

# The *glossy1* Locus of Maize and an Epidermis-Specific cDNA from *Kleinia odora* Define a Class of Receptor-Like Proteins Required for the Normal Accumulation of Cuticular Waxes<sup>1</sup>

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Mutations at the *glossy1* (*gl1*) locus of maize (*Zea mays* L.) quantitatively and qualitatively affect the deposition of cuticular waxes on the surface of seedling leaves. The *gl1* locus has been molecularly cloned by transposon tagging with the *Mutator* transposon system. The *epi23* cDNA was isolated by subtractive hybridization as an epidermis-specific mRNA from *Senecio odora* (*Kleinia odora*). The deduced amino acid sequence of the GL1 and EPI23 proteins are very similar to each other and to two other plant proteins in which the sequences were deduced from their respective mRNAs. These are the Arabidopsis CER1 protein, which is involved in cuticular wax deposition on siliques, stems, and leaves of that plant, and the protein coded by the rice expressed sequence tag RICS2751A. All four proteins are predicted to be localized in a membrane via a common NH<sub>2</sub>-terminal domain, which consists of either five or seven membrane-spanning helices. The COOH-terminal portion of each of these proteins, although less conserved, is predicted to be a water-soluble, globular domain. These sequence similarities indicate that these plant orthologs may belong to a superfamily of membrane-bound receptors that have been extensively characterized from animals, including the HIV co-receptor fusin (also termed CXCR4).

The cuticle is the outermost interface between a plant and its environment, and as such plays a crucial role in the plant's survival (Martin and Juniper, 1970; Kolattukudy, 1981). It consists of cutin embedded in a complex mixture of lipids commonly referred to as the cuticular waxes. Cutin is a polymer of fatty acid derivatives such as  $\omega$ -, di-, and trihydroxy and hydroxy epoxy fatty acids and dicarboxylic fatty acids. These monomers are polymerized by

intermonomeric ester linkages. Cuticular waxes are a complex mixture of lipids, including hydrocarbons (*n*-alkanes, branched alkanes, cyclic alkanes, and alkenes), ketones, ketols, alcohols, aldehydes, diols, acids, and esters. The majority of the lipid compounds in the cuticle are derived from cellular fatty acids (for reviews, see Kolattukudy and Walton, 1973; Kolattukudy et al., 1976; Tulloch, 1976; Kolattukudy, 1980). Early biochemical investigations have identified reactions by which many of the various components of the cuticular wax are synthesized (Kolattukudy and Walton, 1973; Kolattukudy et al., 1976; Tulloch, 1976; Kolattukudy, 1980). However, the isolation and characterization of enzymes postulated to be involved in these processes has been technically difficult to achieve because plant epidermal tissue, the site of cuticular wax biosynthesis (Kolattukudy et al., 1976), is difficult to obtain in large quantities. To overcome this technical barrier, a molecular genetic approach was taken to isolate a gene involved in cuticular wax biosynthesis. Using the *Mutator* transposon system, we have cloned the *glossy1* locus of maize (*Zea mays* L.) via transposon tagging (Chandler and Hardeman, 1992). The sequence of the GL1 protein is similar to a *Kleinia odora* protein in which the transcript accumulates mainly in the epidermis. These proteins have predicted structures similar to those of a class of membrane receptors characterized in animals.

## MATERIALS AND METHODS

The original, stable reference allele of *gl1* (*gl1-ref*) was obtained from the collection of D. Robertson (Iowa State University, Ames). A maize (*Zea mays* L.) *Mutator* (*Mu*) stock containing a *gl1* allele (*gl1-755048*) was previously described (Schnable et al., 1994). This stock was crossed to the *gl1-ref* stock to confirm allelism, and out-crossed to a non*Mutator* recurrent parent (standard Q60, a hybrid of the two inbreds Q66 and Q67) for three generations to generate a stock that carried *gl1-755048* and few copies of the *Mu* transposons. Additional independent *gl1-Mu* alleles were generated by crossing a *Mu* stock with the *gl1-ref* stock and

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Abbreviation: EST, expressed sequence tag.

screening for rare glossy seedlings among the progeny (Schnable et al., 1994). The *Mu* stocks used in this transposon-tagging experiment have a genetic background derived primarily from standard Q60 and standard B70. Standard B70 is an F<sub>1</sub> hybrid of the inbreds B77 and B79.

The upper and lower epidermal layers of young, greenhouse-grown leaves of *Kleinia odora* (Forssk) DG. (or *Senecio odora* [Forssk] Defl.) were isolated by peeling (Kollattukudy, 1968). The epidermal layers and nonepidermal (internal) tissues containing mesophyll and vascular cells were separately frozen in liquid nitrogen and stored at -70°C.

Probes specific for the transposons *Mu1*, *Mu3*, and *Mu8* were isolated from plasmids pRB1 (Robertson et al., 1988), pKD121, and p713 (Chandler and Hardeman, 1992), respectively. A maize B73 cDNA library prepared from 2-week-old green seedlings was provided by Alice Barkin (University of Oregon, Eugene). Genomic and cDNA fragments were subcloned in pBluescript plasmid vectors (Stratagene).

### Scanning Electron Microscopy

Scanning electron microscopy examinations of the surfaces of maize seedlings were conducted at the Bessey Microscopy Facility (Iowa State University of Science and Technology, Ames). Samples were frozen in liquid nitrogen using a cryo-system, coated with gold, and observed at 15 kV using a scanning electron microscope (JSM-35, JEOL, Akishima, Japan).

### Isolation and Analysis of Nucleic Acids

Maize, plasmid, and phage DNAs were isolated by the cetyltrimethylammonium bromide (Rogers and Bendich, 1985), alkaline lysis (Zhou, 1990), and plate lysis (Maniatis et al., 1982) methods, respectively.

DNA was digested with restriction enzymes, as suggested by the manufacturers. DNA restriction fragments were fractionated by electrophoresis and subjected to Southern-blot analyses by standard protocols (Southern, 1975; Wahl et al., 1979). DNA fragments were radioactively labeled by the incorporation of <sup>32</sup>P from [ $\alpha$ -<sup>32</sup>P]dCTP using a random priming method of DNA synthesis (Feinberg and Vogelstein, 1983).

RNA was isolated from plant tissues by an SDS-phenol extraction procedure (Jones et al., 1994). Isolated RNA was fractionated by electrophoresis in formaldehyde-containing agarose gels and subjected to northern-blot analyses by standard methods (Maniatis et al., 1982).

### Molecular Cloning

DNA isolated from maize leaves was digested with *Hind*III, size-fractionated by agarose electrophoresis, and cloned into the  $\lambda$  phage vector  $\lambda$ DASHII (Stratagene).

Poly(A)<sup>+</sup> RNA samples (5  $\mu$ g) extracted separately from the epidermis and the epidermal tissues of *K. odora* leaves were converted to double-stranded, blunt-ended cDNAs using the Librarian cDNA Synthesis kit (Invitrogen, San Diego, CA). Whereas the cDNA synthesized from nonepidermal tissue was digested with *Alu*I and *Rsa*I, the cDNA

synthesized from epidermal tissue was ligated to the *Eco*RI adaptors (Invitrogen). After denaturation, epidermal cDNA (0.1  $\mu$ g) was hybridized with a 50-fold excess of nonepidermal tissue cDNA in a 50% formamide solution containing 5 $\times$  SSPE, 10 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% SDS, and 0.2 mg/mL yeast tRNA at 37°C for 18 h. The hybridization mixture was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. The precipitated DNA was collected by centrifugation, dissolved in water, and ligated to *Eco*RI-digested  $\lambda$ gt11 arms (Stratagene). In this manner cDNAs representing mRNAs that accumulate to higher levels in the epidermis of *K. odora* leaves were cloned into  $\lambda$ gt11.

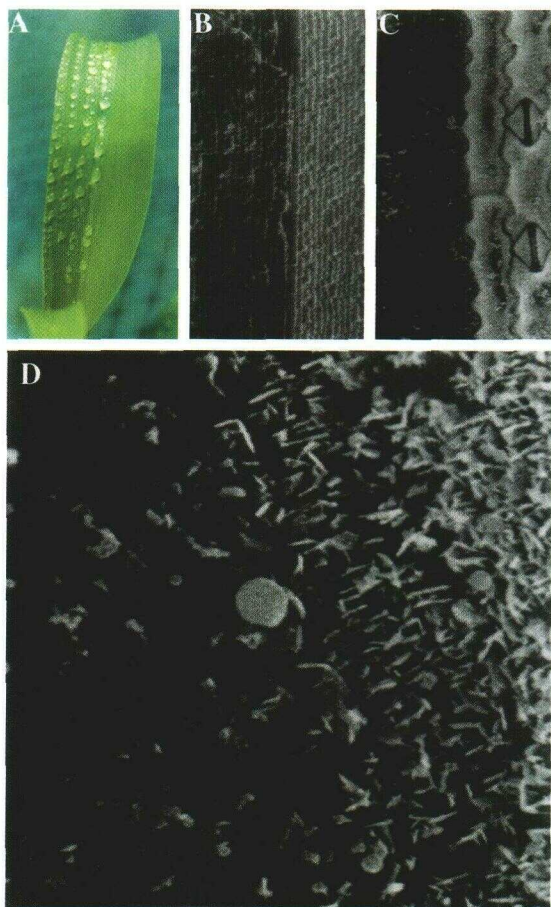
The resulting cDNA library was plated at a density of approximately 5000 plaques on each plate, and duplicate lifts made on nitrocellulose membranes were subjected to hybridization screening with two different probes. The probes were the <sup>32</sup>P-labeled first-strand cDNAs synthesized from the epidermal or the nonepidermal poly(A)<sup>+</sup> RNAs using reverse transcriptase (BRL) and [ $\alpha$ -<sup>32</sup>P]dATP. Prehybridization and hybridization were carried out by standard techniques (Maniatis et al., 1982). Plaques that gave stronger signals with the epidermal probe were purified for further analysis.

### DNA Sequencing

Both strands of all clones were sequenced either at the Iowa State University Nucleic Acids Facility with automated ABI sequencing equipment (using either T3 or T7 primers), or with a Sequenase kit (version 2.0, United States Biochemical). Sequence data were analyzed by the Genetics Computer Group software package (University of Wisconsin, Madison).

## RESULTS

The isolation of the *gl1-ref* allele (Hayes and Brewbaker, 1928) defined a new class of maize mutants that accumulate less cuticular wax on the surfaces of their seedling leaves than do wild-type seedlings. To date, 18 *gl* loci have been defined by mutations in maize (Schnable et al., 1994). Seedlings homozygous for any of these mutations have bright-green leaves because they have less of the whitish wax bloom that is present on wild-type seedlings. In addition, the leaves of such seedlings retain applied water droplets on their surfaces (Fig. 1A). In contrast, relatively forcefully applied water "runs and bounces off" the leaves of wild-type seedlings. The degree of water adherence on the surface of mutant leaves is characteristic of mutants of a particular locus, e.g. *gl1* and *gl8* seedlings are readily identifiable as glossy, but *gl18* "glossiness" is considerably more subtle. Scanning electron microscopy observations (Fig. 1; Lorenzoni and Salamini, 1975; Schnable et al., 1994) and chemical analyses of the cuticular wax component of *gl* plants (Bianchi et al., 1989) have revealed that mutations at each *gl* locus alter not only the amount of cuticular wax on the surface of the seedling but also the composition and physical appearance of the wax crystals.



**Figure 1.** Visualization of the *gl1* phenotype. The *gl1* phenotype is expressed on the first five or six leaves of maize seedlings and is most easily visualized by the retention of water droplets on the surfaces when these leaves are sprayed with water. A, Water was sprayed on the first leaf of a heterozygous (*G1/g1-ref*) maize seedling. Within this seedling a somatic mutation of an unknown nature generated leaf sectors within which the mutant phenotype was revealed; tissue expressing the *gl1* phenotype retains water droplets (left side of the leaf), whereas water is repelled from the surface of wild-type tissue (right side of the leaf). Examination of the interface between wild-type (right side of scan) and mutant (left side of scan) tissue of the leaf shown in A by scanning electron microscopy at 24 $\times$  (B), 160 $\times$  (C), and 4000 $\times$  (D) magnification. The whitish material that is present on the right side of each panel is the cuticular wax, which is essentially absent from the surface of the mutant tissue.

To obtain a molecular and biochemical understanding of cuticular wax biosynthesis, we collected mutant alleles of all of the known *gl* loci, and undertook an extensive transposon mutagenesis program to isolate the new alleles of many of these loci (Schnable et al., 1994). This collection includes 15 independently generated *gl1* alleles isolated from genetic stocks that carry the *Mutator* transposon system.

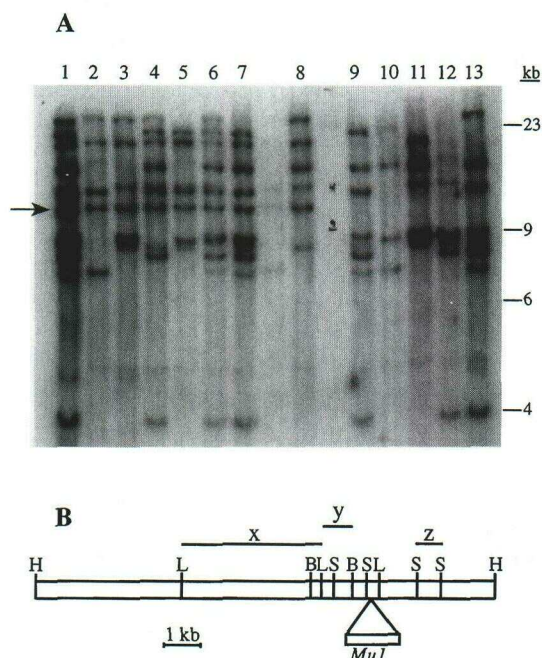
#### Molecular Cloning of the *gl1* Locus of Maize

A *Mutator* maize line carrying the *gl1*-755048 allele (Schnable et al., 1994) was used for more extensive studies. Because this allele was isolated from a *Mutator* stock it may have arisen via the insertion of a *Mu* transposon. Since

*Mutator* maize lines contain multiple copies of *Mu* transposons, to molecularly isolate the *gl1*-755048 allele it was first necessary to identify the individual *Mu* transposon responsible for the mutation. To reduce the number of *Mu* copies in the line containing the *gl1*-755048 allele, backcrosses to a non*Mutator* line were performed for three generations. In each generation the individual progeny plant that carried the *gl1*-755048 allele and the fewest number of *Mu1* transposons was selected to continue the backcrossing program. The presence of the *gl1*-755048 allele was assayed via test crosses or self-pollinations. The number of *Mu1* transposons carried by individual plants was determined by Southern hybridization using a *Mu1*-specific probe. Following the backcrossing procedure, DNA samples from individual progeny of cross 1 were digested with various restriction enzymes that do not cut within *Mu* transposons and subjected to Southern hybridization analyses probing with individual *Mu* transposon-specific DNA fragments (*Mu1*, *Mu3*, or *Mu8*).

#### Cross 1: *gl1*-755048/*G1* $\times$ *G1*/*G1*

A 12.5-kb *Hind*III restriction fragment that hybridizes to *Mu1* and co-segregates with the *gl1*-755048 allele was identified among the progeny of cross 1 (Fig. 2A). This frag-



**Figure 2.** Identification of a *Mu1*-containing 12.5-kb *Hind*III DNA fragment that co-segregates with the *gl1*-755048 allele. A, Southern blot of *Hind*III-digested maize DNA probed with a *Mu1*-specific probe. The arrow indicates the 12.5-kb DNA fragment, which is present in all plants carrying the *gl1*-755048 allele and absent in sibling plants that do not carry this allele. Lanes 1 through 8, DNA from individual plants of genotype *gl1*-755048/*G1*; lanes 9 through 13, DNA from individual plants of genotype *G1*/*G1*. B, Restriction map of the 12.5-kb *Mu1*-containing *Hind*III insert from  $\lambda$ 1211. DNA fragments x, y, and z were used as the probes in later analyses. H, *Hind*III; L, *Sal*I; B, *Bam*HI; and S, *Sac*I.

ment was present in 96 individual progeny plants from cross 1 that carried the *gl1*-755048 allele and absent in 20 siblings that did not carry the *gl1*-755048 allele.

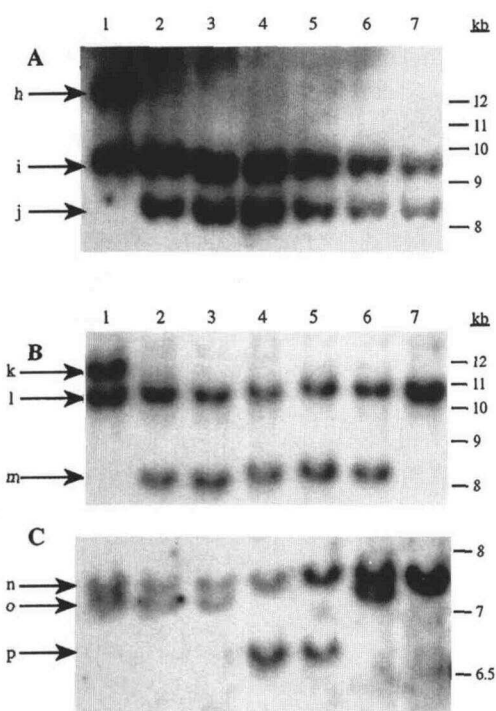
To clone the *Mu1*-containing *Hind*III fragment that co-segregates with the *gl1*-755048 allele, maize genomic DNA from a plant (92g4632) with the genotype *gl1*-755048/*gl1*-755048 was digested with *Hind*III and fractionated by agarose gel electrophoresis. DNA fragments between 11 and 13 kb were purified and cloned into the bacteriophage vector  $\lambda$ DASHIII. This size-selected partial genomic library was screened by plaque hybridization, and recombinant phage that hybridized to *Mu1* were isolated and further analyzed. From approximately 500,000 clones, 3 *Mu1*-hybridizing phage clones were found to contain the identical 12.5-kb *Hind*III insert. A restriction map of this cloned fragment is shown in Figure 2B.

Since most mutations caused by *Mutator* are the result of the insertion of a *Mu* transposon into a gene sequence (Brown et al., 1989), the DNA flanking the *Mu1* transposon in the cloned *Hind*III fragment may represent the *gl1* gene. When used as a probe on co-segregation blots of maize genomic DNA (such as the one in Fig. 2A), fragments y and z (Fig. 2B) each detect the 12.5-kb *Mu1*-containing *Hind*III fragment that is present in all plants carrying the *gl1*-755048 allele, whereas an approximately 1-kb-smaller fragment is detected in the sibling plants that do not carry this allele (data not shown). These data confirm that the cloned DNA fragment is the *Mu1*-hybridizing fragment that co-segregates with the *gl1*-755048 allele.

To verify that the cloned *Mu1*-containing fragment encompasses the *gl1* gene, DNA fragments flanking the *Mu1* transposon (fragments x and z in Fig. 2B) were used as probes in allelic cross-referencing experiments. In these experiments DNA from plants carrying independently generated *gl1*-*Mu* alleles and their progenitor *G11* allele were examined by Southern-blot analysis using DNA from the putative *gl1* clone as a probe. The alleles *gl1*-91g130 and *gl1*-91g132 were recovered as rare glossy plants from cross 2.

#### Cross 2: *G11/G11 Mutator* $\times$ *gl1-ref/gl1-ref*

In addition, wild-type siblings, which had the genotype *G11/gl1-ref*, were also recovered. Based on the genetic background of our *Mutator* stocks, any individual female parent of cross 2 carried potentially two different wild-type *G11* alleles (*G11*+Q66, *G11*+Q67, *G11*+B77, or *G11*+B79). One of these is the direct progenitor of the *gl1*-91g130 and *gl1*-91g132 alleles. DNA samples from individual progeny sibling plants from cross 2, with the genotypes *gl1*-91g132/*gl1-ref* or *G11/gl1-ref*, were subjected to Southern-blot analyses using fragment z as a probe (Fig. 3A). The wild-type progeny of cross 2 used in this experiment are segregating for the two wild-type *G11* alleles present in the female parent of the cross. As described above, one of these alleles must be the progenitor of *gl1*-91g132. The 9.5-kb *Hind*III fragment (band i) that hybridizes with fragment z and that is present in all of the plants that were examined represents the *gl1-ref* allele. The 8.5-kb *Hind*III fragment (band j), which is common to all plants that display a wild-type *G11* phenotype, represents one or both of the wild-type *G11*



**Figure 3.** Southern-blot analyses of allelic cross-referencing experiments. Appearance of a novel DNA fragment polymorphism detected by the putative *gl1* clone (fragment z from Fig. 2B) coincident with the *Mu*-induced mutations at the *G11* locus to create the *gl1*-91G132 (A) and *gl1*-91G130 (B) alleles. A, DNA samples from individual sibling plants with the genotypes *gl1*-91G132/*gl1-ref* (lane 1) or *G11/gl1-ref* (lanes 2–7) were digested with *Hind*III and subjected to Southern-blot analysis. Bands h, i, and j represent the alleles *gl1*-91G132, *gl1-ref*, and *G11*, respectively. B, DNA samples from individual sibling plants with the genotypes *gl1*-91G130/*gl1-ref* (lane 1) or *G11/gl1-ref* (lanes 2–6) were digested with *Hind*III and subjected to Southern-blot analysis. Lane 7 contains similarly analyzed DNA from a plant homozygous for the *gl1-ref* allele. Bands k, l, and m represent the alleles *gl1*-91G130, *gl1-ref*, and *G11*, respectively. C, DNA from the identical plants used in B, digested with *Kpn*I, subjected to Southern-blot analysis, and probed with fragment x (Fig. 2B). Band n represents the *gl1-ref* allele, and bands o and p represent two wild-type alleles (*G11*-Q66, *G11*-Q67, *G11*-B77, or *G11*-B79).

alleles derived from the female parent of cross 2. This hybridizing band is not present in plants carrying the *gl1*-91g132 allele and is replaced by a >12-kb hybridizing band (band h). Hence, band h may represent the *gl1*-91g132 allele.

Similar analyses of sibling plants from the family in which the *gl1*-91g130 allele was recovered are shown in Figure 3B. In these analyses band l, which is common to all siblings of this family (lanes 1–6) and to a plant homozygous for the *gl1-ref* allele (lane 7), represents the *gl1-ref* allele; band m, which is common to all siblings with the genotype *G11/gl1-ref* (lanes 2–6), represents one or both of the wild-type *G11* alleles derived from the female parent of cross 2; and the novel band k, which is present only in the *Mutator*-derived *gl1* plant (lane 1), probably represents the *gl1*-91g130 allele.

If these allelic cross-referencing experiments are to prove that fragment z hybridizes to the *gl1* locus, they must

demonstrate that the hybridizing fragment associated with a particular *gl1-Mu* allele differs from its wild-type progenitor. Figure 3, A and B, illustrates that DNA fragments that are detected by fragment z and that are present in seedlings carrying the *gl1-91g130* and *gl1-91g132* alleles differ from those present in their respective siblings that do not carry a *gl1-Mu* allele. However, within each family, all of the sampled siblings that did not carry a *gl1-Mu* allele exhibit the same hybridization pattern, whereas, based on the pedigree of our *Mutator* stocks, they would have been expected to have segregated for two potentially different wild-type alleles. Hence, the results of the allelic cross-referencing experiments can be interpreted in two ways.

First, it is possible that the data presented in Figure 3, A and B, merely illustrate incomplete sampling of the maternal chromosomes from cross 2. That is, the novel bands present in the seedlings that carry *gl1-91g130* and *gl1-91g132* may represent the segregation of the alternative wild-type allele contributed by the maternal parent of cross 2, which was not sampled in the five or six siblings that do not carry a *gl1-Mu* allele.

Alternatively, the novel bands present in the seedlings that carry *gl1-91g130* and *gl1-91g132* may indeed be associated with the novel alleles. If so, the siblings that do not carry a *gl1-Mu* allele must carry one or the other of the two wild-type alleles that are not distinguishable with the restriction probe/enzyme combination fragment z/*Hind*III.

To distinguish between these two interpretations, the DNA samples used in Figure 3, A and B, were analyzed further in an effort to identify a probe/enzyme combination that would detect two wild-type alleles among the sampled plants in each family. To conduct these experiments we utilized a probe (fragment x) that, although within 0.2 kb of the *gl1* gene, does not encompass it (see Fig. 2B and "Discussion").

Three hybridizing fragments (bands n, o, and p in Fig. 3C) were detected when the DNA samples used in Figure 3B were digested with *Kpn*I and probed with fragment x. The approximately 7-kb band n that was present in all of the examined plants was derived from the chromosome that carried the *gl1-ref* allele. The two remaining bands of 6.2 and 6.7 kb (bands p and o, respectively) segregate as alleles and are derived from two chromosomes from the female parent of cross 2. This result indicates that both maternal chromosomes from cross 2 are represented in this gel-blot analysis (and the blot shown in Fig. 3B). Hence, this finding demonstrates that the novel hybridizing band in lane 1 of Figure 3B represents the *gl1-91g130* allele. Similar data obtained with siblings of plants carrying the *gl1-91g132* allele (data not shown) established that the novel hybridizing band in lane 7 of Figure 3A represents the *gl1-91g132* allele. Hence, the finding that fragment z detects DNA rearrangements, which occur coincident with mutations at the *gl1* locus, demonstrates that fragment z cross-hybridizes with DNA fragments that include the *gl1* locus.

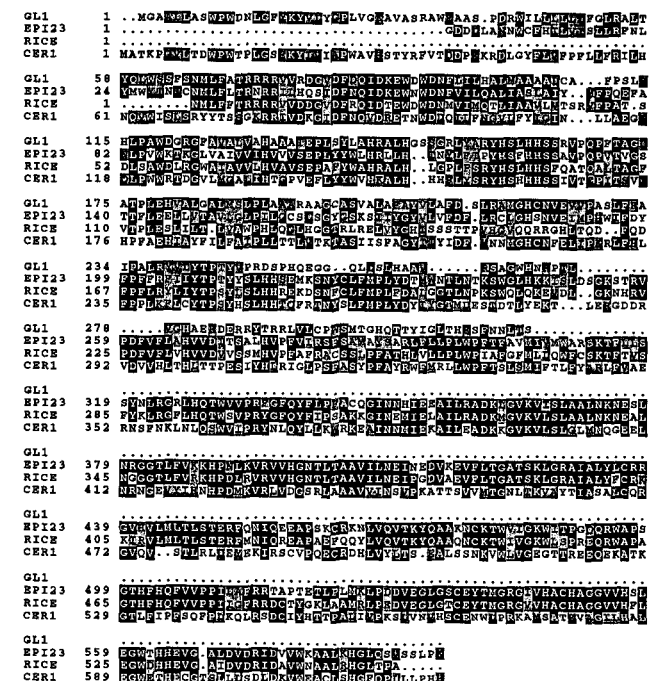
To determine the primary structure of the protein coded by the *gl1* gene, a cDNA library made from mRNA isolated from seedlings of the maize inbred B73 was screened by hybridization using a mixture of fragments y and z (Fig.

2B) as a probe. Two classes of cDNA clones, containing either 0.8- or 1.6-kb inserts, were isolated from a total of approximately 500,000 recombinant phage. Restriction mapping, cross-hybridization experiments, and partial sequence analysis established that the 0.8-kb cDNA is a partial clone that encompasses the 3' end of the 1.6-kb cDNA. All subsequent investigations were performed using the 1.6-kb cDNA clone.

The nucleotide sequence of this cDNA was determined and the amino acid sequence of the encoded protein deduced. The cDNA is 1585 bp in length and its sequence has all of the characteristics of a near-full-length copy of the *gl1* mRNA. The 3' end of the *gl1* mRNA is defined by a poly(A) sequence consisting of 18 nucleotides, 23 nucleotides upstream of which is the sequence ACCAAA, which is probably the polyadenylation signal (Wu et al., 1994). In addition, the 5'-most Met codon occurs 185 nucleotides from the 5' end of the clone. Furthermore, all three translational frames upstream of this codon contain stop codons. This Met codon initiates the longest open reading frame present on this clone and codes for a protein of 319 amino acids (Fig. 4) with an estimated molecular mass of 35.3 kD and a theoretical pI of 9.38.

### Molecular Cloning and Sequence of the *epi23* cDNA of *K. odora*

Since the epidermis is the site of synthesis of cuticular lipids (Kolattukudy, 1968), it should have unique mRNAs that code for enzymes involved in the biosynthesis of



**Figure 4.** Comparison of the deduced amino acid sequences of the proteins coded by maize *gl1* (GL1), *K. odora* *epi23* (EPI23), Arabidopsis *CER1* (CER1), and the rice EST RICS2751A (RICE). Identical residues are boxed in black; conservative substitutions are boxed in gray. Dots represent gaps that were inserted to maximize the alignment.

cuticular lipids. Therefore, in addition to the genetic studies described above, a parallel study was undertaken to biochemically identify and clone mRNAs that accumulate specifically in the epidermal cells of plant leaves. Since the epidermal layer of cells can be readily excised from the leaves of *K. odora* (Kolattukudy, 1968), mRNAs from these separated tissues were used to generate a cDNA library that represents the transcripts expressed in the epidermis.

This epidermis-enriched cDNA library was prepared as described in "Materials and Methods." Using differential hybridization screening, clones that hybridized strongly to an epidermis-derived probe were identified. One such clone was designated *epi23*. Subsequent northern analyses confirmed that this cDNA is a clone of an mRNA that preferentially accumulates in the epidermis of the leaf (see "Discussion").

The nucleotide and deduced amino acid sequences of the *epi23* clone were determined. The *epi23* clone contains an open reading frame coding for a polypeptide of 594 amino acids that shows a high degree of sequence similarity to the GL1 protein (Fig. 4). It is not clear whether the EPI23 open reading frame is complete. It lacks NH<sub>2</sub>-terminal sequences present in the *gl1* clone, and although there are two Met residues at the 25th and 33rd codons, the region immediately upstream of these residues is still in-frame with the open reading frame. The *epi23* cDNA lacks a consensus polyadenylation signal (AATAAA), but several other AT-rich regions are present that may serve this function. One of these potential polyadenylation signals is situated 25 nucleotides upstream from the 3' end of the *epi23* cDNA clone.

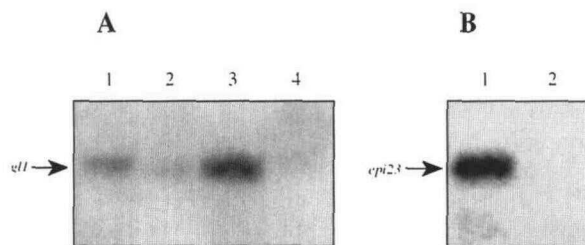
### Expression of *Gl1* and *epi23* in Leaves

To determine the effect of *gl1* mutations on the expression of this gene, RNA was isolated from seedling leaves (two-leaf stage) of the maize inbred B73 and standard Q60 that were homozygous for either a wild-type *Gl1* allele or the mutant *gl1-ref* or *gl1-755048* alleles, respectively. These RNAs were subjected to northern-blot analyses and probed with the *gl1* cDNA. A *gl1* mRNA of about 1.6 kb is readily detectable in plants carrying wild-type *Gl1* alleles; however, its accumulation is considerably reduced (at least 5-fold) in plants carrying either of the two mutant *gl1* alleles (Fig. 5A). In some experiments, the *gl1* cDNA hybridized to two transcripts; the origin of the second (larger) transcript, which was not consistently observed, is unclear.

With the ability to separate the epidermis from the internal tissues of *K. odora* leaves, we were able to examine the epidermis-specific accumulation of the *epi23* mRNA. Northern analysis of RNAs isolated from epidermal and nonepidermal tissues revealed that the 2.2-kb *epi23* mRNA accumulates to approximately 50-fold higher levels in the epidermis relative to the internal tissues of the leaf when probed with the *epi23* cDNA (Fig. 5B).

### Predicted Structure of GL1, EPI23, and Other Similar Proteins

The GL1 and EPI23 proteins show sequence similarity to the proteins encoded by the Arabidopsis EST 178C3T7 and



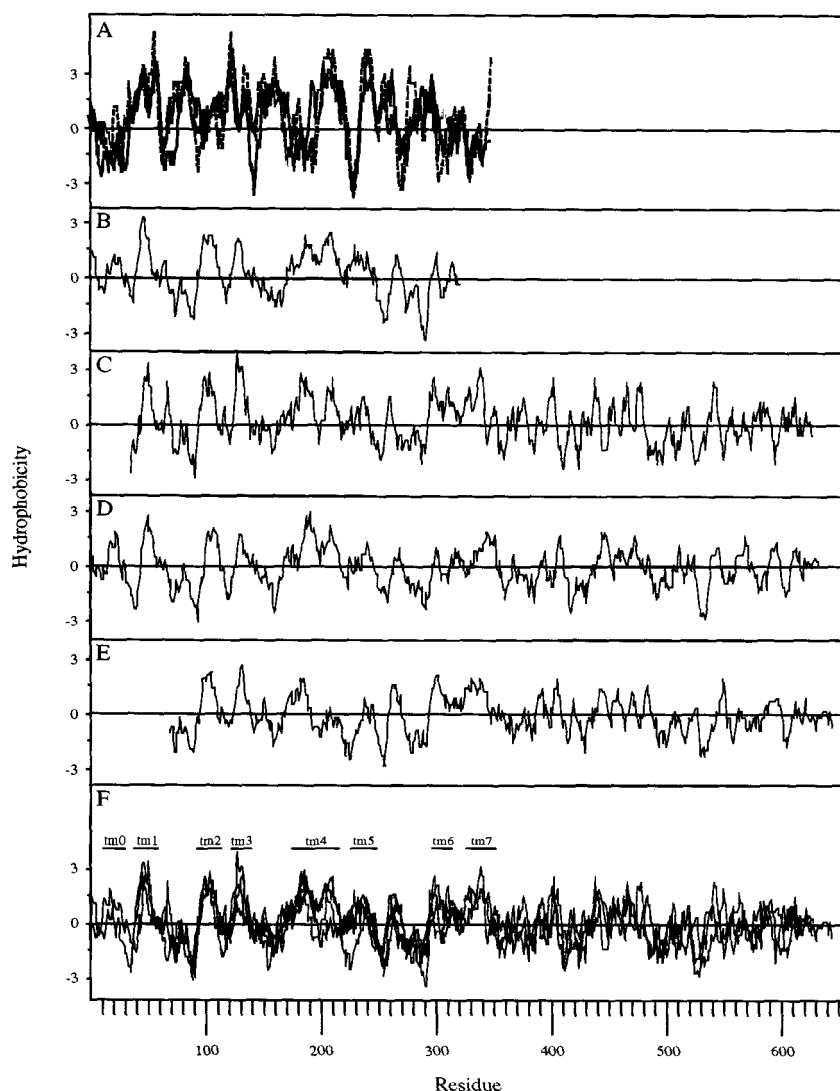
**Figure 5.** A, Effects of mutations at the *gl1* locus on GL1 mRNA accumulation. Northern blot of RNA from seedlings of the maize inbred B73 (lanes 1 and 2) and standard Q60 (lanes 3 and 4). These seedlings were homozygous at the *gl1* locus for wild-type *Gl1* (*Gl1*+B73, lane 1; *Gl1*+Q60, lane 3) or mutant (*gl1-ref*, lane 2; *gl1-755048*, lane 4) alleles. B, Epidermis-specific accumulation of *epi23* mRNA. Northern blot of RNA from epidermal (lane 1) or nonepidermal (lane 2) tissues of *K. odora*.

the rice EST RICS2751A. These two clones were completely sequenced and the amino acid sequences of the encoded proteins were deduced. Aarts et al. (1995) have shown that the Arabidopsis EST 178C3T7 is the product of the *CER1* locus, a gene that affects cuticular wax biosynthesis in that organism.

Comparisons among these sequences indicate that the RICS2751A clone, like *epi23*, lacks the 5' sequences related to GL1. Furthermore, these comparisons establish that, whereas the NH<sub>2</sub>-terminal sequences that are present in all four proteins are highly conserved, the COOH end of the GL1 protein is different from the COOH end of the other proteins. Namely, the GL1 protein has 319 amino acids, whereas the longer CER1 (625 amino acids), EPI23 (594 amino acids), and RICS2751A (555 amino acids) proteins have a conserved COOH-terminal portion of about 286 amino acids, which is not present on the GL1 protein.

Despite this heterogeneity in the lengths of these proteins, there are considerable sequence similarities among the four (Fig. 4). These similarities in the primary sequence of these proteins results in very similar hydrophobicity plots for each protein (Fig. 6).

Additional analyses with the TMPredict algorithm (Hofmann and Stoffel, 1993) and comparison of this output to the Kyte-Doolittle hydrophobicity plots (Kyte and Doolittle, 1982) identified eight highly hydrophobic helices that can potentially span a membrane. These helices are identified in Figure 6F as tm0 through tm7. These analyses predict that these proteins are integral membrane proteins, and indeed the PSort algorithm (Nakai and Kanehisa, 1992) identifies the NH<sub>2</sub>-terminal 40 residues of the GL1 protein and the corresponding region of the CER1 protein as plasma-membrane-targeting signal peptides. The hydrophobic helix tm0 is part of this putative signal peptide. The less well conserved COOH-terminal portions of each of these proteins have hydrophobicity profiles that are characteristic of a globular, water-soluble protein. The TMPredict algorithm predicts that this globular domain is located on the cytoplasmic side of the plasma membrane.



**Figure 6.** Hydrophobicity profiles of GL1 and orthologous proteins. Hydrophobicity profiles of the proteins were generated by the algorithm of Kyte and Doolittle (1982). A, Composite hydrophobicity profiles of the human seven-transmembrane receptor proteins, fusin (solid line) (GenBank accession no. X71635), CKR-5 (gray line) (GenBank accession no. U54994), and CKR-3 (dashed line) (GenBank accession no. U28694). B, Hydrophobicity profile of the maize GL1, C, the *K. odora* EPI23, D, the Arabidopsis CER1 and E, the rice RICS2751A proteins. F, The composite of the hydrophobicity plots D and E was aligned to maximize profile similarity. Predicted transmembrane helices are indicated with the bars tm1 to tm7. tm0 represents the hydrophobic signal peptide.

## DISCUSSION

The cuticular wax of wild-type maize seedlings is composed of long-chain alcohols (63%), aldehydes (20%), alkanes (1%), and esters that are composed of alcohols and long-chain fatty acids (16%). The alcohols and aldehydes are predominantly 32 carbons in length, whereas the main alkane is 31 carbons in length. The predominant esters consist of a 32-carbon alcohol esterified to fatty acids of between 22 and 26 carbons in length (Bianchi et al., 1989). Mutations at the *gl1* locus reduce the amount of wax on the surfaces of maize seedlings to about one-fourth that of wild-type levels (Bianchi et al., 1989).

To obtain a molecular and biochemical understanding of the nature of the genetic lesion that leads to the observed phenotype of *gl1* plants, and, thus, enhance the understanding of cuticular wax biosynthesis, a collection of *Mutator*-induced *gl1* alleles was generated (Schnable et al., 1994). From this collection a 12.5-kb *HindIII* restriction fragment that contains a *Mu1* transposon and co-segregates with the *gl1*-755048 allele was identified and molecularly

cloned. The representation of this fragment on the *gl1* locus was shown by allelic cross-referencing experiments. Northern-blot and sequencing analyses led to the identification of the *gl1* locus within the cloned *HindIII* fragment.

Consistent with the identification of this cloned fragment as the *gl1* locus, northern analyses of RNA probed with this fragment revealed that the *gl1* mRNA accumulates to significantly lower levels in *gl1* maize seedlings relative to wild-type seedlings. Furthermore, the *K. odora* ortholog of *gl1*, *epi23* mRNA, was isolated by an independent approach and accumulates predominantly, if not exclusively, in the epidermis of leaves. This result is consistent with the expression pattern of another cuticular wax gene, *CER2* of Arabidopsis (Xia et al., 1996), which accumulates specifically in the epidermis. These results are consistent with (but do not prove) EPI23 having a functional role in cuticular wax biosynthesis in *K. odora*.

Comparisons to sequence databases revealed that the GL1 and EPI23 proteins are similar in sequence to the rice EST RICS2751A and the Arabidopsis EST 178C3T7. The

latter has been shown to be a cDNA clone of the *CER1* locus (Aarts et al., 1995).

The *CER1* gene of *Arabidopsis* has been predicted to code for an aldehyde decarbonylase (Aarts et al., 1995), an enzyme required for the production of the alkane fraction of the cuticular waxes of this species. This prediction is based on the fact that mutations at the *CER1* locus cause a relative enrichment of the aldehydes and a depletion of the alkanes and alkane-derived metabolites (ketones and secondary alcohols) of the cuticular wax of stems (Hannoufa et al., 1993; McNevin et al., 1993). Furthermore, the *CER1* protein contains three His-rich motifs (HX<sub>3</sub>H, HX<sub>2</sub>HH, and HX<sub>2</sub>HH, where X stands for any amino acid) that are conserved among a number of fatty acyl desaturases, alkane hydroxylase, and xylene monooxygenase, three enzymes that catalyze mechanistically similar reactions (Fox et al., 1994; Shanklin et al., 1994). Functionally, these three His-rich motifs are thought to bind a di-iron-oxo cluster, and these motifs are absolutely essential for the fatty acyl desaturation reaction. Aarts et al. (1995) speculate that the putative aldehyde decarbonylase encoded by the *CER1* gene also contains such a di-iron-oxo cluster, which may be required for the decarbonylation reaction.

Despite the sequence similarity between *CER1*, *EPI23*, *RIC2751A*, and the *GL1* proteins, we think that *gl1* does not code for an aldehyde decarbonylase. This conclusion is based on three lines of reasoning. First, the assignment of the *GL1* protein as an aldehyde decarbonylase is inconsistent with the chemical composition of the waxes found on seedlings homozygous for *gl1* mutations. Namely, it is difficult to envision how a mutation that would affect the conversion of aldehydes to alkanes can globally affect the accumulation of the ester, alcohol, and aldehyde fractions of the cuticular waxes of maize seedlings. Second, the *GL1* protein sequence lacks the third His-rich motif (the corresponding sequence in *GL1* is VPRDS), and in *EPI23* and *RIC2751A* this third His-rich domain is incomplete (YS-LHH and LSLHH, respectively). Thus, it would be doubtful if these proteins can function as di-iron-oxo-cluster-containing decarbonylases, if such a structure is essential for decarbonylase activity. Third, alkanes account for a very small portion of the cuticular waxes of wild-type maize seedlings (about 1%), which is inconsistent with the fact that the *gl1* mRNA is a relatively abundant transcript.

Therefore, even though *GL1* (and *EPI23* and *RIC2751A*) and *CER1* proteins share similar structures, they, in fact, may perform different functions in cuticular wax biosynthesis. This is consistent with the fact that the *CER1* protein is considerably larger than the *GL1* protein, and with the fact that mutations in these two genes confer distinct phenotypic changes on the composition of the cuticular waxes of maize and *Arabidopsis*, respectively.

Finally, there is a possibility that *CER1* may not be an aldehyde decarbonylase, and that both proteins, *CER1* and *GL1*, may perform similar, but as yet unidentified function(s). There are a number of observations that may be interpreted to support this last hypothesis. First, as pointed out by Aarts et al. (1995), the His-rich clusters in *CER1* do not mirror the spacing of these clusters in other di-iron-

oxo-containing proteins (Fox et al., 1994; Shanklin et al., 1994). Although the functional consequence of these differences between *CER1* and the other di-iron-oxo-containing proteins is unclear, it may be significant that *CER1* and *ERG3* exhibit altered spacing between the His-rich motifs.

The second observation that may suggest that *CER1* is not a decarbonylase is the characterization of a partially purified aldehyde decarbonylase from the colonial alga *Botryococcus braunii*. This enzyme requires cobalt as a cofactor rather than iron (Dennis and Kolattukudy, 1992). However, as pointed out by Aarts et al. (1995), this may reflect a difference between plant and algal decarbonylases.

Finally, some of the phenotypic changes associated with mutations at the *CER1* locus cannot be readily explained by a block in decarbonylase activity. The alterations in the composition of cuticular waxes on stems of *cer1* plants (relative to wild type) have been interpreted as a block in the conversion of aldehydes to alkanes, namely a decreased accumulation of alkanes (and metabolites of alkanes) and an increased accumulation of aldehydes (Jenks et al., 1995). However, in addition to these changes, the *cer1* mutation also decreases the accumulation of the primary alcohol constituents of the wax (Jenks et al., 1995). This effect is not expected from a simple block at the aldehyde decarbonylase step. Because aldehydes are precursors of both alkanes and primary alcohols, a block in the conversion of aldehydes to alkanes could conceivably result in an increase in the accumulation of primary alcohols. However, it is difficult to explain how such a block could reduce the accumulation of primary alcohols. The observed decrease in the accumulation of primary alcohols can only be explained by invoking more complex models in which mutations at the *CER1* locus have pleiotropic effects on the pathway. In addition, the effect of the *cer1* mutation on leaf wax composition does not provide any evidence for a block in the decarbonylase reaction. Namely, the leaf waxes of *cer1* plants have reduced alkanes and primary alcohols, without an increase in aldehydes (Jenks et al., 1995). Taken together, these observations could be interpreted as indicating that *CER1* may not be an aldehyde decarbonylase.

Thus, there are two alternative hypotheses to explain the structural similarity between the *GL1* and *CER1* proteins. Further molecular and biochemical investigations will be required to distinguish between them.

Regardless of the biochemical function of *CER1*, the secondary structure predictions and hydrophobicity profiles of the four proteins *GL1*, *EPI23*, *RIC2751A*, and *CER1* indicate that they are integral membrane proteins. Furthermore, the PSort algorithm (Nakai and Kanehisa, 1992) predicts that these proteins are targeted to a membrane, possibly the plasma membrane. Whereas the *GL1* protein is predicted to be integrated into the membrane by five membrane-spanning helices, the *EPI23*, *CER1*, and *RIC2751A* proteins are predicted to be integrated into the membrane by seven membrane-spanning helices. In all instances, the transmembrane helices occur at the NH<sub>2</sub>-terminal portions of these proteins, and all four proteins are predicted to have a COOH-terminal globular, water-soluble domain that is on the cytosolic side of the membrane.

The secondary structure and membrane topological predictions of these orthologs indicate that they may belong to a superfamily of integral membrane receptors. Such receptors have previously been well characterized in animal systems and are composed of a hydrophobic NH<sub>2</sub>-terminal domain and a globular, water-soluble COOH-terminal domain. These receptors bind ligands at the NH<sub>2</sub>-terminal domain and transduce this fact via a G-protein interaction with their COOH-terminal domain (Probst et al., 1992).

A small subset of such receptors that show significant sequence similarity (40% similarity) to the GL1 protein and its plant orthologs are fusin, also called CXCR4 (Loetscher et al., 1994; GenBank accession no. X71635), and the  $\beta$ -chemokine receptors CKR-5 (Raport et al., 1996; GenBank accession no. U54994), CKR-3 (Combadiere et al., 1995; GenBank accession no. U28694), and CKR-2A and CKR-2B (Charo et al., 1994; GenBank accession nos. U03882 and U03905). These receptors act as co-receptors for the HIV receptor CD4, and are essential for HIV-mediated fusion of cellular membranes, a process required for the initial infection and subsequent cell-to-cell movement of the virus (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). These  $\beta$ -chemokine receptors are proteins of about 350 amino acid residues, similar in size to the GL1 protein. Furthermore, the similarities between the hydrophobicity plots of the  $\beta$ -chemokine receptors and of the GL1 protein may indicate that they have similar membrane topologies (Fig. 6). These similarities may suggest that the GL1 protein mediates membrane fusion. Such a function could have a role in transporting intermediates or end products of cuticular wax biosynthesis (Kolattukudy, 1996). This hypothesis could provide an explanation for how mutations in orthologs (*gl1* or *CER1*) can result in diverse effects on wax composition. Namely, the phenotypic effects of these mutations would depend on both the molecular specificities of the individual transporter molecules and the substrate pools available to them.

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