# Characterization of the *FIDDLEHEAD* Gene of Arabidopsis Reveals a Link between Adhesion Response and Cell Differentiation in the Epidermis

**Alexander Yephremov,[1] Ellen Wisman,[2] Peter Huijser, Casper Huijser,[3] Kirsten Wellesen, and Heinz Saedler**

Max-Planck-Institut für Züchtungsforschung, Carl von Linné Weg 10, 50829 Cologne, Germany

We report the isolation of the *FIDDLEHEAD (FDH)* gene of Arabidopsis by transposon tagging. Three mutant alleles of *FDH* carrying insertions of the *Enhancer/Suppressor-mutator* transposon and one stable allele with a transposon footprint were generated in the Arabidopsis ecotype Columbia genetic background. Closer examination of the adaxial epidermis of rosette leaves revealed that in addition to provoking the previously described fusion phenotype in leaves and floral organs, mutations in *FDH* have a deleterious effect on trichome differentiation. *FDH* transcripts were detected exclusively in the epidermis of young vegetative and floral organs. Plants overexpressing *FDH* under control of the cauliflower mosaic virus 35S promoter segregated *fdh* phenocopies, wild-type individuals, and plants showing severe retardation of growth and development. The dwarf plants displayed the most *FDH* expression, the *fdh* phenocopies generally the least. The protein product of *FDH* shows similarity to condensing enzymes involved in lipid biosynthesis, particularly those of the *FATTY ACID ELONGATION* family.

## INTRODUCTION

The epidermis of plants is made up of a single sheet of cells derived from the L1 of meristems. In this layer, cell division takes place only in the anticlinal plane, that is, parallel to the surface; as a result, the cells that form the epidermis of the plant have a common lineage (Satina et al., 1940; Satina and Blakeslee, 1943; Stewart and Burk, 1970). Meristematic cells of the L1 differentiate into several distinctive types of epidermal cells during ontogenesis. However, wounding and grafting both fail to promote dedifferentiation of the epidermis into the parenchymatous cells characteristic of callus tissue (Moore, 1984).

Strikingly, epidermal cells, except those that form the septum of the gynoecium, are not adhesion competent and do not respond to surface contacts by developing shoots. Moreover, the presence of epidermis—even on only one of a pair of graft partners—provides a barrier and prevents underlying parenchymal cells from dedifferentiating into callus. The presence of the cuticle on the outer cell walls of the epidermis is not responsible for this failure of grafting because its removal by abrasion does not promote callus formation

or the union of tissues (Walker and Bruck, 1985; Bruck et al., 1989). These results suggest that the barrier properties of the epidermis cannot be attributed solely to the cuticle and its epicuticular waxes; therefore, other features of the extracellular matrix of the epidermis must account for the failure of cells to adhere to it.

Once established as the protoderm at the embryo stage (Bruck and Walker, 1985), L1 cells can undergo a change in identity when confronted with new positional information—both in rare cases of aberrant periclinal divisions in the L1 and regularly during certain developmental processes (Szymkowiak and Sussex, 1996). For instance, the tips of petals are formed by periclinal divisions of L1 cells that redifferentiate into parenchymal cells, as has been demonstrated by using chimeras (Satina, 1944; Perbal et al., 1996). Another example of the redifferentiation of L1 cells is provided by the union of carpels in angiosperm flowers, the consequence, to various degrees, of two types of fusion events (Baum, 1948a, 1948b, 1949). The first type, known as congenital fusion, involves overgrowth of the intercalary meristem on carpel borders caused by cell divisions in the L2 and L3; as a result, a cuplike structure is formed at the beginning of pistil development. The second type, postgenital fusion, involves a true surface fusion on the adaxial side of the carpels at protruding septum bulges or along their margins, depending on the species; this fusion is induced in L1 cells by a change in responsiveness to cell contact. In Arabidopsis, opposing epidermal cells adhere and redifferentiate into cells of the central transmitting tissue of the septum

according to their new position. However, the single layer of epidermal cells maintained by the developing septum acquires several distinctive features: they retain the ability to form cell wall fusions with the growing pollen tubes that emerge from the central transmitting tissue onto the surface of the septum, and they provide positional information that is used by the pollen tubes to navigate toward the ovule (Lennon et al., 1998).

Several maize and Arabidopsis mutants that display ectopic fusion or adhesion of the aerial portions of plants have been characterized (Sinha, 1998; Lolle, 1999). The cloning of one such locus from maize, *CRINKLY4*, which affects the differentiation of epidermal cells, has been reported (Becraft et al., 1996). The *CRINKLY4* gene encodes a protein similar to transmembrane receptor kinases, particularly members of the animal tumor necrosis factor receptor gene family. Analysis of other mutants is needed before the mechanism of fusion and redifferentiation of the epidermis in plants can be understood.

Here, we report the isolation of three insertion alleles of the *FIDDLEHEAD* (*FDH*) gene of Arabidopsis (Lolle et al., 1992, 1997; Lolle and Cheung, 1993) tagged with the *Enhancer/Suppressor-mutator* (*En/Spm*) transposable element of maize and the subsequent cloning of the *FDH* gene. Examination of the epidermis of *fdh-3940S1*, a new, stable mutant allele, revealed that the number of trichomes initiated on rosette leaves is reduced to nearly one-half that of the wild type. Characterization of the *FDH* gene implies that changes in lipid metabolism may account for the ability of epidermal cells to adhere and undergo differentiation; furthermore, this process appears to be essentially independent of the biosynthesis of epicuticular wax.

## RESULTS

### Identification of the *fdh-3940* Allele Carrying an *En/Spm* Insertion

Mutant *3940* was isolated from a population of Arabidopsis ecotype Columbia (Col) loaded with *En/*Spm transposable elements of maize (Wisman et al., 1998). The mutant demonstrated postgenital fusions between floral organs and some leaves, which resulted in malformations (Figure 1) resembling the phenotype of the *fdh* mutant described in the Landsberg *erecta* (L*er*) ecotype (Lolle et al., 1992). Different leaves of *3940* plants exhibited different degrees of fusion. Although a low seed set was obtained after manual pollination that used mutant plants as male or female parents, the vast majority of the plants remained sterile; therefore, the mutant was maintained by self-pollination of heterozygotes.

The phenotype of mutant *3940* segregated as a single recessive trait. To test for possible allelism, L*er fdh-1* het-

erozygotes were crossed with heterozygotes for the Col *3940* allele and obtained progeny consisted of 80 wild-type Col plants and 16 *fdh* Col plants, indicating that the progeny of this cross segregates 3:1 ($\psi^2 = 2.02$; P > 0.1) and that the mutations are allelic. The new *fdh* allele was designated *fdh-3940*.

The presence of a transposon insertion in the *fdh-3940* locus was indicated by the occurrence of somatic reversions, which were easily recognized by the appearance of wild-type fertile siliques or entire shoots on sterile *fdh* plants (Figure 1). The R$_1$ progeny grown from seeds of such siliques
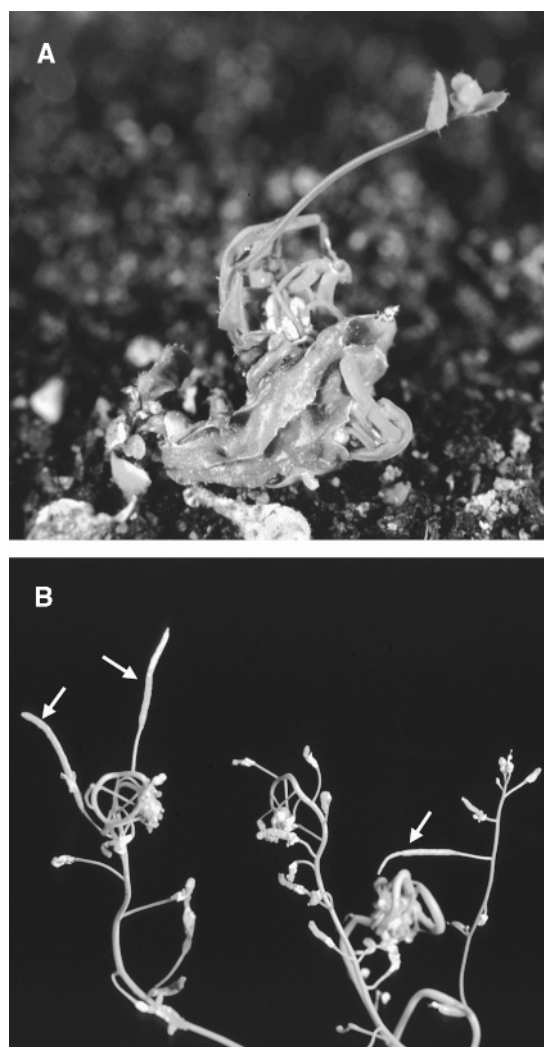


**Figure 1.** Phenotypes of the *fdh-3940* Mutant.

**(A)** Fusion of leaves in plants homozygous for the *fdh-3940* allele.
**(B)** Revertant siliques (arrows) on shoots of *fdh* mutants caused by *En/Spm* excisions from the *fdh-3940* allele.

showed a double excess of *fdh* mutants over wild-type plants (see below). Nevertheless, in the $R_2$ progeny of wild-type $R_1$ plants, the expected 3:1 ratio of wild-type to *fdh* plants was restored, and it was maintained in the next generation in the two revertant families that were analyzed.

### Isolation of Transposon-Flanking Sequences and Analysis of *fdh-3940* Revertants

Amplification of transposon-flanking sequences was performed by using the transposon insertion display (TID) procedure first reported for the retrotransposon *Cin4*, which accumulates in many copies in maize genome (Yephremov and Saedler, 1995). Briefly, a restriction fragment length polymorphism (RFLP) in transposon-flanking sequences is detected by using a restriction endonuclease with a four-base recognition sequence, in a procedure similar to the published amplified fragment length polymorphism approach (Vos et al., 1995). Flanking sequences are amplified by ligation-mediated polymerase chain reaction (PCR) with biotinylated primers, and streptavidin-coated paramagnetic particles are used to capture the biotinylated products. Finally, end-labeled products are separated on a sequencing gel. In principle, identification of a band of interest in TID is similar to cosegregation analysis on DNA gel blots. The band of interest can then be excised from the gel and reamplified by PCR. Details of this procedure will be described elsewhere (A. Yephremov, manuscript in preparation).

Revertants of the *fdh-3940* mutant allele were an important tool in the cloning strategy because they were derived from material that indisputably carries the *En/Spm* transposon insertion in the *FDH* gene. Eleven $R_1$ plants raised from two independent reverted $R_0$ shoots were tested for segregation of the mutant allele in their progeny. One homozygous $R_1$ wild-type plant and four heterozygous $R_1$ plants segregating not more than six transposon copies were selected for TID. A single band that was common to all the heterozygous plants but absent in the homozygous wild-type plant was identified on a sequencing gel. Reamplification and sequencing of the corresponding fragment, designated F250, demonstrated that it accounted for part of the *En/Spm* transposon portion and a flanking sequence, as expected.

The flanking sequence of F250 was used as a probe for DNA gel blots that had been prepared with DNA from $R_1$ plants taken for TID and from their siblings and with DNA from the $R_2$ plants bearing two copies of the *En/Spm* transposon (Figure 2). Cosegregation of F250-hybridizing fragments with the mutant allele of the *FDH* locus was observed in the progeny of the revertants, verifying that fragment F250 contains a part of the *FDH* gene. Sequencing the three revertant alleles of *fdh-3940* demonstrated that all of them were identical to the wild-type *FDH* allele at the site of *En/Spm* insertion.

### *FDH* cDNA and Genomic Clones

A computer search using the F250 flanking sequence revealed several Arabidopsis expressed sequence tags (ESTs) that would likely be identical to the query in the GenBank and EMBL databases. Two of them, accession numbers T04345 and Z26809, were sequenced and appeared to be derived from transcripts of the same gene. A probe prepared from EST T04345 hybridized with all of the fragments that had been detected with the F250 flanking probe in DNA gel blots loaded with genomic DNA from the offspring of the revertant shoots. From the results of DNA gel blot analysis of Col and L*er* genomic DNA digested with various enzymes, it was concluded that *FDH* is a single-copy gene in the Arabidopsis genome. Taken together, these results clearly demonstrate that ESTs T04345 and Z26809 correspond to the F250 fragment. In addition to the ESTs, cDNA clones were isolated from a cDNA library and the two longest clones were sequenced. The *FDH* cDNA was found to be 2055 bp long and contained a single open reading frame.

*FDH* genomic clones were isolated and compared with the cDNA clones by using PCR, tracking, and sequencing. The *FDH* gene was found to contain three exons and two
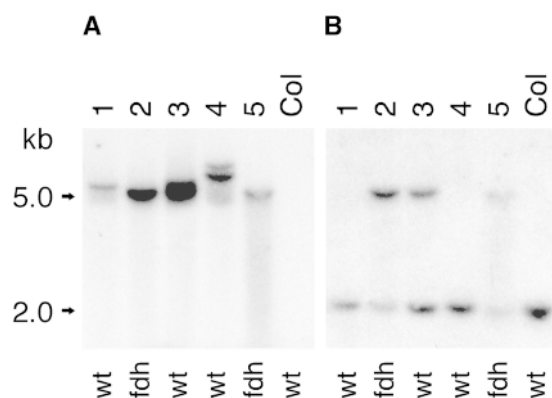


**Figure 2.** Example of DNA Gel Blot Analysis of the Progeny of a *fdh-3940* Revertant.

Each lane on the blot contains 1 $\mu$g of genomic DNA prepared from the $R_2$ progeny of revertant shoot 7625 and digested with DraI. Phenotypes of the plants are indicated as wt (wild type) or fdh (fiddlehead).

**(A)** Hybridization with the EN-600 probe (Wisman et al., 1998) derived from the 5′ portion of the *En/Spm* transposon.

**(B)** The same filter hybridized with the flanking probe derived from F250 by cycle-labeling with the En1R transposon primer located at the very end of *En/Spm*. A band of 5.0 kb corresponds to the *fdh-3940* allele. Note that the mutant plants in lanes 2 and 5 also display a band of 2.0 kb corresponding to the wild-type allele because of somatic excisions of *En/Spm*.

```
ACATTAACTACCTCTCACCAACCACCAAACCCAATCCCCACAATATTACCATTACTCTCA        60
                                     ⟶
TATAACTACACATATTCATATTTACATTTTTTGCCAACACAACTCCTTATAAGATATACA       120
CTTCATCACCTATAGATCTCACTCACATAATCAACCTACAAAACAAAAACAAGAATGGG       180
                                                          M G      2
TAGATCCAACGAGCAAGATCTGCTCTCTCACCGAGATCGTTAATCGTGGGATCGAACCATC      240
 R  S  N  E  Q  D  L  L  S  T  E  I  V  N  R  G  I  E  P  S       22
CGGTCCTAACGCCGGCTCACCAACGTTCTCGGTTAGGGTCAGGAGACGTTTGCCTGATTT      300
 G  P  N  A  G  S  P  T  F  S  V  R  V  R  R  R  L  P  D  F       42
TCTTCAGTCGGTGAACTTGAAGTACGTGAAACTTGGTTACCACTACCTCATAAACCATGC      360
 L  Q  S  V  N  L  K  Y  V  K  L  G  Y  H  Y  L  I  N  H  A       62
                                 FDH::En3940
                                     ▼
GGTTTATTTGGCGACCATACCGGTTCTTGTGCTGGTTTTTAGTGCTGAGGTTGGGAGTTT      420
 V  Y  L  A  T  I  P  V  L  V  L  V  F  S  A  E  V  G  S  L       82
AAGCAGAGAAGAGATTTGGAAGAAGCTTTGGGACTATGATCTTGCAACTGTTATCGGATT      480
 S  R  E  E  I  W  K  K  L  W  D  Y  D  L  A  T  V  I  G  F      102
CTTCGGTGTCTTTGTTTTAACCGCTTGTGTCTACTTCATGTCTCGTCCTCGCTCTGTTTA      540
 F  G  V  F  V  L  T  A  C  V  Y  F  M  S  R  P  R  S  V  Y      122
TCTTATTGATTTCGCTTGTTACAAGCCCTCCGATGAACACAAGgtacgtcccaacttttc      600
 L  I  D  F  A  C  Y  K  P  S  D  E  H  K                        136
catagaggaaatagtctaaattacttttacccaaaaaaaaaaaaaaaaaaaaatctaaa        660
ttaagtatacttaaganattataattagatttgtcaaaaaataataattataattagatg      720
gattagttgttatagggctgcctaaataaaatnaaattttgcctttgcatgtgttacgt      780
tagtaattattttcaggtatatataaaaagtaattattttgcaaaaccttttagatattg      840
gttacgtttgatttaaaaccgaatggtttcgtagaaatttgagaaagtagataacctaaa      900
aactccgattaaangaaaccggtttgacttatataatttaactggtttctgttttcatt      960
ttatttttataaaaaaaacaatccaaatttacgacctataatcaaaggagattgataggaa     1020
ccggactgataattaaatgaagctgaatcaaaccaaacaaaagttcatttaattccggtt     1080
ctctcgggtttaatctcttttttgcattggattggtttagGTGACAAAAGAAGAGTTCATA     1140
                                          V  T  K  E  E  F  I     143
GAACTAGCGAGAAAATCAGGGAAGTTCGACGAAGAGACACTCGGTTTCAAGAAGAGGATC     1200
 E  L  A  R  K  S  G  K  F  D  E  E  T  L  G  F  K  K  R  I      163
TTACAAGCCTCAGGCATAGGCGACGAGACATACGTCCCAAGATCCATCTCTTCATCAGAA     1260
 L  Q  A  S  G  I  G  D  E  T  Y  V  P  R  S  I  S  S  E        183
AACATAACAACGATGAAAGAAGGTCGTGAAGAAGCCTCTACAGTGATCTTTGGAGCACTA     1320
 N  I  T  T  M  K  E  G  R  E  E  A  S  T  V  I  F  G  A  L      203
GACGAACTCTTCGAGAAGACACGTGTAAAACCTAAAGACGTTGGTGTCCTTGTGGTTAAC     1380
 D  E  L  F  E  K  T  R  V  K  P  K  D  V  G  V  L  V  V  N      223
TGTAGCATTTTCAACCCGACACCGTCGTTGTCCGCAATGGTGATAAACCATTACAAGATG     1440
 C  S  I  F  N  P  T  P  S  L  S  A  M  V  I  N  H  Y  K  M      243
AGAGGGAACATACTTAGTTACAACCTTGGAGGGATGGGATGTTCGGCTGGAATCATAGCT     1500
 R  G  N  I  L  S  Y  N  L  G  G  M  G  C  S  A  G  I  I  A      263
                           FDH::En5Q5
                              ▼
ATTGATCTTGCTCGTGACATGCTTCAGTCTAACCCTAATAGTTATGCTGTTGTTGTGAGT     1560
 I  D  L  A  R  D  M  L  Q  S  N  P  N  S  Y  A  V  V  V  S      283
ACTGAGATGGTTGGGTATAATTGGTACGTKGGAAGTGACAAGTCAATGGTTATACCTAAT     1620
 T  E  M  V  G  Y  N  W  Y  V  G  S  D  K  S  M  V  I  P  N      303
TGTTTCTTTAGGATGGGTTGTTCTGCCGTTATGCTCTCTAACCGTCGTCGTGACTTTCGC     1680
 C  F  F  R  M  G  C  S  A  V  M  L  S  N  R  R  R  D  F  R      323
CATGCTAAGTACCGTCTCGAGCACATTGTCCGAACTCATAAGGCTGCTGACGACCGTAGC     1740
 H  A  K  Y  R  L  E  H  I  V  R  T  H  K  A  A  D  D  R  S      343
TTCAGgtcattcattttggtattaattcgttttacaatctcttgaccgacctagtaac     1800
 F  R                                                           345
taattttgtgtggtttttagGAGTGTGTACCAGGAAGAAGATGAACAAGGATTCAAGGGG     1860
                      S  V  Y  Q  E  E  D  E  Q  G  F  K  G     358
TTGAAGATAAGTAGAGACTTAATGGAAGTTGGAGGTGAAGCTCTCAAGACAAACATCACT     1920
 L  K  I  S  R  D  L  M  E  V  G  G  E  A  L  K  T  N  I  T     378
ACCTTAGGTCCTCTTGTCCTACCTTTCTCCGAGCAGCTTCTCTTCTTTGCTGCTTTGCTC     1980
 T  L  G  P  L  V  L  P  F  S  E  Q  L  L  F  F  A  A  L  L     398
CGCCGAACATTCTCACCTGCTGCCAAAACGTCCACAACCACTTCCTTCTCTACTTCCGCC     2040
 R  R  T  F  S  P  A  A  K  T  S  T  T  T  S  F  S  T  S  A     418
ACCGCAAAAACCAATGGAATCAAGTCTTCCTCTTCCGATCTGTCCAAGCCATACATCCCG     2100
 T  A  K  T  N  G  I  K  S  S  S  S  D  L  S  K  P  Y  I  P     438
GACTACAAGCTCGCCTTCGAGCATTTTTGCTTCCACGCGGCAAGCAAAGTAGTGCTTGAA     2160
 D  Y  K  L  A  F  E  H  F  C  F  H  A  A  S  K  V  V  L  E     458
GAGCTTCAAAAGAATCTAGGCTTGAGTGAAGAGAATATGGAGGCTTCTAGGATGACACTT     2220
 E  L  Q  K  N  L  G  L  S  E  E  N  M  E  A  S  R  M  T  L     478
                            FDH::En3AAR103
                                 ▼
CACAGGTTTGGAAACACTTCTAGCAGTGGAATCTGGTATGAGTTGGCTTACATGGAGGCC     2280
 H  R  F  G  N  T  S  S  S  G  I  W  Y  E  L  A  Y  M  E  A     498
AAGGAAAGTGTTCGTAGAGGCGATAGGGTTTGGCAGATCGCTTTCGGTTCTGGTTTTAAG     2340
 K  E  S  V  R  R  G  D  R  V  W  Q  I  A  F  G  S  G  F  K     518
TGTAACAGTGTGGTGGAAGGCAATGAGGAAGGTGAAGACGAAGCCAACCAGGAACAATCCT     2400
 C  N  S  V  V  W  K  A  M  R  K  V  K  K  P  T  R  N  N  P     538
TGGGTGGATTGCATCAACCGTTACCCTGTGCCCTCTCTAAATTATCATTCTTCTAAATTAA     2460
 W  V  D  C  I  N  R  Y  P  V  P  L  *                          550
ATCAAGTAAGATCTCTAATTACTCCAACCAAAAGATACAGTTTGGTTGGATGATAGGAGT     2520
TATTTACTGATCATTCGTATCTAAGTCTGTTATAAGAATGGATGTGGCTAGAGTCCTGTT     2580
CAGCTTCAACTTGTTTTATTTTTTGTTTGTTCTCTATTGGATCTTCATAAACTTTGAGAG     2640
ATTAAAGAAAAAAACTCTTCCTTTAGTTTGATAGAACAGATGGTCATTGTAATTTCTTTAA     2700
                                            ▽
TATGTCAAAGTAAAACAATTTCTTTTTAAGGCAATCTATATTCAGATACATAATAAATTT     2760
AGTTTACGTGTATAAGAAGATAC                                          2783
```
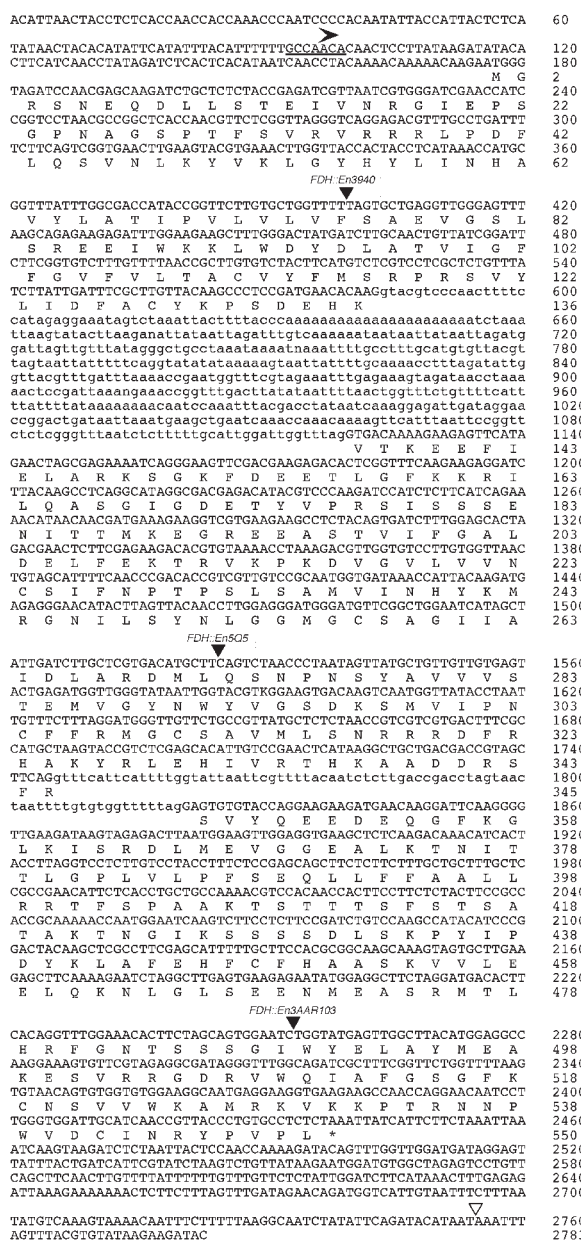
**Figure 3.** Structure of the *FDH* Gene and Its Mutant Alleles Containing *En/Spm* Transposon Insertions.

Intron sequences of *FDH* (GenBank accession number AJ010713) are shown in lowercase letters. The deduced amino acid sequence is shown below the nucleotide sequence of corresponding exons, whereas the putative transcription start is indicated by the arrowhead in the underlined plant consensus. The polyadenylation site is indicated by an inverted empty triangle, and the locations of *En/Spm* insertions are shown by inverted filled triangles: *FDH::En3940* at nucleotide position 401; *FDH::En5Q5* at nucleotide position 1526; and *FDH::En3AAR103* at nucleotide position 2256. The orientation of the transposon is shown by the arrows.

introns of 524 and 72 bp (Figure 3), respectively. The *En/Spm* insertion in *fdh-3940* was located in the coding region of the first exon (Figure 3).

## Other Transposon Insertion Alleles of the *FDH* Gene

Several *fdh*-like mutants were isolated from the *En/Spm* population and were analyzed with the PCR-based assay for the presence of transposons in the *FDH* gene. Two of these mutants, *fdh-5Q5* and *fdh-3AAR103*, had transposon insertions in the second and third exons, respectively, of *FDH*, as revealed by DNA sequencing (Figure 3). Both alleles were recessive and resulted in a mutant phenotype indistinguishable from that of *fdh-3940* (data not shown).

## Mapping of the *FDH* Gene on Chromosome 2

Gel blots prepared from Col and L*er* DNA digested with 30 restriction enzymes did not reveal an RFLP when probed with F250. Because recombinant inbreds could not be used for mapping in this case, the F250 fragment was mapped using the CIC yeast artificial chromosome (YAC) library of Arabidopsis (Creusot et al., 1995). Three YAC clones (CIC10B3, CIC10F1, and CIC10A7) were identified by hybridization, indicating that the F250 fragment is located on chromosome 2 close to the *GPA1* locus. The map position of F250 is consistent with the genetic localization of *FDH* on chromosome 2 (Lolle et al., 1992).

## The FDH Protein Is Similar to Condensing Enzymes Encoded by Genes of the *FATTY ACID ELONGATION* Family

The deduced protein sequence of *FDH* is 550 amino acids long, and the estimated molecular mass of the FDH protein is 62.0 kD, with a calculated pI of 9.14. Computer searches of databases by using the *FDH* cDNA and protein sequences have shown that the genes giving the highest BLAST scores belong to the *FATTY ACID ELONGATION* (*FAE*) family and encode β-ketoacyl-CoA synthases or fatty acid elongases from Arabidopsis, jojoba (*Simmondsia chinensis*), and rapeseed (James et al., 1995; Lassner et al., 1996; Millar et al., 1999; Todd et al., 1999; GenBank accession number U50771). Sequence comparison shows that the five β-ketoacyl-CoA synthases and *FDH* share 54.5 to 56% identical nucleotides, and 73 to 73.5% amino acids are conserved in their products. Multiple sequence alignments reveal remarkable similarity between the proteins along their entire lengths (Figure 4). Other similar sequences in databases showing ~30 to 50% similarity represent exclusively those of fatty acid synthases and polyketide synthases. As do β-ketoacyl-CoA synthases, these condensing enzymes
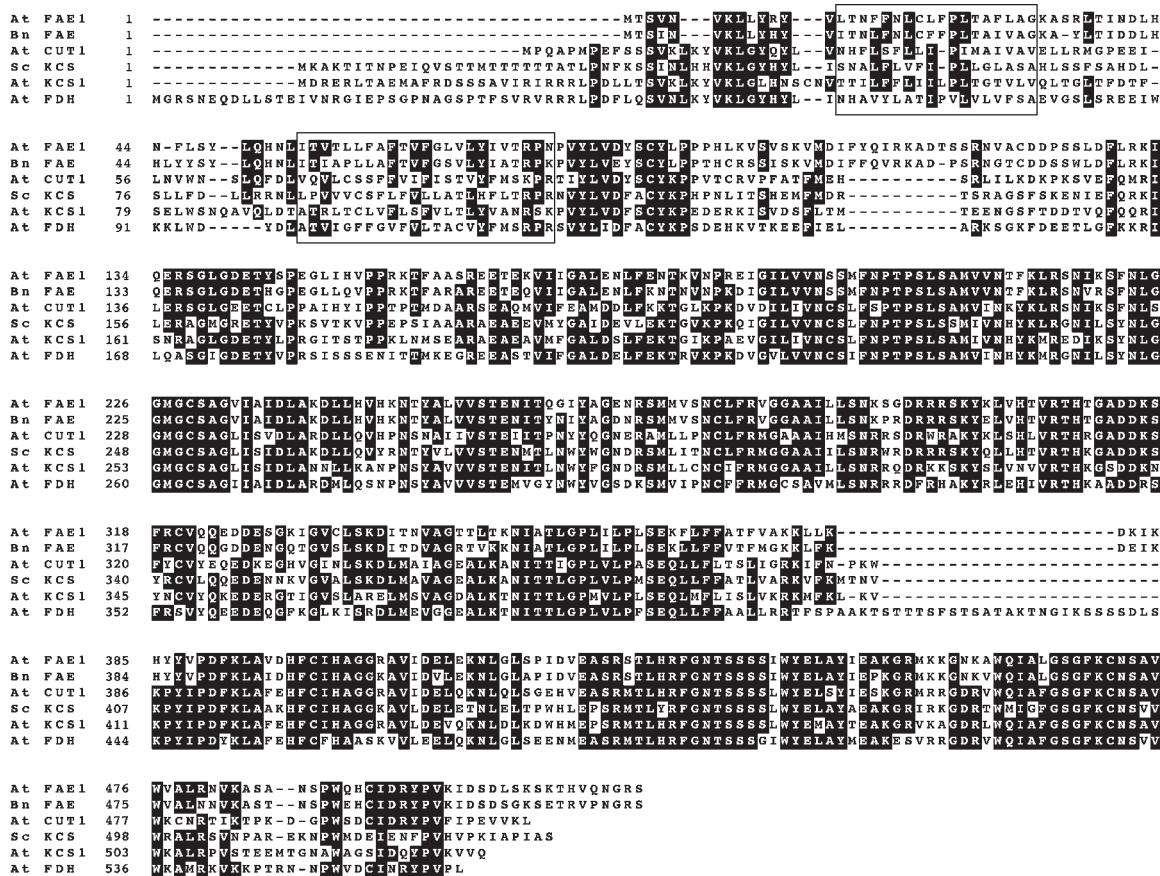
**Figure 4.** Homology between the FDH Protein and the FAE Family of Proteins.

GenBank accession numbers for the proteins in the alignment are as follows: FAE1, U29142; FAE, U50771; CUT1, AAD37122; KCS, U37088; KCS1, AAC99312; and FDH, AJ010713. Multiple alignment of proteins was accomplished by using the PILEUP program from the Genetics Computer Group (Madison, WI) software package. Identical amino acids that are present in at least four of the six sequences are shaded. Amino acids making up the predicted transmembrane domains in all proteins are boxed. At, *Arabidopsis thaliana*; Bn, *Brassica napus* (rapeseed); Sc, *S. chinensis* (jojoba).

catalyze the formation of carbon–carbon bonds in three specific steps (Siggaard-Andersen, 1993). The marked similarity of FDH to condensing enzymes, particularly to fatty acid elongases, suggests that the FDH protein is a condensing enzyme involved in lipid biosynthesis.

The presence of transmembrane domains in FDH was first indicated by the similarity noted between the region at the N terminus and transmembrane domains of various animal receptor proteins (data not shown). Computer analysis implies that the same region probably contains two transmembrane domains at amino acid positions 58 to 75 and 97 to 114, separated by a loop of 24 amino acids. Another putative transmembrane domain in FDH is located between amino acids 382 and 401; however, its predicted transmembrane character is less pronounced. The calculated transmem-

brane domain structures of the FDH and FAE proteins are very similar (data not shown).

**Spatial Pattern of *FDH* Expression in Shoots**

RNA gel blot analysis revealed relatively high amounts of *FDH* transcripts in inflorescences but a very low abundance or even absence of *FDH* transcripts in roots and mature leaves. The more sensitive reverse transcription–PCR (RT-PCR) technique allowed detection of *FDH* transcripts in these latter organs (data not shown). RNA blot analysis showed that *FDH* produces transcripts of ~2.0 kb, which corresponds to the length of the sequenced cDNA (Figure 5).

In situ hybridization analysis of the expression of *FDH*
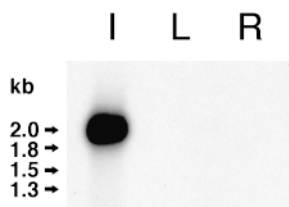
**Figure 5.** Accumulation of *FDH* mRNA in Arabidopsis Tissues Analyzed by Using RNA Gel Blot Hybridization.

The RNA gel blot filter containing 10 μg of total RNA isolated from inflorescences (I), mature leaves (L), and roots (R) was provided by G. Cardon (see Cardon et al., 1997). The filter was probed with $^{32}$P-labeled *FDH* cDNA. Length markers in kilobases are indicated at left.

(Figure 6) shows that *FDH* transcripts are present exclusively in the L1 of vegetative and floral meristems; they are also present in the epidermis of young leaves and coleoptiles, in which the signal is weaker. The trichomes also express *FDH.* Prefusion carpels express the epidermis-specific *FDH* transcripts on both their abaxial and adaxial sides (Figure 6B); transcripts are also detected in the cells of stigmatic papillae, at the margins of ovules, and around the embryo sac (data not shown). The hybridization signal is uniformly distributed in the L1 layer of floral and vegetative meristems; however, it appears to be stronger in the epidermis of younger organs. The expression pattern of *FDH* is compatible with that of the mutant phenotype, which is characterized by epidermal tissue fusions at early stages of shoot development.

## Instability of *fdh-3940* and Generation of a Stable Mutant Allele

The epidermis-specific expression of *FDH* suggests that the phenotype of the shoot is determined by the allelic state at the *FDH* locus in the epidermis. Excisions of the *En/Spm* transposon from the nonfunctional *fdh-3940* allele appear to result in genetic chimeras and mosaics. Because clonally distinct cell lineages are present in shoot meristems, chimeras and mosaics of various types may occur. If the transposon excises in a cell of the L2 or L3, the epidermis remains mutant and defines the phenotype of the *fdh* shoot. Therefore, fragments corresponding to the wild-type allele could be detected by both PCR and DNA gel blot analysis of DNA from *fdh-3940* plants (Figure 2). Excisions in the L1 cells, on the other hand, may lead to periclinal chimeras that show no fusion, although all of their progeny will show the *fdh*-conferred phenotype. This is expected to give rise to an excess of *fdh* plants in the $R_1$ generation, as was indeed observed. Interestingly, $R_0$ plants bearing revertant shoots remain mutant, suggesting that the *FDH* function is cell autonomous or

at most operates only over a short distance. Despite the usefulness of the *FDH* chimeras for the analysis of cell interactions, a stable allele would have helped to avoid the problems associated with mosaicism. Application of the procedure for identification of excision alleles bearing footprints (see Methods) resulted in isolation of the *fdh-3940S1* allele (Figure 7). This stable allele bears a two-base frameshift insertion predicted to result in premature termination of the *fdh-3940S1* gene product. The phenotype of the *fdh-3940S1* mutant is identical to that of the parental mutant *fdh-3940.*

## *fdh* Plants Have Fewer Trichomes on Rosette Leaves

Closer examination of the adaxial epidermis of rosette leaves by cryoscanning electron microscopy confirmed that cell morphology was not noticeably affected in *fdh* mutants (Lolle et al., 1992). The number of trichome cells, however, appeared to be fewer than in wild-type plants (Figure 8). To estimate the statistical significance of this difference, plants segregating the *fdh-3940S1* allele were sampled for analysis after seven or eight rosette leaves had developed but before bolting. At this stage, all mutants display the *fdh*-conferred phenotype and are therefore distinguishable from the segregating sister wild-type plants. Fused and unfused rosette leaves of *fdh* plants demonstrated distorted outlines and jagged leaf blades at maturation, which made direct examination difficult. Therefore, we studied expanding leaves (7 to 10 mm in length) by cryoscanning electron microscopy and calculated the associated trichome density. A nearly twofold decrease in trichome number per square millimeter was found in *fdh* mutants in comparison with the wild type (Table 1), suggesting that *FDH* products contribute to trichome initiation.

## Morphology of Transgenic Plants

To study the effect of constitutive expression of *FDH*, we generated transgenic Arabidopsis plants expressing *FDH* under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

Transgenic plants with pseudo-*fdh* shoots appeared among the $T_1$ transformants; in the $T_2$ generation of 90 plants, these made up ~48% of the total (Figure 9). Compared with *fdh-3940S1* mutants, *fdh* phenocopies did not show rigid fusions, particularly of leaves, and produced a few fertile shoots and siliques, similar to reverting *fdh-3940* mutants. Aside from this class and the wild-type plants, both the $T_1$ and $T_2$ generations segregated plants that were severely retarded in growth and development. These dwarf plants constituted a minority (5.5%) of the $T_2$ progeny of all seven independent $T_1$ transformants. In both $T_2$ dwarfs and $T_2$ *fdh* phenocopies, the epicuticular waxes and the trichomes were apparently normal. However, the leaves on the dwarf plants were darker in color, inflexible, and more rigid
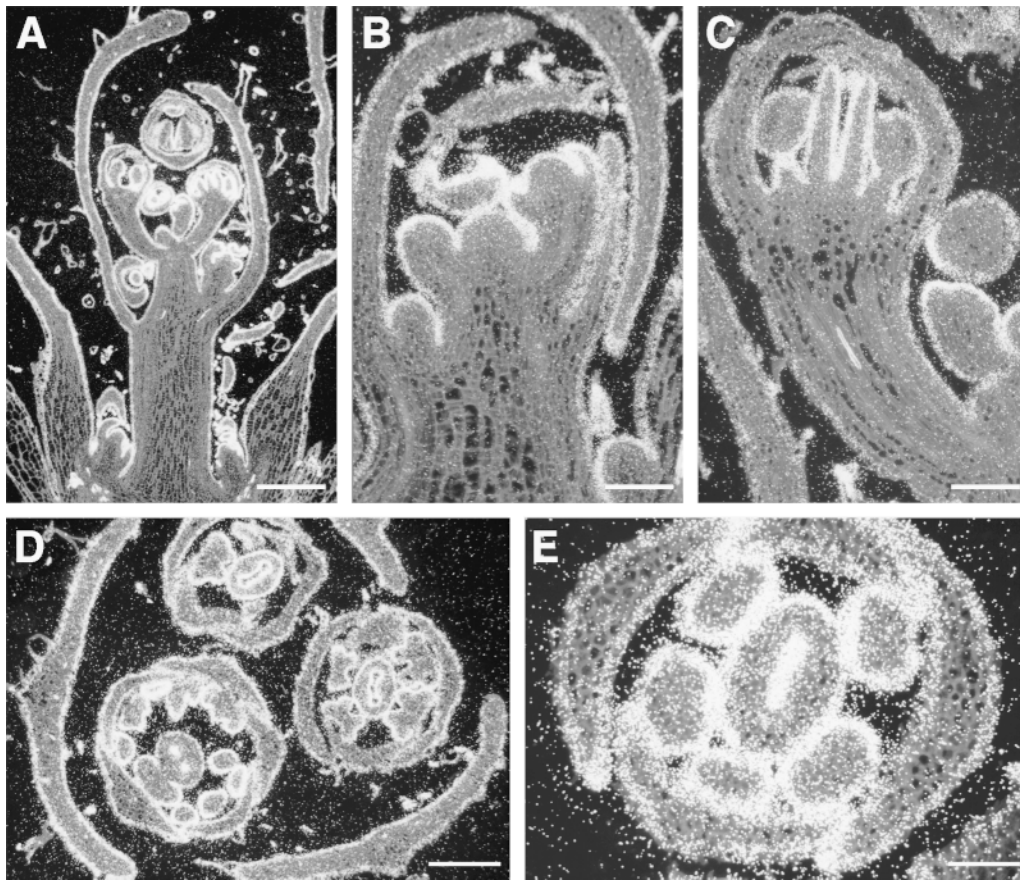
**Figure 6.** In Situ Localization of *FDH* mRNA.

All sections were prepared from wild-type Col plants.
**(A)** Longitudinal section through a bolting plant. Strong *FDH* transcriptional activity is detected in the L1 of apical and lateral meristems as well as in the L1 of organ primordia. A strong hybridization signal is also detected in the epidermis of the shoot and the young organs. Bar = 400 $\mu$m.
**(B)** Longitudinal section through a shoot apex. Note that the hybridization signal declines at later stages of development of organs. Bar = 100 $\mu$m.
**(C)** Longitudinal section of a floral bud, at approximately stage 7 of development (Smyth et al., 1990), showing hybridization signal in the carpel epidermis on the adaxial and abaxial sides. The signal is strongest in the epidermis surrounding the youngest parts of the flower. Bar = 100 $\mu$m.
**(D)** Cross-section through young flowers at the inflorescence apex. *FDH* transcription can be detected in the epidermis of both the adaxial and abaxial sides of all floral organs. Bar = 400 $\mu$m.
**(E)** Cross-section through a floral bud, at approximately stage 7 of development. Bar = 50 $\mu$m.

to the touch. All 96 $T_3$ plants obtained from fertile shoots of four $T_2$ *fdh* phenocopies exhibited fusions in their inflorescences. Unlike $T_2$ plants, the $T_3$ plants rarely produced fertile siliques, and very few seeds could be collected.

## Variability of *FDH* Expression in Transgenic Plants

To assess the expression of *FDH* in the $T_2$ transgenic plants, we isolated total RNA from floral buds and assayed it by quantitative RT-PCR, using the 18S rRNA as an internal standard (Table 2). The assay was performed in the linear range of PCR, as established by using samples of low and high rank (see Methods).

Dwarf plants displayed the most *FDH* expression, whereas *fdh* phenocopies exhibited the least expression among transgenic plants. This result supports the notion that the appearance of *fdh* phenocopies in progeny of transgenic plants may result from cosuppression of *FDH*, whereas the dwarf phenotype correlates with overexpression of *FDH*. Transgenic plants having a wild-type appearance exhibited intermediate amounts of *FDH* expression. These plants represented the most variable group with respect to the extent of expression, indicating that some
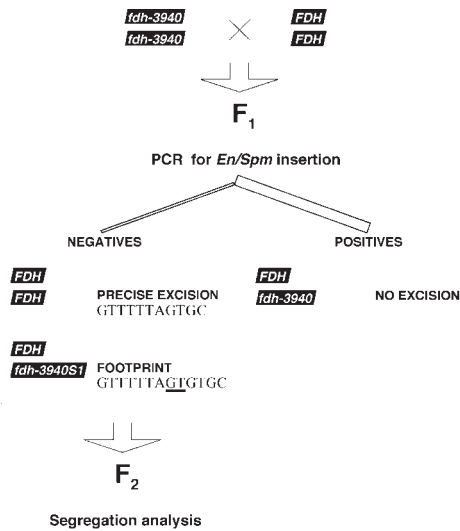
**Figure 7.** Strategy Used to Identify Loss-of-Function Footprints Generated by *En/Spm* in the *FDH* Gene.

Two criteria were sequentially applied for selection. First, F$_1$ heterozygotes of interest had to give a negative result in PCR; second, their progeny had to segregate *fdh* as a recessive trait. The stable *fdh-3940S1* allele detected contains two extra nucleotides, underlined in the sequence GTTTTTAGTGTGC, compared with the sequence in the wild-type allele (GTTTTTAGTGC).

plants retain the wild-type phenotype despite expressing increased amounts of *FDH* mRNA, whereas others remain wild type in morphology, despite partial cosuppression.

DNA gel blot analysis revealed the presence of multiple copies of the transgene (two to six), although no correlation was observed between copy number and phenotype.

## DISCUSSION

### Function of the *FDH* Gene in Development

Mutations in the *FDH* gene result in a complex phenotype. We found that in addition to the fusion of shoot organs (Lolle et al., 1992), the *fdh* mutant exhibits a trichome phenotype. The number of trichomes, which in Arabidopsis develop from single cells of the L1, is reduced on the mutant rosette leaves. This feature was not reported earlier, possibly because of the presence of the *rtn (REDUCED TRICHOME NUMBER)* allele in the *fdh-1* plants in the L*er* genetic background. L*er* plants carrying the *RTN* locus have only one-third the number of trichomes on rosette leaves compared to the Col ecotype. Apparently, the *rtn* allele shortens the period during which epidermal cells continue to initiate trichomes, whereas the rate of trichome initiation remains unchanged (Larkin et al., 1996).

*FDH* expression, as demonstrated by in situ hybridization, begins in L1 cells before trichome initiation and continues throughout their development. However, the epidermal cells ordinarily committed to develop as trichomes do reach their mature form on *fdh-3940S1* mutant leaves. Therefore, although the mode of action of the *fdh-3940S1* allele has not been established, initiation of trichome cells appears to be the main target of the mutation.

The sequence similarity of FDH to the family of condensing enzymes involved in lipid biosynthesis suggests that *fdh* mutations may result in quantitative alterations in lipid composition in the epidermis. However, the glaucous shoot surface of the *fdh* mutant implies that this change is not crucial for the production of epicuticular waxes, which requires the biosynthesis of very long chain fatty acids (VLCFAs). Nevertheless, the *fdh* mutation obviously affects the choice of cell fate in the L1.

Moreover, cells of the mutant epidermis acquire the ability to adhere to other cells with which they make contact. This could mean they display cell surface molecules that are not exposed in wild-type cells. Although we are not able to define the nature of these molecules at this point, we speculate that this role is played by adhesion molecules similar to those in the extracellular matrix of animal cells. Protein–carbohydrate and carbohydrate–carbohydrate interactions could likewise be exploited for adhesion by plant cells.

Epidermal cells of all floral organs respond to the loss of *FDH* function by acquiring the ability to cohere, despite marked differences in their morphology. This suggests that *fdh* mutations affect a pathway shared by various cell types in the epidermis.

Considering that FDH enzymatic activity may be implicated at a branching point in lipid metabolism, *FDH* conceivably could act through distinct pathways to control
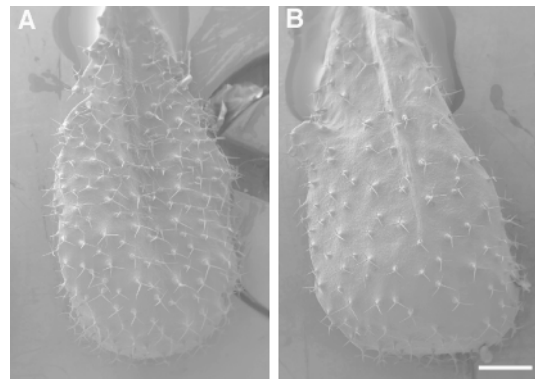


**Figure 8.** Effect of the *fiddlehead* Mutation on Trichome Differentiation in the Col Genetic Background.

**(A)** Cryoscanning electron microscopy of a wild-type leaf.
**(B)** Cryoscanning electron microscopy of a *fdh* leaf.
Bar in **(B)** = 1 mm for **(A)** and **(B)**.

**Table 1.** Average Density of Trichomes on Expanding Leaves in *fdh* and Wild-Type Sister Plants

| Phenotype | No. of Leaves Examined[a] | Density of Trichomes[b] per 1 mm$^2$ |
|---|---|---|
| Wild type | 10 | $1.97 \pm 0.12$ |
| fdh | 20 | $1.07 \pm 0.10$ |

[a] One leaf was taken from each plant for analysis.

[b] Mean $\pm$SE. The null hypothesis is rejected at $P < 0.001$ with the calculated *t* value of 5.38.

epidermal fusions and trichome initiation. Despite this, the fact that cell fate choice depends on the *FDH* function implies that the fusions in *fdh* mutants may be the result of alterations in the developmental state of epidermal cells in meristems.

Analysis of CaMV 35S–*FDH* transgenic plants indicates that ectopic expression of *FDH* is tolerated in many plants without any obvious effect. Some plants, however, are delayed in growth and development, suggesting a link with the pathways controlled by conventional growth compounds. The dwarf phenotype is reminiscent of that seen in some transgenic CaMV 35S–*FAE1* plants (Millar et al., 1998).

**Biochemical Function of the FDH Protein**

The predicted amino acid sequence of the FDH protein reveals significant similarity to various condensing enzymes and most closely resembles the products of genes of the *FAE* family. These genes are thought to encode β-ketoacyl-CoA synthases involved in the biosynthesis of VLCFAs. The FAE proteins catalyze multiple sequential condensing reactions, adding two carbons to the $C_{16}$, $C_{18}$, $C_{20}$, and $C_{22}$ acyl-CoAs by using malonyl-CoA as a donor (Lassner et al., 1996). The *CUT1* and *KCS1* genes encode epidermis-specific β-ketoacyl-CoA synthases, which are required for elongation of fatty acids having >22 carbons (Millar et al., 1999; Todd et al., 1999).

VLCFAs in the epidermis are mainly used for biosynthesis of intracuticular and epicuticular wax, which are mixtures of many aliphatic lipids, including those having backbones up to 32 carbons long (Walton, 1990; Hannoufa et al., 1993; McNevin et al., 1993; Jenks et al., 1995). Condensing enzymes play a crucial role in determining the length of carbon chains, both in seeds and in the epidermis (Millar and Kunst, 1997). β-Ketoacyl-CoA synthases are likely to comprise a small family of proteins in plants, members of which are thought to have different substrate specificities, particularly with respect to the length of the substrate. At least two VL-CFA synthase activities have been purified from microsomes of the leek epidermis (von Wettstein-Knowles,

1993; Cassagne et al., 1994; Evenson and Post-Beittenmiller, 1995).

Most of the Arabidopsis ESTs with sequence similarities to β-ketoacyl-CoA synthases resemble CUT1, KCS1, and FAE1 more than they do FDH (data not shown). In addition, an N-terminal extension of 44 amino acids is specific for
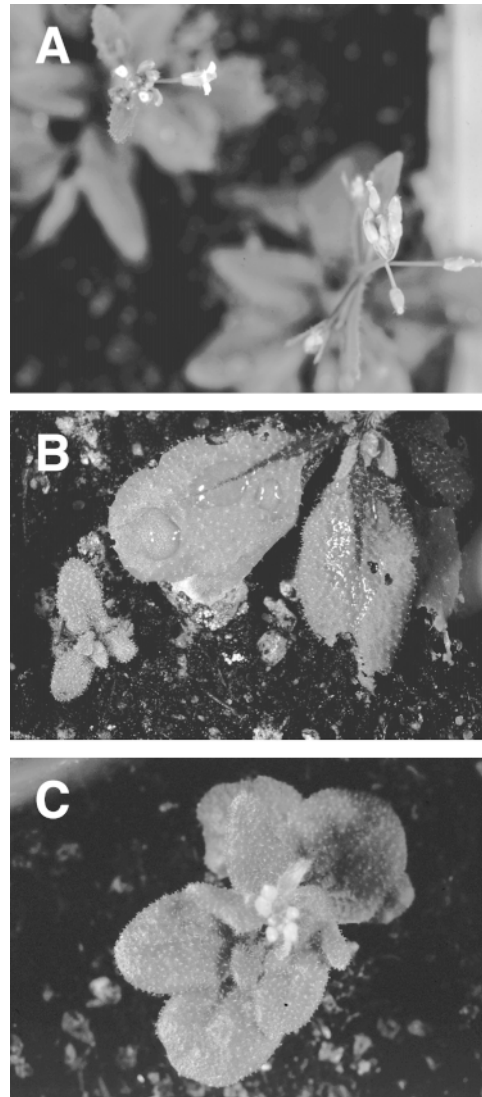


**Figure 9.** Phenotypes of T$_2$ Transgenic Plants Carrying the *FDH* Gene Fused to the CaMV 35S Promoter.

**(A)** Inflorescence of a wild-type plant and a plant showing fusions of floral organs without the leaf phenotype. These moderate phenocopies of the *fiddlehead* mutant made up the majority of T$_2$ plants.
**(B)** A dwarf plant next to the leaf of a wild-type plant taken at the same time as **(A)**.
**(C)** A flowering dwarf plant exhibiting reduced elongation of the stem and leaves of rotund shape.

**Table 2.** Expression of *FDH* in Col and 35S-*FDH*–Transformed Plants as Estimated by Using Relative RT-PCR with the 18S rRNA as an Internal Standard

| Phenotype | No. of Plants | *FDH* Expression Relative to the Internal Standard (%)[a] |
|---|---|---|
| 35S-*FDH* dwarf | 4 | 103.6 ± 2.6 |
| 35S-*FDH* wild type | 11 | 86.6 ± 3.8 |
| 35S-*FDH fdh* phenocopy | 15 | 75.6 ± 1.7 |
| Col | 8 | 69.2 ± 1.7 |

[a] Mean ±SE; see Methods for details. Assays were repeated three times, and reaction tubes were randomized each time in a thermocycler. Repeated measures factor was not found to be significant by analysis of variance; therefore, the data were combined (not shown).

FDH. On the other hand, genes present in rice and *Antirrhinum majus* encode similar motifs at the N termini of their products (A. Yephremov, unpublished data). The *FDH* gene also contains two introns, whereas the known *FAE* genes have none. Finally, CaMV 35S–*FDH* transformants display a range of phenotypes that differs from those demonstrated by CaMV 35S–*FAE1* (Millar et al., 1998) and CaMV 35S–*CUT1* (Millar et al., 1999) transformants. In particular, morphologically altered CaMV 35S–*FAE1* plants exhibit a glossy phenotype we have never observed in CaMV 35S–*FDH*—transformed plants. CaMV 35S–*CUT1* plants also display a severe glossy phenotype, in which the stem wax load is reduced to ~7% of the wild-type amount (Millar et al., 1999).

These differences suggest that FDH could have evolved for specialized roles and could have substrate preferences distinctly different from those of many FAE-like proteins. For instance, the degree of unsaturation and hydroxylation of the carbon chain, in addition to its length, could be important for substrate recognition by the FDH-condensing enzyme. Direct evidence that the FDH protein is an elongase involved in the biosynthesis of fatty acids remains to be provided. Expression of *FDH* in seeds of transgenic plants should help investigators to identify its specific function in lipid biosynthesis, because all the characteristic peaks of the storage oils are well defined.

## Functions of Extracellular Lipids in Adhesion and Fusion

The epidermis is a site of active biosynthesis of various lipids required for deposition of the cuticle and waxes on exterior cell walls. The major role of this lipid layer as a hydrophobic boundary is well established. On the other hand, the barrier function of the epidermis in development cannot be attributed solely to its cuticle and epicuticular waxes, given the inability of epidermal cells with abraded cuticle to undergo redifferentiation during grafting or to con-

vey appropriate signals to underlying cells (Walker and Bruck, 1985; Bruck et al., 1989). The idea is also supported by the fact that exterior epidermal lipids are permeable for signaling substances that induce redifferentiation of the responsive abaxial and adaxial carpel epidermis, as shown by grafting (Siegel and Verbeke, 1989; Verbeke, 1992). Finally, this notion is reinforced by the absence of any explicit correlation between the wax load and organ fusions in multiple mutants of maize and Arabidopsis.

Of the several Arabidopsis *eceriferum* mutants lacking epicuticular waxes (Hannoufa et al., 1993; McNevin et al., 1993; Jenks et al., 1995, 1996a), only *cer10* and *cer13* show slight adhesion or fusion of organs in the shoot. Additional Arabidopsis mutants identified by a visual screen for changes in the texture of the leaf surface (Jenks et al., 1996a) have altered leaf shapes, and two of them, *wax1* and *wax2*, demonstrate fusions of aerial organs. These last two mutants, together with *cer10*, *cer13*, and *deadhead* (Lolle et al., 1998), are the only ones known to display organ fusions or adhesions among the ~30 wax mutants described so far for Arabidopsis (Jenks et al., 1995, 1996a, 1996b). On the other hand, *fdh* plants and other epidermal fusion mutants found in Arabidopsis (Lolle et al., 1998; E. Wisman, unpublished data) are glaucous in appearance and do not have glossy leaves or stems. The *deadhead* locus is worth mentioning separately, because some allelic combinations of this locus did not show wax and fusion phenotypes together (Lolle et al., 1998).

A similar picture is given by phenotypes of maize mutants. Of the 59 mutants in the current maize database that exhibit some kind of a fusion between the aerial organs, only five show any obvious reduction in amounts of wax on leaves, namely, *glossy N203C*, *aberrant seedling N594B*, *adherent N377B*, *adherent N512B*, and *adherent N785B*. Hence, only ~10% of the mutants that show fusion phenotypes are affected in wax cover, a clear demonstration that the visible absence of waxes does not inevitably result in graft-like fusions. At the same time, the occurrence of mutants showing both features implies the existence of an interaction between the two processes. The 1:10 ratio observed is likely to increase because the effect of some mutations on the wax load and its composition may not be immediately apparent to the naked eye and will require a more precise evaluation.

Taking these data into consideration, we may anticipate that external lipids play a subsidiary role in producing epidermal fusions. Conceivably, for instance, impaired cuticle polymerization permits cuticles of adjacent primordia to fuse into one sheet, which consequently leads to an adhesion phenotype. In some mutants, cell wall adhesion molecules may be exposed in L1 cells of meristems and thus play a part in surface fusions. Indeed, the organ fusions exhibited by the various mutants are variable in both pattern and degree. Some display weak organ adhesion phenotypes and may include cuticular mutants that have not yet been described in plants.

The analysis of organ fusions in mutants and the cloning

of genes that, when mutated, result in epidermal fusions should allow us to dissect the relevant pathways and signal transduction cascades.

## METHODS

### Plant Material and DNA Stocks

The *Arabidopsis thaliana* population containing autonomous *Enhancer/Suppressor-mutator* (*En/Spm*) transposons from maize has been described previously (Wisman et al., 1998). The *fdh* mutant generated by ethyl methanesulfonate mutagenesis in the ecotype Landsberg *erecta* (L*er*) was obtained from S. Lolle (Harvard University, Cambridge, MA). Arabidopsis plants were grown in a greenhouse at 22 to 23°C or in growth chambers at 16 to 18°C under 16 hr of daylight.

### Nucleic Acid Isolation and Hybridization Analysis

Genomic DNA was extracted according to Rodgers and Bendich (1988), with slight modifications. DNA samples (1 to 2 $\mu$g) were digested with restriction enzymes in buffers provided by the manufacturers (Boehringer Mannheim, Mannheim, Germany; New England Biolabs, Beverly, MA; and MBI Fermentas, Vilnius, Lithuania) and were subjected to gel electrophoresis in 0.8% SeaKem (FMC, Rockland, ME) agarose. Treatment of gels and membranes (Hybond+; Amersham; Qiabrane; Qiagen) for DNA gel blot hybridization was performed mainly according to Sambrook et al. (1989), except that the cross-linking by exposure to UV light was done in a Stratalinker (Stratagene, La Jolla, CA). Filters were hybridized at 65°C with $\alpha$-$^{32}$P-dCTP–labeled probes (Amersham) prepared by oligolabeling with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) or by cycle-labeling with Taq polymerase (Boehringer Mannheim); the final wash was conducted with 0.2 $\times$ SSPE (1 $\times$ SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) containing 0.1% SDS at 65°C for 20 min. Before hybridization, the labeled probe was separated from unincorporated radioactivity with a QIAprep nucleotide removal kit (Qiagen).

The probes complementary to the 5′ end or the 3′ end of *En/Spm* transposons were prepared by polymerase chain reaction (PCR) as described (Wisman et al., 1998). The filter used for RNA gel blot analysis, which contained 10 $\mu$g of total RNA per lane, was provided by G. Cardon (see Cardon et al., 1997). The blot was hybridized with the complete *FDH* cDNA probe from pYAY$\Delta$A4 (see below), as described in Cardon et al. (1997). $\gamma$-$^{32}$P-ATP (Amersham) end-labeled PCR fragments were cut out from the transposon insertion display (TID) sequencing gel by using disposable syringe needles and were eluted overnight in 100 $\mu$L of TE buffer (TE is 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.6). Before PCR reamplification, the fragments were precipitated with linear polyacrylamide as an inert carrier (Gaillard and Strauss, 1990). Plasmid DNA was extracted by using the QIAprep spin plasmid kit (Qiagen). $\lambda$ Phage DNA was isolated from plate lysates according to Qiagen protocols by using Qiagen-tip 20 columns.

Total RNA for reverse transcription (RT)–PCR assays was isolated with total RNA reagent (Biomol, Hamburg, Germany) and dissolved in formamide (Chomczynski, 1992).

### Oligonucleotides and PCR

Oligonucleotides were either synthesized on a DNA/RNA synthesizer (model 392; Applied Biosystems, Foster City, CA) or purchased from MWG-Biotech (Ebersberg, Germany) or Eurogentec (Seraing, Belgium).

PCRs were performed with Taq polymerase (Boehringer Mannheim) under the conditions recommended by the manufacturer; primers were used at 1 $\mu$M unless otherwise stated. The typical PCR profile started with 1 min at 85°C to preheat samples before the first denaturation step. The thermocyclers used were UNO-Thermoblock and TRIO-Thermoblock (Biometra, Goettingen, Germany) and PCT-200 (MJ Research, Watertown, MA).

### Isolation of *FDH* cDNA and Genomic Clones

cDNA and genomic libraries of the Arabidopsis ecotype Columbia (Col) were screened under the conditions used for DNA gel blot analysis. The cDNA library prepared from flowers in the NM1189 $\lambda$ phage vector was obtained from H. Sommer (Max-Planck-Institut für Züchtungsforschung). The genomic library in the GEM11 $\lambda$ phage vector, prepared by J. Mulligan and R. Davis (Stanford University, Stanford, CA), was obtained from C. Koncz (Max-Planck-Institut für Züchtungsforschung). Arabidopsis expressed sequence tag (EST) clones were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Screening of $5 \times 10^4$ phages from the cDNA library with EST T04345 as a probe resulted in 24 positive clones, which were amplified directly from the phage plaques as described (Gussow and Clackson, 1989), with use of NM17 (5′-AGC-AAGTTCAGCCTGGTTAAG-3′) and NM18 (5′-CTTATGAGTATTTCT-TCCAGGGTA-3′) primers complementary to the *imm434* portion of the $\lambda$gt10 cloning vector (Huynh et al., 1985). Twenty-two library clones were subjected to cycling G- and S-tracking by using primer FA22 (5′-AGAGCGAGGACGAGACATGAAGTAG-3′) on the reverse strand of *FDH* cDNA. Of those, the six longest clones were partially sequenced from the 5′ end to determine the start of the *FDH* transcript, and two of these were sequenced completely, together with EST T04345 and EST Z26809, by primer "walking" on both strands.

To define the intron–exon structure of the *FDH* gene, we compared the genomic and cDNA clones by PCR, using a set of primers distributed along the sequence of the gene. Positions of introns and their lengths were defined by G- and S-tracking (see below). Two introns of 524 and 72 bp were so identified and sequenced.

### Cycle Sequencing, Tracking, and Labeling

The *f-mol* cycle sequencing kit (Promega) was used on plasmids and PCR products according to the enclosed protocol, except that the annealing temperature for sequencing was 5°C below that recommended for PCR.

Mapping of introns and 5′ ends of cDNA clones was performed by G-tracking, which is similar to T-tracking (Sanger et al., 1980; Deininger, 1983), or by S-tracking, using primers labeled at the 5′ end with $\gamma$-$^{32}$P-ATP (Amersham). The reaction conditions were similar to those for cycle sequencing, except that the G-mix from the *f-mol* cycle sequencing kit and all four deoxynucleotide triphosphates (dNTPs) at 20 nM were used for G-tracking and S-tracking, respectively. The S-tracking reaction is similar to the G-tracking reaction, in which premature termination of DNA synthesis occurs after incorporation of ddGTP in a sequence-specific manner. The S-tracking

reaction yields similar results without added dideoxynucleotides because Taq polymerase does not copy a template uniformly, and polymerization is reproducibly interrupted at certain positions along the template. Analysis on a sequencing gel allows one to map the exon–intron and insert–phage junctions and to select appropriate clones and primers (Deininger, 1983).

Cycle-labeling reactions were performed with the PCR products generated by TID, because these are relatively short and contain part of the transposon in addition to the flanking portion. Labeling was done by extension of the En1R primer (5′-ACTCCTTTGACGTTTTCT-TGTGTAGTG-3′) located at the very end of the *En/Spm* transposon under conditions similar to those for PCR. Thus, only the flanking sequence was used as a template for the single-stranded radioactive probe. The reaction was conducted by mixing on ice 2 μL of 5 × *fmol* sequencing buffer (Promega) containing 250 mM Tris-HCl, pH 9.0, and 10 mM MgCl$_2$; 0.8 to 1 μL (16 to 20 pmol) of the specific primer; 1 μL (10 to 100 ng) of the PCR fragment; 1 μL of 10 × C-Mix (20 μM dATP, 20 μM dGTP, and 20 μM dTTP); 0.5 μL (2.5 units) of Taq polymerase; and 5 μL (16 pmol) of α-$^{32}$P-dCTP or ReadyView α-$^{32}$P-dCTP (Amersham) and then running a standard PCR program on samples kept under mineral oil, with annealing at 50°C for 25 to 30 cycles.

## Computer Analysis of Sequences and Database Searches

Management of sequence data was accomplished by using MacVector and AssemblyLIGN programs (Oxford Molecular Group, Campbell, CA). Protein molecular mass and pI were estimated with the Compute pI/Mw program, transmembrane domains were located by using PredictProtein, and the coiled-coil structure was predicted by using the Coils program, all at ExPASy (http://expasy.hcuge.ch/www/tools.html). Homology searches in databases were performed with the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). Estimates of sequence similarity and multiple sequence alignments were obtained by using the Genetics Computer Group (Madison, WI) software package.

Descriptions of the maize mutants showing fusion and adhesion were found in the maize genome database (http://teosinte.agron.missouri.edu/top.html).

## In Situ Hybridization

In situ hybridization was performed as described by Huijser et al. (1992). The DraI-EcoRV fragment containing the poly(A) tail was deleted from the T04345 EST clone, taking advantage of the presence of a unique DraI site at the beginning of the poly(A) stretch and the EcoRV site in the polylinker of the host pBluescript SK− plasmid (Stratagene). The resulting plasmid, pYAYΔA4, containing the complete *FDH* cDNA sequence was used to prepare the α-$^{35}$S-UTP–labeled sense and antisense RNA probes.

## Ectopic Expression of *FDH* in Transgenic Arabidopsis Plants

The complete *FDH* cDNA from pYAYΔA4 was cloned as an XbaI-SalI fragment into the cauliflower mosaic virus (CaMV) 35S promoter–terminator expression cassette of the binary vector pDHFR-35S. This vector, a derivative of pGPTV-DHFR (Becker et al., 1992), allows selection of transgenic plants on medium containing 50 μg/mL methotrexate (G. Cardon, unpublished data). Plasmids were transferred

into *Agrobacterium tumefaciens* strain GV3101 (C58C1, rifampicin resistant) pMP90 (gentamycin resistant) (Koncz and Schell, 1986) by electroporation with an Electroporator II device (Invitrogen) under the conditions recommended by the manufacturer (*Escherichia coli* transformation protocol). Transgenic Arabidopsis plants were obtained by using the vacuum infiltration procedure (Bechtold et al., 1993).

## Identification of the Stable *fdh-3940S1* Mutant

To identify new *fdh* mutant alleles containing footprints that result in a frameshift after imprecise excision of the transposon from the *fdh-3940* allele, we used a PCR-based strategy (Figure 7). The presence of the *fdh-3940* allele in F$_1$ heterozygotes was indicated by the amplification of a 363-bp fragment with the FA22 *FDH* primer and the En205R transposon primer (5′-AGAAGCACGACGGCTGTAGAA-TAGGA-3′). One plant among the 42 analyzed did not contain the *En/Spm* insertion in the gene, although its progeny segregated *fdh* mutants. The offspring were subjected to a second round of PCR with the FA11 (5′-TACCGAGATCGTTAATCGTGGGATC-3′) and FA22 *FDH* primers to amplify the fragment containing the remaining footprint. Direct sequencing of the resulting 329-bp product of the *fdh-3940S1* allele revealed an additional 2 bp at the site of the *En/Spm* insertion that was not present in the sequence of the *FDH* allele (Figure 7).

## Screening for Other Transposon Insertions in the *FDH* Gene

DNAs of several mutant plants showing phenotypes similar to that of the *fdh* mutants were analyzed by PCR for the presence of transposon insertions in *FDH*; to do this, we used the FA11 and FA22 *FDH* primers in all possible combinations with the En205R and En8130 (5′-GAGCGTCGGTCCCCACACTTCTATAC-3′) transposon primers. The conditions for PCR were standard, as described above, except that the annealing temperature was 60°C and the extension time was 2 min. After amplification, PCR products were separated on 1% agarose gels and blotted onto filters.

The gene probe was prepared by labeling *FDH* cDNA from pYAYΔA4 with digoxigenin, for which we used the DIG High Prime DNA labeling and detection kit II (Boehringer Mannheim). The DNA (1 μg) was labeled overnight in a 20-μL reaction, which was then diluted 1:10 with the TE buffer. To each 10 mL of hybridization solution was added 2 μL of diluted sample. Blotting, hybridization, and washing were performed as described for genomic DNA gel blot analysis, and detection was done according to the manufacturer's instructions. The *fdh-5Q5* mutant allele was identified from the specific PCR product obtained with the En8130 and FA11 primers, the *fdh-4AAR103* mutant allele from the product of the En205R and FA11 primers. Corresponding products were reamplified for sequencing by using the appropriate combination of gene and transposon primers. Sequencing reactions were performed as described above with use of the En91R (5′-TGCAGCAAAACCCACACTTTTACTTC-3′) and En8130 transposon primers.

## Cryoscanning Electron Microscopy

Leaves were frozen and vacuum-dried on the cryostage of the preparation chamber, sputter-coated with gold, and examined at 5 kV with a scanning electron microscope (model DSM 940; Zeiss).

## Quantitative RT-PCR Assay with 18S rRNA as an Internal Standard

Aliquots (2 $\mu$g) of the samples of total RNA were precipitated from formamide with 4 volumes of ethanol, washed with absolute ethanol, and dried. RNA pellets were dissolved in 10 $\mu$L of $H_2O$, mixed with 2 $\mu$L of random hexamer primers (60 ng/$\mu$L), and heated at 70°C for 5 min. The RT reaction was initiated by adding 4 $\mu$L of 5 × first-strand buffer (Gibco BRL), 2 $\mu$L of 0.1 M DTT, 1 $\mu$L of 10 mM dNTPs, and 1 $\mu$L of Superscript II RT enzyme (Gibco BRL); the samples were incubated at 25°C for 10 min and then at 42°C for 60 min. The RT reaction products were diluted with 20 $\mu$L of $H_2O$ before storage.

Multiplex PCR was performed in 20-$\mu$L reaction volumes by mixing 2 $\mu$L of the RT reaction product solution, 0.2 $\mu$L of $\alpha$-$^{32}$P-dCTP, PCR buffer components, 1 unit of PLATINUM Taq DNA polymerase (Gibco BRL), and primers. The proprietary antibody complexed with the Taq polymerase allowed for an automatic "hot start" to the PCR. Suppression of amplification of the 315-bp 18S rRNA internal standard to the level of *FDH* was optimized by using a 1:10 ratio of plant 18S rRNA primers to Competimers (Ambion, Austin, TX): 18S rRNA primers modified at their 3′ ends to block extension by DNA polymerase. For PCR, 2 $\mu$L of the mixture of primers was taken. To amplify the *FDH* cDNA-specific product of 258 bp, F2intF (5′-CGAACTCATAAGGCTGCTGACGACC-3′) and F2intR (5′-GGTGGCGGAAGTAGAGAAGGAAGTG-3′) primers were added to the multiplex PCR at 0.4 $\mu$M each. The primers were chosen to anneal at the borders of the second intron of 72 bp, thus allowing DNA contamination to be monitored; no significant contamination was noted under our conditions. PCR was performed as follows: 94°C for 30 sec and 18 cycles at 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min, and then 72°C for 3 min. In a separate experiment using samples with low and high amounts of transcript, the number of cycles chosen was shown to be in the linear range of the reaction. Radioactive PCR products were separated on nondenaturing polyacrylamide gels, which were dried onto blotting paper and exposed to x-ray film. Labeled bands were excised and quantified with a scintillation counter. Relative expression of *FDH*, calculated as log (counts per minute for the *FDH* band)/log (counts per minute for the 18S rRNA band) × 100%, ranged from 65 to 125%. Assays were repeated three times, and data were analyzed by standard procedures in the STATISTICA package (StatSoft, Tulsa, OK).

## REFERENCES

**Baum, H.** (1948a). Postgenitale Verwachsung in und zwischen Karpell- und Staubblattkreisen. Sitzungsber. Oesterr. Akad. Wiss. Math.-Naturwiss. Kl. Abt. 1 **157**, 17–38.

**Baum, H.** (1948b). Über die postgenitale Verwachsung in Karpellen. Oesterr. Bot. Z. **95**, 86–94.

**Baum, H.** (1949). Zur Frage des schrittweisen Überganges vom apokarpen zum coenokarpen Gynözeum. Osterr. Bot. Z. **95**, 470–474.

**Bechtold, N., Ellis, J., and Pelletier, G.** (1993). In planta *Agrobacterium* gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Ser. III Sci. Vie **316**, 1194–1199.

**Becker, D., Kemper, E., Schell, J., and Masterson, R.** (1992). New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol. Biol. **20**, 1195–1197.

**Becraft, P.W., Stinard, P.S., and McCarty, D.R.** (1996). CRINKLY4—A TNFR-like receptor kinase involved in maize epidermal differentiation. Science **273**, 1406–1409.

**Bruck, D.K., and Walker, D.B.** (1985). Cell determination during embryogenesis in *Citrus jambhiri.* II. Epidermal differentiation as a one-time event. Am. J. Bot. **72**, 1602–1609.

**Bruck, D.K., Alvarez, R.J., and Walker, D.B.** (1989). Leaf grafting and its prevention by the intact and abraded epidermis. Can. J. Bot. **67**, 303–312.

**Cardon, G.H., Hoehmann, S., Nettesheim, K., Saedler, H., and Huijser, P.** (1997). Functional analysis of the *Arabidopsis thaliana* SBP-box gene *SPL3*: A novel gene involved in the floral transition. Plant J. **12**, 367–377.

**Cassagne, C., Lessire, R., Bessoule, J.J., Moreau, P., Creach, A., Schneider, F., and Sturbois, B.** (1994). Biosynthesis of very long chain fatty acids in higher plants. Prog. Lipid Res. **33**, 55–69.

**Chomczynski, P.** (1992). Solubilization in formamide protects RNA from degradation. Nucleic Acids Res. **20**, 3791–3792.

**Creusot, F., et al.** (1995). The CIC library: A large insect YAC library for genome mapping in *Arabidopsis thaliana.* Plant J. **8**, 763–770.

**Deininger, P.L.** (1983). Approaches to rapid DNA sequence analysis. Anal. Biochem. **135**, 247–263.

**Evenson, K.J., and Post-Beittenmiller, D.** (1995). Fatty acid–elongating activity in rapidly expanding leek epidermis. Plant Physiol. **109**, 707–716.

**Gaillard, C., and Strauss, F.** (1990). Ethanol precipitation of DNA with linear polyacrylamide as carrier. Nucleic Acids Res. **18**, 378.

**Gussow, D., and Clackson, T.** (1989). Direct clone characterization from plaques and colonies by the polymerase chain reaction. Nucleic Acids Res. **17**, 4000.

**Hannoufa, A., McNevin, J., and Lemieux, B.** (1993). Epicuticular waxes of *eceriferum* mutants of *Arabidopsis thaliana.* Phytochemistry **33**, 851–855.

**Huijser, P., Klein, J., Lönnig, W.E., Meijer, H., Saedler, H., and Sommer, H.** (1992). Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *SQUAMOSA* in *Antirrhinum majus.* EMBO J. **11**, 1239–1249.

**Huynh, T.V., Young, R.A., and Davis, R.W.** (1985). Constructing

and screening cDNA libraries in λgt10 and λgt11. In DNA Cloning: A Practical Approach, D.M. Glover, ed (Oxford, UK: IRL Press), pp. 49–78.

**James, D.W., Jr., Lim, E., Keller, J., Plooy, I., Ralston, E., and Dooner, H.K.** (1995). Directed tagging of the *Arabidopsis FATTY ACID ELONGATION1* (*FAE1*) gene with the maize transposon *Activator*. Plant Cell **7**, 309–319.

**Jenks, M.A., Tuttle, H.A., Eigenbrode, S.D., and Feldman, K.A.** (1995). Leaf epicuticular waxes of the *eceriferum* mutants in *Arabidopsis*. Plant Physiol. **108**, 369–377.

**Jenks, M.A., Rashotte, A.M., Tuttle, H.A., and Feldmann, K.A.** (1996a). Mutants in *Arabidopsis thaliana* altered in epicuticular wax and leaf morphology. Plant Physiol. **110**, 377–385.

**Jenks, M.A., Tuttle, H.A., and Feldmann, K.A.** (1996b). Change in epicuticular waxes on wild type and *eceriferum* mutants in *Arabidopsis* during development. Phytochemistry **42**, 29–34.

**Koncz, C., and Schell, J.** (1986). The promoter of $T_L$-DNA gene 5 controls the tissue specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. **204**, 383–396.

**Larkin, J.C., Young, N., Prigge, M., and Marks, M.D.** (1996). The control of trichome spacing and number in *Arabidopsis*. Development **122**, 997–1005.

**Lassner, M.W., Lardizabal, K., and Metz, J.G.** (1996). A jojoba β-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell **8**, 281–292.

**Lennon, K.A., Roy, S., Hepler, P.K., and Lord, E.M.** (1998). The structure of the transmitting tissue of *Arabidopsis thaliana* (L.) and the path of pollen tube growth. Sex. Plant Reprod. **11**, 49–59.

**Lolle, S.J.** (1999). Epidermal cell interactions: A case for local talk. Trends Plant Sci. **4**, 14–20.

**Lolle, S.J., and Cheung, A.Y.** (1993). Promiscuous germination and growth of wild type pollen from *Arabidopsis* and related species on the shoot of the *Arabidopsis* mutant *fiddlehead*. Dev. Biol. **155**, 250–258.

**Lolle, S.J., Cheung, A.Y., and Sussex, I.M.** (1992). *Fiddlehead*: An *Arabidopsis* mutant constitutively expressing an organ fusion program that involves interactions between epidermal cells. Dev. Biol. **152**, 383–392.

**Lolle, S.J., Berlyn, G.P., Engstrom, E.M., Krolikowski, K.A., Reiter, W.D., and Pruitt, R.E.** (1997). Developmental regulation of cell interactions in the *Arabidopsis fiddlehead-1* mutant: A role for the epidermal cell wall and cuticle. Dev. Biol. **189**, 311–321.

**Lolle, S.J., Hsu, W., and Pruitt, R.E.** (1998). Genetic analysis of organ fusion in *Arabidopsis thaliana*. Genetics **149**, 607–619.

**McNevin, J.P., Woodward, W., Hannoufa, A., Feldmann, K.A., and Lemieux, B.** (1993). Isolation and characterization of *eceriferum* (*cer*) mutants induced by T-DNA insertions in *Arabidopsis thaliana*. Genome **36**, 610–618.

**Millar, A.A., and Kunst, L.** (1997). Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J. **12**, 121–131.

**Millar, A.A., Wrischer, M., and Kunst, L.** (1998). Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. Plant Cell **10**, 1889–1902.

**Millar, A.A., Clemens, S., Zachgo, S., Giblin, E.M., Taylor, D.C., and Kunst, L.** (1999). *CUT1*, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell **11**, 825–838.

**Moore, R.** (1984). Cellular interactions during the formation of approach grafts in *Sedum telephoides* (*Crassulaceae*). Can. J. Bot. **62**, 2476–2484.

**Perbal, M.C., Haughn, G., Saedler, H., and Schwarz-Sommer, Z.** (1996). Non-cell-autonomous function of the *Antirrhinum* floral homeotic proteins DEFICIENS and GLOBOSA is exerted by their polar cell-to-cell trafficking. Development **122**, 3433–3441.

**Rodgers, S.O., and Bendich, A.J.** (1988). Extraction of DNA from plant tissues. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–10.

**Sambrook, J., Fritsch, E.F., and Maniatis, T.** (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

**Sanger, F., Coulson, A.R., Barrel, B.G., Smith, A.J.H., and Roe, B.A.** (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**, 161–178.

**Satina, S.** (1944). Periclinal chimeras in *Datura* in relation to development and structure (A) of the style and stigma (B) of calyx and corolla. Am. J. Bot. **31**, 493–502.

**Satina, S., and Blakeslee, A.F.** (1943). Periclinal chimeras in *Datura* in relation to the development of the carpel. Am. J. Bot. **30**, 453–462.

**Satina, S., Blakeslee, A.F., and Avery, A.G.** (1940). Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. Am. J. Bot. **27**, 895–905.

**Siegel, B.A., and Verbeke, J.A.** (1989). Diffusible factors essential for epidermal cell redifferentiation in *Catharanthus roseus*. Science **244**, 580–582.

**Siggaard-Andersen, M.** (1993). Conserved residues in condensing enzyme domains of fatty acid synthases and related sequences. Protein Sequences Data Anal. **5**, 325–335.

**Sinha, N.** (1998). Organ and cell fusion in the *adherent1* mutant in maize. Int. J. Plant Sci. **159**, 702–715.

**Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M.** (1990). Early flower development in *Arabidopsis*. Plant Cell **2**, 755–768.

**Stewart, R.N., and Burk, L.G.** (1970). Independence of tissues derived from apical layers in ontogeny of the tobacco leaf and ovary. Am. J. Bot. **57**, 1010–1016.

**Szymkowiak, E.J., and Sussex, I.M.** (1996). What chimeras can tell us about plant development. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**, 351–376.

**Todd, J., Post-Beittenmiller, D., and Jaworski, J.G.** (1999). *KCS1* encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. Plant J. **17**, 119–130.

**Verbeke, J.A.** (1992). Fusion events during floral morphogenesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43**, 583–598.

**von Wettstein-Knowles, P.M.** (1993). Waxes, cutin and suberin. In Lipid Metabolism in Plants, T.S. Moore, ed (Boca Raton, FL: CRC Press), pp. 128–166.

**Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van De Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M.** (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. **23,** 4407–4414.

**Walker, D.B., and Bruck, D.K.** (1985). Incompetence of stem epidermal cells to dedifferentiate and graft. Can. J. Bot. **63,** 2129–2132.

**Walton, T.J.** (1990). Waxes, cutin and suberin. In Methods in Plant Biochemistry, Vol. 4, J.L. Harwood and J.R. Boyer, eds (New York: Academic Press), pp. 105–158.

**Wisman, E., Cardon, G.H., Fransz, P., and Saedler, H.** (1998). The behaviour of the autonomous maize transposable element *En/Spm* in *Arabidopsis thaliana* allows efficient mutagenesis. Plant Mol. Biol. **37,** 989–999.

**Yephremov, A., and Saedler, H.** (1995). A PCR-based transposon insertion display (TID) for gene tagging and evolutionary studies. In 37th Annual Maize Genetics Conference, B. Sheridan, ed (Grand Forks, ND: University of North Dakota), p. 69.