The Plant Cell, Vol. 12, 2001–2008, October 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

Alterations in *CER6*, a Gene Identical to *CUT1*, Differentially Affect Long-Chain Lipid Content on the Surface of Pollen and Stems

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Very long chain lipids contribute to the hydrophobic cuticle on the surface of all land plants and are an essential component of the extracellular pollen coat in the Brassicaceae. Mutations in Arabidopsis *CER* genes eliminate very long chain lipids from the cuticle surface and, in some cases, from the pollen coat. In Arabidopsis, the loss of pollen coat lipids can disrupt interactions with the stigma, inhibiting pollen hydration and causing sterility. We have positionally cloned *CER6* and demonstrate that a wild-type copy complements the *cer6-2* defect. In addition, we have identified a fertile, intragenic suppressor, *cer6-2R*, that partially restores pollen coat lipids but does not rescue the stem wax defect, suggesting an intriguing difference in the requirements for CER6 activity on stems and the pollen coat. Importantly, analysis of this suppressor demonstrates that low amounts of very long chain lipids are sufficient for pollen hydration and germination. The predicted CER6 amino acid sequence resembles that of fatty acid–condensing enzymes, consistent with its role in the production of epicuticular and pollen coat lipids >28 carbons long. DNA sequence analysis revealed the nature of the *cer6-1*, *cer6-2*, and *cer6-2R* mutations, and segregation analysis showed that *CER6* is identical to *CUT1*, a cDNA previously mapped to a different chromosome arm. Instead, we have determined that a new gene, *CER60*, with a high degree of nucleotide and amino acid similarity to *CER6*, resides at the original *CUT1* locus.

INTRODUCTION

The Arabidopsis extracellular pollen coat is essential for pollen recognition by the stigma surface. *cer6-2* mutations inhibit pollen hydration by depleting pollen coat lipids and proteins (Preuss et al., 1993), both of which are functionally important (Wolters-Arts et al., 1998; Mayfield and Preuss, 2000). *cer6* pollen is viable, and the sterile phenotype is reversed at high humidity, where atmospheric water bypasses the need for hydration by the stigma. In addition, *cer6* mutations act specifically at pollen hydration without disrupting pollen adhesion (Zinkl et al., 1999). Here, we report positional cloning of *CER6* and analysis of an intragenic suppressor of *cer6-2*, elucidating the role of *CER6* in pollen coat composition and maintenance.

The Arabidopsis *CER* genes form a large family, originally identified by the glossy, bright-green appearance of *cer* mutant stems (Koornneef et al., 1989; McNevin et al., 1993). CER proteins, and their homologs in species such as maize and barley, play a role in forming a hydrophobic waxy cuticle that is critical for reducing water loss, averting pathogen attack, and minimizing damage by UV irradiation (reviewed in Post-Beittenmiller, 1996). *CER* genes also contribute to the synthesis of the lipid-rich pollen coat—several Arabidopsis *cer* mutants (*cer1*, *cer2, cer3*, and *cer6*) exhibit defective pollen recognition, failed pollen hydration, and consequently, male sterility (Preuss et al., 1993; Hulskamp et al., 1995). The lipids in both the pollen coat and cuticle consist of a mixture of very long chain fatty acids (VLCFAs) and their derivatives (Post-Beittenmiller, 1996). VLCFAs are synthesized by the repeated transfer of two-carbon units from malonyl-acyl carrier protein to C_{16} and C_{18} fatty acid precursors. After reaching an appropriate length, VLCFAs are modified to generate alkanes, esters, primary and secondary alcohols, aldehydes, and ketones.

All classes of lipids are detected in *cer6* mutants, indicating that this gene is not required for forming fatty acid derivatives. Instead, CER6 is probably involved in lipid elongation, because the quantities of all derivatives >28 carbons long are substantially decreased in *cer6* pollen, stems, and leaves (McNevin et al., 1993; Preuss et al., 1993; Jenks et al., 1995). Elongation of fatty acids requires four enzymatic steps: condensation, reduction, dehydration, and a second reduction (reviewed in von Wettstein-Knowles, 1982). The first step, condensation, is thought to be rate-limiting (Millar and Kunst, 1997). Recently, Millar et al. (1999) identified

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CUT1, an Arabidopsis cDNA encoding a putative condensing enzyme. Plants overexpressing *CUT1* have a sense-suppressed phenotype strikingly similar to *cer6*: they lack stem epicuticular waxes, have substantial decreases in lipids >28 carbons long, and exhibit conditional sterility. However, because mapping *CUT1* showed it was not linked to *CER6*, the two were considered to be distinct (Millar et al., 1999).

As described below, we positionally cloned *CER6*, sequenced the *cer6-1* (Koornneef et al., 1989) and *cer6-2* alleles (Preuss et al., 1993), identified and sequenced an intragenic suppressor of *cer6-2*, placed the *CER6* gene on several high-resolution genetic maps, and demonstrated that the cloned gene complements *cer6* mutant phenotypes. Unexpectedly, we found that *CER6* can exhibit differential activity in pollen and stems; fertility, but not stem wax, was completely restored in the suppressor, as well as in some complemented mutants. Furthermore, we showed that *CUT1* and *CER6* correspond to the same locus and that the mapping data described by Millar et al. (1999) point to a novel *CER6*-like gene, *CER60.*

RESULTS

Mapping *CER6*

We positionally cloned the *CER6* gene by analyzing the segregation of polymerase chain reaction (PCR)–based markers in F_2 plants derived from a cross of a Landsberg *cer6-2* homozygote to wild-type Columbia (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). After analyzing 490 recombinant chromosomes, we mapped *cer6-2* between the molecular markers mi185 and AP1 (see www.Arabidopsis.org/maps.html), consistent with its previously described (Koornneef, 1994) position on chromosome 1 at ~107 centimorgans (cM) (see Figure 1). We refined the location of *CER6* by generating additional PCR-based markers, narrowing the region to 45 kb contained entirely within the sequenced bacterial artificial chromosome (BAC) T26J14 (GenBank accession number AC011915; Figure 1).

Annotation of the 45-kb region revealed seven predicted genes (GenBank accession number AC011915), four of which are associated with expressed sequence tags (ESTs) (Figure 1). Moreover, this region included a gene, T26J14.10, for which the predicted exon sequence was entirely identical to that of the *CUT1* cDNA, which had been previously mapped to 38 cM on chromosome 1, near the marker ZFPG (Millar et al., 1999). Sense-suppressed *CUT1* plants display phenotypes similar to *cer6* mutants, and as described below, we found that *CUT1* actually maps to the *CER6* region. Consistent with the Arabidopsis gene nomenclature standards (Meinke and Koornneef, 1997), we hereafter refer to *CUT1* as *CER6*.

We used PCR to amplify the *CER6* sequence, identified a polymorphism within the gene (AF-1), and verified that this polymorphism cosegregated with the *cer6-2* mutant pheno-

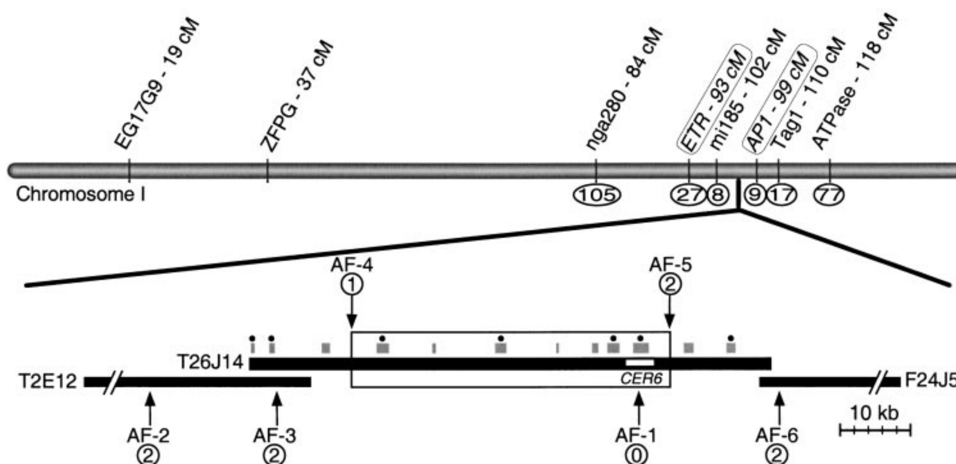

Figure 1. Genetic and Physical Mapping of *CER6* on Chromosome 1 and T26J14.

cer6-2 was positionally mapped between mi185 and AP1 on chromosome 1 by scoring PCR-based markers; the indicated positions in centimorgans correspond to the map generated from recombinant inbred lines (http://nasc.nott.ac.uk/new_ri_map.html). New PCR-based markers were generated at *ETR* and *AP1*; the centimorgan positions correspond to the phenotypic marker map (Koornneef, 1994) and within the BAC clones T2E12, T26J14, and F24J5 (AF-1 through AF-6). Circled numbers indicate the number of recombination events detected between each marker and *CER6*. The 45-kb region between AF-4 and AF-5 (open box) defines the boundaries of the region containing *CER6*; annotated genes (gray boxes), some of which correspond to ESTs (filled circles), are indicated. The white line within the T26J14 BAC indicates the fragment used for complementation experiments.

type: every homozygous mutant plant from the *cer6-2* mapping population contained Landsberg DNA at this locus (Figure 1). In addition, we used tetrad analysis to monitor the segregation of AF-1 in 32 plants derived from eight pollen tetrads (Copenhaver et al., 1998). Because analysis of the segregation of >100 markers in these tetrads has revealed the locations of all the crossover sites, any new marker can be placed precisely on the genetic map. Using this method, we placed *CER6* on chromosome 1 between ETR (94 cM) and TAG-1 (110 cM) and excluded the possibility that the gene lies near ZFPG. Finally, by scoring AF-1 segregation on the same recombinant inbred population used by Millar et al. (1999), we placed *CER6* uniquely on chromosome 1 at 107 cM, with an LOD score of 3.0 (http://nasc.nott.ac.uk/RI_data/html/chrom1.html).

The mapping data we obtained are consistent with the virtually complete genomic sequence generated by the Arabidopsis Genome Initiative. Only one gene identical to *CER6* has been found, consistent with the high-stringency DNA gel blot analysis preformed by Millar et al. (1999). In an attempt to account for the previous erroneous placement of *CUT1*, we investigated the genomic sequence near ZFPG (BAC clone F2J7; GenBank accession number AC079281) and found a novel gene that shares 79 and 85% nucleotide identity with *CER6* in exons 1 and 2, respectively, but showed no similarity in the upstream, downstream, or intron sequences (Figure 2A). The similarity in DNA sequence between the 38- and 107-cM regions on chromosome 1 could have confounded the previous mapping experiments with restriction fragment length polymorphisms. Because no previously described *cer* defect has been mapped to this region, we termed this gene *CER60.* BLAST searches of the Arabidopsis EST database revealed three high-quality matches for *CER60* and defined the splice junction (GenBank accession numbers AV527833, BE527614, and AI997914). At the protein level, CER6 and CER60 share 88% identity and 94% similarity (Figure 2B). Despite this high degree of conservation, however, the presence of wild-type *CER60* does not eliminate the phenotype of *cer6* mutants. Although ESTs from both genes are found in whole-plant libraries, their tissue specificities could differ. Furthermore, ∼10-fold more ESTs corresponding to *CER6* than to *CER60* are known, suggesting that the former gene is expressed at higher levels than the latter.

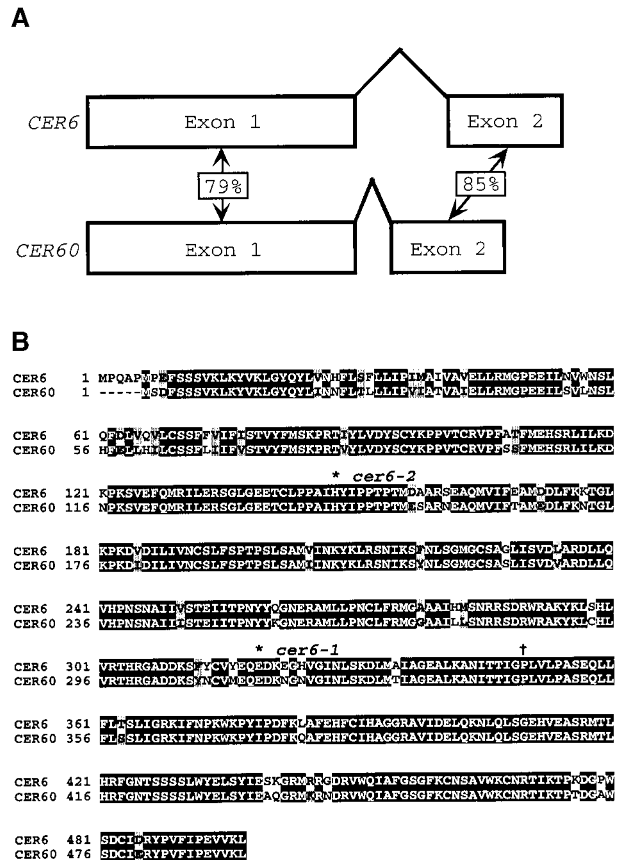

Figure 2. Sequence Analysis of *CER6* and *CER60*.

(A) Scale drawing of the *CER6* and *CER60* genes, indicating the percentage of DNA sequence identity for each exon.

(B) Sequence alignment of CER6 and CER60, generated by using ClustalW 1.8 (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and Boxplot (http://www.ch.embnet.org/software/BOX_form.html). (*), residues altered by the mutations; black shading, identical residues; gray shading, similar residues; (†), position of the exon1/exon2 splice junction. Dashes were introduced to optimize alignment.

Analysis and Complementation of *cer6* Mutations

We sequenced the *cer6-1* and *cer6-2* alleles and identified the corresponding lesions. Because both were Landsberg-derived, we sequenced the wild-type Landsberg *CER6* gene and showed that it differed by only two silent substitutions from Columbia: F361 (TTC to TTT) and K467 (AAA to AAG). *cer6-1* is a three-base deletion eliminating the E319 codon; *cer6-2* is an A-to-C transversion that changes H148 to proline (Figure 2B). The *cer6-1* deletion lies in a region conserved among other Arabidopsis VLCFA-condensing enzymes (Millar et al., 1999); the H148 altered by *cer6-2* is not conserved, but the introduction of a proline residue may alter the protein structure. Interestingly, of the two alleles, *cer6-2* has the stronger phenotype, in terms of both stem epicuticular lipids and pollen coat morphology (Preuss et al., 1993).

We introduced a genomic fragment containing T26J14.10 into *cer6-2* mutants (Figure 1), identified the transformed plants carrying the gene, and analyzed fertility and stem wax phenotypes (Table 1). All 13 transformants containing *CER6* produced at least 30-fold more seed than did the *cer6-2* mutants, and most exhibited deposits of epicuticular stem lipids. Transformants containing only the T-DNA vector were

identical to the *cer6-2* parent. These results confirmed that T26J14.10 complements *cer6-2*; differences in the extent of complementation may reflect effects of position on gene expression or differences in insertion number.

Differential Requirements for *CER6* in Transgenic Plants and Suppressors

The *cer6* mutations, as with many other *cer* defects, reduce the abundance of long-chain lipids both in the pollen coat and on the stem surface (Preuss et al., 1993). Interestingly, both defects were not rescued equally by introducing a wild-type *CER6* allele into *cer6-2* mutants: some transformed plants with restored fertility still exhibited a strong defect in stem wax, whereas the opposite result, sterile transformants with abundant stem lipids, was not observed (Table 1). This unexpected finding suggests that *CER*6 activity may be greater in reproductive tissues than in stems or that restoring fertility requires less lipids than are required to restore stem wax structures.

Table 1. Complementation of Fertility and Stem Cuticle Phenotype of *cer6-2* Mutants with *CER6*

Line[a]	Stem Epicuticular Wax[b]	Seeds (*n*)[c]	Kan Resistance[b,d]	WT *CER6*[b,e]
L*er*	+++	27.2 ± 2.9 (23)	NA	+
cer6-2	−	0.3 ± 0.6 (14)	NA	−
6-2 FJ	+++	29.5 ± 2.0 (12)	+	+
6-2 FG	+++	27.4 ± 4.7 (15)	+	+
6-2 FI	+++	24.4 ± 6.0 (18)	+	+
6-2 R1	+++	22.2 ± 2.9 (15)	+	+
6-2 FF	+++	20.8 ± 2.7 (18)	+	+
6-2 RA	++	28.3 ± 3.3 (19)	+	+
6-2 F4	++	21.6 ± 4.1 (19)	+	+
6-2 FK	+	21.8 ± 5.1 (8)	+	+
6-2 F5	+	11.5 ± 3.0 (13)	+	+
6-2 FH	−/+	26.1 ± 3.3 (19)	+	+
6-2 F3	−/+	23.1 ± 3.1 (16)	+	+
6-2 RB	−	26.3 ± 2.2 (16)	+	+
6-2 FD	−	15.9 ± 3.3 (14)	+	+
6-2 FA	−	0.4 ± 1.0 (10)	+	−
6-2 FB	−	0.4 ± 0.7 (10)	+	−

[a] Lines 6-2 FJ through 6-2 FD denote *cer6-2* lines transgenic for a wild-type copy of *CER6*, ranked by their stem cuticle phenotypes. Lines 6-2 FA and 6-2 FB contain vector sequences but lack *CER6*. L*er*, Landsberg *erecta*.
[b] (+++), wild-type wax levels; (++), (+), (−/+), intermediate wax; (−), no wax.
[c] Mature seeds per half seed pod and standard deviation.
[d] The presence of the kanamycin resistance gene was determined by PCR. NA, not applicable.
[e] The presence of wild-type (WT) *CER6* was assessed using two dCAPS markers.

Additional support for this differential activity was obtained from the analysis of six intragenic *cer6-2* suppressors that were fertile but lacked abundant epicuticular waxes. All six suppressors were dominant: crosses to *cer6-2* mutants yielded fertile progeny with glossy stems. Crosses to the wild type confirmed that all six suppressors were tightly linked to *cer6-2*: one-quarter of the F_2 plants had glossy stems, and of those, 782 of 800 were fertile (as opposed to the 600 of 800 expected if the suppressor were not linked). The few sterile glossy plants observed in the F_2 population reflected unrelated sterile mutations detected in the mutagenized background. Although the suppressors were isolated as independent M_1 mutants, DNA sequencing surprisingly revealed the same reversion event in all six lines. This suppressing mutation, which alters the *cer6-2* lesion, is designated *cer6-2R*. As described above, *cer6-2* changes codon 148 from CAT (His) to CCT (Pro); the suppressing mutation is a C-to-T transition that generates a TCT (Ser) codon at position 148.

Ultrastructural and chemical analyses of the suppressors confirmed that partial restoration of CER6 was sufficient for a functional pollen coat but not for the development of a normal epicuticular wax layer. Transmission electron microscopy of *cer6-2* pollen revealed an unevenly distributed pollen coat with few lipid droplets (Preuss et al., 1993), whereas both wild-type and *cer6-2R* pollen grains were completely surrounded by an abundant pollen coat containing many lipid droplets (Figure 3). Furthermore, as shown in Figure 4, gas chromatography/mass spectrometry analysis of the pollen coat lipids demonstrated that the C_{29} and C_{30} lipids were partially restored in *cer6-2R* but not to wild-type quantities. Although no epicuticular wax was apparent on the stems of *cer6-2R* plants, scanning electron microscopy revealed a slight restoration of wax crystals relative to *cer6-2* stems (Figure 5). Thus, the suppressing mutation that alters the *cer6-2* P148 codon is unable to restore enough CER6 activity to generate a normal stem cuticle, but it is able to generate a morphologically and functionally normal pollen coat, even with C_{29} and C_{30} lipids in less than wild-type amounts. Additionally, because *cer6-2*/*cer6-2R* heterozygous plants are also fertile, we conclude that one copy of CER6-2R generates sufficient lipid for a functional pollen coat.

DISCUSSION

Here, we describe the cloning of *CER6*, a lipid biosynthesis gene critical for Arabidopsis pollination, the identification of the *cer6-1* and *cer6-2* lesions, and the characterization of an intragenic suppressor of *cer6-2* sterility. *CER6* is identical to *CUT1*, a cDNA conferring a *cer6*-like phenotype through sense suppression (Millar et al., 1999). Using a molecular marker and three independent mapping populations, we showed that *CER6* maps to chromosome 1 at 107 cM. Fur-

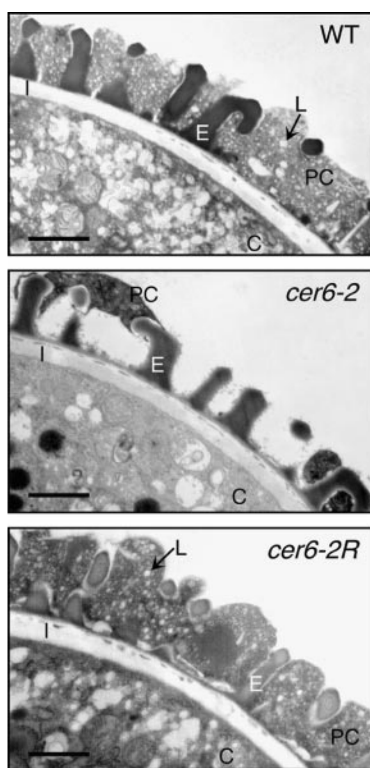

Figure 3. Morphology of the *cer6-2R* Pollen Coat.

Transmission electron microscopy of wild-type (WT), *cer6-2*, and *cer6-2R* pollen grains, showing the resemblance between *cer6-2R* pollen grains and the wild type. C, cytoplasm; E, exine; I, intine; L, lipid droplet; PC, pollen coat. Bars = 1 μm.

thermore, because *CUT1* was previously mapped to 38 cM on chromosome 1, we analyzed the DNA sequence of the surrounding region and identified a related gene, *CER60*. This gene is the likely source of error in the previous attempt to map a *CUT1* restriction fragment length polymorphism.

The stem cuticle and pollen coat of *cer6-2* mutants are deficient in all classes of very long chain lipids (Preuss et al., 1993), and the predicted CER6 amino acid sequence is highly similar to that of VLCFA-condensing enzymes and to other condensing enzymes such as chalcone synthase (Millar et al., 1999). Sense-suppressed mutants overexpressing the *CER6* message can display even stronger lipid synthesis defects (Millar et al., 1999), raising the possibility that such mutants are deficient in messages from both *CER6* and the closely related gene, *CER60.* Although *CER60* probably encodes another VLCFA-condensing enzyme, understanding the role of this gene in plant development will require analysis of mutants that are defective only in *CER60*.

In Arabidopsis, a family of at least 22 *CER* genes controls the formation of cuticular lipids (Koornneef et al., 1989; McNevin et al., 1993). To date, the genes corresponding to four *cer* mutants have been cloned from Arabidopsis: *CER1* (Aarts et al., 1995), *CER2* (Negruk et al., 1996; Xia et al., 1996), *CER3* (Hannoufa et al., 1996), and *CER6* reported here. *cer1* mutants accumulate C_{30} aldehyde and are deficient in C_{29} alkane, secondary alcohols, and ketones (Hannoufa et al., 1993); the *CER1* gene shares several characteristics with decarbonylases, indicating a role in the conversion of aldehydes to alkanes. Like *cer6* mutants, *cer2* plants accumulate shorter lipids and lack longer ones (Hannoufa et al., 1993; Jenks et al., 1995); CER2 shares sequence similarity with GL2, a maize protein having an unknown enzymatic role required for lipid biosynthesis. Neither the sequence of *CER3* nor the phenotype of the *cer3* mutant suggests a role for this putative nuclear protein in lipid biosynthesis.

In addition to cloning *CER6,* we identified an intragenic suppressor, *cer6-2R*, that only partially restores pollen coat lipids but fully restores fertility. These data indicate that pollen hydration and germination can proceed when the coat contains only a small amount of very long chain lipids. Moreover, the epicuticular wax defects seen with the *cer6-2R*

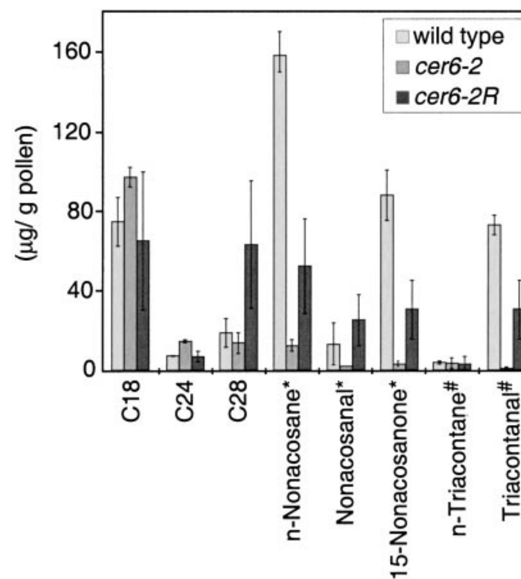

Figure 4. Lipid Contents in the Pollen Coats of Wild-Type, *cer6-2*, and *cer6-2R* Pollen.

Chloroform-extracted lipids were analyzed by gas chromatography/mass spectrometry. Lipids constituting >1% of wild-type pollen coat lipids are reported. Quantities were normalized to an internal control. All classes of lipids for a given carbon chain length—18, 24, or 28—were pooled for that length. Error bars indicate standard deviation; for the wild type (derived from Landsberg and Columbia), $n = 2$; for *cer6-2*, $n = 2$; and for *cer6-2R*, $n = 8$. (*), C_{29} lipids; (#), C_{30} lipids.

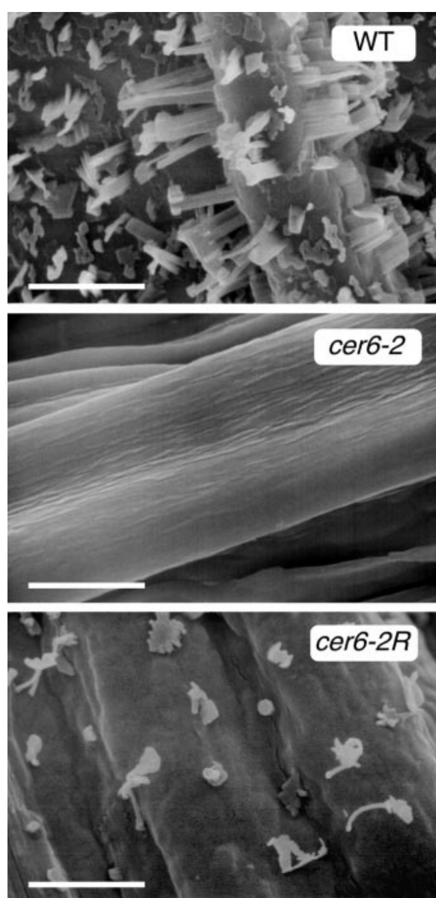

Figure 5. Presence of Lipid Crystals on *cer6-2R* Stems.

Scanning electron microscopy of wild-type (WT), *cer6-2*, and *cer6-2R* stems shows fewer crystals on *cer6-2R* stems than on those of the wild type. Bars = 5 μm.

suppressor, and in some *cer6-2* mutants transformed with *CER6*, apparently indicate different requirements for very long chain lipids in the assembly of the pollen coat and in the stem cuticle. Possibly, *cer6-2R* is the only mutation that can fully restore *cer6-2* fertility without producing normal amounts of stem lipids. We independently isolated *cer6-2R* six times and found no mutations that upregulate *cer6-2*, alter *CER60*, or modify any other Arabidopsis lipid-condensing enzyme. Consequently, we conclude that the proline residue at position 148 in CER6 is detrimental to its overall structure and function. Because the abundance of very long chain lipids in *cer6-2R* plants is much less than in the wild type, this suppressor provides an important opportunity to dissect the requirements for lipids in the assembly of the pollen coating, interaction with pollen coat proteins, and migration of the coating onto the stigma surface. Moreover, this allele will be of use in elucidating the role of different classes and quantities of long-chain lipids in the formation of epicuticular wax structures.

METHODS

Arabidopsis thaliana Strains

cer6-1 was generated by fast neutron mutagenesis (Koornneef et al., 1989); *cer6-2* was generated with ethyl nitrosourea (Preuss et al., 1993). Both lines are available from the Arabidopsis Biological Resource Center (Ohio State University; http://aims.cps.msu.edu/aims). Recombinant inbred lines were also provided by the Arabidopsis Biological Resource Center (Lister and Dean, 1993). *cer6-2R* was generated by mutagenizing *cer6-2* seeds with 1% ethyl methanesulfonate; fertile M_1 plants were identified as described previously (Ohad et al., 1996).

Molecular Markers and DNA Sequencing

All polymerase chain reaction (PCR)–based genetic markers used in this study are available from the Arabidopsis Information Resource (http://www.arabidopsis.org/aboutcaps.html). The identity of the recombinant inbred lines was verified by scoring the markers AthATPase and ZFPG (chromosome 1, 118 and 37 cM, respectively), nga361 (chromosome 2, 63 cM), and ca72 (chromosome 5, 30 cM). Primers for the AF-1 marker, which corresponds to *CER6*, were AF-1F (5′-GGCTAGAAGCGAGGCTCAGATGG) and AF-1R (5′-GCCAGCTTGAAATCCGGTATGTATG). Digestion of the AF-1 PCR product with MnlI revealed an additional cleavage site in Columbia DNA compared with Landsberg. Two dCAPS markers (Neff et al., 1998) were designed; because each uses the same forward primer (dCER6-F, 5′-TCGTGTCCCCTTCGCAACTTTCAT) with the reverse primer dCER6-2R (5′-GTCCATGGTTGGTGTGGGAGGAATCTAA) or dCER6wt-R (5′-GTCCATGGTTGGTGTGGGAGGAATATCA), detection of the *cer6-2* or wild-type *CER6* allele, respectively, is unambiguous. Digestion of the dCER6-F/dCER6-2R PCR product with DdeI cleaves the mutant allele, whereas digestion of the dCER6-F/dCER6wt-R PCR product with NlaIII cleaves the wild-type allele.

The 3.6-kb region that includes *CER6* was sequenced as six overlapping regions from wild-type Landsberg, *cer6-1*, *cer6-2*, and *cer6-2R* genomic DNA. Fragments were amplified with Pfu Turbo (Stratagene, La Jolla, CA), cloned into the pCR2.1 vector by using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA), and transformed into TOP10F′ cells (Invitrogen). At least two clones from independent PCR reactions were sequenced.

Mutant Analysis and Complementation

Pollen coat collection, lipid extraction, gas chromatographic/mass spectrometric lipid analysis (Mayfield and Preuss, 2000), and scanning and transmission electron microscopy (Preuss et al., 1993) were performed as previously described.

A 3988-bp fragment containing the entire *CER6* coding sequence, 1143 bp 5′, 650 bp 3′, and no other predicted genes, was generated

by EcoRV digestion of the bacterial artificial chromosome (BAC) T10H19. This fragment was introduced into the SmaI site of a *lacZ*-containing version of pBI-121 (Clonetech, Palo Alto, CA), generating pBI-*CER6*. These *CER6*-containing clones were electroporated into *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101, and Agrobacterium-carrying pBI-*CER6* were introduced into *cer6-2* plants by vacuum infiltration (Bechtold and Pelletier, 1998). To obtain seeds, infiltrated *cer6-2* plants were exposed to humid conditions as described previously (Preuss et al., 1993). Transformed seedlings were selected on agar plates containing 0.5 × Murashige and Skoog (1962) medium, 50 mg/mL kanamycin (Kan), and 25 mg/mL benomyl; Kan-resistant plants were transferred to soil after 4 weeks. The presence of the Kan resistance gene was confirmed by PCR; the presence of a wild-type *CER6* allele was monitored by using the dCAPS markers. The stem cuticle of the transformants was assessed visually, and fertility was assessed by counting the mature seeds per half seedpod.

ACKNOWLEDGMENTS

We thank Brooke Bevis, Natalie Cullinane, Sarah Kniss, and Annette Weglinski for assistance in the mapping of this gene; Bonnie Bartel for sharing markers; Chris Town and Samir Kaul for sharing DNA sequences; and members of the Preuss, Greenberg, and Malamy laboratories for helpful discussions. This work was supported by Grant No. MCB-9513511 from the National Science Foundation awarded to D.P., Grant No. 97-35304-4941 from the U.S. Department of Agriculture awarded to R.L.F., and a predoctoral fellowship from the National Science Foundation awarded to A.F. Pollen lipid analysis by the Michigan State University Mass Spectrometry Facility was supported by Grant No. DRR-00480 from the National Institutes of Health.

Received June 21, 2000; accepted August 21, 2000.

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