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Sequence Analysis of the Cloned *glossy8* Gene of Maize Suggests That It May Code for a β-Ketoacyl Reductase Required for the Biosynthesis of Cuticular Waxes[1]

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The *gl8* locus of maize (*Zea mays* L.) was previously defined by a mutation that reduces the amount and alters the composition of seedling cuticular waxes. Sixty independently derived *gl8* mutant alleles were isolated from stocks that carried the *Mutator* transposon system. A DNA fragment that contains a *Mu8* transposon and that co-segregates with one of these alleles, *gl8-Mu3142*, was identified and cloned. DNA flanking the *Mu8* transposon was shown via allelic cross-referencing experiments to represent the *gl8* locus. The *gl8* probe revealed a 1.4-kb transcript present in wild-type seedling leaves and, in lesser amounts, in other organs and at other developmental stages. The amino acid sequence deduced from an apparently full-length *gl8* cDNA exhibits highly significant sequence similarity to a group of enzymes from plants, eubacteria, and mammals that catalyzes the reduction of ketones. This finding suggests that the GL8 protein probably functions as a reductase during fatty acid elongation in the cuticular wax biosynthetic pathway.

Surfaces of the aerial portions of plants are covered with a complex mixture of lipids termed cuticular waxes, the majority of which are derivatives of fatty acids (Kolattukudy and Walton, 1973; Kolattukudy et al., 1976; Tulloch, 1976; Kolattukudy, 1980). These waxes provide a hydrophobic barrier between a plant and its environment. Despite their biological significance, little is known about the biochemical and molecular genetic mechanisms that regulate the biogenesis of cuticular waxes.

The composition of cuticular waxes is species-specific. For example, the cuticular waxes on wild-type maize (*Zea mays* L.) seedling leaves are composed of long-chain alcohols (63%), aldehydes (20%), alkanes (1%), and esters (16%) formed between the alcohols and VLCFAs. The alcohols and aldehydes are predominantly 32 carbons in length, i.e. *n*-dotriacontanol (99% of the alcohols) and dotriacontaldehyde (96% of the aldehydes). The alkane fraction is mainly 31 carbons in length, i.e. hentriaconate (Bianchi et al., 1985).

The synthesis of VLCFAs is thought to require the sequential operation of two fatty acid biosynthetic systems: de novo fatty acid biosynthesis, which occurs in plastids, and fatty acid elongation, which occurs on the microsomal membranes (Agrawal et al., 1984; Agrawal and Stumpf, 1985; Lessire et al., 1985a, 1985b, 1985c, 1989).

In maize 17 loci (the *glossy* or *gl* loci) have been identified that affect the quantity and/or the composition of cuticular waxes on the surfaces of seedling leaves (Schnable et al., 1994). For example, mutations at the *gl8* locus reduce the amount of wax on these surfaces to about one-third that of wild-type levels (Bianchi et al., 1979; Fig. 1). Mutant seedlings can be readily distinguished from wild-type seedlings. Because of the alterations in the cuticular wax load, applied water forms droplets on the leaf surfaces of mutants (Coe and Neuffer, 1977; Bianchi, 1978; Bianchi et al., 1985), presenting a "glossy" appearance.

Comparisons between the chemical compositions of the waxes produced by seedlings homozygous for each of the various *gl* mutants and wild-type seedlings have been used to putatively identify the biochemical steps encoded by some of the *gl* genes (Bianchi et al., 1985; von Wettstein-Knowles, 1987). However, the chemical phenotype associated with mutations at the *gl8* locus makes such an assignment difficult. The aldehyde, alcohol, and ester components of the cuticular waxes of *gl8* seedlings accumulate to 17, 23, and 70% of wild-type levels, respectively. It is interesting that, although the chain lengths of the alcohol and acid moieties of the wax esters produced by *gl8* mutant seedlings are reduced relative to wild-type seedlings (from 32 and 22–26 carbons, respectively, to less than

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Abbreviations: ACP, acyl carrier protein; fabG-Ec, *Escherichia coli* 3-ketoacyl-ACP reductase; HSDH-Hs, human estradiol 17-β-hydroxysteroid dehydrogenase; KAR-At, *Arabidopsis thaliana* 3-ketoacyl-ACP reductase; KAR-Cl, *Cuphea lanceolata* ketoacyl-ACP reductase; VLCFA, very-long-chain fatty acid; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

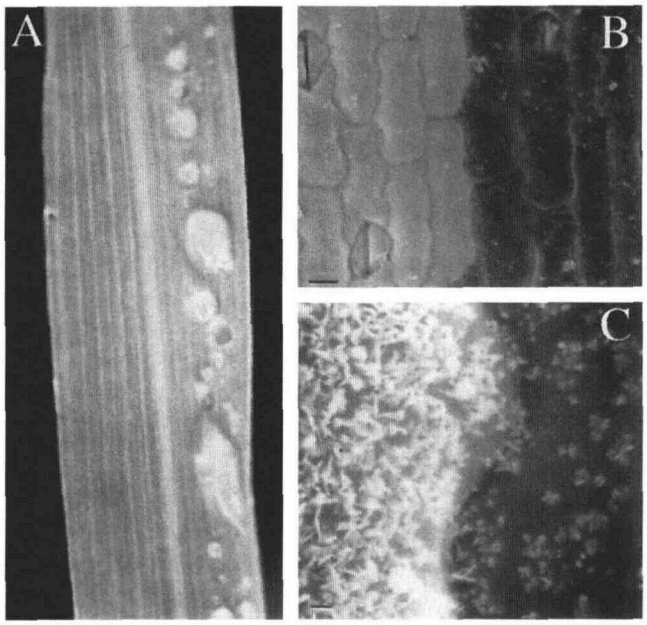

Figure 1. Visualization of the *gl8* phenotype. A, Chimeric seedling leaf that contains wild-type (left) and *gl8* mutant (right) sectors. The leaf was sprayed with water to better distinguish the wild-type from mutant tissue. B, A portion of this chimeric seedling was examined under scanning electron microscopy (×300). The wild-type sector (left) is covered with wax crystals; the mutant sector (right) has considerably fewer crystals on its surface. Bar equals 20 μm. C, Higher magnification (×5000) of same region as shown in B. Bar equals 1 μm.

24 carbons), similar effects are not observed on the free alcohols and aldehydes (Bianchi et al., 1979). Hence, although it has been hypothesized that the *gl8* locus is required for the elongation step in cuticular wax biosynthesis (Bianchi et al., 1985; von Wettstein-Knowles, 1987), a clear picture of its specific role has not emerged from these studies. A more direct means to determine the function of this gene would be to isolate and characterize the GL8 protein.

Cuticular waxes are thought to be synthesized by the cells of the epidermal tissue, which account for less than 10% of the cells of the organism. Hence, a purely biochemical approach to the isolation and characterization of the enzymes involved in the biosynthesis of cuticular waxes has been hampered by the difficulty in isolating epidermal tissues. However, transposon tagging provides a means to isolate genes, such as those involved in cuticular wax biosynthesis, for which the gene product is unknown but for which mutants can be readily isolated. Lines carrying the *Mutator* transposon system exhibit a mutation rate 50-fold higher than the spontaneous rate and the rate observed in lines carrying other transposon systems (Robertson and Mascia, 1981; Robertson, 1983). Most of the new *Mutator*-induced mutants arise via the insertion of *Mu* transposons (Chandler and Hardeman, 1992).

In this report we describe the transposon tagging, isolation, and initial characterization of the *gl8* locus of maize and the isolation of *gl8* orthologs from other plant species, such as barley (*Hordeum vulgare* L.), leek (*Allium porrum* L.), and Arabidopsis. These experiments suggest that the role of the GL8 protein in the cuticular wax biosynthetic pathway is to serve as a reductase during fatty acid elongation.

MATERIALS AND METHODS

Genetic Stocks

The origins and maintenance of *Mutator* transposon stocks, the F_1 hybrid Q60, and the *gl-ref* alleles used in this study have been described previously (Stinard et al., 1993; Schnable et al., 1994).

Isolation of Transposon-Tagged Alleles of the *gl8* Locus

Large numbers of progeny from cross 1 can readily be generated by the isolation-plot technique (Peterson, 1978), which involves planting the two parental genotypes in isolation from other maize (*Zea mays* L.) plants and detasseling (emasculating) the female parent (listed first in cross 1). Hence, the only pollen in the isolation plot comes from the male parent and carries the *gl8-ref* allele.

To analyze the *gl8-Mu* alleles isolated via directed tagging, it was necessary to sequester them from the *gl8-ref* allele that was used to uncover them in cross 1. To facilitate this process the *pr* marker allele was incorporated into the male (*gl8-ref*) parent that was used in cross 1. The recessive *pr* mutant, which is 1 centimorgan away from *gl8*, conditions a red kernel phenotype, in contrast to the wild-type purple (*Pr*), making it a readily scorable marker. In glossy progeny from cross 1, the *gl8-ref* allele could be distinguished from newly induced *gl8-Mu* alleles because the former was in coupling with *pr*, whereas the latter were in coupling with *Pr*.

Construction and Screening of Recombinant DNA Libraries

Two cDNA libraries, both of which were prepared from mRNA isolated from greenhouse-grown 2-week-old maize B73 seedlings, were used. One was prepared using the vector λZAPII (Stratagene) and was a gift from Alice Barkan (University of Oregon, Eugene, personal communication). Details regarding the construction of the second cDNA library, prepared by Yiji Xia using the pAD-GAL4 vector (Stratagene), will be published elsewhere. cDNA libraries prepared from mRNA isolated from sprouted barley (*Hordeum vulgare* L. var Morex) and peeled leek (*Allium porrum* L.) epidermal tissues cloned into the *Eco*RI site of λZapII (Stratagene) were gifts from Ron Skadsen (U.S. Department of Agriculture, Madison, WI) and Chris Somerville (Carnegie Institute, Stanford, CA), respectively.

A maize partial genomic library was constructed using the λ vector Charon 33 (Leonen and Blattner, 1983). Following the digestion of vector DNA with the restriction enzyme *Hin*dIII, the vector arms and stuffer fragments were separated by Suc gradient centrifugation (Sambrook et al., 1989). Maize *Hin*dIII DNA fragments ranging in size from 7 to 9 kb were gel-purified and ligated with the vector

arms. The ligation reaction was packaged in vitro using commercial packaging extracts (Stratagene).

A 713-bp *Hin*dIII/*Eco*RI *Mu8*-specific probe was isolated from plasmid p713(Mu8) (Fleenor et al., 1990).

Recombinant libraries were screened via in situ hybridization (Sambrook et al., 1989) at 68°C (maize) or 50°C (leek and barley). Hybridizations were performed in 6× SSPE. Final washes were performed with 5 mM Tris (maize) or 0.1× SSPE (leek and barley).

Isolation and Analysis of Nucleic Acids

Maize DNAs were isolated from seedlings or immature ears by the methods of Dellaporta et al. (1983) or Saghai-Maroof et al. (1984). Maize DNAs were digested using commercially available restriction enzymes, according to the manufacturers' specifications. Digested DNAs were fractionated by electrophoresis in agarose gels and transferred to nylon membranes (Micron Separations, Westboro, MA), according to standard methods (Sambrook et al., 1989). Membranes were subjected to hybridization and washed according to the manufacturer's specifications.

RNAs were isolated from a variety of frozen tissues following the method of Dean et al. (1985). Leaves and roots were harvested from greenhouse-grown maize seedlings at the two-leaf stage (1 week after planting). Each seedling RNA sample was prepared from a bulk of between 6 and 10 seedlings. Mature roots, leaves, husks, and unfertilized ears were harvested from a greenhouse-grown Q60 F_2 plant at the time of anthesis. Immature endosperms (inbred A636) were harvested 20 d after pollination, and all harvested tissues were quickly frozen in liquid nitrogen and stored at −70°C.

RNAs were fractionated by electrophoresis in formaldehyde-containing agarose gels and blotted onto nylon membranes according to standard procedures (Sambrook et al., 1989). Ethidium bromide-stained rRNAs were used as the internal loading control.

Plasmid and phage DNAs were isolated by standard methods (Sambrook et al., 1989). DNA probes were prepared by random hexamer priming using ^{32}P-labeled dCTP (Feinberg and Vogelstein, 1983).

DNA Sequencing and Analysis

Clones were sequenced at The Iowa State University Nucleic Acid Facility on an automated DNA sequencer (ABI 373A, Applied Biosystems). In all instances, both DNA strands were completely sequenced. Analyses of the resulting DNA sequences were performed using version 8 of the Genetics Computer Group (Madison, WI) sequence analysis software. The output of the sequence alignments was produced using version 3.21 of the Boxshade algorithm.

RESULTS

Isolation of Transposon-Tagged Alleles of the *gl8* Locus

Because a transposon-tagged allele by definition contains a transposon insertion, and such an insertion often results in a mutation, it is possible to identify tagged alleles by screening for mutations within large populations that carry an active transposon system. Two general kinds of populations carrying the well-studied *Mutator* transposon system were screened for *gl8* mutations. The first population type was generated via cross 1 and is an example of a directed tagging experiment. In the absence of a mutation, the seeds produced by the female parent of this cross (listed first) will all be heterozygous (*Gl8/gl8-ref*) and will therefore yield wild-type seedlings. Only if a gamete from the female (*Mutator*) parent carries a newly generated mutant allele (e.g. via the insertion of a *Mu* transposon) will a glossy seedling be obtained (*gl8-Mu/gl8-ref*). The screening of more than 100,000 seedlings from this population resulted in the isolation and final recovery of 46 *gl8-Mu* alleles (data not shown).

Cross 1: *Mutator Gl8 Pr/Gl8 Pr* × *gl8-ref pr/gl8-ref pr*

In contrast to the directed tagging experiment described above (which generates mutants at a defined locus), random tagging experiments generate mutants at all loci, which yield a glossy phenotype when mutated. At least 12 of a set of 42 *gl** mutants isolated via random tagging were allelic to *gl8* (Schnable et al., 1994). Together, the two tagging approaches generated 60 mutant alleles of *gl8*.

Identification of a *Mu8* Transposon That Co-Segregates with *gl8-Mu3142*

Candidate *Mu* transposon insertions at the *gl8* locus can be identified by co-segregation analysis. Among progeny from a plant heterozygous for a given *gl8-Mu* allele, the *Mu* transposon inserted in that *gl8-Mu* allele would be present in all progeny that carry the *gl8-Mu* allele but would be absent in all siblings that do not. Because large numbers of *Mu* transposons are present in the active *Mutator* stocks from which the *gl8-Mu* alleles were derived, lines carrying these alleles were first backcrossed (cross 2) to a non-*Mutator* stock for several generations to simplify the process of identifying the *Mu* transposon that co-segregates with a particular *gl8-Mu* allele. The F_1 hybrid Q60 was used as the recurrent parent for these backcrosses because it carries relatively few *Mu* transposons (data not shown).

Cross 2: *Gl8/Gl8* × *gl8-Mu/Gl8*

At each generation plants were selected that carried the appropriate *gl8-Mu* allele and that carried the fewest number of *Mu* transposons (as determined via DNA gel-blot analyses). Following four generations of backcrossing, progeny families, each of which was segregating for a particular *gl8-Mu* allele, were obtained. The genotypes of individual plants within these progenies were established via test crosses. DNAs from individual siblings within each progeny family were digested with *Hin*dIII (which does not cut within most of the *Mu* transposons) and subjected to DNA gel blotting using *Mu* transposon-specific probes.

In a family carrying the *gl8-Mu3142* allele, an 8.6-kb *Hin*dIII, *Mu8*-containing DNA fragment was identified that

co-segregated with the *gl8-Mu3142* allele in 88 progeny of cross 2 (Fig. 2A). This fragment was carried by all 59 heterozygous (*gl8-Mu3142/Gl8*) progeny, whereas none of the 29 homozygous (*Gl8/Gl8*) progeny carried this fragment.

Cloning the *gl8* Locus

*Hin*dIII-digested DNA fragments that ranged in size from 7.0 to 9.0 kb were isolated from a plant with the genotype *gl8-Mu3142/Gl8* and cloned into the λ vector Charon 33. Two *Mu8*-hybridizing clones were isolated from the resulting library. Because these two clones appeared identical at the resolution afforded by endonuclease restriction enzyme recognition site mapping, only one, λ515, was further analyzed. The restriction site map of λ515 is shown in Figure 2B.

To confirm that the insert of λ515 is the specific *Mu8*-containing DNA fragment that co-segregates with the *gl8-Mu3142* allele, the 3.6-kb *Hin*dIII-*Sac*I fragment from λ515 was used as a hybridization probe against the original co-segregation DNA blot shown in Figure 2A. A portion of the resulting exposure is shown in Figure 3. Two hybridizing fragments were revealed with this probe. One of the hybridizing fragments is about 7.2 kb in size. Because this fragment is carried by all plants in both genotypic classes,

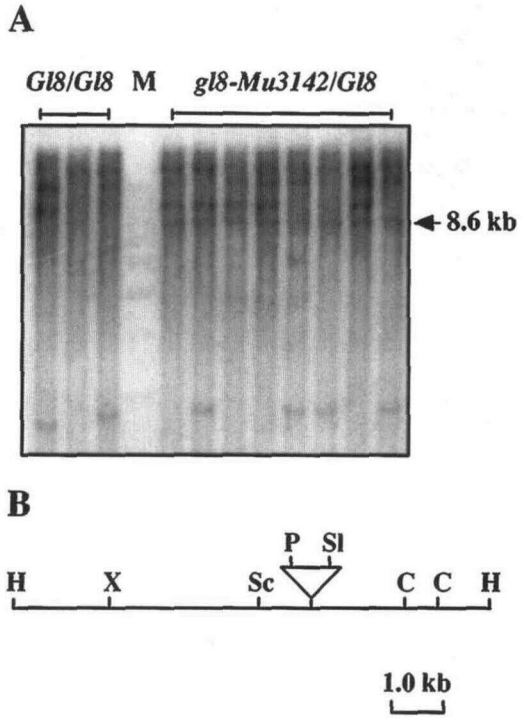

Figure 2. Identification and cloning of a *Mu8*-containing fragment that co-segregates with the *gl8-Mu3142* mutant allele. A, DNA samples isolated from progeny of the cross *gl8-Mu3142/Gl8* × *Gl8/Gl8* were digested with *Hin*dIII, electrophoresed on agarose gels, transferred to a nylon membrane, and hybridized with a 713-bp fragment from the *Mu8* transposon. M indicates the marker lane. B, Restriction site map of the λ515 clone, the 8.6-kb *Hin*dIII fragment of the *gl8–3142* allele. H, *Hin*dIII; X, *Xho*I; Sc, *Sac*I; P, *Pst*I; Sl, *Sal*I; and C, *Cla*I. The triangle indicates the position of the *Mu8* transposon.

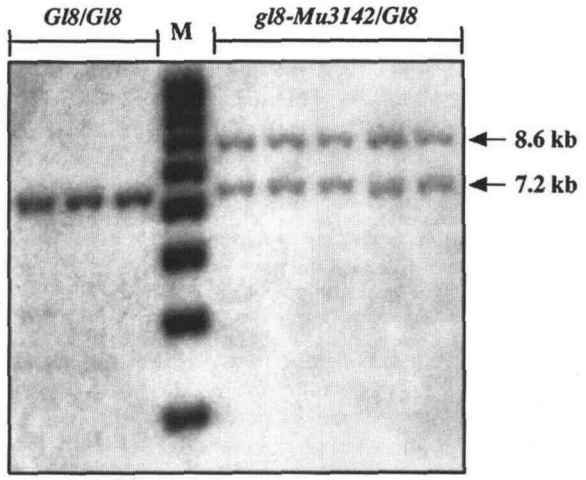

Figure 3. DNA gel-blot analysis using λ515. The 3.6-kb *Hin*dIII-*Sac*I fragment of λ515 was used as a hybridization probe against the original co-segregation DNA gel blot illustrated in Figure 2A. M indicates the marker lane.

it must be derived from the *Gl8*-containing chromosomes contributed by the female parent of cross 2. This parent was heterozygous for two wild-type *Gl8* alleles, *Gl8*+Q66 and *Gl8*+Q67. Hence, these results establish that the *Gl8*+Q66 and *Gl8*+Q67 alleles do not display an restriction fragment length polymorphism under these conditions. The second hybridizing fragment is 8.6 kb and is present only in plants that carry the *gl8-Mu3142* allele. Hence, this fragment must be derived from the *gl8-Mu3142*-containing chromosome contributed by the male parent of cross 2.

Although these results confirm that λ515 is a clone of the *Mu8*-containing DNA fragment that co-segregates with the *gl8-Mu3142* allele, they do not unambiguously establish that this clone is derived from the *gl8* locus. To determine whether the DNA fragment cloned as λ515 indeed represents the *gl8* locus, the 3.6-kb *Hin*dIII-*Sac*I fragment of this clone was used as a hybridization probe in allelic cross-referencing experiments. The rationale for these experiments is that if this probe is derived from the *gl8* locus, it would be expected to be able to detect restriction fragment length polymorphisms between *gl8* mutants and their respective wild-type progenitor alleles.

To conduct these experiments, DNAs from 12 rare glossy seedlings derived from cross 1 (each of which carried an independently derived *gl8-Mu* allele) were compared via gel-blot analyses with DNAs from their wild-type siblings. These analyses demonstrated that λ515 does cross-hybridize with the *gl8* locus. Figure 4 illustrates an analysis of one of these 12 families that gave rise to the mutant allele *gl8-Mu91g210*. DNAs from the glossy seedling and its corresponding wild-type siblings were digested with *Eco*RI and subjected to gel-blot analysis. The genotypes of the glossy seedling and its wild-type siblings were *gl8-Mu91g210/gl8-ref* and *Gl8/gl8-ref*, respectively. Hybridization with the 3.6-kb *Hin*dIII-*Sac*I fragment from λ515 revealed three hybridizing fragments in each DNA sample.

Two common bands were present in all DNA samples and were therefore derived from the *gl8-ref* allele, which was

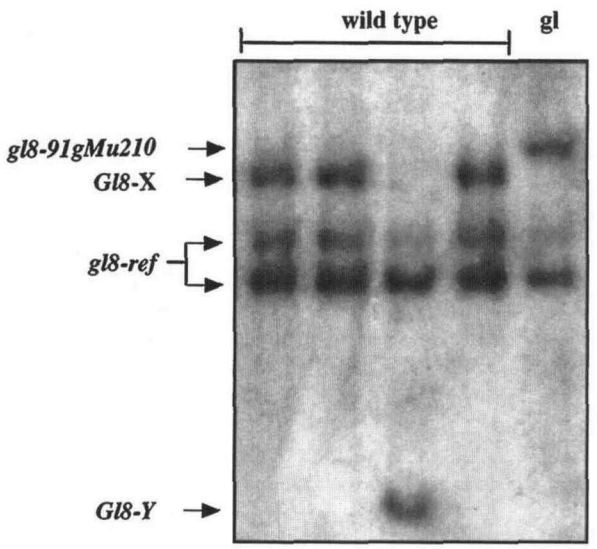

Figure 4. Allelic cross-referencing experiments. DNA samples isolated from a plant that carried a newly induced mutant allele (*gl8-Mu9lg210/gl8-ref*, designated gl) and its wild-type siblings (*Gl8-X/gl8-ref* or *Gl8-Y/gl8-ref*, designated wild-type) were digested with *Eco*RI, electrophoresed on agarose gels, and transferred to a nylon membrane. Hybridization with the 3.6-kb *Hin*dIII-*Sac*I fragment of λ515 revealed a size polymorphism associated with the *gl8-Mu9lg210* allele relative to both of the two wild-type alleles (*Gl8-X* and *Gl8-Y*).

carried by all plants. Because each wild-type sibling had one of two forms of the third band, these two bands represent two distinct wild-type alleles derived from the female parent of cross 1. The segregation of two wild-type alleles in this family is not unexpected, based on the origin of the *Mutator* stocks used in cross 1, and many of the female parents were heterozygous for any two of the following four alleles: *Gl8+Q66*, *Gl8+Q67*, *Gl8+B77*, or *Gl8+B79*. One of the two wild-type *Gl8* alleles that was segregating among the wild-type seedlings is the direct progenitor of *gl8-Mu91g210*. Significantly, the exceptional glossy seedling, which had the genotype *gl8-Mu91g210/gl8-ref*, carried a band different in size from those associated with both wild-type *Gl8* alleles in this family. This result demonstrates that, coincident with the mutation at the *gl8* locus that generated the *gl8–91g210* allele, a DNA rearrangement occurred in the fragment detected by the 3.6-kb *Hin*dIII-*Sac*I fragment from λ515. Analogous results were obtained in 8 of 11 additional comparisons between independently derived *gl8-Mu* alleles and their direct wild-type progenitors. Therefore, these allelic cross-referencing experiments demonstrate that λ515 contains at least part of the *gl8* locus.

Expression of the *gl8* Gene

The expression of the *gl8* gene was analyzed by RNA gel-blot analyses. RNAs were isolated from seedlings with the following genotypes: *Gl8/Gl8* (Q60 F2), *gl8-ref/gl8-ref*, and *gl8-Mu3142/gl8-Mu3142*. The *gl8-ref* and *gl8-Mu3142* alleles in this analysis had both been backcrossed to Q60 for two generations to place the alleles in a near-uniform genetic background. Using the 3.6-kb *Hin*dIII-*Sac*I fragment of λ515 as a probe, we detected a single 1.4-kb mRNA in RNAs isolated from the wild-type and mutant seedlings (Fig. 5A). This mRNA accumulates to significantly lower levels in RNAs isolated from the *gl8-ref* and *gl8-Mu3142* mutant seedlings, as compared with wild-type seedlings.

The accumulation of the *gl8* mRNA was also analyzed in various organs of a homozygous wild-type plant at various stages of development (Fig. 5B). The *gl8* mRNA accumulates in all stages of development and in all organs analyzed. However, this accumulation is significantly lower than in seedling leaves.

Isolation of cDNAs Derived from the Maize *gl8* Gene and *gl8* Orthologs from Barley, Leek, and Arabidopsis

The 3.6-kb *Hin*dIII-*Sac*I fragment of clone λ515 was used to screen approximately 400,000 plaques from a cDNA library prepared by Alice Barkan from greenhouse-grown 2-week-old maize B73 seedlings. Eight *gl8*-hybridizing clones, representing three size classes (0.5, 0.8, and 1.2 kb) were isolated. A 1.2-kb cDNA was partially sequenced and found to include an unspliced intron (data not shown). A 0.8-kb cDNA clone was sequenced and shown to contain an open reading frame that extended to the 5′ end and encoded 172 amino acid residues. This clone was named p88m and used to isolate an apparently full-length 1.4-kb *gl8* clone from a second cDNA library also prepared from B73 seedlings. This clone was also sequenced and shown to encode a 327-amino acid protein with a predicted molecular mass of 36 kD.

Orthologs of the maize *gl8* gene were isolated by screening approximately 200,000 plaques each from barley and

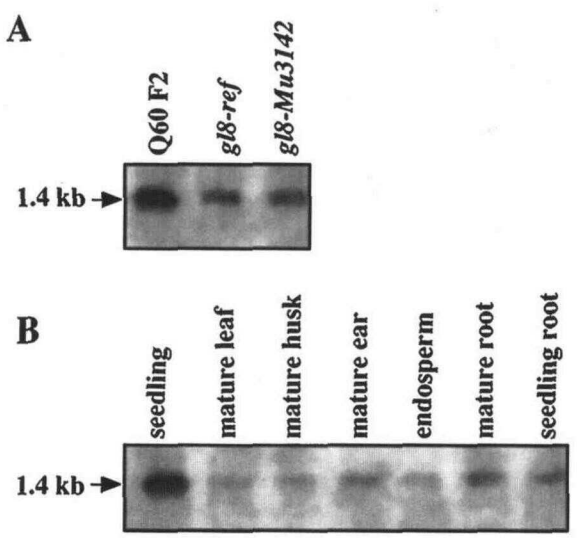

Figure 5. RNA gel-blot analyses with the 3.6-kb *Hin*dIII-*Sac*I fragment of λ515. A, The accumulation of the *gl8* transcript was analyzed in RNA isolated from seedlings with the following genotypes: *Gl8/Gl8* (Q60F2), *gl8-ref/gl8-ref*, and *gl8-Mu3142/gl8-Mu3142*. B, The accumulation of the *gl8* transcript was analyzed in RNA isolated from various organs of wild-type plants at both mature and seedling stages.

leek cDNA libraries with the 0.8-kb *gl8* cDNA. Based on its alignment with the maize *gl8* sequence, the 1.1-kb barley cDNA clone appears to be full length (Fig. 6). It contains an open reading frame that encodes a 325-amino acid protein. Because the leek cDNA is only 872 bp in length and encodes a 173-amino acid protein, it is presumably a partial cDNA (Fig. 6).

A BLAST (Altschul et al., 1990) search of the dbEST database identified an Arabidopsis ortholog of the maize *gl8* gene. The corresponding cDNA clone (ID no. 105H4T7) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH) and sequenced. Clone 105H4T7 is 896 bp in length and has an open reading frame that encodes a 253-amino acid protein (Fig. 6).

These *gl8* orthologs are well conserved. The protein encoded by the full-length barley *gl8* ortholog is 96% similar and 92% identical with the maize GL8 protein. The proteins encoded by the leek and Arabidopsis *gl8* orthologs are about 87 and 77% similar and 77 and 59% identical, respectively, to the maize GL8 protein.

Structure and Function of the GL8 Protein

Based on an analysis using the PSORT algorithm (Nakai and Kanehisa, 1992), the deduced GL8 protein has an N-terminal cleavable signal sequence, which is predicted to target this protein to the ER (Fig. 7). This finding is consistent with the recovery of elongase activity from microso-

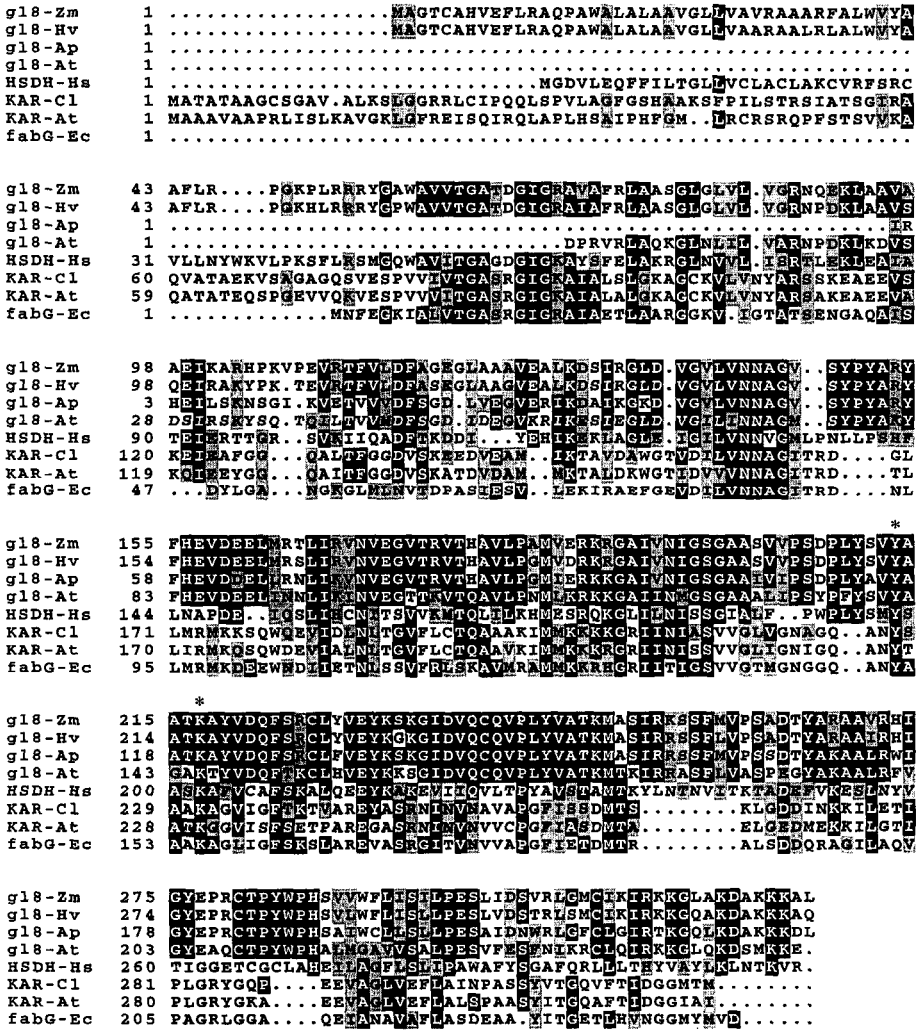

Figure 6. Predicted sequences are shown for the maize *gl8* gene (gl8-Zm, GenBank accession no. U89509); barley (*H. vulgare*), leek (*A. porrum*), and Arabidopsis orthologs of *gl8* homolog sequence (gl8-Hv, gl8-Ap, and gl8-At, respectively); HSDH-Hs, EC 1.1.1.62, GenBank accession no. 1169300; KAR-Cl, EC 1.1.1.100, GenBank accession no. 119791; KAR-At, EC 1.1.1.100, GenBank accession no. 462049; and fabG-Ec, EC 1.1.1.100, GenBank accession no. 119792. The sequences of the leek and Arabidopsis *gl8* orthologs are partial. The GenBank accession numbers of the *gl8* orthologs from barley, leek, and Arabidopsis are U89510, U89511, and U89512, respectively. The conserved Tyr and Lys residues within the active domain are indicated by asterisks. Amino acid identities in the alignments are indicated by black boxes with white letters. Similar amino acids are indicated by gray boxes. Dots indicate gaps introduced to optimize the alignments.

mal preparations (Agarawal et al., 1984; Agrawal and Stumpf, 1985; Lessire et al., 1985a, 1985b, 1985c, 1989). The predicted cleavage site is located at the carboxyl side of residue 29. The barley *gl8* ortholog has a nearly identical signal sequence (with only one amino acid difference out of 29), indicating that the final compartmentalization of the two proteins is probably the same.

The PSORT algorithm predicted that GL8 is a type Ia membrane protein with a single putative transmembrane helix that spans residues 71 to 87. Hence, this algorithm would indicate that the C terminus of the mature GL8 protein is located within the cell.

The TM predict algorithm (Hofmann and Stoffel, 1993) identified four potential transmembrane domains. One of these transmembrane domains is at essentially the same position as that found by PSORT (i.e. between residues 68 and 86). Another spans the predicted signal peptide, i.e. between residues 15 and 34. The remaining two are located between residues 191 and 215 and between residues 282 and 304. Hence, both algorithms predict that the mature GL8 protein is an integral membrane protein.

Identification and Analysis of Proteins that Exhibit Sequence Similarity to the GL8 Protein

The deduced amino acid sequence of the maize GL8 protein was compared with the GenBank database using the BLAST algorithm (Altschul et al., 1990). These analyses revealed significant amino acid sequence similarities to a group of enzymes from plants, eubacteria, and mammals, all of which catalyze the reductions of ketones (Fig. 6). Included in this group are: ketoacyl-ACP reductases (EC 1.1.1.100), which are required for de novo fatty acid biosynthesis; keto reductases (EC 1.3.1.-), which are required in polyketide biosynthesis; a putative oxidoreductase involved in the modification of a *Rhizobium* nodulation signal molecule; acetoacetyl-CoA reductases (EC 1.1.1.36); hydroxysteroid reductases (EC 1.1.1.62); tropinone reductase (EC 1.1.1.236); and glucitol-6-phosphate dehydrogenase (EC 1.1.1.140). All of these enzymes belong to the short-chain alcohol dehydrogenase family defined in the Prosite database (Bairoch et al., 1995). A defining characteristic of this enzyme family is the presence of a signature pattern that contains two perfectly conserved residues, a Tyr and a Lys. The former residue has been shown to be important for the catalytic activity and/or subunit binding in several members of this family (Ensor and Tai, 1991). The positions of these two invariant residues are indicated in Figure 6.

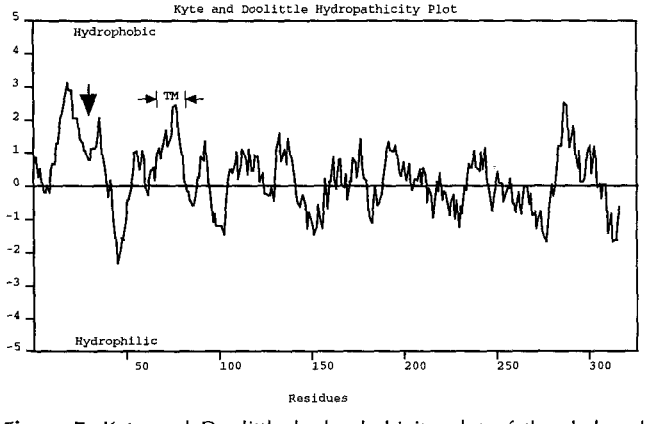

Figure 7. Kyte and Doolittle hydrophobicity plot of the deduced amino acid sequence of the GL8 protein. The large arrow indicates the predicted N-terminal cleavable signal peptide. The single transmembrane domain predicted by PSORT (from amino acid residues 71–87) is indicated by TM.

DISCUSSION

Relatively few of the genes involved in cuticular wax accumulation have been cloned. Furthermore, it has been difficult to assign precise molecular functions to those that have been cloned. The apparent orthologs, *CER1* and *gl1*, are thought to code for a decarbonylase (Aarts et al., 1995) or a transmembrane receptor-like protein (Hansen et al., 1997), respectively. The *gl2* (Tacke et al., 1995) and *CER2* (Negruk et al., 1996; Xia et al., 1996) orthologs code for novel proteins, as does the *CER3* gene (Hannoufa et al., 1996).

The *gl8* gene was defined more than 60 years ago (Emerson et al., 1935), when it was discovered that mutations at this locus interrupted the normal accumulation of cuticular waxes on maize seedling leaves. Although it has been possible to deduce some characteristics of the function of the GL8 protein via comparative chemical analysis of waxes extracted from wild-type and mutant seedlings, a detailed understanding of the function of this gene in cuticular wax biosynthesis or deposition was hampered by the difficulty in isolating the GL8 protein. In this report we describe a genetic approach by which the *gl8* gene was cloned and the sequence of its encoded protein deduced.

The Paup algorithm (Swofford, 1991) revealed that proteins that exhibit high degrees of sequence similarity to the GL8 protein fall into three classes (Fig. 8). The first class consists of the plant *gl8* orthologs isolated in this study and HSDH-Hs. The second class consists of a diverse collection of reductases that includes several *Streptomyces* reductases involved in polyketide biosynthesis, 7-hydroxysteroid dehydrogenase, tropinone reductase, and sorbitol-6-phosphate 2-dehydrogenase. The third class consists of acetoacetyl-CoA reductases, *nodG*, and ketoacyl-ACP reductases of type II, dissociable fatty acid synthases. This latter group includes *fabG* and several ketoacyl-ACP reductases that are involved in de novo fatty acid biosynthesis in the plastids of higher plants. These predicted evolutionary relationships probably reflect the fact that *gl8*, its plant orthologs, and the 17-hydroxysteroid dehydrogenase (Geissler et al., 1994) are all (or are predicted to be) integral membrane proteins. In contrast, the ketoacyl-ACP reductases in class 3 that are involved in the elongation of fatty acids in the de novo pathway are more distantly related because they are soluble globular proteins (Rawlings and Cronan, 1992).

The findings that the GL8 protein has a high degree of sequence similarity to a family of enzymes, all of which catalyze the reduction of a ketone group, and that *gl8* mutant seedlings accumulate less cuticular wax and reduce

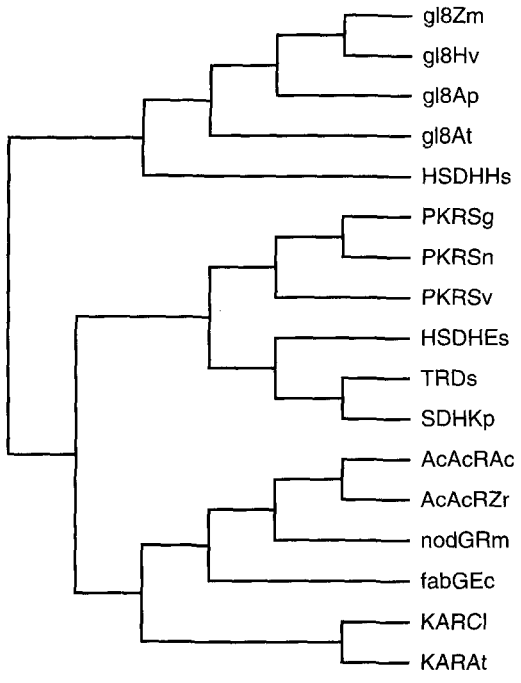

Figure 8. Phylogenetic tree of the GL8 protein and related proteins. The single most parsimonious tree obtained using the heuristic method (midpoint rooting) of the Paup 3.0 algorithm is illustrated. gl8Zm, gl8Hv, gl8Ap, and gl8At designate the maize GL8 protein and its orthologs from barley, leek, and Arabidopsis, respectively; HSDHHs, EC 1.1.1.62, GenBank accession no. 1169300; PKRSg, polyketide ketoreductase from *Streptomyces griseus,* GenBank accession no. 1076100; PKRSn, polyketide ketoreductase from *Streptomyces nogalater,* GenBank accession no. 1181158; PKRSv, granaticin/polyketide synthase putative ketoacyl reductase from *Streptomyces violaceoruber,* GenBank accession no. 118571; HSDHEs, 7-α-hydroxysteroid dehydrogenase from *Eubacterium VPI-12708,* EC 1.1.1.159, GenBank accession no. 114800; TRDs, tropinone reductase from jimsonweed (*Datura stramonium*), EC 1.1.1.236, GenBank accession no. 539027; SDHKp, sorbitol-6-phosphate 2-dehydrogenase from *Klebsiella pneumoniae,* EC 1.1.1.140, GenBank accession no. 548951; AcAcRAc, acetoacetyl-CoA reductase from *Alcaligenes eutrophus,* EC 1.1.1.36, GenBank accession no. 130015; AcAcRZr, acetoacetyl-CoA reductase from *Zoogloea ramigera,* EC 1.1.1.36, GenBank accession no. 130017; nodGRm, nodulation protein G from *Rhizobium meliloti,* GenBank accession no. 128466; fabGEc, EC 1.1.1.100, GenBank accession no. 119792; KAR-Cl, EC 1.1.1.100, GenBank accession no. 119791; KAR-At, EC 1.1.1.100, GenBank accession no. 462049. The total tree length is 1742, with a consistency index of 0.775 and a retention index of 0.620.

the chain lengths of some components of the wax that does accumulate strongly suggest that the GL8 protein functions to reduce the ketone group of the ketoacyl intermediate that is produced during the elongation of VLCFAs in cuticular wax biosynthesis.

Fatty acids are elongated by the sequential addition of 2-carbon units. In plants de novo fatty acid biosynthesis occurs in the plastids and is catalyzed by a type II, dissociable, fatty acid synthase system (Fig. 9). Fatty acid synthesis is initiated with an acetyl-CoA primer to which the malonyl moiety from malonyl-ACP is condensed to generate a β-ketoacyl-ACP intermediate. Prior to the subsequent addition of another 2-carbon unit, the carbonyl group of this intermediate is reduced to a methylene group. This occurs in three steps. The first of these steps is catalyzed by a β-ketoacyl-ACP reductase. The subsequent reactions are catalyzed by the sequential action of a hydroxyacyl-ACP dehydrase and an enoyl-ACP reductase. The products of de novo fatty acid biosynthesis are fatty acids of 16- and 18-carbon units.

The VLCFAs required for cuticular waxes are products of further elongation of these C16 and C18 fatty acids. These elongation reactions occur outside of the plastids; the enzyme system that catalyzes these reactions is located within microsomal membranes (Agrawal et al., 1984; Agrawal and Stumpf, 1985; Lessire et al., 1985a, 1985b, 1989). Although the quaternary structure of this elongation system has not been defined, the findings that malonyl-CoA is required for these reactions (Agrawal et al., 1984; Agrawal and Stumpf, 1985) and that they produce a β-ketoacyl-CoA intermediate (Lessire et al., 1989) indicate that these microsomal elongation reactions occur in a manner analogous to de novo fatty acid biosynthesis (Fig. 9). 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 seeds) is sequence similar to ketoacyl-ACP synthase III (James et al., 1995), which catalyzes the condensation of acetyl-CoA with malonyl-ACP.

Given its proposed enzymatic function, seedlings that carry *gl8* mutations would be expected to produce waxes

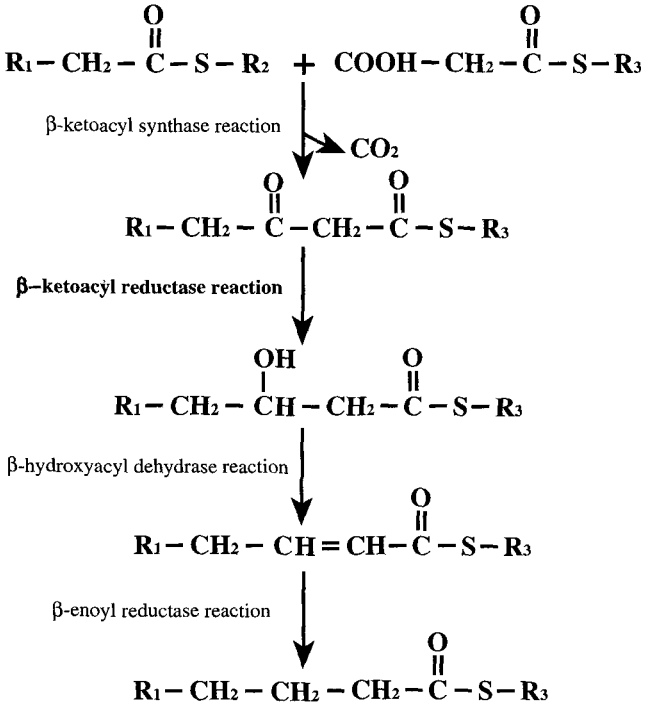

Figure 9. Elongation of VLCFAs. The elongation of VLCFAs destined for incorporation into cuticular waxes is thought to occur as illustrated. The nature of the R1, R2, and R3 groups are not yet defined.

having constituents with shorter carbon chain lengths. Indeed, as discussed earlier, such is the case but only relative to certain components of the wax. The chain lengths of the alcohol and acid moieties of the wax esters produced by *gl8* mutant seedlings are reduced relative to wild-type seedlings (from 32 and 22 to 26 carbons, respectively, to less than 24 carbons); similar effects are not observed on the free alcohols and aldehydes (Bianchi et al., 1979). Hence, the GL8 protein (which we believe to be a β-ketoacyl reductase) must be required for the elongation (from 24 to 32 carbons) of fatty acids destined for the wax esters. Such channeled fatty acid elongations would require the presence of multiple elongation systems. Therefore, there must be at least one other β-ketoacyl reductase responsible for the elongation of fatty acids from 18 to 24 carbons. This, or another reductase, is also required for the further elongation of fatty acids destined for the free alcohols and aldehydes (in which the chain lengths are not affected by mutations at *gl8*). These results are consistent with the hypothesis put forward by von Wettstein-Knowles (1979) and recently reviewed by Post-Beittenmiller (1996) that elongation occurs via a multibranched enzyme system within which substrates destined for specific end products are not freely exchangeable. The presence of multiple β-ketoacyl reductases in maize is consistent with RNA gel-blot experiments that revealed the presence of *gl8* cross-hybridizing mRNAs in *gl8* mutant seedlings.

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