

Cloning and Characterization of *CER2*, an Arabidopsis Gene That Affects Cuticular Wax Accumulation

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Cuticular waxes are complex mixtures of very long chain fatty acids and their derivatives that cover plant surfaces. Mutants of the *ECERIFERUM2* (*cer2*) gene of Arabidopsis condition bright green stems and siliques, indicative of the relatively low abundance of the cuticular wax crystals that comprise the wax bloom on wild-type plants. We cloned the *CER2* gene via chromosome walking. Three lines of evidence establish that the cloned sequence represents the *CER2* gene: (1) this sequence is capable of complementing the *cer2* mutant phenotype in transgenic plants; (2) the corresponding DNA sequence isolated from plants homozygous for the *cer2-2* mutant allele contains a sequence polymorphism that generates a premature stop codon; and (3) the deduced *CER2* protein sequence exhibits sequence similarity to that of a maize gene (*glossy2*) that also is involved in cuticular wax accumulation. The *CER2* gene encodes a novel protein with a predicted mass of 47 kD. We studied the expression pattern of the *CER2* gene by in situ hybridization and analysis of transgenic Arabidopsis plants carrying a *CER2*– β -glucuronidase gene fusion that includes 1.0 kb immediately upstream of *CER2* and 0.2 kb of *CER2* coding sequences. These studies demonstrate that the *CER2* gene is expressed in an organ- and tissue-specific manner; *CER2* is expressed at high levels only in the epidermis of young siliques and stems. This finding is consistent with the visible phenotype associated with mutants of the *CER2* gene. Hence, the 1.2-kb fragment of the *CER2* gene used to construct the *CER2*– β -glucuronidase gene fusion includes all of the genetic information required for the epidermis-specific accumulation of *CER2* mRNA.

INTRODUCTION

The surfaces of the aerial portions of plants are covered by the cuticle, which serves as a barrier between a plant and its environment. The outer portion of the cuticle is composed of a complex mixture of acyl lipids, commonly termed cuticular waxes. Cuticular waxes are believed to play important roles in helping plants resist drought, frost, pathogens, and insects and in protecting them from UV irradiation as well as influencing the retention of applied chemicals (reviewed in Martin and Juniper, 1970).

Cuticular waxes are complex mixtures of very long chain fatty acids (VLCFAs) and their derivatives (reviewed in Tulloch, 1976). The chemical composition of these waxes varies from species to species. For example, the cuticular waxes found on the leaves of Arabidopsis are mainly composed of alkanes, alcohols, and fatty acids (Jenks et al., 1995). In contrast, the waxes found on maize seedling leaves are primarily composed of alcohols, aldehydes, and esters (Bianchi et al., 1985).

The amount of cuticular wax deposited on a surface is strongly influenced by developmental and environmental sig-

nals. For example, wild-type Arabidopsis stems and siliques are covered with a heavy layer of cuticular waxes, but Arabidopsis leaves accumulate considerably less wax (Koornneef et al., 1989; Hannoufa et al., 1993; Jenks et al., 1995). Similarly, as part of the coordinated juvenile-to-adult phase transition that occurs in maize, seedling leaves accumulate considerably more cuticular wax than do adult leaves (Bianchi et al., 1985; Evans et al., 1994; Moose and Sisco, 1994). In addition to altering the amount of wax deposited, developmental signals can affect the composition of cuticular waxes. For example, the composition of the waxes on Arabidopsis stems differs from the composition of those on Arabidopsis leaves (Hannoufa et al., 1993; Jenks et al., 1995). In contrast to the waxes on the leaves, the waxes on Arabidopsis stems are composed mainly of alkanes, ketones, and alcohols (Hannoufa et al., 1993).

Although advances have been made in our understanding of the biosynthesis of specific constituents of cuticular waxes (for reviews, see Kolattukudy et al., 1976; Post-Beittenmiller, 1996), many questions pertaining to the organization and regulation of this pathway remain unanswered (Kolattukudy et al., 1976; von Wettstein-Knowles, 1979; Cheesbrough and Kolattukudy, 1984). The precursor of VLCFAs is thought to be

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the saturated fatty acid stearate (18:0). This fatty acid is synthesized via de novo fatty acid biosynthesis, which occurs in plastids. In epidermal cells, a specific thioesterase releases the stearate molecule from the fatty acid synthase system of enzymes (Liu and Post-Beittenmiller, 1995). Subsequently, stearate is transported to the cytosol where it is elongated via an elongase system(s), which is probably localized on the membranes of the endoplasmic reticulum (Lessire and Stumpf, 1982; Lessire et al., 1982, 1989; Agrawal et al., 1984; Agrawal and Stumpf, 1985; Evenson and Post-Beittenmiller, 1995).

It has been possible to define some of the genes involved in cuticular wax accumulation because mutants of these genes condition a phenotype easily distinguished with the naked eye from the wax bloom that is present on wild-type plants. Such mutants have been identified in a number of species, including maize (reviewed in Schnable et al., 1994), barley (von Wettstein-Knowles, 1979, 1982), *Sorghum bicolor* (Jenks et al., 1992), and oilseed rape (Macey and Barber, 1970). In *Arabidopsis*, at least 21 loci involved in this pathway (the *ECERIFERUM* or *CER* loci) have been identified in this fashion (Koornneef et al., 1989; Hannoufa et al., 1993). The availability of mutants affecting cuticular wax accumulation and the isolation of the corresponding genes will assist in the elucidation of cuticular wax accumulation and the molecular mechanisms by which its production is regulated.

In this study, we report the isolation and initial characterization of the *CER2* gene, one of the *Arabidopsis* genes involved in cuticular wax accumulation. Our analyses demonstrate that the *CER2* gene encodes a novel protein. By using in situ hybridization and transgenic *Arabidopsis* plants, we show that the *CER2* gene is expressed in an organ- and tissue-specific manner consistent with the phenotypic expression of *cer2* mutants.

RESULTS

Chromosome Walking to the *CER2* Locus

As shown in Figure 1A, the *CER2* locus is located on chromosome 4 between the visible markers *IMMUTANS* (*IM*) and *APETALA2* (*AP2*) (Koornneef, 1987). As a first step in cloning the *CER2* locus via chromosome walking (Bender et al., 1983), F_3 families segregating for the genetic markers *AGAMOUS* (*AG*), *IM*, *CER2*, and *AP2* were screened for genetic recombinants (see Methods). In total, 26 recombinants were isolated between *IM* and *CER2* and 92 between *CER2* and *AP2*.

To map the locations of these recombination breakpoints more precisely, plants carrying these recombinant chromosomes were analyzed with several restriction fragment length polymorphism (RFLP) markers. Of the 92 recombinants between *CER2* and *AP2*, 11 occurred between *CER2* and m600. Based on these results, the RFLP marker m600 is ~2.5 centimorgans (cM) from *CER2*. Assuming a value of 185 kb per

cM for *Arabidopsis* chromosome 4 (Schmidt et al., 1995), this interval is estimated to be ~460 kb. Four yeast artificial chromosome (YAC) clones (EG24D9, EW14G12, EW9C10, and EW11E4; Figure 1B) that contain sequences homologous to m600 were identified by screening a number of *Arabidopsis* YAC libraries (Ward and Jen, 1990; Grill and Somerville, 1991). Both ends of each of these YACs were subcloned via either plasmid rescue (Burke et al., 1987) or λ subcloning (see Methods). These YACs were oriented relative to the genetic map and to each other by using the subcloned YAC ends as RFLP markers to analyze the collection of genetic recombinants and in cross-hybridization experiments involving the other YACs. The YAC end closest to the *CER2* locus was then used to isolate another set of YACs closer to the *CER2* locus. This cycle was repeated seven times (Figure 1B).

YAC CIC9C5 was subcloned into the λ insertion vector NM1149 as HindIII fragments of up to 8.5 kb in length (see Methods). The resulting DNA fragments were used as RFLP markers to analyze the genetic recombinants on both sides of *CER2*. These analyses established that two of the 26 recombinants between *IM* and *CER2* have recombination breakpoints between marker C9L (the centromeric end of YAC CIC9C5) and *CER2* (Figure 1B). Similarly, one of the 11 recombinants between *CER2* and m600 has a breakpoint between *CER2* and marker C9-15, which lies ~150 kb from the centromeric end of CIC9C5. Therefore, the *CER2* locus is located within an ~150-kb interval of YAC CIC9C5. The ends of this interval are defined by the positions of markers C9L and C9-15 on YAC CIC9C5.

Complementation of the *cer2-2* Mutant

To define more precisely the chromosomal region that contains the *CER2* gene, DNA fragments from the interval defined by the probes C9L and C9-15 were tested for their ability to complement the *cer2* mutation. DNA fragments derived from the 150-kb interval containing *CER2* were isolated from a λ genomic library prepared with DNA from wild-type *Arabidopsis* ecotype Landsberg *erecta* (*Ler*) (Voytas et al., 1990). Overlapping DNA fragments were subcloned into the binary vector pBI121 and used to transform *Arabidopsis* plants homozygous for the *cer2-2* allele. Initially, this complementation test was performed by using an *Agrobacterium*-mediated *Arabidopsis* root explant transformation system (Huang and Ma, 1992). Although transgenic plants were obtained (data not shown), the resulting plants seldom initiated roots. In addition, because cuticular wax deposition is strongly influenced by tissue culture conditions (Figures 2A and 2D versus Figures 2C and 2F), it was difficult to score the *CER2* phenotype on the resulting regenerated plants. Subsequently, a vacuum infiltration transformation procedure (Bechtold et al., 1993) was successfully used to obtain transgenic seed at a rate of approximately one per 150 seed.

Two small overlapping genomic fragments (pG1H and

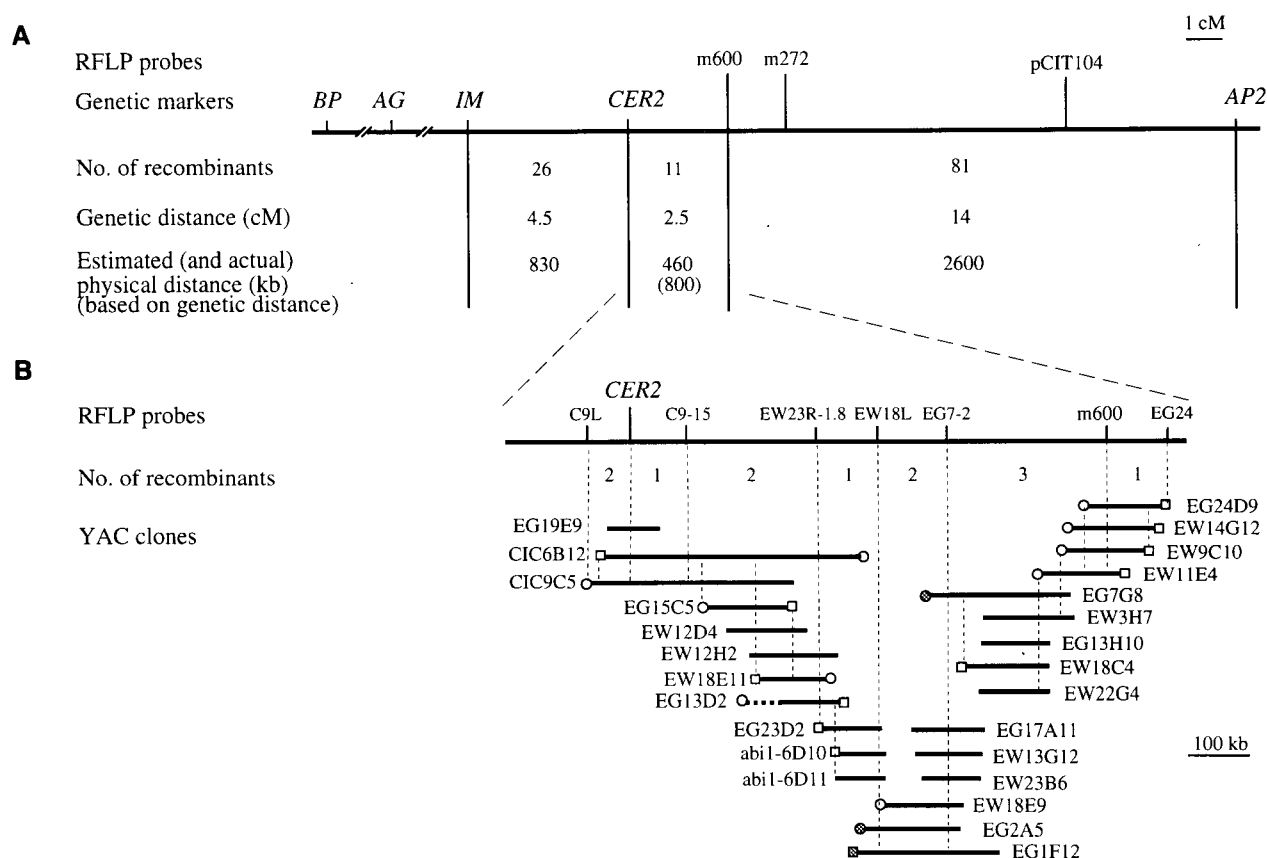


Figure 1. A Chromosome Walk to the *CER2* Locus of Arabidopsis.

(A) A partial map of chromosome 4 that shows the estimated genetic and physical distances between RFLP and visible genetic markers and the numbers of genetic recombinants isolated within each interval. Genetic distances are estimated based on subsets of the recombinants.

(B) A summary of the chromosome walk from the RFLP marker m600 to beyond *CER2*. Vertical dotted lines connecting YACs and chromosome 4 depict cross-hybridization. Probe C9-15 maps to the right of one of the 11 recombination breakpoints recovered between m600 and *CER2*. Similarly, probe C9L maps to the left of two of the 26 recombination breakpoints between *CER2* and IM. These results establish that the *CER2* locus is located within an ~150-kb interval of YAC CIC9C5. The position of the breakpoint associated with one of the 11 recombinants between *CER2* and m600 was not determined. Open circles and squares represent the centromeric and *URA3*-containing ends of the YACs, respectively. Cross-hatched circles and squares indicate YAC ends that contain repetitive sequences. Orientations were not determined for all YACs. The dashed horizontal lines represent chimeric YAC clones.

pG1RSc) isolated from the λ genomic clone G1 are illustrated in Figure 3A. They are capable of complementing the *cer2-2* mutant phenotype (Figures 2G and 2J, and Figures 2H and 2K). Eight of 10 transgenic plants that were transformed with pG1H (representing at least three independent transformation events) and eight of nine plants that were transformed with pG1RSc (representing at least four independent transformation events; see Methods) exhibited a phenotype indistinguishable from that of the wild-type plants when examined with the naked eye.

One of the genomic DNA fragments, pG1H, capable of com-

plementing the mutant phenotype was partially sequenced (GenBank accession number U40894). One end of pG1H contains the 3' portion of the *ATR1* gene, which encodes an NADPH-cytochrome P450 reductase (GenBank accession number X66016). Adjacent to the *ATR1* gene is a sequence with close identity to Arabidopsis expressed sequence tag (EST) 154C7T7 (GenBank accession number T76511). This 1.4-kb cDNA (EST 154C7T7) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH) and subcloned into pBI121 such that its transcription is under the control of the cauliflower mosaic virus 35S promoter (Figure 3A). The

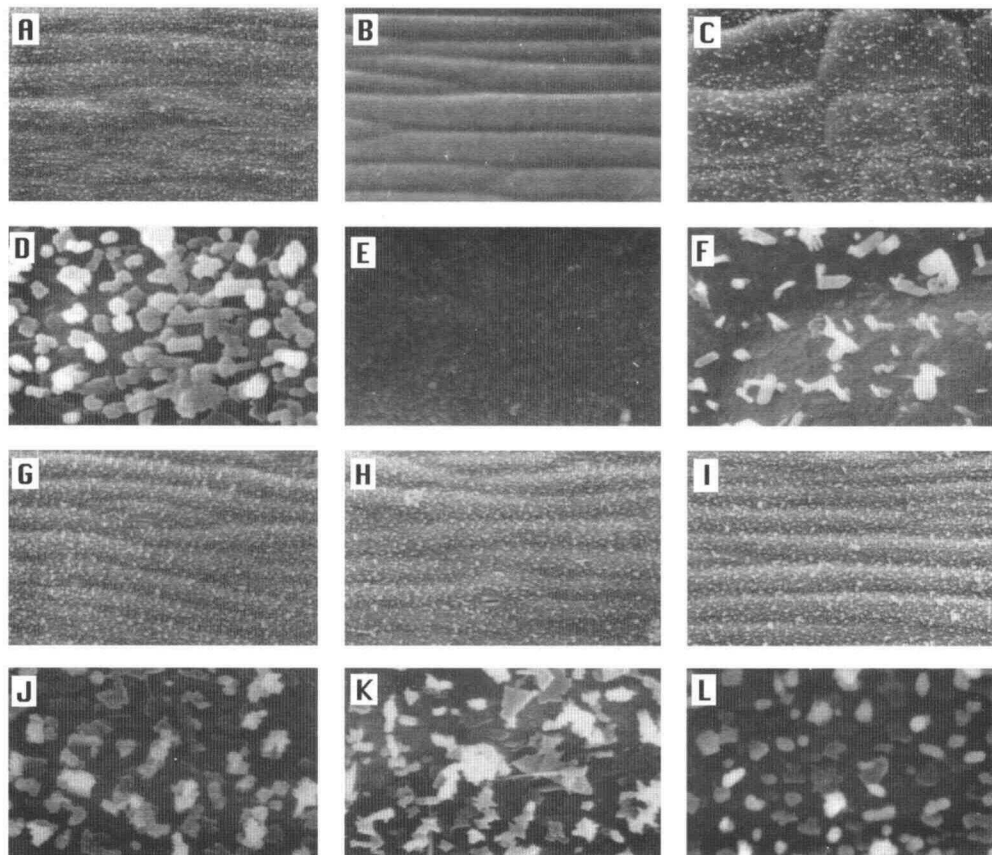


Figure 2. Appearance of Cuticular Wax Crystals on Stems Observed by Scanning Electron Microscopy.

(A) and (D) Soil-grown wild-type *Ler* plant.

(B) and (E) Soil-grown *cer2-2* plant.

(C) and (F) Wild-type *Ler* plant grown in tissue culture.

(G) to (L) Soil-grown transgenic plants (*cer2-2/cer2-2*) harboring *CER2* transgenes. In (G) and (J) is pG1RSc. In (H) and (K) is pG1H. In (I) and (L) is pG1C1. All samples were collected at the base of the first branch at the stage of seed setting.

The magnification for (A) to (C) and (G) to (I) is $\times 260$; the magnification for (D) to (F) and (J) to (L) is $\times 2600$.

resulting construct (pG1C1) was transformed into plants homozygous for the *cer2-2* allele and was found to be sufficient to complement the *cer2* mutant phenotype (Figures 2I and 2L). The EST 154C7T7 therefore represents the *CER2* coding region.

Cuticular wax crystals on stems of wild-type, mutant, and transgenic plants were examined by scanning electron microscopy (Figure 2). Wild-type plants produce a large number of condensed tube-shaped wax crystals (Figures 2A and 2D). In contrast, *cer2* plants produce relatively few crystals (Figures 2B and 2E). Four transgenic *cer2-2* plants transformed with a *CER2* genomic fragment (the pG1RSc or pG1H constructs; Figure 3A) or constitutively expressing the *CER2* cDNA (construct pG1C1; Figure 3A) produce wax crystals in numbers similar to those on wild-type plants (Figures 2G to 2I versus

Figure 2A). Although the crystals present on the plant carrying the pG1H construct differed somewhat in shape from those present on wild-type plants (Figure 2K versus Figure 2D), in the remaining three instances, the shape of the wax crystals was similar to that of the wild type (Figures 2J and 2L versus Figure 2D).

Sequence Analysis of the *CER2* Gene

As shown in Figure 4A, the DNA sequence of the 1407-nucleotide EST 154C7T7 contains a 134-nucleotide 3' untranslated region that includes a putative polyadenylation signal (AATAAA) and a 24-nucleotide poly(A) tail. The size of this

cDNA is indistinguishable from the size of the 1.4-kb mRNA detected when it is used as a probe on RNA gel blots (see below). However, to provide additional evidence that this clone represents a near full-length cDNA, the region of pG1H that includes the *CER2* coding region was sequenced (Figure 4A). Two putative TATA boxes (TATAAG and TATATA) that exhibit a high degree of similarity to the plant TATA consensus sequence (Joshi, 1987) were identified at positions -37 and -61 (relative to the 5' end of the cDNA), respectively. In addition, two putative CAAT boxes were identified at positions -68 and -85. Together, these data support the view that the EST 154C7T7 cDNA is almost full length. The only sequence polymorphism between the cDNA derived from the Columbia ecotype and the genomic clone derived from the *Ler* ecotype is a single conservative nucleotide substitution at position +1495.

The *CER2* cDNA contains an open reading frame that could encode a protein of 421 amino acid residues with a predicted molecular mass of 47 kD. No alternative open reading frames of significant length were identified. Computer-based homology searches using various derivatives of the BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988) algorithms

failed to reveal any significant sequence similarities between the deduced *CER2* protein and entries in the nonredundant nucleotide and protein data bases that have a known biochemical function. However, the deduced *CER2* protein exhibits a high level of sequence similarity (35% amino acid identity and 63% similarity over the entire protein) to that encoded by *glossy2* (*gl2*), a gene that plays an undefined role in cuticular wax accumulation in maize (GenBank accession number X88779; Tacke et al., 1995). The deduced *CER2* protein does not contain a recognizable protein-sorting signal sequence or a transmembrane domain when analyzed with the PSORT algorithm (Nakai and Kanehisa, 1992). However, the TMpred algorithm (Hofmann and Stoffel, 1993) predicts a putative transmembrane domain between residues 147 and 172 of the deduced *CER2* protein (Figure 4B).

The *cer2-2* Mutation Contains a Premature Stop Codon

CER2-specific polymerase chain reaction (PCR) primers (Figure 3B) were used to amplify the *cer2-2* mutant allele from

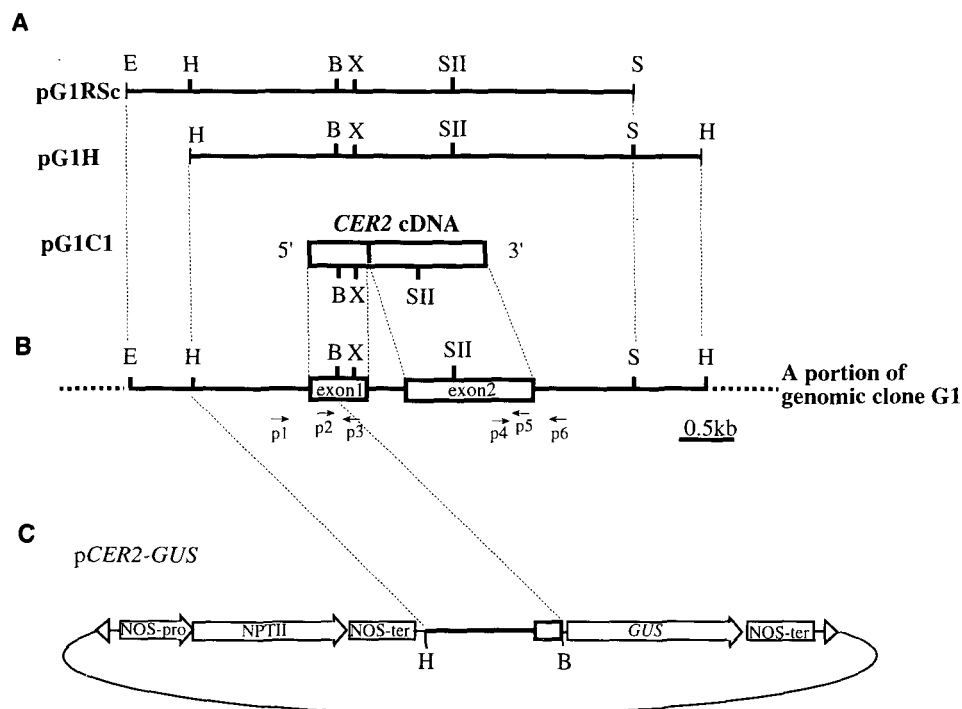


Figure 3. Structure of the *CER2* Gene and Constructs.

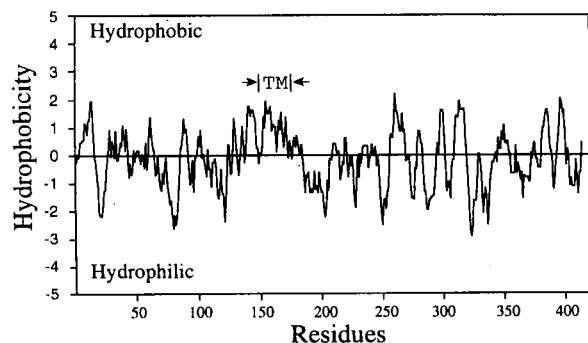
(A) Two genomic fragments were cloned into pBI121 to generate plasmids pG1RSc and pG1H, and a cDNA was cloned into pBI121 to generate plasmid pG1C1.

(B) The *CER2* gene structure is shown. The exons are indicated as boxes. The primer pairs p1 and p3, p2 and p5, and p4 and p6 were used to PCR amplify the *cer2-2* allele.

(C) The chimeric reporter gene construct pCER2-GUS contains the 1.2-kb HindIII-BamHI fragment of pG1H fused in-frame with the *GUS* reporter gene in the vector pBI101.3.

B, BamHI; E, EcoRI; H, HindIII; NOS-pro, promoter region of nopaline synthase; NOS-ter, terminator of nopaline synthase; NPTII, neomycin-phosphotransferase; S, SacI; SII, SacII; X, XhoI.

B



(A) Nucleotide sequence and deduced amino acid sequence of the *CER2* gene. The cDNA sequence is shown in uppercase letters; the

The *CER2* cDNA clone was used as a probe in RNA gel blotting experiments. Total RNA and poly(A)-enriched RNA were

remainder of the nucleotides are shown in lowercase letters. The dashed underlines represent three direct repeat sequences (tggtgtg [R1], taatgtg [R2], and aactca [R3]) present in the upstream region of the *CER2* gene. Putative TATA and CAAT boxes are boxed. A putative polyadenylation signal (AATAAA) is underlined. The underlined amino acid residues represent a putative transmembrane region. The sequence of the cDNA derived from the Columbia ecotype contains a C residue (boldface) at position +1495 instead of a T residue present in the allele derived from the *Ler* ecotype. The *cer2-2* mutant contains a G-to-A (boldface A) substitution at position +1150. This results in a premature stop codon. The GenBank accession number of the *CER2* sequence is U40894. Asterisk indicates the stop codon.

(B) Kyte and Doolittle hydrophobicity plot of the deduced CER2 amino acid sequence. A putative transmembrane (TM) domain is indicated.

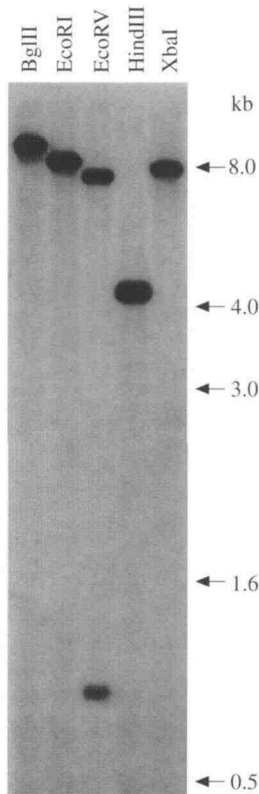


Figure 5. Genomic DNA Gel Blot Analysis of *CER2* Hybridizing Sequences.

Five hundred nanograms of *Ler* genomic DNA digested with the indicated restriction enzymes was probed with the *CER2* cDNA insert derived from EST 154C7T7. The blot was washed with $1 \times$ SSC for 40 min at 65°C. The positions of DNA length markers in kilobases are indicated at right.

isolated from aerial parts of adult wild-type plants and mutants homozygous for the *cer2-2* allele. As shown in Figure 6, these hybridization experiments revealed a single 1.4-kb mRNA in RNA isolated from a pool of leaves, stems, young siliques, and inflorescences. The amount of steady state *CER2* mRNA in the *cer2-2* mutant plants is approximately five- to 10-fold lower than that in wild-type plants.

Expression Patterns of the *CER2* Gene

Based on the phenotype associated with *cer2* mutations, the *CER2* mRNA would be expected to accumulate in the epidermis of siliques and stems. To test this hypothesis, in situ hybridizations were performed on cross-sections of stems and siliques. As shown in Figures 7E and 7G, strong hybridization was detected only in the epidermis of cross-sections of young

stems. Similar results were obtained from cross-sections of siliques (data not shown).

These studies were extended using a β -glucuronidase (*GUS*) reporter construct in transgenic *Arabidopsis* plants. The 1.2-kb HindIII-BamHI fragment of pG1H that includes positions -1009 to position +234 of the *CER2* gene was fused in-frame with the *GUS* reporter gene in the binary vector pBI101.3. The resulting construct (p*CER2-GUS*; Figure 3C) was transformed into *Arabidopsis cer2-2* plants that were derived from a cross of *Ler* marker line and Columbia *IM* marker line (see Methods). Samples from six individual transgenic plants (T_1) and their derivatives (T_2) representing at least three independent transformation events (see Methods) were stained for *GUS* activity with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid. All six transgenic plants showed similar expression patterns; a typical pattern is shown in Figure 7A. The *CER2-GUS* chimeric gene is expressed in siliques and stems; the most prominent expression occurs in mature ovaries and young siliques. Only the upper portions of stems and older siliques (arrow in Figure 7A) stained for *GUS* activity. Hence, the localization of *GUS* activity roughly corresponds to those regions that are undergoing elongation. The expression of the *CER2-GUS* gene was examined in cross-sections of *GUS*-stained stems and siliques. When *GUS* activity was assayed in the presence of ferric ions (see below), *GUS* staining was detected only in the epidermal layers of these organs (Figures 7B and 7D; data not shown). Expression of the *CER2-GUS* gene was not detected in rosette leaves (before or after bolting), cauline leaves, sepals, petals, or roots (data not shown).

In initial reporter gene experiments, *GUS* staining was conducted without the addition of ferric ions. Under these conditions, *GUS* staining was observed not only in the epidermis of stems and siliques but also in the vascular system and anthers (Figure 7A; data not shown). In subsequent experiments, *GUS* staining was conducted in the presence of ferric ions, as suggested by De Block and Debrouwer (1992).

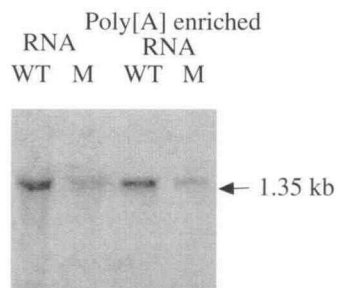


Figure 6. RNA Gel Blot Analysis of *CER2* mRNA.

Fifteen micrograms of RNA and 150 ng of poly(A)-enriched RNA from aerial portions of adult wild-type *Ler* *Arabidopsis* plants and *cer2-2* plants were probed with the cDNA insert of EST 154C7T7. The position of an RNA length marker in kilobases is indicated at right. WT, wild type; M, *cer2-2* mutant.

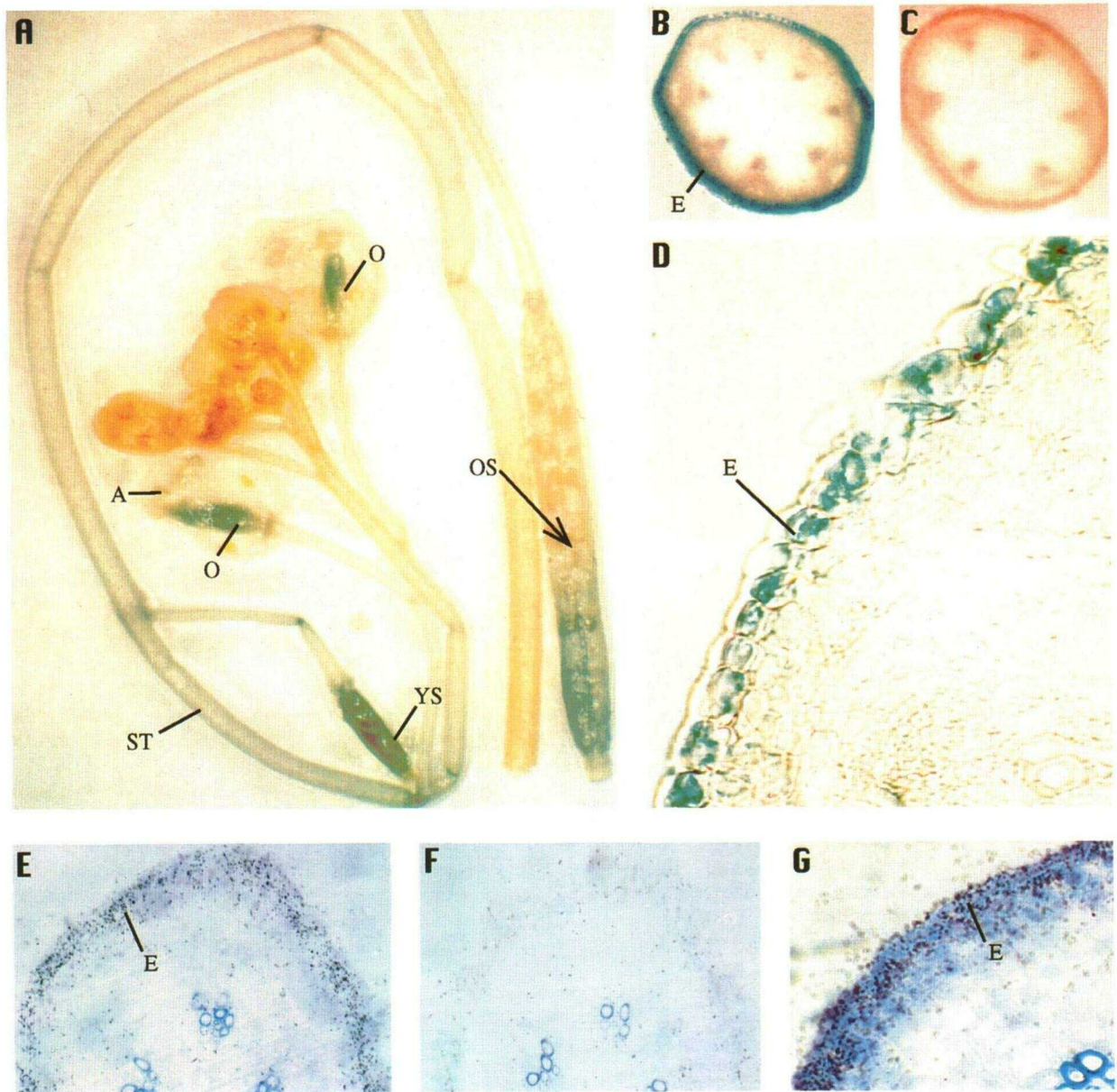


Figure 7. Tissue-Specific Expression of the *CER2* Gene.

(A) to (D) show the histochemical localization of *CER2* promoter activity in the aerial portions of transgenic plants harboring the *CER2*–*GUS* gene. (E) to (G) show the in situ localization of the *CER2* mRNA in cross-sections of stems.

(A) GUS activity is detectable in stems (ST), silques (OS and YS), ovaries (O), and anthers (A). The arrow indicates a silique (OS) on which only the upper but not the lower portion exhibits GUS staining. OS and YS designate silques of ~6- and 1.5-mm lengths, respectively. This *Arabidopsis* stem was folded into a spiral and stained in the absence of ferric ions.

(B) In a freehand-cut cross-section of a stem, GUS activity can be detected in the epidermis (E) after staining in the presence of ferric ions.

(C) GUS activity cannot be detected in a freehand-cut cross-section of a stem from a nontransgenic plant.

(D) Shown is a close-up view of GUS staining in the epidermis (E) of a 12-μm-thick section of a stem.

(E) Shown is in situ hybridization of a ³⁵S-labeled *CER2* antisense RNA probe with a cross-section of a stem. Hybridization signals are visible as dark silver grains in the epidermis (E); the blue color is the result of staining with toluidine blue for 1 min to reveal histological features (see Methods).

(F) In situ hybridization of a stem cross-section with a ³⁵S-labeled *CER2* sense RNA probe is shown. Hybridization signals are not detectable. For histological visualization, the specimen was stained with toluidine blue for 1 min.

(G) Shown is a close-up view of in situ hybridization of a ³⁵S-labeled *CER2* antisense RNA probe with a cross-section of a stem. Hybridization signals are detected in the epidermis (E). For histological visualization, the specimen was stained with toluidine blue for 2 min.

The magnification for (A) is $\times 4.7$, for (B) and (C) $\sim \times 45$, for (E) and (F) $\sim \times 80$, and for (G) $\sim \times 200$.

Under these conditions, GUS staining was not detectable in the vascular system and was barely detectable in anthers (data not shown); however, GUS staining confirmed that the *CER2-GUS* gene is expressed in the epidermis of developing siliques and those portions of stems that are undergoing elongation.

DISCUSSION

CER2 Encodes a Novel Protein

We conducted a chromosome walk on chromosome 4 from the RFLP marker m600 to beyond the *CER2* locus. The resulting 2.5-cM YAC contig spans ~800 kb. Three lines of evidence support the identification of the *CER2* gene from this contig. First, two overlapping genomic fragments and a cDNA encoded by these fragments complement the *cer2* phenotype upon transformation into *cer2-2* Arabidopsis plants. Second, the DNA sequence of the *cer2-2* mutant allele contains a nucleotide substitution relative to the wild-type allele that results in a premature stop codon in the encoded mRNA. Third, the *CER2* sequence is similar to *g/2* (Tacke et al., 1995), a maize gene involved in cuticular wax accumulation. While this paper was being revised, Negruk et al. (1996) reported the cloning of the *CER2* locus via a T-DNA tagging approach.

The nucleotide sequence of the *CER2* gene is predicted to encode a 421-amino acid protein. Apart from GL2, this protein does not exhibit sequence similarity to any known sequence with a defined biochemical function. The *CER2* protein does not appear to contain a protein-sorting signal sequence. Although one algorithm predicts that the *CER2* protein contains a putative transmembrane region, this region contains several charged and polar residues, making it unlikely to be part of an integral membrane protein. Hence, it is most likely that the *CER2* protein is localized in the cytoplasm. We are currently testing this hypothesis via immunolocalization experiments.

The *cer2-2* allele carries a mutation that results in a truncated protein that is most likely nonfunctional. Hence, *cer2-2* probably is a null allele. The steady state level of *CER2* transcripts is considerably reduced in *cer2-2* plants relative to that of wild-type plants. Because the only difference between the sequenced *cer2-2* allele and the wild-type allele is the nucleotide substitution at position +1150, it is likely that the premature stop codon in the *cer2-2* mutant allele results in a transcript with reduced stability relative to the wild-type transcript.

Function of the *CER2* Gene in Cuticular Wax Accumulation

In plants, de novo fatty acid biosynthesis occurs in plastids, using acetyl-coenzyme A and malonyl-acyl carrier protein (ACP) as precursors. The acetate moiety is elongated, two carbons at a time, to stearate via a series of condensation reactions

catalyzed by the fatty acid synthase system of enzymes (reviewed in Ohlrogge et al., 1993). Each elongation cycle includes the condensation of acyl-ACP with malonyl-ACP, the reduction and subsequent dehydration of the derived β -ketoacyl-ACP, and finally the reduction of the enoyl-ACP. The condensation reactions involved in the elongation of fatty acids from two to 18 carbons are catalyzed by the sequential action of three isozymes of keto-acyl synthase (KAS, i.e., KASIII, KASI, and KASII). The acyl group is then released from ACP by a thioesterase, thereby terminating the elongation reactions. The preferred substrate of the thioesterase present in most tissues is oleoyl-ACP. However, epidermal cells contain an additional thioesterase with a preference for stearoyl-ACP (Liu and Post-Beittenmiller, 1995). Thus, in epidermal cells, the released stearate serves as a substrate for elongation to form VLCFAs. These elongation reactions are thought to be catalyzed by an elongase system in a manner similar to de novo fatty acid biosynthesis (Lessire et al., 1989; Evenson and Post-Beittenmiller, 1995). This view is supported by the findings that the Arabidopsis gene *FAE1* (which is involved in the elongation of oleate [18:1] to 11-eicosenoate [20:1] and erucate [22:1] in seed) has sequence similarity to the gene coding for KASIII (James et al., 1995), and the maize gene *g/8* (which is involved in cuticular wax biosynthesis) has sequence similarity to the genes coding for β -ketoacyl reductases (X. Xu, C. Dietrich, M. Delledonne, T.-J. Wen, Y. Xia, D.S. Robertson, B.J. Nikolau, and P.S. Schnable, manuscript in preparation). Thus, these findings indicate that the elongase system required for the biosynthesis of VLCFAs is composed of heteromeric subunits. However, because this system has not been purified, its structural organization remains unknown.

The predominant constituents of cuticular wax isolated from Arabidopsis stems are derived from a fatty acid with a chain length of 30 carbons (Hannoufa et al., 1993; Jenks et al., 1995). These constituents include (listed in descending order of their contribution to total wax) alkanes, primary alcohols, symmetric ketones, aldehydes, fatty acids, and secondary alcohols (Hannoufa et al., 1993). According to the current view, these fatty acid derivatives are biosynthesized via a bifurcated pathway in which fatty acids are reduced to the corresponding aldehyde, which can then be reduced further to a primary alcohol or decarbonylated to an alkane. Subsequent modifications of the alkanes give rise to the symmetric ketones and secondary alcohols.

The total amount of cuticular wax present on stems of *cer2* plants is ~40% of that found on wild-type stems (Hannoufa et al., 1993). This reduction occurs primarily in aldehyde, alkane, secondary alcohol, and ketone constituents. In contrast, additional fatty acids accumulate, and the amount of primary alcohols remains approximately unchanged. The predominant chain length of all of these constituents is two to four carbons shorter than that of the wild type (Hannoufa et al., 1993).

Hence, it appears that the *cer2* mutation blocks the terminal or the terminal two steps of VLCFA elongation. The effect of this block is to reduce the chain lengths of all the fatty

acid-derived constituents. Because the absolute amounts of the alkanes, ketones, and secondary alcohols are reduced by the *cer2* mutation, it appears that the decarbonylase is specific for C30 aldehyde. This prediction is further supported by the near-absence of C28-derived alkanes, ketones, and secondary alcohols in wild-type wax, even though C28 aldehydes constitute a significant portion (20%) of the aldehyde pool in this wax (Hannoufa et al., 1993).

In contrast, it appears that the aldehyde reductase is not as sensitive to reductions in the chain length of its substrate. The evidence for this hypothesis is the finding that the total amount of primary alcohol is relatively unchanged by the *cer2* mutation, even though the average chain length of the aldehyde pool is reduced. Additional evidence for this hypothesis is provided by the finding that a significant proportion of the primary alcohols present in wild-type wax have chain lengths of C28 and C26 (Hannoufa et al., 1993).

Based on the observation that chain length is reduced in all of the constituents of *cer2* wax, it has been proposed that the *CER2* gene encodes an elongase (McNevin et al., 1993). In contrast, Jenks et al. (1995) have proposed that the *CER2* gene product may be a stem-specific regulator of the elongase system. Because the predicted *CER2* protein does not have sequence similarity to any of the enzymes required for de novo fatty acid biosynthesis (genes for all of which have been cloned), either the *CER2* protein catalyzes one of the component reactions of the elongase, but has a novel structure, or it encodes a novel molecular function, perhaps of a regulatory nature. Because the *FAE1* and *GL8* proteins exhibit high degrees of sequence similarity to the corresponding enzymes involved in de novo fatty acid biosynthesis, the first of these possibilities is unlikely. In either instance, the function of the *CER2* protein must be relatively specific for the terminal elongation steps.

The predominant constituents of the cuticular wax that accumulate on maize seedling leaves are derived from 32-carbon fatty acids. Seedlings homozygous for the *gl2* mutation accumulate waxes that are two to four carbons shorter (Bianchi, 1978). Hence, even though the chemical compositions of the cuticular waxes of maize seedling leaves and *Arabidopsis* stems and siliques are quite different, the mutations at the *gl2* and *CER2* loci of both maize and *Arabidopsis* affect the terminal elongation reactions in VLCFA biosynthesis. Thus, the structurally similar *CER2* and *GL2* proteins share a similar (but not identical) function. This is particularly interesting because even though the cuticular waxes on *Arabidopsis* leaves are derived from 32- (like those of maize) and 34-carbon fatty acids (Hannoufa et al., 1993; Jenks et al., 1995), the *cer2* mutation does not affect the constituents of leaf waxes. This specificity is further supported by the inability of the *CER2* gene promoter to express the *GUS* reporter gene to detectable levels in leaves.

Expression of the *CER2* Gene Is Tissue and Organ Specific and Developmentally Regulated

Expression of the *CER2* gene was analyzed via in situ hybridization and in transgenic *Arabidopsis* plants harboring a

chimeric construct consisting of the *CER2* promoter and part of the *CER2* coding region fused to the *GUS* reporter gene. In situ RNA localization experiments demonstrated that the *CER2* mRNA accumulates specifically in the epidermis. Analyses of the reporter gene construct revealed that the *CER2-GUS* gene is expressed in the epidermis of developing siliques and in those portions of stems that are undergoing elongation. This demonstrates that the tissue-specific accumulation of *CER2* mRNA is correlated generally with the phenotype conditioned by *cer2* mutations. Because in situ hybridization reveals the same tissue specificity as does *GUS* staining of transgenic plants, the 1.2-kb HindIII-BamHI fragment of the *CER2* gene used to construct p*CER2-GUS* must include all of the genetic information required for the epidermis-specific accumulation of *CER2* mRNA.

The *cer2* mutation does not affect leaf waxes (Jenks et al., 1995). Consistent with this observation, *GUS* activity was undetectable in the leaves of transgenic plants harboring the *CER2-GUS* gene. Together, these results suggest that the *CER2* gene is not involved in the accumulation of the cuticular waxes of leaves. However, because the cuticular wax of wild-type *Arabidopsis* leaves consists of VLCFAs and their derivatives having chain lengths of up to 34 carbons, we expect a *CER2*-like function to be required. However, based on the inability to readily detect *CER2*-cross-hybridizing sequences in DNA gel blots, this analogous function is not encoded by a gene with a high degree of sequence similarity to *CER2*.

METHODS

Plant Materials and Isolation of Genetic Recombinants

An *Arabidopsis thaliana* Landsberg *erecta* (*Ler*) genetic marker line carrying the recessive markers *BREVIPELCELLUS* (*BP*), *ECERIFERUM2* (*CER2*), and *APETALA2* (*AP2*) (Koornneef, 1987) was crossed to a Columbia stock carrying the mutant *IMMUTANS* (*IM*) (Wetzel et al., 1994). The resulting *F*₁ plants were allowed to self-pollinate. Random *F*₂ plants were also allowed to self-pollinate. The resulting *F*₃ families were scored for recombinant phenotypes. Codominant cleaved amplified polymorphic sequences (CAPS) mapping using primers based on the *AGAMOUS* (*AG*) sequence (see Konieczny and Ausubel, 1993 for primer sequences and conditions) was used in some instances to score *F*₂ plants and thereby identify recombinants with breakpoints between *AG* and *CER2*. The selected *F*₂ plants were allowed to self-pollinate. Analysis of the resulting *F*₃ families provided genotypic data for the *IM* locus. The *cer2-2* mutant allele was generated via ethyl methanesulfonate mutagenesis of the *Ler* ecotype (Koornneef et al., 1989) and was obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH) (stock No. CS8). The *Arabidopsis* ecotypes *Ler* and Columbia, the *Ler* marker line, and the Columbia *IM* marker line were obtained from S. Roderick and D. Voytas (Iowa State University). *Arabidopsis* plants were grown at 23°C, under 16 hr of daylight and 8 hr of dark.

Isolation and Analysis of *Arabidopsis* Nucleic Acids

Arabidopsis DNA was isolated from 20- to 30-day-old plants by using a modified cetyltrimethylammonium bromide procedure (Saghai-Maroo

et al., 1984). Digestions with restriction enzymes were conducted according to the manufacturer's specifications (Promega). Genomic DNA (0.5 µg) was loaded per lane and subjected to electrophoresis. RNA was isolated from the aerial parts of soil-grown adult plants, including leaves, stems, young siliques, and inflorescences via the method of Dean et al. (1985). Poly(A)-enriched RNA was isolated using the Poly-A-Tract mRNA Isolation System III (Promega, Madison, WI). Fifteen micrograms of RNA and 150 ng of poly(A)-enriched RNA were loaded per lane and subjected to electrophoresis. Labeling of DNA fragments with phosphorus-32, electrophoresis, blotting of nucleic acids, and hybridizations were conducted according to standard procedures (Sambrook et al., 1989).

Isolation of λ Clones

An Arabidopsis λ library prepared from genomic DNA isolated from the *Ler* ecotype (Voytas et al., 1990) was screened according to standard procedures (Sambrook et al., 1989).

Yeast Artificial Chromosome (YAC) Manipulations and Subcloning of YAC Ends

The EG1, EW, ABI-1, and CIC YAC libraries (Ward and Jen, 1990; Grill and Somerville, 1991; Creusot et al., 1995) were screened as described by Gibson and Somerville (1992). Yeast chromosomes were separated as described previously (Ausubel et al., 1994). The YAC DNA from agarose slices was purified by electroelution (Sambrook et al., 1989) or by using the GeneClean Kit (Bio-101, Vista, CA). YAC ends were subcloned via plasmid rescue (Burke et al., 1987) or λ subcloning. For λ subcloning, the recovered YAC DNA was digested by using the restriction enzyme HindIII, ligated to the λ insertion vector NM1149 (Murray, 1983), and in vitro packaged by using Gigapack II (Stratagene, La Jolla, CA). To isolate the subclones containing the YAC ends, the resulting λ subclone libraries were screened by using DNA fragments of pYAC4 flanking the cloning site as the centromeric end (the 0.5-kb HindIII-EcoRI fragment of pYAC4) and *URA3* containing end (the 3-kb HindIII-EcoRI fragment of pYAC4) probes following standard procedures (Sambrook et al., 1989).

Polymerase Chain Reaction Primers and Conditions

The primers and conditions for CAPS mapping at the *AG* locus were described by Konieczny and Ausubel (1993). The paired primers p1 and p3, p2 and p5, and p4 and p6 were used to polymerase chain reaction (PCR) amplify the *cer2-2* mutant allele. The primers were synthesized at the Iowa State University Nucleic Acid Facility by using a 394 DNA/RNA synthesizer from Applied Biosystems (Foster City, CA). The sequences of the *CER2* primers (and their laboratory designations) are as follows: p1 (*cer2*-up), 5'-AGGTGGACGTAATAAGTGTG-3'; p2 (yx757), 5'-GGTGTGCCTGCTTCTTTGGTA-3'; p3 (yx758), 5'-AAATCGAACCCTTCCCACTG-3'; p4 (yx1293), 5'-GAGGATGATAGAACAAGATAAGG-3'; p5 (yx1136), 5'-GGCATCACTACACTTACATCCT-3'; and p6 (*cer2*-down), 5'-CAGTGACACCAACAAGAACA-3'.

Amplification reactions were conducted in 50-µL volumes containing 50 to 100 ng of genomic DNA and 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 150 µM deoxynucleotide triphosphates, and 0.5 µM of each primer. The reactions were overlaid with 100 µL of mineral oil and denatured at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 54°C (for

the p1/p3 primer pair) or 58°C (for the p2/p5 and p4/p6 primer pairs) for 45 sec, and extension at 72°C for 1 to 2 min. The reactions were given a final extension at 72°C for 10 min to complete the elongation.

Construction of pG1H, pG1RSc, pG1C1, and p*CER2-GUS* and Plant Transformation

DNA fragments from the genomic clone G1 and the entire insert from the cDNA clone (expressed sequence tag [EST] 154C7T7) were subcloned in pBI121 (Clontech, Palo Alto, CA) to generate pG1H, pG1RSc, and pG1C1. To construct p*CER2*- β -glucuronidase (*GUS*), the 1.2-kb HindIII-BamHI fragment of pG1H was subcloned into the HindIII-BamHI cloning sites of pBI101.3 (Clontech). The resulting plasmids were introduced into *Agrobacterium tumefaciens* C58C1 (Koncz and Schell, 1986) via a freeze-thaw transformation procedure (An et al., 1988).

Agrobacterium-mediated root explant transformation was conducted according to the procedure of Huang and Ma (1992). For the vacuum infiltration transformation, a laboratory protocol from the P. Green Laboratory (Michigan State University, East Lansing) (A. van Hoof and P. Green, manuscript submitted for publication), an adaptation of the method of Bechtold et al. (1993), was used. Seed harvested from the vacuum infiltration-treated plants (*T*₀) in same pot were bulked. Transgenic plants (*T*₁) from different bulks were considered to represent independent transformation events.

Analysis of *GUS* Activity

Histochemical analyses of *GUS* activity with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid were performed as described previously (Jefferson, 1987) with modifications proposed by De Block and Debrouwer (1992). To monitor *CER2-GUS* expression in cross-sections, stems were harvested, cut into 0.5- to 1-cm lengths, and incubated for 10 hr in the presence of 2 mM potassium ferricyanide to reduce background staining in vascular tissues caused by peroxidase activity. Chlorophyll was removed with ethanol from freehand-cut sections. For thinner sections, stained stems were washed in 50 mM phosphate buffer, pH 7.0, fixed in 20% ethanol, 5% formaldehyde, and 5% acetic acid for 2 hr, dehydrated with an ethanol and *t*-butanol series, embedded in Paraplast+ (Fisher, Itasca, IL), and cut into 12-µm sections with a microtome.

In Situ Hybridization

Stems were harvested and cut into 0.5- to 1-cm sections, fixed with FAA (50% ethanol, 5% acetic acid, 10% formalin), dehydrated with an ethanol and *t*-butanol series, and embedded in Paraplast+. Sections (8 µm) were cut with a microtome and placed on poly-L-lysine-coated slides. To prepare the riboprobes, the 0.47-kb XhoI-SacII fragment of pG1C1 was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA) to generate pG1C1XSc. T3 (antisense transcript) and T7 (sense transcript) polymerases were used to generate 35S-labeled riboprobes from linearized pG1C1XSc following the manufacturer's protocol (Promega). Prehybridization treatment, hybridization conditions, and post-hybridization treatments were performed as described previously (Raikhel et al., 1989). Slides were coated with Kodak NBT-2 emulsion and exposed at 4°C. Specimens were developed after a 5-day exposure and counterstained with 0.015% toluidine blue for 1 or 2 min.

DNA Subcloning, Sequencing, and Analysis

For sequencing, various fragments of the *CER2* genomic clone G1 were subcloned into pBluescript KS+ and pBluescript SK+ (Stratagene). The cDNA clone (EST 154C7T7) was subcloned into the Sall-HindIII sites of pGem3fz(-) (Promega). PCR-amplified fragments for sequencing were purified by electroelution (Sambrook et al., 1989). DNA sequencing of plasmids and PCR products was performed at the Iowa State University Nucleic Facility by using the double-stranded dye terminator technique on an automated DNA sequencer (model 373; Applied Biosystems). In all instances, both DNA strands were sequenced. Sequence comparisons and analyses were performed using the Genetics Computer Group (Madison, WI) sequence analysis software package (version 8) and the PSORT, TMpred, BLAST, and FASTA algorithms, as described previously (Pearson and Lipman, 1988; Altschul et al., 1990; Nakai and Kanehisa, 1992; Hofmann and Stoffel, 1993).

Scanning Electron Microscopy

Scanning electron microscopy examinations of stem cuticular wax crystals were conducted at the Iowa State University Bessey Microscopy Facility. Samples were frozen in liquid nitrogen by using a cryo-system (model SP2000A; EMScope Laboratories, Kent, UK), coated with gold, and observed with a scanning electron microscope (model JSM-35; JOEL, Tokyo, Japan) at 15 kV. Samples were analyzed from two *Ler* wild-type plants, one *cer2-2* mutant plant, one transgenic plant harboring pG1H and another harboring pG1RSc, and two transgenic plants harboring pG1C1.

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