudy, 1975). The primary function of epicuticular wax deposition is to reduce water loss through the epidermis (Hall and Jones, 1961), a feature contributing to drought tolerance. In addition, this outer layer has a major function in the interaction with herbivorous insects and plant pathogenic fungi (Thompson, 1963; Städer, 1986; Podila et al., 1993; Eigenbrode and Espélie, 1995). An unexpected function of wax has been described recently by Preuss et al. (1993), who found pollen wax composition and structure to be important factors for a proper pollen--pistil interaction.

The wax composition is determined by the various biochemical steps of wax biosynthesis. Plant wax layer mutants, which unravel these biochemical steps, are available (von Wettstein-Knowles, 1979; Kolattukudy, 1980; Bianchi et al., 1985) and have led to a basic outline of the wax biosynthetic pathway (von Wettstein-Knowles, 1979; Bianchi et al., 1985; Lemieux et al., 1994; von Wettstein-Knowles, 1994). Starting with hexadecanoic acid, long chain fatty acids with an even carbon number (in general, C20 to C22) are produced by elongation. These fatty acids are reduced to fatty aldehydes and primary alcohols or reduced and decarbonylated to yield alkanes with an uneven carbon number (Cheesbrough and Kolattukudy, 1984). The latter can be further converted to secondary alcohols and ketones (Kolattukudy, 1980).

Many genetic loci influencing wax deposition have been identified in maize, barley, Brassica spp, and Arabidopsis (Baker, 1974; von Wettstein-Knowles, 1979; Bianchi et al., 1985; Koornneef et al., 1989; McNevin et al., 1993). Mutants with altered wax production or wax composition are in general characterized by a bright green phenotype. In Arabidopsis, there is an additional effect on fertility for some mutants, and the 21 different genetic wax layer or ECERIFERUM (CER) loci identified in Arabidopsis have been grouped into four classes based on fertility and visual degree of glossiness of the mutants (Koornneef et al., 1989; McNevin et al., 1993). Isolation of these cer genes will contribute to the understanding of pollen--pistil interactions as well as plant--herbivorous insect interactions. Furthermore, it may provide tools for the manipulation of wax composition in crop species to generate a source of broad host range resistance against herbivorous insects. A highly active transposon tagging system based on the autonomous Enhancer or Suppressor-mutator (EnSpm) and nonautonomous Inhibitor or defective Suppressor-mutator (IdSpm) elements from maize has been developed in our laboratory for Arabidopsis (Aarts et al., 1993, 1995), and we used it to generate transposon-mutagenized populations that were screened for cer mutants.

In this article, we report the identification and phenotypic analysis of an IdSpm transposon-tagged cer1 mutant and the
subsequent isolation and characterization of the CER1 gene involved in wax biosynthesis and pollen fertility. Stem wax of cer1 mutants has been =analyzed previously (Hannouna et al., 1993; McNevin et al., 1993; Lemieux et al., 1994) and found to be especially rich in aldehydes but lacking alkanes, suggesting that the CER1 protein is involved in the conversion of aldehydes to alkanes. We present evidence for the function of the CER1 protein as an aldehyde decarbonylase.

RESULTS

Phenotypic and Genetic Analysis of a Transposon-Induced cer Mutant

To isolate genes involved in epicuticular wax biosynthesis, we used an ldsSpm transposon tagging approach to generate and screen Arabidopsis lines containing the EnSpm–ldsSpm transposon tagging system (see Methods; Aarts et al., 1995). Among the screened lines, we selected one line with multiple ldsSpm elements and the TEn2 transposase T-DNA (Aarts et al., 1995), which revealed some bright green semisterile mutants among normal wild-type plants. Phenotypically, these mutants strongly resemble a known class of cer mutants with a glossy stem and reduced fertility (Koornneef et al., 1989). Complementation tests with the cer1, cer3, cer6, and cer10 mutants in this class revealed that the transposon-induced mutant is allelic to cer1-7. No clear phenotypic differences were found between our cer1 mutants (which we called cer1-m) and cer1-1 mutants. Both display a strong glossy stem and fruit phenotype, without any visible sign of wax production. Wax production on other organs of the plant was apparently not altered, but epicuticular wax production in wild-type plants is mainly confined to stem and fruit surface and not visually detectable on other parts of the plant.

Mutation of cer1 not only alters the wax deposition on stem and fruit but also has a pleiotropic effect on plant fertility. A similar effect has been observed for cer6-2 or pop1 (defective in pollen–pistil interactions), described as a conditionally male-sterile cer mutant, but was never characterized for cer1 mutants; therefore, we decided to examine the cause of sterility in much the same way as was performed for cer6-2 (Preuss et al., 1993). Although normal amounts of pollen are produced by the cer1-1 and cer1-m mutants, they were completely self-sterile under dry conditions (30 to 40% relative humidity). Crossing cer1-m mutant flowers with wild-type Landsberg erecta pollen led to a normal seed set. Self-fertility was increased to the wild-type level by growing the plants under high humidity (90 to 100% relative humidity), indicating that, as with cer6, the self-sterility is an environmentally controlled form of male sterility (Preuss et al., 1993). We tested germination of cer1-m and Landsberg erecta wild-type pollen on mutant and wild-type stigma papillae (see Methods) and found that mutant pollen does not germinate on either wild-type or cer1-m pistils, whereas the pollinations with wild-type pollen were normal, yielding normal seed set. Pollen from cer1-m mutants germinates similarly to wild-type pollen in vitro (data not shown). The inability of cer1-m pollen grains to germinate in vivo coincided with the inability to rehydrate on the stigma surface, as was also reported for the cer6-2 mutant. Rehydration of wild-type pollen grains occurred within minutes after deposition on the stigma papillae, during which time the pollen grain swelled to about twice its original size (Preuss et al., 1993; M.G.M. Aarts, unpublished observations). The accumulation of callose on the stigma surface in response to pollination, which was observed for cer6-2 pollen (Preuss et al., 1993), was also seen when fully receptive wild-type or cer1-m stigmas were pollinated with cer1-m pollen but not when pollinated with oilseed rape or petunia pollen (data not shown).

In terms of pollen–pistil interaction, the cer1 mutants are very similar to cer6-2 mutants. For the latter mutant, conditional male sterility is explained based on an alteration of the tryphine layer covering the pollen grain (Preuss et al., 1993). When examined in a scanning electron microscope, exines of mature, shed pollen of wild type, cer1-m, and cer1-1 all appeared to contain tryphine, which generally was found covering the entire pollen grain (Figure 1A). Transmission electron microscopy indicated that the amount of lipid in the tryphine of the three genotypes examined was comparable. However, lipid droplets in the tryphine of cer1-m and cer1-1 pollen (Figures 1C and 1D) were more numerous and considerably smaller than in tryphine of wild-type pollen (Figure 1B).

cer1-m Mutant Is Tagged by an ldsSpm Element

The cer1-m mutant was found in a line with transposing ldsSpm elements and was presumably caused by insertion of an ldsSpm element. To determine whether cer1 was tagged, large offspring populations from mutants were screened for progeny that had reverted to the wild-type phenotype. This is a phenomenon typical of transposon-induced mutations. Germinal reversions were found at a frequency between 1 in 50 and 1 in 300 in four independent progenies, indicating that the unstable mutation was indeed due to a transposon insertion in the CER1 gene.

DNA blot analysis of segregating progeny (see Methods) revealed one ldsSpm insert (ldsSpm89) cosegregating with the cer1-m mutant phenotype. The flanking DNA of this ldsSpm89 insert was amplified by inverse polymerase chain reaction (IPCR) and cloned. Based on the DNA sequence, primers were designed for PCR amplification of wild-type and revertant ldsSpm89 excision alleles. Three independently derived germinal revertant plants all contained an excision allele, demonstrating that the cer1-m mutant was indeed tagged by the ldsSpm89 insertion, creating a cer1::ldsSpm89 allele. Excision of ldsSpm89 elements normally creates short base pair deletions and additions (Aarts et al., 1993), but in these three cases, the DNA sequences of the revertant alleles were iden-
Figure 1. Tryphine on the Pollen Grains of Wild-Type Plants and cer1-1 and cer1-m Mutants.

(A) Scanning electron microscopy of a cer1-1 pollen grain with tryphine covering its entire surface. Bar = 1 µm.
(B) Transmission electron microscopy showing detail of a cross-section through a Landsberg erecta wild-type pollen grain. Tryphine can be observed filling the exine. Bar = 500 nm.
(C) Transmission electron microscopy showing comparable detail of a cross-section through a cer1-1 pollen grain. Bar = 500 nm. Tryphine in cer1-1 and cer1-m appears more granulated than in the wild type and has more but smaller lipid droplets and gas inclusions. Exine (e), intine (i), gas inclusions (thick arrows), and lipid droplets (thin arrows) are indicated in (B) to (D). Gas inclusions are the white spherical or irregularly shaped spaces within the exine cavities. Lipid droplets are spherical or oval-shaped gray inclusions in the exine, which do not contrast very well with the rest of the tryphine but can be distinguished due to the amorphous structure of their contents.

tical to the wild-type DNA sequence, suggesting insertion of *HdSpm89* at a vital position of the gene.

The availability of a transposon-tagged cer1 mutant allowed an easy screen for determining whether the *CER1* gene acts cell autonomously. The presence of clearly cell-specific somatic reversion sectors would mean a cell-autonomous expression. Somatic reversions were observed on the stem of ~20% of the mutant plants. Such variegated plants showed one or more very small glaucous gray sectors of wax deposition on a bright green background (Figure 2A). The distinct boundaries of the wild-type revertant sectors on the stem suggested a cell-autonomous expression of *CER1*. Scanning electron micrographs of these sectors revealed individual epidermal cells with overlaying wax deposition shown as the rodlike wax structures typical for Arabidopsis (Figure 2B; Koornneef et al., 1989). At higher magnification, it was clear that the effect of reversion was not completely cell autonomous, because some wax structures had formed on epidermal cells flanking revertant cells (Figure 2C). Either the *CER1* gene product or the wax components generated by this gene product had diffused a short distance away from the producing cell.

The TEn2 En-transposase, present in the cer1-m mutants, induces in general a low frequency of transposition, and therefore, we combined the *cer1::HdSpm89* allele with TEn5, a different, more active En/Spm transposase locus (Aarts et al., 1995; see Methods), which increased approximately fivefold the number of variegated mutants and the size and number of wild-type sectors per plant. To determine the effect of *CER1* expression in different layers, we searched for large excision sectors that extended over whole inflorescences. One such sector was found, and to our surprise, this sector now had wild-type stem wax and displayed a concomitant reversion to wild-type fertility. To test whether the reversion had occurred in the L2 layer, 22 offspring descending from the reverted inflorescence were sown. All offspring were found to be cer7 mutants, suggesting that the excision was L1 rather than L2 layer specific.

Cloning of *CER1*

Using the *HdSpm89* flanking genomic DNA as a probe, a homologous cDNA clone and a 17-kb genomic clone were isolated from the respective DNA libraries. To confirm that the cDNA clone originated from the *CER1* locus, part of the insert DNA was used as a probe and hybridized to a blot of *cer1::HdSpm89* mutant and revertant plants (Figure 3). All mutants were homozygous for a fragment containing the *HdSpm89* insert,
Figure 2. Phenotype of a Transposon-Tagged cer1 Mutant.

(A) Bright green stem of a cer1::ldSpm mutant. The black arrowhead indicates a glaucous wild-type somatic excision sector.
(B) Scanning electron microscopy of a wild-type excision sector similar to the one shown in (A). Bar = 100 μm.
(C) One-cell-wide excision sector with wild-type epicuticular wax production. The presence of the typical wild-type Arabidopsis epicuticular wax rods is not limited to the surface directly above the epidermal cells carrying the excision, but some wax structures are formed on neighboring cells. Bar = 10 μm.
(D) Stem of a wild-type (CER1cer1::ldSpm89) plant with a cer1 mutant sector (white arrowheads).
although excision could be observed, and all revertants were either hemizygous for the \textit{iIdSpm89} insert or lacked the insert.

Conclusive proof that the isolated gene was indeed the \textit{CER1} gene involved in epicuticular wax formation was obtained from the analysis of a plant with wild-type phenotype except for a small mutant cer sector (Figure 2D). In the course of \textit{cer1::iIdSpm89} analysis, three such plants were found in various progeny. The mutant sector in one of these plants, hemizygous for \textit{iIdSpm89}, ended in a small leaf, from which DNA was isolated for PCR analysis. Combinations of an \textit{iIdSpm}-specific terminal primer and different \textit{CER1}-specific primers (see Methods) were used for PCRs with DNA from the cer sector and from the wild-type rosette leaves of the same plant (Figure 4A). Two cer sector-specific DNA fragments were amplified for two primer combinations. The new cer sector-specific \textit{iIdSpm} insertion was positioned within the coding region of the cloned gene, 1.0 kb upstream of the \textit{iIdSpm89} insert (Figure 4B). Because a new insertion of an \textit{iIdSpm} element into the cloned gene resulted again in a mutant cer phenotype, we conclude that the cloned gene is indeed \textit{CER1} involved in epicuticular wax biosynthesis.

**Analysis of the \textit{CER1} cDNA**

Epicuticular waxes are found mainly on the stem and fruit epidermis of Arabidopsis, and the isolated \textit{CER1} gene should be expressed in these organs. \textit{CER1} transcription was therefore tested by RNA gel blot hybridization, and as expected, the \textit{CER1} transcript was found in wild-type stem and fruit tissue. Additional strong expression was detected in Arabidopsis flowers, in which expression of the \textit{CER1} gene could be expected based on the male-sterile phenotype of the mutant. Arabidopsis has little wax formation on the leaves, explaining the low level of leaf transcript (Figure 5A). Transcription of the \textit{CER1} gene was blocked in \textit{cer1::iIdSpm89} mutant flowers, whereas transcription in \textit{cer1-1} flowers was not affected (Figure 6A).
The mutant phenotype of the chemically induced cer1-1 mutant is probably due to a minor rearrangement such as a point mutation. In flowers of the F1 hybrid between cer1::ldSpm and cer1-1, the transcription level of the gene was the intermediate of the two parents (Figure 5C).

The 2109-bp CER1 cDNA contains an open reading frame of 625 amino acids (Figure 6A). Part of the corresponding genomic DNA sequence was determined, and an in frame stop codon was found 33 bp upstream of the ATG start codon, indicating that the cDNA clone comprises the complete open codon was found 33 bp upstream of the ATG start codon, indicating the cDNA clone comprises the complete open reading frame. A putative TATA transcription initiation sequence is present 72 bp upstream of the ATG start codon in the genomic DNA sequence. The predicted protein has an apparent molecular mass of 72.3 kD and a pI of 8.23. Analysis of the amino acid sequence with the PC/Gene computer package classifies the protein as an integral membrane protein. Two putative transmembrane helices are predicted, stretching from amino acid positions 178 to 213 and 325 to 350 (Figure 6A), and additional membrane-associated helices cover amino acid positions 7 to 27, 45 to 65, 99 to 119, and 126 to 146. Two possible Asn glycosylation sites are found at positions 258 and 456. Insertion of ldSpm89 disrupts the reading frame from Thr (amino acid position 272) onward (Figure 6A).

CER1 Homologs Are Present in Other Species

Wax production is common to many plant species, and genes involved in wax biosynthesis may well be conserved among species. This was confirmed as data base searches, performed with the CER1 cDNA and predicted amino acid sequences, revealed significant homologies with cDNA and expressed sequence tag (EST) sequences from both dicot and monocot species (see Methods). The predicted amino acid sequence of the EST ATTS1001 cDNA isolated from flower buds of Arabidopsis showed 53.8% identity with the C-terminal region (210 amino acids) of the predicted CER1 amino acid sequence (Figure 6A). In addition, a B. campestris flower bud EST was found with 49.1% predicted amino acid sequence identity (117 amino acids), a potato epidermal EST with 67.4% amino acid identity (46 amino acids), and a Senecio odorus epidermal cDNA with 31.3% amino acid identity (513 amino acids) (Figure 6A).

This family of related sequences could be extended to the monocot species maize and rice. A maize vegetative meristem EST showed 52.7% amino acid identity over 110 amino acids. The homology of two rice callus cDNAs sequenced from their 5’ end started exactly at the N terminus of the predicted CER1 amino acid sequence extending over the entire length of sequenced cDNA, representing ~80 amino acids (37.5% overall identity; Figure 6B). Interestingly, the predicted amino acid sequence of another rice cDNA with a short stretch of amino acid similarity with the predicted CER1 amino acid sequence showed additional homology in this region with the C-5 sterol desaturase protein of yeast encoded by the ERG3 gene (Arthington et al., 1991). These two short stretches of homology are conserved among CER1, SOLIPTBR, rice EST D23996, and ERG3, and a part of it is also found in maize EST T70657 (Figure 7). Each stretch of homology reveals a short motif with the consensus sequence Tyr-His-Ser/Thr-X-His-His (where X stands for any amino acid).

A cer1-like gene is closely linked to the cer1 gene but transcribed differently

Part of the genomic sequence upstream of the CER1 gene was determined, and when used in a DNA data base search, DNA sequences between 1.0 and 1.7 kb upstream of the CER1 start codon surprisingly were found to be identical with the ATTS1001 EST sequence (data not shown). The DNA sequence of ATTS1001 shows only 62.2% identity with the CER1 cDNA (compared with the 53.8% amino acid identity found previously), and when used as a probe, it does not cross-hybridize to the CER1 cDNA sequence. From these data, we conclude
DISCUSSION

To confirm that this gene is transcribed in Arabidopsis and to examine any correlation between CER1 and CER1-like expression, we tested CER1-like transcription by RDA blot hybridization using the ATTS1001 EST insert probe (Figures 5B and 5D). In contrast to the strong transcription of CER1 in stem, flower, and fruit, transcription of the CER1-like gene was restricted to flowers but with a similar mRNA size and transcription level as the CER1 gene (Figure 5B). Transcription of the CER1-like gene was not affected by the cer1 mutation, as seen by the similar level of CER1-like transcription in the flowers of cer1::ildSpm89, cer1-1, and the F1 (Figure 5D), demonstrating the difference in transcriptional regulation between CER1 and CER1-like. The transcription of both the CER1 and CER1-like gene in chemically or radiation-induced cer2 to cer9 flowers was the same as in the wild type (data not shown), suggesting that none of these transcriptional regulators.

CER1 Protein Has a Function in Wax Alkane Biosynthesis

We have cloned and characterized the CER1 gene from Arabidopsis involved in epicuticular wax biosynthesis and pollen fertility. CER1 is a cell autonomously expressed gene, mainly active in stem, flower, and fruit and at a low level in leaf. The cer1 mutant is one among four of the cer mutants with a drastically changed epicuticular wax phenotype, for which a biochemical function has been proposed to the corresponding wild-type gene. CER2 and CER6 are thought to encode components of fatty acid elongation, and CER4 is suggested to be involved in fatty aldehyde elongation (Hannoufa et al., 1993; Lemieux et al., 1994; Jenks et al., 1995). Biochemical studies (Hannoufa et al., 1993; McNevin et al., 1993; Lemieux et al., 1994) have shown that cer1 mutants are blocked in the conversion of stem wax C29 aldehydes (triacontanal) to C29 alkanes (nonacosane) and that they also lack the secondary alcohols (14- and 15-nonacosanol) and ketones (15-nonocacnone) derived thereof. Alkanes, secondary alcohols, and ketones indicate that the SOLIPTRB and ATTS1001 cDNAs are partial and that the N-terminal ends of the complete amino acid sequences are missing.
gous sequences found after searching the sequence data bases with fatty acid desaturases, an alkane hydroxylase, and a xylene monoxygenase (TBLASTN (top) and motifs present in a number of membrane-bound proteins (bottom; Shanklin et al., 1994). The CER1 homologous sequence shown are the stearoyl-CoA desaturase from rat (rat; Thiede et al., 1986) and yeast (yeast; Stukey et al., 1990), the A12 fatty acid desaturase from Arabidopsis (FAD2 and FAD3; Okuley et al., 1994; Arondel et al., 1992), the A6 fatty acid desaturase from Synechocystis (D6; Reddy et al., 1993), the alkane hydroxylase from Pseudomonas oleovorans (ALKB; Kok et al., 1989), and the xylene monooxygenase from P. putida (XYLM; Suzuki et al., 1991). Similar and identical residues are as indicated in Figure 6.

CER1 Protein Contains an Iron Binding Motif

Because no genes encoding fatty alcohol decarbonylases have been cloned previously, this biochemical function has not been described at the sequence level. The homologies we observed between CER1 and other cDNA or EST sequences in the sequence data bases describe a family of related proteins, but they are not very useful in defining a biochemical function for these proteins. The S. odoratus and potato partial cDNA sequences encoding CER1 homologous amino acid sequences are described as epidermis specific, which corresponds to a function for CER1 in epicuticular wax biosynthesis. The only homology we found with a functional protein is with the C-5 sterol desaturase from yeast. This homology is actually confined to a very short sequence, centered around three histidine-rich motifs ordered as HX3H + HX2HH + HX2.3HH motifs, were recently found to be conserved among a number of integral membrane fatty acid desaturases from mammals, fungi, insects, higher plants, and cyanobacteria as well as bacterial membrane alkane hydroxylase and xylene monooxygenase (Figures 7A and 7B). All eight conserved histidine residues are essential for the enzymatic function, as was tested with complementation studies in yeast using site-specific mutagenized rat stearoyl-CoA desaturase genes (Shanklin et al., 1994).

Apart from the histidine-rich motifs, there is no apparent homology with other regions of these proteins (Figures 7A and 7B), suggesting that the reported proteins with these motifs (including the C-5 sterol desaturase and the protein encoded from Synechocystis (D6; Reddy et al., 1993), the alkane hydroxylase from Pseudomonas oleovorans (ALKB; Kok et al., 1989), and the xylene monooxygenase from P. putida (XYLM; Suzuki et al., 1991). Similar and identical residues are as indicated in Figure 6.

B) Comparison of the third histidine-rich motif HXp.3HH present in S. pseudomonas oleovorans (ALKB; Kok et al., 1989), and the xylene monooxygenase from P. putida (XYLM; Suzuki et al., 1991). Similar and identical residues are as indicated in Figure 6.

C) Hydrophobicity plot of the predicted CER1 amino acid sequence according to Kyte and Doolittle (Devereux 1984), indicating the location of putative transmembrane sequences (horizontal bars labeled 1 and 2) and the histidine-rich motifs (H). Transmembrane sequence 1 is long enough to span the membrane twice (a and b).
by the partial cDNA clone from rice) are likely to share some biochemical properties rather than performing the same biochemical function. The presence of closely spaced histidine residues is typical for metal binding motifs, and based on the requirement of fatty acid desaturases for iron, the histidine motifs are strongly suggested to be involved in the binding of iron ions (Okuley et al., 1994). The spacing separating the first two histidine motifs from the third motif, which is reported to be conserved among membrane desaturases and among the alkane hydroxylase and the xylene monoxygenase, is similar albeit slightly smaller in the CER1, SOLIPTRB, D23996, and ERG3 proteins (Figures 7A and 7B). This distance appears to be conserved within families of related proteins, because it is also smaller in the alkane hydroxylase and the xylene monoxygenase compared with the desaturases. One reason there is some variation in the spacing between the first two and the third histidine motifs among protein families is that the structure of this protein part, rather than the spacing between the motifs, is important for function of the proteins. All proteins with the histidine-rich motifs described so far were found to contain long hydrophobic domains between the first two and the third histidine motifs (Shanklin et al., 1994). These domains are able to span a membrane twice. The same is found for the CER1, SOLIPTRB, and ERG3 protein, in which both HX2HH motifs are separated by a predicted transmembrane sequence that can be divided into two parts long enough to satisfy this requirement (Figure 7C).

Based on all these characteristics, the CER1 protein closely resembles a class of structurally and perhaps evolutionarily related integral membrane enzymes that share the preference for highly hydrophobic substrates, the presence of metal binding histidine-rich motifs, and the need for electron donors to perform their catalytic function.

**CER1 Protein as Part of an Aldehyde Decarbonylase**

The CER1 protein has been proposed to function in the decarbonylation of aldehydes to alkanes (Hannoufa et al., 1993; Lemieux et al., 1994). So far, two plant aldehyde decarbonylases (from pea and a green colonial alga, *Botryococcus braunii*) have been studied in some detail (Cheesbrough and Kolattukudy, 1984; Dennis and Kolattukudy, 1992). Both are integral membrane proteins, with the pea decarbonylase suggested to be located in the cuticular cell membrane and the alga decarbonylase in the microsomal membranes. Both use highly hydrophobic fatty aldehydes as substrate and need metal ions for their function. The metal identity is known only for the purified decarbonylase from *B. braunii*, which interacts with cobalt present in a cobalt–porphyrin or corrin structure (Dennis and Kolattukudy, 1992). The partially purified decarbonylase from pea is merely known to depend on metal ions, because the activity is severely inhibited in the presence of metal ion chelators (Cheesbrough and Kolattukudy, 1984). All enzymatic proteins with the histidine-rich motifs identified so far are proposed to have nonheme iron-containing active sites (Shanklin et al., 1994). This is in contrast with the observed interaction of *B. braunii* decarbonylase with porphyrin cobalt, but to assume all plant decarbonylases interact with heme-bound metals based on the characterization of only one (lower) plant decarbonylase would be a bit premature. It can still be envisaged that decarbonylases from higher plants use nonheme iron rather than heme cobalt. The observed biochemical properties of the partially purified aldehyde decarbonylase from pea (an integral membrane protein processing highly hydrophobic molecules in the presence of metal ions) underpin the properties ascribed to the CER1 protein on the basis of homology with some functional integral membrane enzymes. This is strong supporting evidence for the CER1 protein acting as the catalytic iron-containing part of a fatty aldehyde decarbonylase enzyme involved in the Arabidopsis epicuticular wax and pollen trypine alkane biosynthesis.

The aldehyde decarbonylase from pea is thought to be present in the extracellular or cuticular matrix of the epidermis (Cheesbrough and Kolattukudy, 1984). This is also the case for certain nonspecific lipid transfer proteins suggested to be involved in cuticle formation (Sterk et al., 1991; Thoma et al., 1993; Pyee et al., 1994). In the absence of a signal sequence, the CER1 protein may be transported post-translationally to the cell membrane, where the putative membrane-spanning helices allow it to be anchored, with the histidine motifs probably located on the intracellular side. Wax biosynthesis at the site of wax deposition outside the cell can explain the diffusion in wax structures from the surface of stem epidermal cells with a functional CER1 gene to cells without the gene, as we observed in a wax layer–variegated Arabidopsis plant (Figure 2C).

**CER1 Protein Is Needed for Pollen Fertility**

In addition to stem wax alkane synthesis, the CER1 gene has an essential function in pollen development. cer1 mutants have a stem wax and male sterility phenotype very similar to that of cer3 and cer6 mutants. As in cer1 mutants, both the stem and leaf wax alkane content of cer3 and cer6 mutants is low (Lemieux et al., 1994; Jenks et al., 1995). For cer6-2 mutants, the pollen lipid content has been examined biochemically and was found to be equally low in alkanes and wax components derived thereof (Preuss et al., 1993). The similarity in phenotype and the concomitant absence of stem alkane in cer1 and cer6 mutants lead us to conclude that the CER1 protein performs a similar function in pollen trypine synthesis and in stem wax synthesis. The low pollen alkane content of the cer6-2 mutants was accompanied by the virtual absence of a trypine layer deposited on the mature pollen grain (Preuss et al., 1993). Conditional male sterility of cer1::IdSpm89 and cer1-1 mutants was not linked to a reduction of the trypine deposition on the pollen grains. Although the lipid in the trypine of both cer1 mutants was more dispersed than in trypine of the wild-type pollen grains, the total amount of (visible) lipids in the trypine
was similar for all three genotypes. As a whole, the entire tryphine consistently showed a more granular appearance in the two cer7 mutants. This might have to do with a further state of tapetal cytoplasm degeneration before tryphine deposition. Also, the capillary process of tryphine transfer to the exine layer of pollen grains might influence the final texture of the tapetal remnants. As with both cer1 mutants, the conditionally male sterile cer6-1 mutant also does not show a reduction in tryphine deposition (Preuss et al., 1993; C.J. Keijzer, unpublished observations), in contrast with the cer6-2 mutant. It must be noted that a correct observation of this phenomenon requires a strictly timed fixation: shortly before anthesis, tryphine can be found only inside the tapetal membrane sacs, and as late as a few hours before anther dehiscence, it is transferred to the exines of the pollen grains. Analyzing still closed anthers (sometimes even found in open flowers) might lead to the incorrect conclusion that pollen grains of a given plant lack tryphine.

Based on the electron microscopic analyses, pollen sterility in cer7 mutants is clearly not a mechanistic problem associated with lack of tryphine as it might be in cer6-2 mutants. Tryphine deposition is a passive process following degradation of tapetal cells. The differences in tryphine composition and ultrastructure must therefore be a direct consequence of differences in tapetum between the wild type and cer1 or cer6 mutants. Lack of tapetal CER1 or CER6 activity results in a different constitution of tapetal remains, and depending on the severity of tapetal alterations caused by cer1 or cer6 mutations, the tryphine composition and ultrastructure show less or more visible differences with wild-type tryphine. Our observations with cer7 mutants emphasize the hypothesis proposed by Preuss et al. (1993) that long chain lipid molecules, in particular alkanes, are needed in the tryphine layer for proper pollen-pistil signaling. These lipids are produced in the tapetum, and because they are probably not needed for proper tapetal functioning, their absence will not affect tapetal appearance but is visible only as an altered tryphine ultrastructure in the cer1 and cer6 mutants. A secondary effect of the altered tryphine, which may be stress induced, is the accumulation of callose in stigma papillae in response to cer1 and cer6 pollen grains. No callose accumulation was observed when pollinating wild-type or cer7 pistils with nonspecific oilseed rape or petunia pollen, and the response thus is not a general mechanism to prevent pollination by other species.

We used a cell layer chimera to study expression of CER1 in flowers. One cer1::ildSpm89 plant with a fertile revertant inflorescence sector containing phenotypically normal stem wax was found. In plant development, the reproductive cells, including the tapetum, descend from the L2 layer (Goldberg et al., 1993), and the epidermis descends from the L1 layer. We therefore tested offspring from the sector, which were all cer1 mutants, showing that the cer1::ildSpm89 to CER1 reversion did not occur in the L2 layer but only in the L1 layer. Unexpectedly, CER1 expression in the L1 layer alone was enough to restore a fertile phenotype, inferring that there is some mechanism for transporting the CER1 protein or its products to the tapetum or pollen wall.

### The CER1 Gene Is Conserved among Plant Species

Genes performing steps in such a general plant biosynthetic pathway as wax synthesis are expected to be found in many plant species. This is indeed the case for the CER1 gene; we found homologs from a related Brassicaceae species, from the unrelated Compositae S. odoratus, and from the even more distantly related monocot species rice and maize. By comparing the derived amino acid sequences, it is clear that all members of the CER1 gene family we have found encode equally divergent homologous proteins. The level of identity among these proteins (between 30 to 70%) is in the same range as the homology found among fatty acid desaturases from different species, irrespective of the position of the double bond they introduce (Iba et al., 1993; Okuley et al., 1994; Sakamoto et al., 1994).

Based on the level of identity, the CER1 homologous proteins are presumably all performing a similar function. At least one additional CER1 family member is present in Arabidopsis. The CER1-like gene, as we termed it, is physically (and genetically) linked to CER1, with a physical distance of only 1 kb. This CER1 gene cluster most likely originated from an ancient gene duplication. Since then, both genes have evolved to such an extent that the ATTS1001 partial cDNA clone derived from the CER1-like gene is not recognized by the CER1 cDNA probe upon DNA gel blot hybridization. Also, their expression patterns have changed. The CER1-like gene is transcribed only in flowers and is therefore not involved in stem wax biosynthesis. A function of the CER1-like gene in epicuticular wax biosynthesis on flower petals or sepals is unlikely, given the visual absence of wax structures on flower organs; this contrasts with the high expression of the CER1-like gene. By analogy to the range of fatty acid desaturases that use different unsaturated fatty acids as substrate, the CER1-like protein may have a function similar to that of the CER1 protein in pollen development, for instance, in the production of alkenes in tryphine. Alternatively, it may be expressed in another part of the flower. The lipid transfer protein LTP1 is known to be expressed in stigma papillae, where it can function in the secretion of stigma lipids (Thoma et al., 1994), and the CER1-like protein may be involved in the production of these lipids.

### Conclusions

The isolation of CER1 is a first step in understanding the biosynthesis of epicuticular wax components. The CER1 protein is part of an integral cell membrane enzyme that we deduce to be an aldehyde decarbonylase. The presence of histidine-rich motifs HX24H or HX23HH, described as iron binding sites in fatty acid desaturases, may help to identify domains of the protein that interact with cofactors, which will provide additional knowledge of the biochemical properties of decarbonylases. The identification of CER1 homologs allows their testing in the manipulation of wax alkane contents in other species. This can...
be important for influencing drought resistance or insect re-

sistance. For instance, in rice, drought tolerance is associated

with high wax lines, especially rich in C29, C33, and C35

alkanes (O'Toole and Cruz, 1983; Haque et al., 1992), and a

higher ratio of long chain to medium long chain aldehydes or

alkanes promotes resistance to brown planthoppers (Woodhead

and Padgham, 1988). The CER7 gene is the first gene isolated

that is responsible for pollen–pistil interaction in the self-

compatible species Arabidopsis, and with it, the role of lipids

in pollen–pistil signaling can be analyzed further. Meanwhile,

the isolation of other wax biosynthesis genes by transposon
tagging in Arabidopsis continues, providing more tools to study

and modify the wax biosynthesis pathway.

METHODS

Transposon Tagging Lines and eceriferum Mutants

All experiments were performed with the Landsberg erecta ecotype
of Arabidopsis thaliana, which was also the genetic background
of the chemically or physically induced eceriferum (cer) mutants tested
for phenotypic complementation (all mutants were provided by M.
Koornneef, Wageningen Agricultural University). For screening, 25
transposon tagging lines of 12 plants, each with Enhancer/Suppressor-
mutator transposases genes and nonautonomous inhibitor-defective
Suppressor-mutator elements that constitute the EnlSpm-lldSpm sys-
tem, were grown individually in the greenhouse and examined for cer
mutations. All lines were obtained after two generations of self-
pollination, starting with one plant containing the TEN2 Enhancer trans-
posase T-DNA locus along with several transposed lldSpm elements
(Aarts et al., 1995). The original cer1:lldSpm89 transposon–tagged
mutant was found in line H12.1.6.2, containing ~15 different lldSpm
elements and homozygous for the TEN2 T-DNA. TEN5 is another
Enhancer transposase homolog and lacking cer1 T-DNA. Ten5 was screened
for cer1 allele and no other lldSpm elements. This line was crossed to a
cer1::lldSpm99 plant, and cer1 F2 plants were screened for excision
sectors. All plants grown for progeny were kept in Aracon containers
(BetaTech, Gent, Belgium) to prevent cross-pollination. Fertility of cer
mutants was conditioned by keeping the plants enclosed in a plastic
bag to increase relative humidity (Koornneef et al., 1989).

Identification of a cer1-m Cosegregating lldSpm99 Element and
Isolation of Flanking Genomic DNAs

The original cer1-m mutant was backcrossed to Landsberg erecta wild

type for two generations. Genomic DNA was isolated from second back-
cross offspring plants and tested for the presence of lldSpm elements.
All plants were allowed to self, and their progeny were tested for segre-
gation of the cer1 phenotype to confirm linkage of an lldSpm element
with the cer1 phenotype in the second backcross offspring. Genomic DNA
from plants containing the cer1-linked lldSpm99 element and a
few other unlinked lldSpm elements was used to obtain DNA flanking
both sides of lldSpm99 after lldSpm-specific inverse PCR (IPCR;
Masson et al., 1991). Additional PCR amplification using primer T
(5'-GACACTCTTAGATCTTTCCTTGAGAGT-3'), setting both terminal in-
verted repeats of lldSpm, enabled the isolation of fragments with
minimal transposon DNA. Based on lldSpm99 flanking sequences,
Microscopy

Germination of cer1-m, cer1-1, and wild-type pollen was examined by bright-field and fluorescent microscopy after staining with aniline blue according to Preuss et al. (1993). Revertant excision sectors and mutant insertion sectors on 4- to 5-week-old stems were examined with a preparation microscope. For a more detailed observation, fresh 1-cm-long stem parts were excised and mounted on stubs; the excision wounds were closed with rapidly drying cyano-acrylate glue. Subsequently, they were transferred to a scanning electron microscope (model No. 5200; JOEL Ltd., Tokyo, Japan), partially dehydrated for 5 min in the vacuum of this microscope, and finally photographed at 15 kV. Closing the wounds prevented excessive water loss from the specimens, which would have disturbed the vacuum of the microscope; the (gentle) dehydration step was introduced to better expose the epidermis cell boundaries.

For scanning and transmission electron microscopy of tryphine on pollen grains, freshly opened anthers were fixed in 3% glutaraldehyde in 0.7 M cacodylate buffer for 15 min, rinsed in the buffer several times, postfixed in 1% osmium tetroxide in the same buffer for 15 min, and stepwise dehydrated into ethanol 100%; all treatments were performed at room temperature. For scanning electron microscopy, specimens were critical point dried via carbon dioxide, mounted on stubs, coated with platinum, and observed in a JEOL 6300 field-emission scanning electron microscope at 80 kV. For transmission electron microscopy, the 100% ethanol-saturated specimens were stepwise infiltrated with resin (Spurr, 1969), polymerized, ultrathin sectioned, stained with lead citrate and uranyl acetate, and observed in a JEOL 1200 transmission electron microscope at 5 kV. For transmission electron microscopy of tryphine on pollen grains, fresh anthers were fixed in 3% glutaraldehyde, rinsed in buffer, postfixed in 1% osmium tetroxide, and dehydrated stepwise into ethanol 100% and then with 100% ethanol in hexane. For transmission electron microscopy of tryphine on pollen grains from revertant excision sectors and mutant insertion sectors on 4- to 5-week-old stems, specimens were fixed with 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer, then postfixed in 1% osmium tetroxide, and stepwise dehydrated into ethanol 100%; all treatments were performed at room temperature. For transmission electron microscopy, specimens were critical point dried via carbon dioxide, mounted on stubs, coated with platinum, and observed in a JEOL 6300 field-emission scanning electron microscope at 80 kV. For transmission electron microscopy of tryphine on pollen grains from revertant excision sectors and mutant insertion sectors on 4- to 5-week-old stems, specimens were critical point dried via carbon dioxide, mounted on stubs, coated with platinum, and observed in a JEOL 6300 field-emission scanning electron microscope at 80 kV.

ACKNOWLEDGMENTS

We thank Maarten Koornneef for sharing his experience with cer mutants and for critically reading the manuscript. We thank Christiane Leonardis-Schippers for helpful discussions, Jan-Peter Nap for reading the manuscript critically, and Gerrit Stunnenberg and Gerard Scholten for taking care of the plants in the greenhouse. We also thank the members of our European Scientists Sequencing Arabidopsis (ESSA) DNA sequencing team for their support in DNA sequencing and Bateau Ystrada for his advice on the analysis of pollen germination. Our colleagues at the DLO-Centrum voor Fotografie en Beeldverwerking are gratefully acknowledged for making the photographs of cer1 and CER1 sectors and for their help in preparing the other figures. This work was supported by the European Union BRIDGE/BIOTECH project (Grant No. BIOT-CT90-0207) on Development and Use of Arabidopsis thaliana as a Tool for Isolating Genes of Agronomic Importance.

Received July 12, 1995; accepted October 4, 1995.

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*Plant Cell* 1995;7:2115-2127

DOI 10.1105/tpc.7.12.2115

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