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CUT1, an Arabidopsis Gene Required for Cuticular Wax Biosynthesis and Pollen Fertility, Encodes a Very-Long-Chain Fatty Acid Condensing Enzyme

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Land plants secrete a layer of wax onto their aerial surfaces that is essential for survival in a terrestrial environment. This wax is composed of long-chain, aliphatic hydrocarbons derived from very-long-chain fatty acids (VLCFAs). Using the Arabidopsis expressed sequence tag database, we have identified a gene, designated *CUT1*, that encodes a VLCFA condensing enzyme required for cuticular wax production. Sense suppression of *CUT1* in transgenic Arabidopsis plants results in waxless (*eceriferum*) stems and siliques as well as conditional male sterility. Scanning electron microscopy revealed that this was a severe waxless phenotype, because stems of *CUT1*-suppressed plants were completely devoid of wax crystals. Furthermore, chemical analyses of waxless plants demonstrated that the stem wax load was reduced to 6 to 7% of wild-type levels. This value is lower than that reported for any of the known *eceriferum* mutants. The severe waxless phenotype resulted from the downregulation of both the decarbonylation and acyl reduction wax biosynthetic pathways. This result indicates that *CUT1* is involved in the production of VLCFA precursors used for the synthesis of all stem wax components in Arabidopsis. In *CUT1*-suppressed plants, the C24 chain-length wax components predominate, suggesting that CUT1 is required for elongation of C24 VLCFAs. The unique wax composition of *CUT1*-suppressed plants together with the fact that the location of *CUT1* on the genetic map did not coincide with any of the known *ECERIFERUM* loci suggest that we have identified a novel gene involved in wax biosynthesis. *CUT1* is currently the only known gene with a clearly established function in wax production.

INTRODUCTION

Waxes are major constituents of the cuticle, a hydrophobic barrier covering the aerial portions of land plants. They are embedded within the cuticular matrix (intracuticular waxes) and also form the outermost layer of the cuticle (epicuticular waxes). The chemical and physical properties of waxes determine functions vital for plant life, such as regulation of nonstomatal water loss and protection against UV radiation (Reicosky and Hanover, 1978). Waxes also help plants resist bacterial and fungal pathogens (Jenks et al., 1994) and play a role in plant–insect interactions (Eigenbrode and Espelie, 1995). In addition, waxes found in the tryphine layer of pollen grains are essential for proper pollen–stigma signaling required for fertilization (Preuss et al., 1993).

Cuticular waxes are complex mixtures of lipids, and their composition differs widely among plant species as well as among the organs and tissues of a single plant (Post-Beittenmiller, 1996). They are composed mainly of long-chain aliphatic hydrocarbons derived from saturated very-long-chain fatty acids (VLCFAs; chain length is $>$18 carbons). The chain length of these hydrocarbons is species dependent, but typically they are 26 to 34 carbons long.

VLCFAs, the precursors for wax biosynthesis, are formed by a microsomal fatty acid elongation (FAE) system. FAE involves sequential additions of C2 moieties from malonyl–coenzyme A (CoA) to preexisting C16 or C18 fatty acids derived from the de novo fatty acid synthesis (FAS) pathway of the plastid. By analogy to FAS, each cycle of FAE is accomplished by a series of four enzymatic reactions: (1) condensation of malonyl–CoA with a long-chain acyl–CoA; (2) reduction to β-hydroxyacyl–CoA; (3) dehydration to an enoyl–CoA; and (4) reduction of the enoyl–CoA, resulting in the elongated acyl–CoA (Fehling and Mukherjee, 1991). Together, these four activities are termed the elongase (von Wettstein-Knowles, 1982). VLCFAs in the epidermal cells are then converted through several biosynthetic pathways to all of the other wax components.

In Arabidopsis, there are two principal wax biosynthetic

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pathways, a decarbonylation pathway and an acyl reduction pathway (Figure 1). The decarbonylation pathway is initiated by the production of aldehydes from VLCFA precursors by a fatty acyl–CoA reductase, followed by decarbonylation by an aldehyde decarbonylase to yield odd-chained alkanes (Cheesbrough and Kolattukudy, 1984). Alkanes can then undergo sequential oxidations to form secondary alcohols and ketones. The acyl reduction pathway is also initiated by a reduction of VLCFAs to aldehydes, followed by a further reduction of aldehydes by an aldehyde reductase to produce even-chained primary alcohols (Kolattukudy, 1971). Recently, Vioque and Kolattukudy (1997) have shown that in pea, these two reduction steps are conducted by one reductase without the release of the aldehyde intermediate. Primary alcohols can then combine with free fatty acids to form wax esters (von Wettstein-Knowles, 1979). Cell fractionation studies have shown that many of the enzyme activities of wax biosynthesis are located in the microsomal fraction of the cell (Bognar et al., 1984; Bessoule et al., 1989; Vioque and Kolattukudy, 1997). However, very little else is known about the enzymes of these biosynthetic pathways, the regulation of wax production, and the secretion of wax components onto the surface of the plant.

Because epicuticular waxes influence light refraction, mutations in genes involved in wax accumulation can be detected visually. Consequently, mutants have now been isolated in a number of plant species, including barley, Arabidopsis, maize, and *Brassica napus*. The mutant loci in barley and Arabidopsis are termed *eceriferum* (*cer*), whereas loci identified in maize and *B. napus* are referred to as *glossy* (*gl*). Scanning electron microscopy has shown that the epicuticular wax crystals on many of these mutants are absent or exhibit particular morphological changes. Chemical analyses support scanning electron microscopic observations, demonstrating that mutants have lower wax loads than do wild-type plants and/or that they contain wax with a dramatically altered chemical composition. Barley is the most extensively studied species, with 85 identified *cer* loci (von Wettstein-Knowles, 1987). In Arabidopsis, there are currently 22 known *cer* loci (Koornneef et al., 1989; McNevin et al., 1993). However, only one allele has been identified for eight of the Arabidopsis loci, suggesting that not all of the genes involved in wax accumulation have been found.

A number of *CER* and *GL* genes have been cloned. For example, *CER1*, *CER2*, and *CER3* have been isolated from Arabidopsis (Aarts et al., 1995; Hannoufa et al., 1996; Negruk et al., 1996; Xia et al., 1996). Based on the wax composition of the *cer1* mutants (Hannoufa et al., 1993) and the histidine-rich motifs of the protein sequence (Aarts et al., 1995), *CER1* has been proposed to encode an aldehyde decarbonylase. However, other possible functions for CER1 have been suggested (Hansen et al., 1997). The *CER2* gene is likely to encode a biosynthetic enzyme, because it shares sequence similarity with acyl transferases (St-Pierre et al., 1998). Its precise function is not known. It was hypothesized to be involved in the elongation of C28 to C30 fatty acids

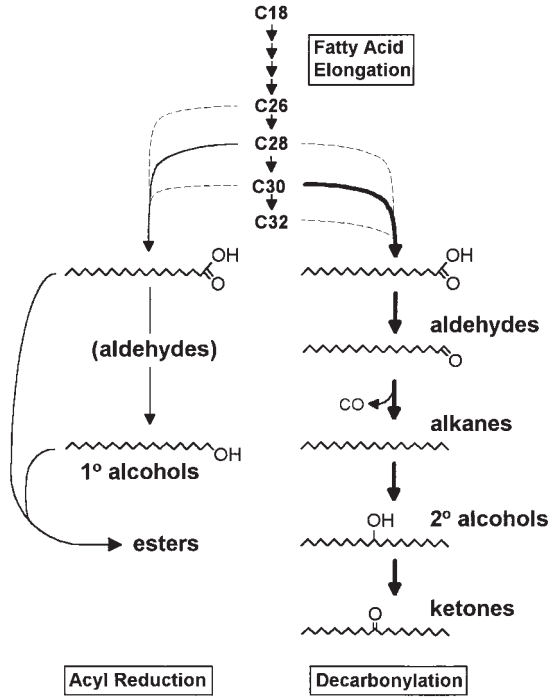

Figure 1. Proposed Metabolic Pathways for Wax Biosynthesis in Arabidopsis Stems.

VLCFAs in the epidermal cells are converted through two biosynthetic pathways to all of the other wax components. The decarbonylation pathway produces aldehydes, alkanes, secondary (2°) alcohols, and ketones, whereas the acyl reduction pathway yields primary (1°) alcohols and esters. Widths of the arrows indicate relative metabolic fluxes through different reactions.

(Hannoufa et al., 1993) but subsequently has been shown to be localized in the nucleus (Xia et al., 1997). The *CER3* gene product lacks significant sequence similarity with any known proteins. However, nuclear localization sequences have prompted the suggestion that it may have a regulatory function (Lemieux, 1996). Of the cloned *GL* genes from maize, *GL1* and *GL2* share sequence similarity with *CER1* and *CER2*, respectively (Tacke et al., 1995; Hansen et al., 1997). On the other hand, the *GL8* gene of maize may encode a reductase involved in fatty acid biosynthesis (Xu et al., 1997). However, similar to the cloned *CER* gene products from Arabidopsis, the exact biochemical function of all of the GL proteins remains to be determined.

Recently, an Arabidopsis gene involved in VLCFA biosynthesis, *FATTY ACID ELONGATION1* (*FAE1*), was isolated (James et al., 1995). It encodes a seed-specific condensing enzyme that catalyzes the first reaction of the microsomal FAE system necessary for the production of C20 and C22 VLCFAs of the seed storage lipids. Using *FAE1* in experiments with transgenic plants, we demonstrated that the levels of expression and specificity of the condensing enzyme

determine the amounts and chain lengths of VLCFAs produced by the microsomal FAE system, respectively (Millar and Kunst, 1997). Based on evidence that condensing enzymes are pivotal control points of VLCFA biosynthetic pathways and that the majority of wax constituents are derived from VLCFAs, we proposed that condensing enzymes would play key roles in the synthesis of waxes (Millar and Kunst, 1997). In this study, we confirm this prediction by identifying and characterizing *CUT1*, a gene encoding an epidermis-specific condensing enzyme involved in VLCFA production for cuticular wax biosynthesis.

RESULTS

Identification of a *FAE1*-like Gene Expressed in the Epidermal Cells of Arabidopsis Stems

A TBLASTN search for related sequences (Altschul et al., 1990) in the database of expressed sequence tags (ESTs) of anonymous Arabidopsis cDNA clones (Newman et al., 1994; Cooke et al., 1996), using the deduced amino acid sequence of FAE1, revealed that >15 ESTs had open reading frames with significant sequence similarity. These ESTs did not correspond to known condensing enzymes, such as chalcone synthase or 3-ketoacyl–acyl carrier protein synthase III (KASIII), which had lower similarity scores. Thus, a family of *FAE1*-related genes exists in Arabidopsis. These genes are likely to encode condensing enzymes, which may be involved in the synthesis of VLCFAs.

The expression patterns of several of these *FAE1*-related genes were examined by using RNA gel blot analyses. One gene, represented by EST T76616, was expressed in stems, leaves, and flowers, but it was not expressed in roots (data not shown). Furthermore, in situ hybridization analysis demonstrated that the transcript corresponding to this gene specifically accumulated in the epidermal cells of the Arabidopsis stem (Figure 2). These are the cells in which wax biosynthesis occurs, suggesting that this gene, *CUT1*, might be a good candidate for a condensing enzyme involved in wax production. DNA sequencing revealed that the *CUT1* cDNA from this T76616 clone was 1829 nucleotides long, which is a length similar to that of the *FAE1* transcript (James et al., 1995). The longest open reading frame encodes a 497–amino acid protein, assuming that the ATG furthermost upstream is the correct start codon. There is an in-frame stop codon, TAA, 15 nucleotides upstream of this ATG. If this assumption is correct, the *CUT1* cDNA has a 1494-bp coding region, which is preceded by 58 bp of 5′ untranslated region and followed by a 277-bp 3′ untranslated region, containing a 23-bp poly(A) tail. This cDNA sequence analysis, together with the fact that the FAE1 protein is 506 amino acids in length, suggests that we have identified a full-length cDNA. Alignment of the predicted CUT1 amino acid sequence with the FAE1 protein revealed that they share 50.0% amino acid identity and 74.7% amino acid similarity (Figure 3A), confirming that the protein encoded by *CUT1* is a putative condensing enzyme, possibly involved in VLCFA biosynthesis. DNA gel blot analysis demonstrated that *CUT1* is a single-copy gene (Figure 4), although there was a faintly hybridizing second band when the experiment was performed at low stringency (data not shown).

A 35S–*CUT1* Transgene Can Confer a Waxless Phenotype and Lead to Conditional Male Sterility

In an attempt to elucidate the function of *CUT1*, we generated transgenic Arabidopsis plants harboring the binary vector p35S–*CUT1*, in which the complete *CUT1* cDNA was subcloned in sense orientation behind the cauliflower mosaic virus 35S promoter. The expression of this transgene in Arabidopsis could result in either the constitutive expression of the CUT1 protein or the sense suppression of the expression of the endogenous *CUT1* gene, which occurs efficiently when transgenes are transcribed from a strong promoter (Elmayan and Vaucheret, 1996).

In our transformation experiment, we obtained 46 kanamycin-resistant Arabidopsis plants, transferred them to soil, and grew them to maturity. Instead of the typical waxy or glaucous appearance of wild-type inflorescence stems, 36 of the transgenic plants had stems with a shiny bright green appearance, indicating the absence of the epicuticular wax layer (Figure 5A). Scanning electron microscopy revealed that the surfaces of these stems completely lacked wax crystals (Figure 6A), an appearance that is characteristic of

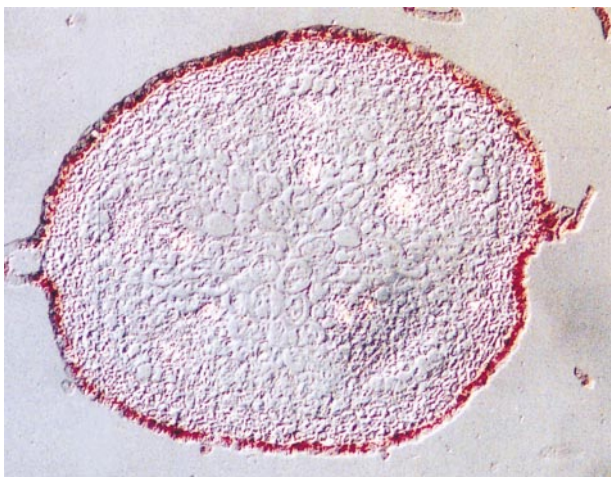

Figure 2. Distribution of the *CUT1* Transcript in a Cross-Section of an Arabidopsis Stem.

The section was hybridized with the digoxigenin-labeled antisense *CUT1* transcript, which is shown by the formation of a dark red-brown precipitate.

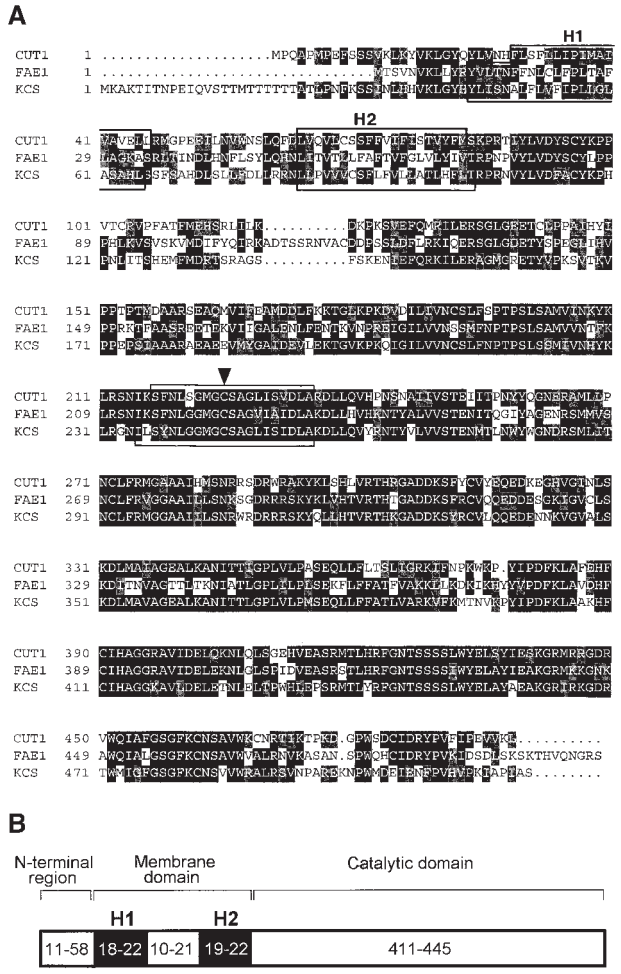

Figure 3. Amino Acid Sequence Alignment of the Microsomal Condensing Enzymes.

(A) Sequences were aligned using the CLUSTAL W program. Identical amino acids are highlighted on a black background; similar amino acids are shown on a gray background. Gaps introduced for the alignment are indicated by dots. Boxed segments H1 and H2 correspond to putative transmembrane regions, as determined in Figure 9. The boxed region with an arrowhead indicates a highly conserved region that contains the predicted active site cysteine. The FAE1 sequence is from James et al. (1995), and the jojoba β-ketoacyl–CoA synthase (KCS) sequence is from Lassner et al. (1996). The nucleotide sequence of *CUT1* has GenBank accession number AF129511.
(B) Schematic representation of the possible domains in the FAE1-like class of proteins. The putative transmembrane domains are shown. Numbers shown inside the boxes correspond to the size variation in each domain among the different FAE1-like proteins.

the Arabidopsis *cer1*, *cer2*, and *cer6* mutants. Furthermore, of these 36 waxless primary transformants, 32 had seedless short siliques (Figure 5B), whereas four were partially sterile, producing <200 seeds each. In contrast, the remaining 10 primary transformants that had wild-type wax blooms were all fully fertile. Male sterility has been previously associated with waxless mutants in which the absence of waxes in the trypine layer of the pollen grain disrupts pollen–pistil interactions (Preuss et al., 1993). However, this sterility is conditional and can be relieved when the pollen grain germinates in a humid environment (Preuss et al., 1993). Similarly, when the 32 waxless 35S–*CUT1* transgenic plants were placed in a highly humid environment, two to 30 seeds were obtained from 14 of the previously sterile primary transformants. Furthermore, cross-pollination of sterile waxless plants with wild-type pollen resulted in normal seed set. In reciprocal crosses, using pollen from waxless lines to pollinate wild-type stigmas, no seed was produced. Thus, similar to some of the Arabidopsis *cer* mutants, the waxless 35S–*CUT1* transgenic plants are conditionally male sterile. Other phenotypic traits of the *cer* mutants, such as reduced height and slenderness (Koornneef et al., 1989), were not apparent in the 35S–*CUT1* waxless transformants.

Seed obtained from these waxless transgenic lines were sown, and the progeny were analyzed. In some lines, the T_4 progeny also had a waxless phenotype, whereas in other lines, none was observed. In many of the transgenic lines in which T_4 waxless plants were obtained, there was no simple Mendelian genetic ratio of wild-type–to–waxless plants. Furthermore, wild-type and waxless stems could occur on the same plant. In other cases, the bottom half of the stem was

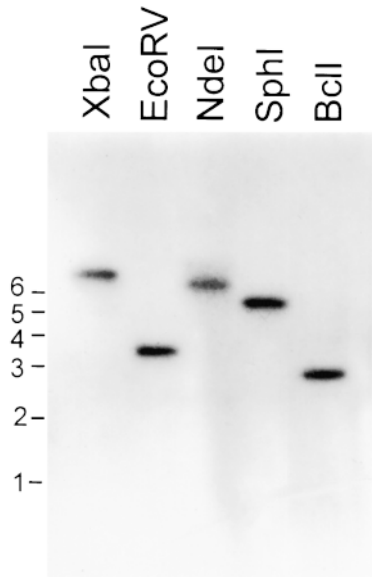

Figure 4. Genomic DNA Gel Blot Analysis of *CUT1* Hybridizing Sequences.

Ten micrograms of Arabidopsis wild-type DNA (ecotype Columbia-4) was digested with the indicated restriction enzyme. The blot was hybridized with the complete *CUT1* coding region derived from the EST clone T76166. The positions of molecular length markers in kilobases are indicated at left.

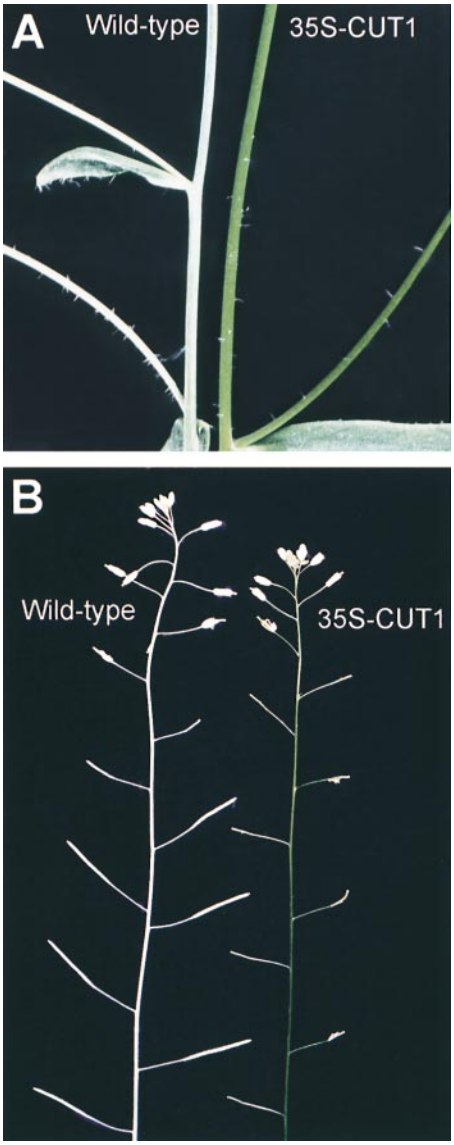

Figure 5. 35S–*CUT1* Plants Display a Waxless Phenotype and Are Male Sterile.

(A) Comparison of the wax load on the stems of wild-type (left) to transgenic 35S–*CUT1* (right) plants. In strong light, wild-type plants appear white because the light is reflected off the wax crystals. In contrast, the stems of the 35S–*CUT1* plants have no coating of wax and thus have a bright green appearance.

(B) 35S–*CUT1* plants are sterile, as evident from siliques failing to develop. A fertile wild-type plant is shown at left.

waxless, whereas the top half was covered in wax. Thus, it appeared that the waxless phenotype conferred by the 35S–*CUT1* transgene was unstable. Seed from the 10 primary transformants that were not waxless were sown, and the T_4 seedlings were analyzed. Two of these lines, 2 and 5, had T_4 progeny that exhibited the waxless phenotype. In contrast to the primary transformants with a waxless phenotype in which seed was difficult to obtain, these two primary transformants provided an abundant seed supply and a constant source of T_4 plants with a waxless phenotype for our analyses. For this reason only, we have used line 5 for the majority of experiments reported in this article.

Waxless Phenotype in Line 5 Is Associated with the Homozygous State of the Transgene and Results from the Suppression of the Steady State Level of the *CUT1* Transcript

The ratio of the waxless–to–wild-type phenotype of the T_4 progeny of line 5 was 12:39. This is approximately a 1:3 ratio ($\chi^2 = 0.0065$; $P > 0.90$), suggesting that there is one segregating transgene that, when in a homozygous state, confers the waxless phenotype. We investigated this possibility further by germinating seed on a selective kanamycin medium. The ratio of kanamycin-sensitive to kanamycin-resistant plants was 65:243, again approximately a 1:3 ratio ($\chi^2 = 2.28$; $P > 0.10$). After selection, 143 kanamycin-resistant plants were transferred to soil and grown to maturity. Of these plants, 51 had the waxless phenotype, whereas 92 appeared wild type. This is in good agreement with a 1:2 ratio ($\chi^2 = 0.25$; $P > 0.50$). Thus, the T_4 progeny were segregating in a 1:2:1 ratio of waxless plants that were kanamycin resistant (putative homozygotes) to wild-type-looking plants that were kanamycin resistant (putative hemizygotes) to kanamycin-sensitive plants. This ratio is consistent with one segregating transgene. This result was confirmed by analyses of the T_5 progeny. Thus, only when the transgene is in a homozygous state was the waxless phenotype observed.

One explanation for the generation of transgenic lines with the waxless phenotype is that the endogenous gene corresponding to *CUT1* is being cosuppressed due to the presence of the 35S–*CUT1* transgene. In the case of line 5, this occurs only when the transgene is in a homozygous state. Similarly, a β-1,3-glucanase transgene confers cosuppression only when in a homozygous state (de Carvalho et al., 1992). To investigate this possibility, the steady state levels of the *CUT1* transcripts were examined by RNA gel blot analysis (Figures 7A and 7B). Total RNA isolated from wild-type stems and hybridized with a *CUT1* probe resulted in a single band of ~1.8 kb. The abundance of the *CUT1* transcript in the hemizygous line 5 plants (lane 1) was much higher than in the wild type (lane 3), indicating that the gene has been successfully expressed by the 35S promoter. However, the band in lane 1 is much wider than that in lane 3 (Figure 7). This reflects the fact that two different-sized transcripts are being made in this transgenic line—the endogenous transcript and a slightly longer transcript corresponding to the transgene, which contains a 67-bp-long Ω translational enhancer. In lane 2 containing the RNA from the homozygous line 5 plants, the abundance of the *CUT1*

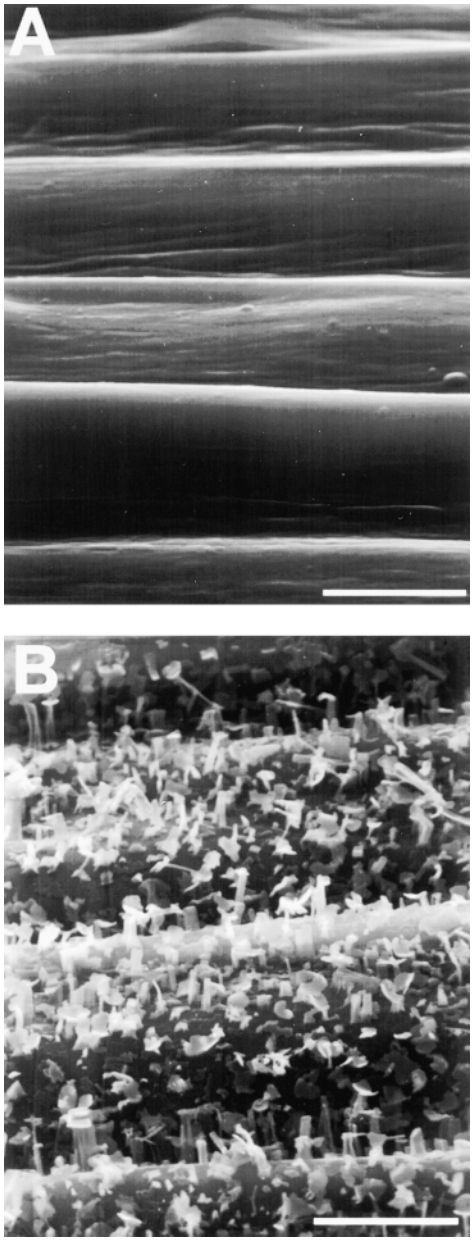

Figure 6. Stems of Transgenic 35S–*CUT1* Plants Are Completely Devoid of Wax Crystals.

(A) Scanning electron microscopy of the surface of stems from 35S–*CUT1* plants displaying the waxless phenotype.
(B) Stems from wild-type plants.
Bars in **(A)** and **(B)** = 10 μm.

transcript is significantly lower than in either wild-type or hemizygous line 5 plants. Furthermore, the trace amounts of transcript that are present correspond to the transgene and not the endogenous gene, demonstrating that transcription from the endogenous *CUT1* gene has been suppressed. Thus, the abolished expression of the *CUT1* gene has resulted in the waxless phenotype.

Both Decarbonylation and Acyl Reduction Pathways Are Downregulated in *CUT1*-Suppressed Plants

Using gas–liquid chromatography, we analyzed the composition and quantity of wax on the stems of the Arabidopsis ecotype Columbia (Figure 8A). In stems, the decarbonylation pathway is the major pathway of wax production. We found that its principal products were the C30 aldehyde, C29 alkane, C29 secondary alcohol, and C29 ketone, which accounted for >90% of the wax. In contrast, primary alcohols, which are made by the acyl reduction pathway, comprise

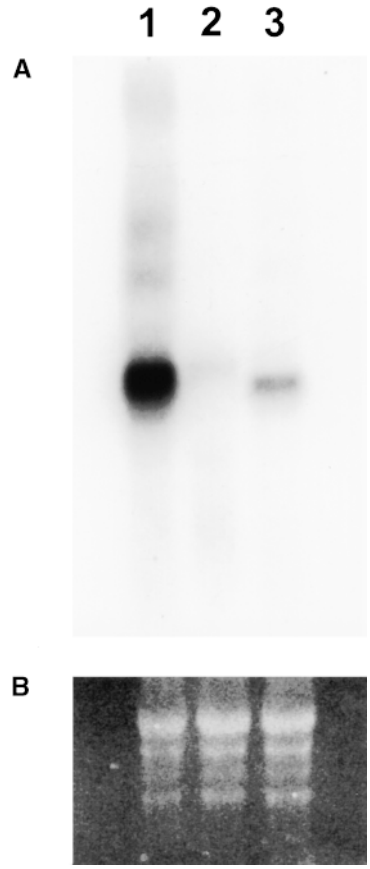

Figure 7. The Waxless Phenotype Is Associated with the Suppression of the *CUT1* Transcript.

(A) RNA gel blot analysis of total RNA prepared from hemizygous 35S–*CUT1* plants from line 5 displaying a wild-type phenotype (lane 1), homozygous 35S–*CUT1* plants from line 5 displaying a waxless phenotype (lane 2), and wild-type Columbia-4 Arabidopsis plants (lane 3). The blot was hybridized with the complete *CUT1* coding region derived from EST clone T76166.
(B) Ethidium bromide–stained gel of the corresponding lanes in **(A)**.

only ~5% of the total stem wax. Furthermore, our measurements indicate that fatty acids, which are the precursors for these two pathways, make up <5% of the total wax load. Our data are in good agreement with wax composition analyses previously reported for other Arabidopsis ecotypes (Jenks et al., 1995; Rashotte et al., 1997). The only major difference is that the level of C30 fatty acid is higher than that previously reported, which is due to the fact that the accumulation of this fatty acid increases dramatically between 25-day-old plants used in previous studies and senesced stems used here (data not shown).

The wax load on the stems of wild-type Arabidopsis, defined as the sum of all the measured wax components, reaches on average 7106 ± 1184 μg of wax per g dry weight. In contrast, wax loads on the stems of all *CUT1*-suppressed lines are severely reduced. For example, the wax load on the stems of line 5 plants totaled 483 ± 83 μg of wax per g dry weight (Figure 8B), which is only 6 to 7% of the wax load of wild-type plants (Figure 8A). Wax composition analyses of *CUT1*-suppressed plants revealed that the decarbonylation pathway was almost completely inactive (Figure 8B). The C30 aldehyde, C29 alkane, C29 secondary alcohol, and C29 ketone reached only 3.5, 2.2, 1.4, and 2.2%, respectively, of the levels found on the stems of wild-type plants. Furthermore, there was no corresponding increase in alkanes, secondary alcohols, or ketones with shorter chain lengths. *CUT1* suppression also had a pronounced effect on the acyl reduction pathway. The major products of this pathway in wild-type stems are the C30 and C28 primary alcohols. In *CUT1*-suppressed plants, both alcohols were reduced to only 6 to 7% of their wild-type levels. However, in contrast to the products of the decarbonylation pathway, increases in the levels of C22 and C24 primary alcohols were observed, with the C24 alcohol being the most abundant primary alcohol in *CUT1*-suppressed plants. The overall levels of primary alcohols in *CUT1*-suppressed plants were ~45% that of the wild type. This reduction is not as dramatic as that measured for the decarbonylation pathway. This is due mainly to the accumulation of the shorter alcohol species.

Similar to primary alcohols, the levels of C28 and C30 fatty acids and aldehydes were drastically reduced in *CUT1*-suppressed stems relative to those of the wild type. Like the primary alcohols, the C24 products are the most abundant species of aldehydes and fatty acids. In total, wax components comprised of C24 chains were the most common in the wax of *CUT1*-suppressed plants, and their levels had increased by more than sevenfold compared with levels in wild-type plants. These data suggest that the CUT1 condensing enzyme is needed for the synthesis of fatty acyl chains longer than 24 carbons. Even though all of the wax composition changes were described for transgenic line 5, they were consistent in 12 other *CUT1*-suppressed lines analyzed. In all, these 13 lines represent at least seven independent transformation events.

The wax load and composition were also determined for the hemizygous line 5 plants overexpressing the *CUT1* gene

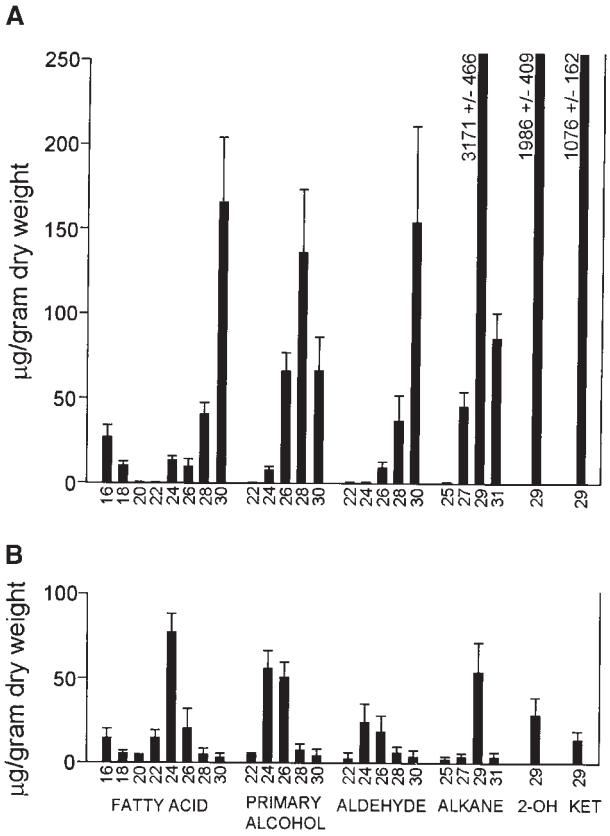

Figure 8. Both Decarbonylation and Acyl Reduction Pathways Are Downregulated in the Stems of *CUT1*-Suppressed Plants.

(A) Composition of the stem cuticular wax of wild-type Columbia-4 plants. Chemical classes and chain length distributions are labeled on the x axis. When the amount of a component is off the scale of the y axis, a number designating the actual value is presented next to the bar. These data were published as Figure 5A in Millar et al. (1998). Data for wild-type, *CUT1* **(B)**, and 35S–*FAE1* (Millar et al., 1998) plants were obtained simultaneously.

(B) Transgenic *CUT1*-suppressed Columbia-4 plants.

Each value is the mean of eight independent measurements of individual plants. Error bars indicate standard deviations. KET, ketone; 2-OH, secondary alcohol.

(Figure 7). In this case, the wax load (6012 ± 1369 μg of wax per g dry weight) and wax composition (data not shown) were not significantly different from that found on the wild-type stems. Thus, overexpression of *CUT1* under the control of the 35S promoter did not result in a higher wax load or an altered wax profile.

CUT1 Genetic Map Position Does Not Correspond to Any of the Mapped *cer* Loci

To determine whether *CUT1* corresponds to one of the 10 previously mapped *cer* mutants, we mapped it using the

Arabidopsis recombinant inbred lines (Lister and Dean, 1993). The total lack of stem wax crystals and the male sterility observed in *CUT1*-suppressed plants are phenotypic characteristics of the *cer1*, *cer2*, and *cer6* mutants of Arabidopsis (Koornneef et al., 1989; Preuss et al., 1993). However, the *CER1* and *CER2* genes have been isolated previously. Therefore, the most likely candidate was *CER6*.

A comparison of the banding pattern between DNA isolated from the Columbia and Landsberg *erecta* ecotypes of Arabidopsis on a DNA gel blot using the *CUT1* cDNA as a probe identified a ClaI restriction fragment length polymorphism. Using this polymorphism, we mapped *CUT1* to the top arm of chromosome 1, at 35.98 centimorgans (cM). Three different *CER* genes have been mapped to chromosome 1, and on the integrated map of the Arabidopsis genome, their locations are as follows: *CER1* at 11.7 cM, *CER5* at 79.9 cM, and *CER6* at 96.0 cM (Hauge et al., 1993). Thus, *CUT1* maps the closest to *CER1*. However, the sequence of *CER1* (Aarts et al., 1995) is unrelated to the *CUT1* sequence. Even though the *CER6* gene is also on chromosome 1, it is basically unlinked, being ~60 cM away from the map position of *CUT1*. Therefore, *CUT1* does not correspond to any of the mapped *cer* loci.

Structural Analysis of the CUT1 Protein

Isolation of the elongase activity, either from the epidermis or seed, shows that it is located in the microsomal fraction of the cell as a membrane-bound complex (Bessoule et al., 1989; Fehling et al., 1992). To determine whether the CUT1 condensing enzyme has any membrane-spanning domains, the sequence was analyzed using the TMpred algorithm (Hofmann and Stoffel, 1993). We found that both the CUT1 and FAE1 condensing enzymes contain two very distinctive N-terminal hydrophobic regions (Figures 9A and 9B). These regions are likely to be transmembrane domains, implying that both CUT1 and FAE1 are integral membrane proteins. Interestingly, the amino acid sequence comparisons of these strong hydrophobic regions revealed that they are among the most highly variable regions between FAE1 and CUT1 (Figure 3). This trend continues with four other FAE1-like proteins that have been identified by the Arabidopsis genome project. Each of the putative amino acid sequences of AC003105, AC004484, AC023094, and AC002411 share >45% identity with FAE1. In addition, they have hydropathy plots that are very similar to FAE1 and CUT1, with two strong hydrophobic regions at the N terminus that are highly variable in sequence (data not shown).

The hydropathy plots of the N terminus of the FAE1 and CUT1 proteins also show a striking similarity to the N terminus of another integral membrane protein, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; Figure 9C). For HMGR, the N terminus has been demonstrated to contain two membrane-spanning domains that mediate targeting of HMGR to microsomes derived from the endoplasmic reticulum

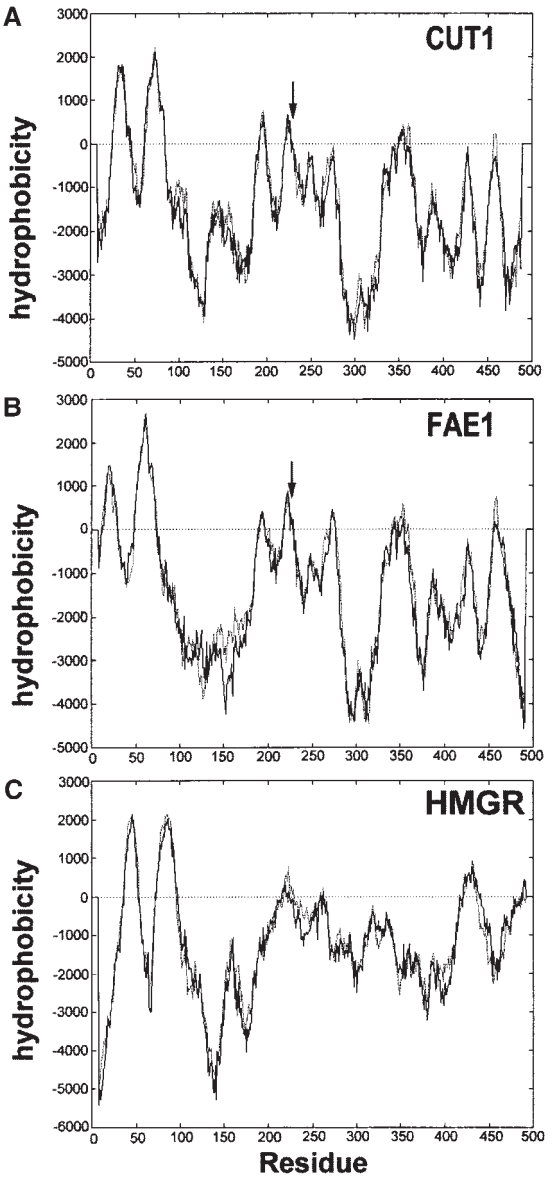

Figure 9. Comparison of the Predicted Transmembrane Regions from Arabidopsis CUT1, FAE1, and HMGR2.

The hydropathy plots of the putative amino acid sequences according to Hofmann and Stoffel (1993). The positions of the predicted active site cysteines are indicated by arrows.
(A) CUT1.
(B) FAE1.
(C) HMGR2.

(Campos and Boronat, 1995). Thus, the N-terminal region in the FAE1-like group of condensing enzymes may be functionally similar, targeting these proteins to specific membrane domains of the endoplasmic reticulum.

Furthermore, four distinct regions have been defined in

the primary structure of the HMGR protein. This includes highly conserved membrane and catalytic domains as well as highly variable (both in sequence and length) N-terminal and linker regions (Campos and Boronat, 1995). CUT1 and the other FAE1-like condensing enzymes seem to have a similar organization (Figure 3B). In these enzymes, a highly variable N-terminal region (both in length and sequence) is followed by the two potential membrane-spanning domains (H1 and H2). After these sequences, there is a variable region (both in length and sequence; Figure 3A); the remaining portions of the proteins are mostly hydrophilic and are highly conserved. The latter highly conserved region contains the predicted active site cysteine (Lassner et al., 1996) and thus probably represents the catalytic domain of the protein.

DISCUSSION

CUT1 Is a VLCFA Condensing Enzyme Required for the Formation of Cuticular Waxes

Several lines of evidence indicate that CUT1 functions as a condensing enzyme in VLCFA biosynthesis for the production of cuticular waxes. (1) CUT1 was identified on the basis of its high sequence identity with FAE1 and jojoba β-ketoacyl–CoA synthase, which are both condensing enzymes involved in VLCFA biosynthesis. (2) The sense suppression of *CUT1* in transgenic plants results in a waxless phenotype, which earmarks CUT1 as a component of the VLCFA biosynthetic pathway for wax synthesis. (3) Changes in the wax composition of *CUT1*-suppressed plants are consistent with a block in an enzyme involved in fatty acid elongation, because shorter chain length wax components accumulate in these plants in comparison with the wild type. (4) *CUT1* is exclusively expressed in the epidermis, which is the site of wax biosynthesis. (5) The presence of two putative transmembrane domains at the N terminus of the protein and the similarity of this region with the endoplasmic reticulum–localized protein HMGR are consistent with the notion that elongase activity of epidermal cells is membrane bound and localized in the microsomal membranes (Bessoule et al., 1989). Immunolocalization experiments are currently under way to verify if CUT1 is indeed located in the membrane of the endoplasmic reticulum.

Cosuppression of *CUT1* Results in a Severe Waxless Phenotype

Scanning electron microscopy revealed that there are no wax crystals on the surface of the stems of *CUT1*-suppressed plants. Of the Arabidopsis *cer* mutants, only *cer1*, *cer2*, and *cer6* completely lacked wax crystals, and these mutants had strong waxless phenotypes with wax loads of ~20, 36, and 19% that of wild-type plants, respectively (Jenks et al., 1995). The wax load of *CUT1*-suppressed plants was even lower, reaching only 6 to 7% of that of the wild type. There have been no *cer* mutants reported (Hannoufa et al., 1993; McNevin et al., 1993; Jenks et al., 1995) with such a low relative wax load. Because a small amount of transcript from the *CUT1* transgene was found in *CUT1*-suppressed plants (Figure 6), it is possible that there was still residual CUT1 activity. Thus, a *cut1* null allele may accumulate even less wax.

There Is Only One Major FAE Pathway for the Synthesis of Waxes in Arabidopsis Stems

The overall perception, based on genetic and biochemical analyses of wax biosynthesis, is that multiple independent fatty acid elongating systems exist to supply the different wax biosynthetic pathways (Post-Beittenmiller, 1996; Vioque and Kolattukudy, 1997). Supporting this notion is the fact that in nature, the predominant alkanes found in cuticular waxes are 29 and 31 carbons long, whereas the most common primary alcohols are 26 and 28 carbons in length. Thus, it has been proposed that the elongation system for the production of alkanes would supply longer fatty acids, and the system for the production of primary alcohols would synthesize shorter ones (Avato, 1987). In addition, channeling was thought to occur so that the substrates destined for specific end products were not freely exchangeable (von Wettstein-Knowles, 1979).

Surprisingly, our results demonstrate that suppression of *CUT1* downregulates both the decarbonylation and acyl reduction pathways of wax synthesis, implying that there is only one major fatty acid elongation pathway providing VLCFAs for wax synthesis in Arabidopsis stems (Figure 1). Because cosuppression of endogenous genes can be achieved by transformation even with closely related sequences (Elkind et al., 1990), it might be argued that the process resulting in the silencing of *CUT1* may also be silencing other homologous genes. We believe that this is unlikely, because DNA gel blot analysis demonstrated that there were no closely related sequences to *CUT1* in the Arabidopsis genome (Figure 4).

Analysis of Metabolism in *CUT1*-Suppressed Plants: Insights into the Regulation of the Wax Biosynthetic Pathways

Blocking an enzyme in a metabolic sequence can cause the accumulation of the immediate precursor. Based on this concept and the fact that several C24 cuticular wax components accumulate in the *CUT1*-suppressed plants, it seems likely that CUT1 is required for the elongation of C24 fatty acids. However, analyses of fatty acid condensing enzymes have shown that they can catalyze multiple sequential reactions. For example, starting at C16, the jojoba β-ketoacyl–CoA synthase can produce acyl chains up to 24 carbons in

length (Lassner et al., 1996), whereas FAE1 uses C16 to C20 acyl groups as substrates (Kunst et al., 1992; Millar and Kunst, 1997). Thus, CUT1 also may be involved in the elongation of either longer or shorter fatty acids than 24 carbons. Our results to date do not allow us to draw definite conclusions concerning the overall specificity of CUT1.

The almost complete absence of wax on *CUT1*-suppressed plants implies that a large block in the flow of carbon through the wax biosynthetic pathway has occurred. Although shorter chain length wax constituents are accumulating to higher levels, their total mass represents only a small fraction of the longer chain length constituents found on wild-type stems. Thus, the amount of carbon (C16 or C18 fatty acids) entering the VLCFA biosynthetic pathway has been dramatically reduced, implying that either CUT1 is also involved in the elongation of C16 to C24 fatty acids or that the absence of CUT1 activity results in the whole pathway shutting down. For instance, accumulation of high levels of C24 fatty acids could lead to feedback inhibition of enzymes earlier in the pathway. This would be analogous to the situation in FAS in plastids, in which C18 fatty acids have been implicated in the feedback regulation of enzymes such as acetyl–CoA carboxylase (Shintani and Ohlrogge, 1995). In either case, this result implies that CUT1 is an important regulatory point in wax biosynthesis.

Although high levels of *CUT1* transcript were present in the hemizygous line 5 35S–*CUT1* plants, they did not translate into greater wax loads than those measured for wild-type plants. This might suggest that some enzyme or factor other than *CUT1* is limiting to wax accumulation. However, to address properly the question of whether *CUT1* overexpression could result in greater wax loads, correct spatial and temporal expression is necessary. For instance, in their attempt to modify lignin composition, Meyer et al. (1998) found that a lignin biosynthesis–related promoter was vastly superior to the 35S promoter, which failed to promote high levels of gene expression in the cells undergoing lignification. Likewise, instead of using the 35S promoter, the expression of *CUT1* under the control of its native promoter or another wax biosynthesis–associated promoter might result in transgenic Arabidopsis with a greater wax load.

The shorter chain length VLCFAs that are generated in the *CUT1*-suppressed plants are better used by the acyl reduction pathway than by the decarbonylation pathway. Primary alcohols of shorter chain length (C24 to C26) accumulate to higher levels than that found in wild-type plants, although the total amount of carbon converted into primary alcohols in the transgenic plants is still significantly lower than in the wild-type plants (124 versus 275 μg per g dry weight). In contrast, the large amount of carbon that is found in the C29 alkanes of wild-type waxes does not accumulate in the form of shorter chain length alkane species. Moreover, in the *CUT1*-suppressed plants, the C29 species are still the predominant products of the pathway. Thus, unlike the acyl reduction pathway, the decarbonylation pathway cannot use fatty acids of shorter chain lengths.

CUT1 Is a New Gene Required for Wax Biosynthesis and Pollen Fertility

Genetic screens for mutants deficient in surface wax accumulation resulted in the identification of 22 *cer* loci in Arabidopsis (Koornneef et al., 1989; McNevin et al., 1993). In addition, alleles of many of these loci have been recovered in other screens for mutants with altered epicuticular wax layers (Aarts et al., 1995; L. Kunst and E. Wisman, unpublished data) or aberrant pollen–stigma interactions (Preuss et al., 1993; Hulskamp et al., 1995). However, despite extensive screening and the striking waxless phenotype of the *CUT1*-suppressed plants, it appears that a mutant with a lesion in the *CUT1* gene has not been recovered. This conclusion is based on evidence indicating that the genetic map position of the *CUT1* gene does not coincide with any of the mapped *cer* loci, *CER1* through *CER10* (Hauge et al., 1993; Lolle et al., 1998), and that the wax composition of the *CUT1*-suppressed plants does not resemble any of the Arabidopsis *cer* mutants. Furthermore, the phenotypic characteristics of the unmapped *cer* mutants are significantly different from the *CUT1*-suppressed plants. Their wax phenotypes are invariably less severe, and they are all fertile (Koornneef et al., 1989). However, the possibility remains that one of these unmapped *cer* loci represents a weak allele of *CUT1*.

In view of the numerous screens for waxless mutants conducted to date, it is somewhat surprising that we may have identified a novel gene with a function in wax production. The fact that the wax load on the *CUT1*-suppressed plants is lower than that on any of the known *cer* mutants suggests that previous screens may not have been able to recover mutants with severe waxless phenotypes. The failure to isolate a *cut1* mutant may relate to the observation that the loss of CUT1 activity results in a dysfunctional pollen grain, which may hinder the recovery of a homozygous *cut1*/*cut1* plant. If the degree of male sterility is related to the amount of wax accumulation, we would predict that a *cut1* mutant would be severely sterile.

Conclusions

Using a reverse genetic approach, with the resources of the Arabidopsis genome project, we have identified a previously unknown gene involved in wax biosynthesis. Judging by the wax load of transgenic Arabidopsis plants, the suppression of the *CUT1* gene results in a more severe waxless phenotype than in any of the *cer* mutants isolated to date. The fact that this gene encodes an enzyme with a known function should help us study the regulation of the wax biosynthetic pathway. In addition, we will be able to determine whether condensing enzymes, like CUT1, will be useful for manipulating wax accumulation and composition in plants. Directed genetic engineering of plant waxes may eventually allow us to increase tolerance of crops to environmental stresses,

such as UV light, heat, and drought, as well as resistance to insects and pathogens.

METHODS

Identification and Analysis of a *CUT1* cDNA Clone

The cDNA corresponding to expressed sequence tag (EST) T76616 was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Sequencing of the cDNA was performed on plasmid DNA by automated sequencing with an Applied Biosystems 373A DNA sequencer (Foster City, CA). Multiple sequence alignments were generated using the CLUSTAL W program (Thompson et al., 1994). The output of the sequence alignments was produced using version 3.21 of the Boxshade algorithm. Transmembrane prediction analysis was performed with the TMpred algorithm (Hofmann and Stoffel, 1993).

Construction of the Transformation Vector and Generation of Transgenic Plants

The *CUT1* EST was obtained in the vector derived from λZipLox (Gibco BRL). The insert was excised with KpnI and BamHI, and the resulting 1.85-kb fragment was directionally subcloned into the KpnI-BamHI sites of pGEM7z(f) (Promega), resulting in the plasmid pGEM–*CUT1*. This plasmid was linearized with XhoI and then partially cleaved with SstI. After running the digest on an agarose gel, the 1.9-kb product was isolated and subcloned into the vector pJD330 (Shaul and Galili, 1992), which had been digested with SalI and SstI. This places the *CUT1* coding region in a sense orientation behind the 35S promoter of the cauliflower mosaic virus and the Ω translational enhancer (Gallie et al., 1987) of pJD330. This vector was then subcloned into the EcoRI and HindIII sites of pBIN19. The resulting binary vector, p35S–*CUT1*, was electroporated into the *Agrobacterium tumefaciens* strain GV3101 containing the plasmid pMP90 (Koncz and Schell, 1986), and transformants were selected on Luria–Bertani medium containing 25 μg/mL gentamycin and 50 μg/mL kanamycin. *Arabidopsis thaliana* transformation was conducted as previously described (Millar and Kunst, 1997). Transgenic plants with a waxless phenotype were enclosed in a polyethylene bag to create a high-humidity environment during growth and development, because this had been previously shown to be beneficial for seed set (Koornneef et al., 1989).

In Situ Hybridization

CUT1 sense and antisense probes were prepared by polymerase chain reaction (PCR) using internal *CUT1* primers with an added T7 polymerase binding site and *CUT1* cDNA in the ZipLox vector (Gibco BRL) as a template. The primer sequences are as follows: for the antisense probe, 5′ primer 5′-ATGCCTCAGGCACCG-3′ and 3′ primer 5′-GCTAATACGACTCACTATAGGGTTATTTGAGTACACC-3′; and for the sense probe, 5′ primer 5′-CCCTATAGTGAGTCGTATTAATGCCTCAGGCACCG-3′ and 3′ primer 5′-TTATTTGAGTACACC-3′. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA) and digoxigenin-11-rUTP labeled according to the Boehringer Mannheim nucleic acid labeling kit. Fixation and embedding of the plant material as well as in situ hybridization and detection of the signal were performed according to Samach et al. (1997).

RNA and DNA Gel Blot Analyses

For RNA gel blot analyses, total RNA from tissues of Arabidopsis was isolated using the RNeasy plant mini kit (Qiagen). Ten micrograms of RNA per lane was separated in agarose gels containing 5.8% formaldehyde and blotted onto Hybond-NX filters (Amersham) in 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate). After baking for 2 hr at 80°C, the membrane was hybridized to the *CUT1* gene, which had been labeled with ^{32}P-dATP by PCR, using Taq polymerase (Gibco BRL) and the oligonucleotides 5′-ACTTCCCATTTCTCAATCCCC-3′ and 5′-TCTAGCTCGGTGAAGCTCAAG-3′. The following PCR program was used: 95°C for 5 min, 30 cycles of denaturation (95°C for 30 sec), annealing (62°C for 60 sec), and extension (72°C for 90 sec), followed by a 6-min incubation at 72°C. Hybridization was performed at 65°C in 0.5 M phosphate buffer, pH 7.2, 7% SDS, and 10 mM EDTA. The blots were washed at high stringency (0.1 × SSC and 0.1% SDS at 65°C) before x-ray film (Du Pont) was exposed to them.

DNA for gel blot analyses was isolated according to the directions of Bethesda Research Laboratories (see volume 12 of *Focus*, pages 13 to 15). Ten micrograms of DNA per lane was separated in a 1% agarose gel. Blotting, hybridization, and washing procedures were the same as those for RNA gel blot analyses.

Determination of *CUT1* Map Position

To determine the position of the *CUT1* locus within the Arabidopsis genome, restriction fragment length polymorphism mapping using the Arabidopsis recombinant inbred lines was employed (Lister and Dean, 1993). DNA gel blot analysis identified a ClaI polymorphism between the Columbia and Landsberg *erecta* ecotypes. This polymorphism was then used to score DNA isolated from 76 recombinant inbred lines.

Scanning Electron Microscopy

Stems from the waxless transgenic plants and the wild type were allowed to air dry, mounted on stubs, and coated with gold particles in a Nanotech SEMPrep2 sputter coater (Nanotech, Manchester, UK). Specimens were then examined in a Cambridge model 250T scanning electron microscope (Leica, Cambridge, UK) at an accelerating voltage of 20 kV.

Wax Extraction and Analysis

Stems were immersed in a 2:1 chloroform–methanol mixture to remove surface waxes. Initially, we tested several different extraction times ranging between 10 sec and 10 min. A 10-sec immersion into the solvent resulted in ~80% efficiency in comparison with the amount of wax extracted from the wild-type stems after 10 min. However, because there were no qualitative differences between the two extraction protocols, we chose to proceed with the 10-sec extractions in our analysis.

After extraction, wax samples were evaporated to dryness under a stream of nitrogen, dissolved in 100 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Pierce, Rockford, IL), and derivatized at 80°C for 1 hr. Samples were then analyzed by gas–liquid chromatography in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector, using either a DB-1 or DB-5 column. Gas–liquid chromatography analyses were performed at the initial temperature of 150°C, followed by ramping at 4°C per min to 320°C, which was maintained for an additional 10 min. Peaks were identified by the comparison of their retention times to known standards and confirmed using gas chromatography–mass spectrometry on a Trio 2000 gas chromatograph mass spectrometer (Micromass, Manchester, UK) in the negative CI mode (Carrier et al., 1995). Quantification of compounds was based on flame ionization detector peak areas, which were converted to mass units by comparison with an internal standard, 17:0 methylester, that was added to each sample before the extraction.

Wax loads were measured initially both on 6-week-old plants (based on fresh weight) and on plants that had senesced and dried (based on dry weight). We found that measurements on the latter were more consistent, and these were used in this study. Only the principal surface lipids were measured: *n*-nonacosane (C29 alkane), 14- and 15-nonacosanol (C29 secondary alcohol), 15-nonacosanone (C29 ketone), C22 to C30 aldehydes, C22 to C30 primary alcohols, and C16 to C30 fatty acids (Hannoufa et al., 1993). The total area of these peaks accounted for >90% of the total area of the detectable peaks from a sample and was considered to be the wax load of the stem. Our measurements were comparable with previously reported wax load measurements of McNevin et al. (1993). They reported a wax load of 1013 ± 55 μg/g fresh weight on 6-week-old wild-type Arabidopsis plants (ecotype Wassilewskija). Our wax load measurement on 6-week-old Arabidopsis plants (ecotype Columbia) was 1339 ± 178 μg/g fresh weight.

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