Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid ω-hydroxylation in development

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We describe *lacerata* (*lcr*) mutants of *Arabidopsis*, which display various developmental abnormalities, including postgenital organ fusions, and report cloning of the *LCR* gene by using the maize transposon *Enhancer*/*Suppressor-mutator* (*En*/*Spm*). The pleiotropic mutant phenotype could be rescued by genetic complementation of *lcr* mutants with the wild-type *LCR* gene. The *LCR* gene encodes a cytochrome P450 monooxygenase, CYP86A8, which catalyzes ω-hydroxylation of fatty acids ranging from C12 to C18:1, as demonstrated by expression of the gene in yeast. Although palmitic and oleic acids were efficient substrates for LCR, 9,10-epoxystearate was not metabolized. Taken together with previous studies, our findings indicate that *LCR*-dependent ω-hydroxylation of fatty acids could be implicated in the biosynthesis of cutin in the epidermis and in preventing postgenital organ fusions. Strikingly, the same pathway seems to control trichome differentiation, the establishment of apical dominance, and senescence in plants.

The epidermis of plants is a composite tissue that comprises several cell types. Some of these, such as stoma cells, trichomes, and papilla cells, can be easily distinguished from the predominating pavement cells by their characteristic morphological features. Other cell types are not readily distinguishable, although they apparently perform specific functions. One example of this is given by epidermal cells on the adaxial side of carpels, which exhibit a unique contact response during elaboration of the pistil and are able to adhere and redifferentiate into parenchymatous cells. Another unique feature of these epidermal cells is their ability to adhere to the growing pollen tube and guide it to the embryo sac (1, 2). In contrast to animals, where selectively established cell adhesions are common and play an enormous role in development (3, 4), examples of regular cell adhesions in higher plants are rare, and indeed may be restricted to the processes cited above. In particular mutants in several plant species fusions of organs occur during development of the shoot, in a process that resembles the regular fusion of carpels. It is not yet known whether the same molecular mechanisms underlie all instances of cell fusions. By comparison with the epidermis cells of fused carpels, epidermis cells at sutures in fusion mutants do not alter their normal anticlinal plane of division and do not redifferentiate in response to the adhesion. Cell differentiation, however, is affected in at least two fusion mutants. The epidermis of *crinkly4* (*cr4*) maize plants contains enlarged, occasionally spherical, cells, which can divide periclinally to give rise to multilayered sectors (5). In the *fiddlehead* (*fdh*) mutant of *Arabidopsis*, the epidermis of rosette leaves displays a 2-fold reduction in the number of trichomes (6). These findings indicate a link between the altered cell differentiation in the epidermis and the fusion of organs in the mutants.

By using transposon tagging, *FDH* and *CR4*, two genes that result in organ fusions when mutant, have been isolated; *FDH* encodes a putative β-ketoacyl synthase (fatty acid elongase), and *CR4* belongs to a large gene family encoding leucine-rich repeat-containing receptor kinases (5–7). The characterization of the *FDH* gene (A.Y., unpublished data) implies that changes in lipid metabolism in the *fdh* mutant might account for the impairment of cell differentiation and for the ability of epidermal cells to adhere to each other. However, the data that have been accumulated so far did not allow us to identify the molecular pathways responsible. Here we describe an *Arabidopsis* organ fusion mutant, called *lacerata* (*lcr*), and the cloning of the *LCR* gene. We demonstrate that *LCR* encodes cytochrome P450 CYP86A8, which functions as a fatty acid ω-hydroxylase. Our findings reveal several different roles for fatty acid ω-hydroxylation in the development of plants.

Materials and Methods

Plant Growth. *Arabidopsis* plants were grown in a greenhouse at 22–23°C or in growth chambers at 16–18°C under 16 h of daylight at 50–60% relative humidity.

Nucleic Acid Isolation and Gel Blot Analysis. Isolation of genomic DNA, DNA gel blotting and hybridization, and isolation of DNA fragments from agarose gels were done as described in ref. 6. Total plant RNA was extracted by using Total RNA reagent (Biomol, Plymouth Meeting, PA) and additionally purified with an RNeasy plant mini kit (Qiagen, Chatsworth, CA). RNA gel blot hybridization was performed as in ref. 8.

The probes for the 3′ and 5′ ends of the *En*/*Spm* transposon (9) and the *LCR* gene were generated by using PCR. Probes were radioactively labeled as described (6) by using random oligolabeling and cycle labeling.

Molecular Isolation of the *lcr-3P77* Transposon Insertion Allele. DNA blot analysis has shown that about 20 copies of the *En*/*Spm* transposon segregate in *lcr-3P77* plants. *lcr* mutants were crossed twice to plants of the Columbia ecotype, and the BC_2 progeny plants obtained were studied by using DNA blot analysis with *Eco*RI and *Eco*RV. Of the 12–16 copies of *En*/*Spm* that were found in BC_2, one transposon insertion cosegregated with the

Abbreviations: SEM, scanning electron microscopy; RACE, rapid amplification of cDNA ends; HFAs, hydroxylated fatty acids.

Data deposition: The sequence of *Arabidopsis thaliana LACERATA* gene reported in this paper has been deposited in the GenBank database (accession no. AC004665).

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lcr-3P77 allele. This result was obtained when the hybridization probe for the 3′ end of the *En/Spm* transposon was used; however, none of the bands detected with the 5′ end hybridization probe cosegregated with the mutant allele. *Eco*RI fragments of 4.8 kb that corresponded to the cosegregating band were isolated from a 1% SeaKem GTG agarose (FMC) gel, which contained 5 μg of *lcr* mutant DNA, by electroelution into a dialysis membrane bag. The DNA fragments were concentrated with a Microcon-100 (Amicon) and washed once with 300 μl of 2 M NaCl/10 mM Tris·HCl, pH 8.0/0.1 mM EDTA and once with 300 μl of 10 mM Tris·HCl, pH 8.0. Finally, they were eluted from the Microcon-100 membrane with 10 mM Tris·HCl, pH 8.0, and diluted to 10 ng/μl with the same buffer. About 20 ng of the fragments was ligated overnight to 200 ng of NM1149 vector at 16°C in a volume of 5 μl. Phages were packaged with Gigapack Gold extract (Stratagene).

PCR and Reverse Transcription PCR. Screening for transposon insertions in the *FDH* and *LCR* genes by using PCR with gene-specific and *En/Spm* transposon primers was conducted as described in ref. 6. Rapid amplification of cDNA ends (RACE) was performed on 1 μg of total RNA by using the Smart PCR cDNA synthesis kit (CLONTECH). 3′ RACE products were analyzed and purified for sequencing by agarose gel electrophoresis. The 5′ products were labeled with [γ-^{32}P]ATP (Amersham Pharmacia) as described for transposon insertion display (10).

Microscopical Techniques. Cryoscanning microscopy was performed as described in ref. 6. For transmission electron microscopy, materials were prepared as described in ref. 11. Ultrathin sections (≈50 nm) were examined with a Zeiss EM 10C microscope.

Expression of *LCR* in Transgenic *Arabidopsis* Plants. To obtain the pBHS binary vector, the *Hin*dIII–*Sac*I fragment of pGPTV-BAR (12) was substituted for the polylinker containing unique sites (*Hin*dIII, *Xba*I, *Bsi*WI, *Xho*I, *Sca*I, *Sma*I, and *Sac*I).

Primers used to amplify the *LCR* gene introduced restriction sites to facilitate cloning into appropriate vectors. The 3,879-bp *LCR* genomic fragment comprising 1,581 bp of the *LCR* promoter and the transcribed sequence was obtained by using PCR; the fragment was cloned into the *Xba*I and *Sac*I sites of pBHS, thus resulting in the p450gen binary vector.

Transformation of *Arabidopsis* using vacuum infiltration with *Agrobacterium tumefaciens* strain GV3101 (13) was performed as described (14).

Expression of *LCR* in Yeast and Enzymatic Assays. The 1,638-bp fragment containing the *LCR* gene was obtained by PCR with the primers CD1C (TTTCCGGATCCATCATGGAGATTTCCACTGC) and CD2A (AATGGAGCTCCGATTGATTCACCTACCC), and it was prepared for cloning by digestion with *Bam*HI and *Sac*I (underlined sites). The fragment was ligated into the *Bam*HI/*Sac*I-digested yeast expression vector pYeDP60, placing it under the transcriptional control of the galactose-inducible hybrid GAL10-CYC1 promoter (15). The LCR protein was expressed in the *Saccharomyces cerevisiae* strain WAT11, which overexpresses the *Arabidopsis* NADH:P450 reductase ATR1 (16). Enzyme activity was determined by using ^{14}C-labeled substrates and yeast microsomes as described (17).

Results

The *lacerata* Mutation Has Pleiotropic Effects on Cell Differentiation and Plant Development. The mutant 3P77, which is characterized by fusions between rosette leaves and in inflorescences, was found in an *Arabidopsis* population of ecotype Columbia harboring *En/Spm* transposons of maize (9). Features of the recessive phenotype of 3P77 suggested that the mutation was not allelic to mutations in the *FIDDLEHEAD* (*FDH*) gene, which we tagged with *En/Spm* and isolated recently (6). In contrast to *fdh* plants, which regularly exhibited severe shoot deformations and sterility, the particularly strong fusion phenotype of 3P77 was observed in rosette leaves. Unlike the sterile *fdh* plants, 3P77 plants generally produced fertile flowers and displayed relatively weak fusions in inflorescences (Fig. 1*A*). PCR assays using *FDH* and transposon primers have demonstrated that there is no transposon insertion in the *FDH* gene in 3P77 plants. Some 3P77 plants exhibited extremely strong fusions resulting in tearing of leaf tissues during their growth. Occasionally, petioles of some fused leaves have been forced to tear; however, the detached leaves did not senesce and remained green for several days (Fig. 1*C*). Tearing of aerial organs distinguishes 3P77; therefore we called the mutant *lacerata* (*lcr*), and the corresponding allele was designated *lcr-3P77*. Detached leaves of *lcr* were apparently able to obtain the required water and mineral nutrients through the

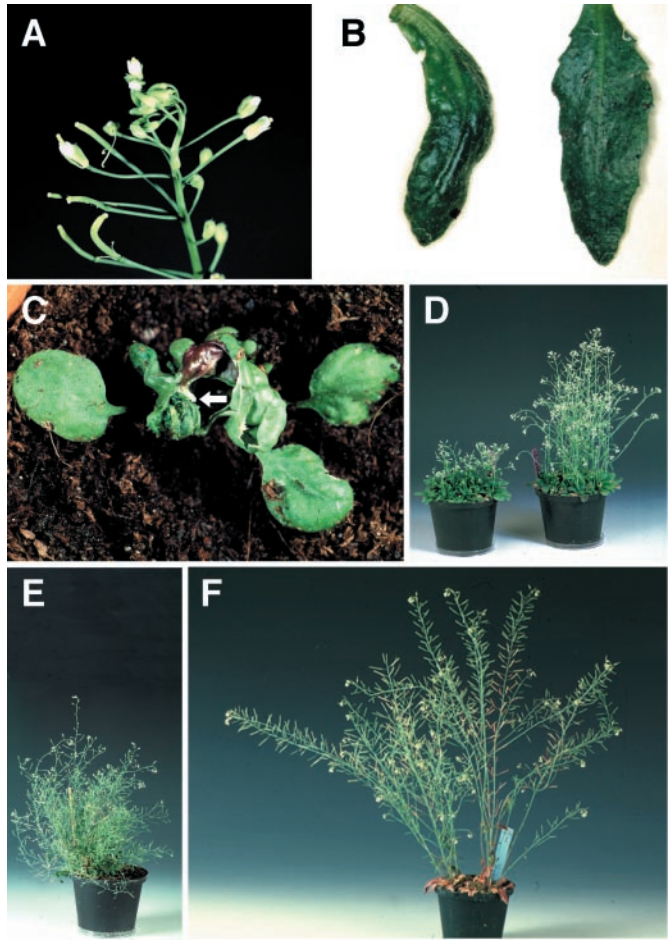

Fig. 1. Features of the phenotype of *lcr* mutants. (*A*) Inflorescence of an *lcr* plant exhibiting organ fusion. Although this feature is typical for *lcr*, its inflorescences manifest less deformation than do those of the *fdh* mutant. The *lcr* mutant is generally fertile, although partially filled siliques are sometimes observed. (*B*) Distorted rosette leaf of *lcr* (*Left*) compared with that of a wild-type plant (*Right*). (*C*) An example of the strong leaf fusion that caused tearing of tissues that were not directly implicated in the fusion during growth of the *lcr* plant. The arrow shows the broken petiole; note that the detached leaf blade did not senesce. (*D*) Reduced height of *lcr* plants (left) in comparison to wild types (right). (*E*) A 3-month-old *lcr* plant demonstrating the extended vegetative growth, extensive branching, and delayed senescence. (*F*) A 2-month-old wild-type plant, which has already ceased to grow.

sutures with contacting leaves, suggesting that the lipid boundary at the exterior epidermal cell walls in *lcr-3P77* mutants becomes permeable or may even disappear upon fusion. To address this question, the sutures were investigated by using transmission electron microscopy (TEM). The analysis demonstrated that the cuticle was not particularly conspicuous, and that contacting cell walls were probably able to adhere directly to each other along the sutures (Fig. 2*G*). No indication of plasmodesmata was found in the sutures analyzed by TEM.

Epidermal cells of organs that had reached different stages of maturation participated in fusions in one *lcr-3P77* shoot. For instance, rosette leaves were found fused to leaf primordia, therefore suggesting that the epidermis of *lcr-3P77* remains competent for fusion over an extended period during development (Fig. 2*E*). Interestingly, it appeared that the morphology and plane of division of epidermis cells that were directly involved in sutures were not noticeably altered in these cases.

Infrequently, plants without fusions were found among the progeny of *lcr-3P77* mutants. The variation in the phenotype of *lcr-3P77* raised the question whether some plants that show no fusions might be revertants resulting from excisions of the *En/Spm* transposon from the *lcr-3P77* allele. However, extensive genetic analysis has demonstrated that the progeny of these plants do not carry the *LCR* allele. The notable variation of the fusion phenotype of *lcr-3P77* therefore must be attributed to other factors.

In addition to the formation of cell bridges between fused organs (Fig. 2 *A* and *B*) and deformations caused by the resulting mechanical stresses, the analysis of the epidermis by scanning electron microscopy (SEM) revealed abnormalities in cell morphology in *lcr-3P77*. These were very often observed as bulges consisting of several cells and as abnormally large roundish cells (Fig. 2*B*). The development of trichomes was also found to be affected in a way that suggested that cell differentiation was retarded in *lcr-3P77* compared with wild-type plants (Fig. 2 *A–D*). As a consequence, young *lcr* plants could be tentatively identified in a segregating population before the fusion phenotype became manifest on the appearance of the second pair of leaves, which are smaller than normal and show reduced pubescence (Fig. 2 *C* and *D*). Some *lcr* leaves displayed regions that virtually lacked all trichomes (Fig. 2*A*).

The ability to support pollen germination on the leaf surface may distinguish many of the fusion mutants from wild types (18). Therefore, we performed a pollen germination test with *lcr* and found that at least two types of the mutant epidermal cells, pavement cells and trichomes, were able to induce pollen germination, as shown in Fig. 2 *H–K*.

In addition to the features described above, *lcr-3P77* plants reached a lower height and had an increased number of shoots, relative to wild type, implying that apical dominance was reduced (Fig. 1*D*); delayed senescence (Fig. 1 *E* and *F*), irregular leaf shapes (Fig. 1*B*), and lower fertility (Fig. 1*A*) compared with wild-type plants were also noted. These growth features were also found in plants that did not display organ fusions, suggesting that they may be caused by a mutation in another gene. Although it appeared upon genetic analysis that the hypothetical second locus is closely linked (or indeed identical) to *LCR*, we carried out complementation experiments to verify that all these traits were a result of the pleiotropy of the *lcr-3P77* mutation.

Transposon Insertion Alleles in a Cytochrome P450 Gene Define *lcr* Alleles. An *En/Spm* transposon insertion that cosegregates with the mutant allele was found in the progeny of *lcr-3P77* plants that were studied by using DNA blot analysis (data not shown). The corresponding fragment isolated from genomic DNA of a mutant plant (see *Materials and Methods*) contained the 3′ end of the *En/Spm* transposon next to an apparently noncoding sequence. Searches in databases demonstrated that this sequence

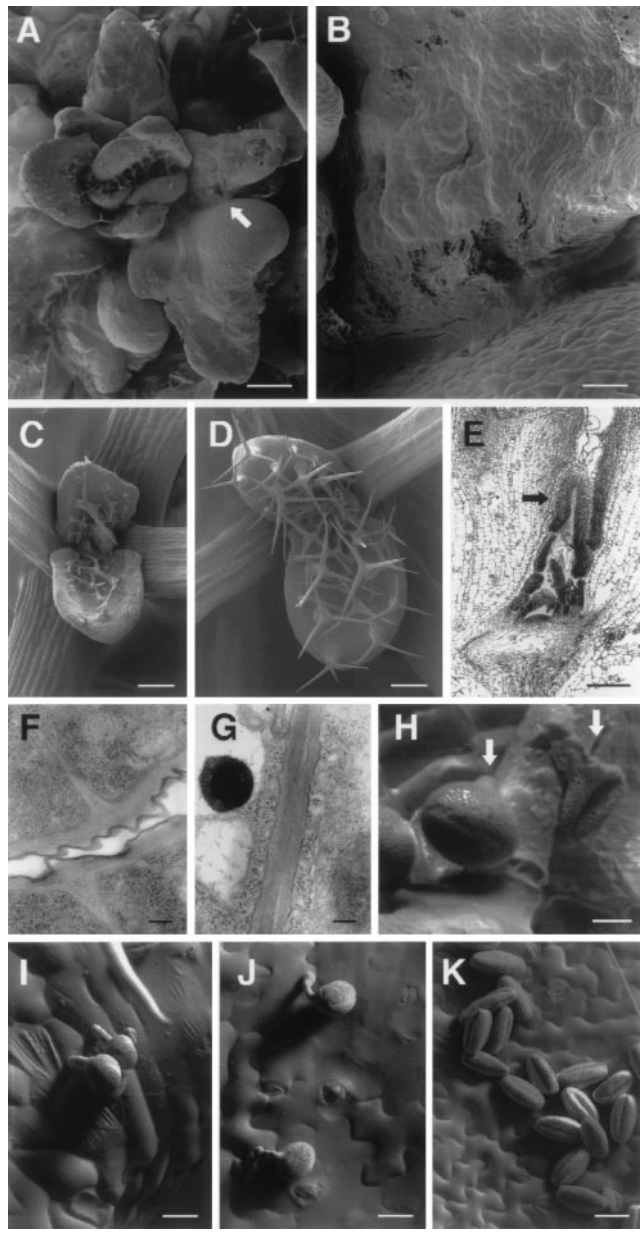

Fig. 2. Microscopic analysis of the epidermis in *lcr* mutants. (*A*) Scanning electron micrograph of a 4-week-old *lcr* plant. Note that trichomes do not appear to be normally differentiated. The arrow indicates the suture region that was examined at higher resolution in *B*. (*B*) Abnormalities in cell differentiation and cell shape in the leaf epidermis of an *lcr* mutant observed with scanning electron microscopy (SEM). Multicellular bulges and spherical cells dominate this spectacular view. Tearing of leaf tissues can be seen next to the fusion. (*C* and *D*) SEM images of the emerging second leaf pair in an *lcr* (*C*) and a wild-type (*D*) plant. Trichomes in the wild type display their normal mature appearance; in contrast, trichomes in *lcr* remain at less advanced stages and exhibit a variety of forms. More developed trichomes are located in the center and along the proximodistal axis of the lamina. (*E*) A longitudinal section stained with toluidine blue and examined by light microscopy, showing fusions between different organs in the *lcr* shoot (arrow). (*F* and *G*) Transmission electron micrographs of two primordia that are in contact in an *lcr* mutant. The cuticle can be seen as a dark layer covering the outermost thick cell walls. In the postgenitally formed suture (*G*), the cuticle is degraded or fails to develop. (*H*) Germination of wild-type pollen on trichomes in *lcr* plants, analyzed by SEM. Arrows indicate emerging pollen tubes. (*I* and *J*) SEM images showing germination of pollen in response to the induction by contact with pavement cells on the adaxial side of leaves in *lcr* plants. The pollen are round as a result of hydration. (*K*) Pollen applied to wild-type leaves remains dormant. (Scale bars are 500 μm in *A*, 100 μm in *B*, 200 μm in *C–E*, 1 μm in *F* and *G*, 10 μm in *H*, and 20 μm in *I–K*.)

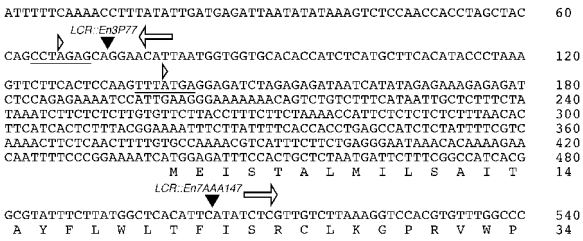

Fig. 3. Structure of the 5′ end of the *LCR* gene and its mutant alleles containing *En/Spm* transposon insertions. The deduced amino acid sequence of *LCR* (GenBank accession no. AJ301678) is shown below the nucleotide sequence. The positions of two transcription starts, TSI and TSII, at nucleotides 67 and 139, as determined by 5′ RACE (see *Materials and Methods*), are indicated by the arrowhead in the underlined plant consensus sequence. The locations of *En/Spm* insertions are shown by ▼: *LCR*::*En3P77* at nucleotide position 72 and *LCR*::*En7AAA147* at nucleotide position 504. Arrows show the orientation of transposons.

appeared to be part of the BAC F4I18 and YAC CIC02E07 clones comprising portions of chromosome 2 of *Arabidopsis*. The only ORF located in the vicinity of the transposon insertion site was that of gene F4I18.5, which encodes a putative cytochrome P450 monooxygenase (GenBank accession no. AC004665). The 1,169-bp copy of *En/Spm* comprising the 3′ end of the transposon was found in the reverse orientation in the 5′ untranslated region of the intronless gene F4I18.5; 5 nucleotides of the otherwise conserved CACTA sequence at the 3′ end were deleted in this transposon copy.

DNAs of several fusion mutants that were found in the population were analyzed by using PCR with primers derived from gene F4I18.5 and *En/Spm.* A copy of the *En/Spm* transposon inserted in the direct orientation into the coding part of gene F4I18.5 was identified in the recessive mutant 7AAA147, which displayed a phenotype essentially identical to that of *lcr-3P77*. This finding strongly suggested that *LCR* might correspond to the gene F4I18.5 encoding a putative cytochrome P450. The new mutant allele was tentatively designated as *lcr-7AAA147* (Fig. 3). Unlike the truncated and, therefore, stable transposon in *lcr-3P77*, *En/Spm* in *lcr-7AAA147* is somatically active, as demonstrated by the PCR amplification of the wild-type fragment from *lcr-7AAA147* DNA. As a result of germinal reversions, 10% of progeny of two *lcr-7AAA147* mutants tested appeared to be heterozygous for the wild-type allele at the *LCR* locus.

Complementation of the *lcr-3P77* Mutation in Transgenic Plants. To address the question whether mutations in other genes contribute to the phenotype of *lcr* plants, the *lcr-3P77* mutant was transformed with a 3,869-bp genomic clone comprising 1,581 bp of the putative promoter region and the transcribed sequence of gene F4I18.5. Eight independent transgenic T_1 p450gen *lcr* plants, carrying 1–8 copies of the T-DNA, manifested a phenotype that was identical to that of wild-type plants (Fig. 4). In contrast, the T_1 progeny of *lcr-3P77* mutants transformed with an empty vector retained the mutant phenotype (Fig. 4 *B* and *C*). This observation implied that the *lcr-3P77* allele could be complemented by the portion of genomic DNA corresponding to gene F4I18.5, thus providing independent proof that the *LCR* gene had been cloned. The complementation experiment also demonstrated that the complex *lcr* phenotype, which comprises, particularly, organ fusions, irregular leaf shapes, reduced apical dominance, and delayed senescence (Fig. 4), is attributable to pleiotropic effects of the mutation in the *LCR* gene encoding cytochrome P450 CYP86A8. Furthermore, since particular features of the mutant are independent of the appearance of organ fusions, *LCR* seems to act in different developmental pathways.

Analysis of *LCR* Transcripts. Using RNA blot hybridization, we detected a 2.2-kb *LCR* transcript in roots, seedlings, and various other organs of flowering plants (Fig. 5). Analysis of the uneven distribution of hybridization signals in organs suggests that *LCR* may be more strongly expressed in the growing parts of a plant. In this respect, *LCR* may be similar to *FDH,* the other organ fusion gene known in *Arabidopsis*; however, in contrast to *FDH* (6), *LCR* is apparently transcribed in roots.

Extensive screening of cDNA libraries failed to detect *LCR* clones. Therefore the 5′ and 3′ ends of *LCR* transcripts were identified by using RACE techniques (19). The sizes of the transcripts deriving for the two transcription start positions, which are marked as TSI and TSII in Fig. 3, appear to be of 2274 and 2202 nucleotides—in good agreement with the results of RNA blot hybridization.

Cytochrome P450 CYP86A8, Encoded by *LCR*, Is a Fatty Acid ω-Hydroxylase. The deduced sequence of the LCR protein corresponds to a putative microsomal cytochrome P450 monooxygenase, which has already been designated as CYP86A8 (see D. Nelson's cytochrome P450 web site, http://drnelson.utmem.edu/Arabfam.html). Fatty acid ω-hydroxylation has previously been established as a biochemical function of CYP86A1, another cytochrome P450 of the CYP86 family (17). To address the question of its function, the LCR protein was expressed in WAT11, a yeast strain that coexpresses an NADPH:P450 reductase from *Arabidopsis* (16) under the control of the galactose-inducible GAL10-CYC1 promoter. In this background-free system, the association between the cytochrome P450 and the NADPH:P450 reductase is reconstituted in yeast microsomes. After induction, P450 was readily detected (data not shown) by carbon monoxide binding difference spectroscopy (20). Very little of the inactive P420 form was discovered, providing evi-

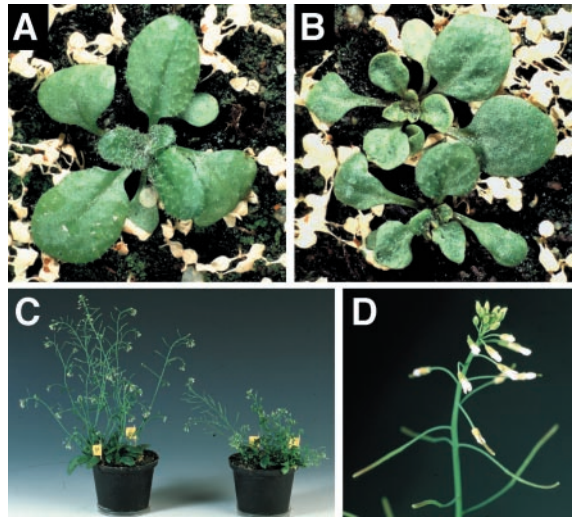

Fig. 4. Complementation experiments with the *LCR* gene in transgenic plants. For complementation of the *lcr* mutation, mutant plants bearing the stable *lcr-3P77* allele were transformed with *Agrobacterium tumefaciens*. One of the plasmid binary vectors, p450gen, carried a portion of the genomic sequence including the gene F4I18.5, which encodes cytochrome P450 CYP86A8; the other, pBHS, was taken as a negative control. (*A*) The herbicide Basta-resistant T_1 transgenic *lcr* plant, which was transformed with p450gen, displays wild-type features with respect to leaf morphology and trichome form. (*B*) In contrast, T_1 transgenic *lcr* plants that were transformed with pBHS exhibit the typical *lcr* phenotype. (*C*) Six-week-old transgenic p450gen *lcr* plants (left) are wild type in appearance with respect to overall plant architecture, whereas transgenic pBHS *lcr* plants (right) remain identical to *lcr* mutants. (*D*) No fusions are observed in inflorescences of transgenic p450gen *lcr* plants. The inflorescence of a T_1 plant is shown.

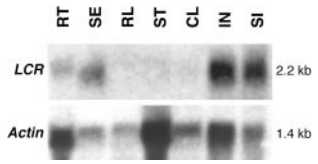

Fig. 5. Accumulation of *LCR* mRNA in various organs of *Arabidopsis* plants. Blot hybridization analysis was performed with 10 μg of total RNA fractionated by agarose gel electrophoresis and transferred to a nylon membrane. RNAs used were from roots (RT), 2-week-old seedlings (SE), rosette leaves (RL), stems (ST), cauline leaves (CL), inflorescences (IN), and siliques (SI). The membrane was sequentially hybridized with the *LCR* and actin probes. Sizes of fragments are indicated to the right.

dence that the hemoprotein was correctly folded. Assays were performed on yeast microsomes by using a set of ^{14}C-radiolabeled substrates (Table 1); no activity with any of the substrates was detected with a vector.

It was found that the LCR protein is able specifically to catalyze ω-hydroxylation of fatty acids ranging from C12 to C18:1. Highest activities were recorded with palmitic and oleic acids as substrates. Interestingly, although oleate was the best substrate, the corresponding epoxide, 9,10-epoxystearate, was not metabolized. This behavior is in sharp contrast to that of the fatty acid hydroxylases of the CYP94 family, which hydroxylate this epoxide more effectively than any other substrate (21). Jasmonate was also not oxidized by LCR, suggesting that the *lcr* phenotype was not caused by a deficiency in breakdown of jasmonic acid by the LCR protein.

Discussion

Cytochrome P450-Mediated ω-Hydroxylation of Fatty Acids in Plants. Cytochromes P450, lipoxygenases, peroxygenases, and hydroxylases contribute to hydroxylation of the acyl chain of fatty acids in plants (22–24). However, cytochromes P450 represent the only known class of enzymes that can introduce a hydroxyl group at the ω-carbon atom of fatty acids.

There is emerging evidence that cytochrome P450s of closely related families constituting clan 86 (http://drnelson.utmem.edu/CytochromeP450.html) are likely to represent a complex group of plant fatty acid hydroxylases, which are distinguished by their substrate specificity, their regional selectivity, and the patterns of their expression. CYP94A1 of *Vicia sativa* was the first fatty acid ω-hydroxylase isolated from plants. As shown by expression in yeast, CYP94A1 catalyzes the ω-hydroxylation of several saturated and unsaturated fatty acids with chain lengths from C10 to C18, but does not modify stearic acid (C18:0) (25). Epoxystearate, and not oleic acid, is the preferred substrate for CYP94A1, which catalyzes the enantioselective ω-hydroxylation of 9,10-epoxystearic acid to 9*R*,10*S*-epoxy-18-hydroxystearic acid (21). This metabolite is the precursor of 9,10,18-trihydroxystearic acid, which is a major monomer of C18 cutins. Besides being cutin monomers, these two latter metabolites may also be involved in signaling during plant–pathogen interaction (21, 26).

Table 1. Catalytic activity of LCR (CYP86A8) in yeast microsomes

Fatty acid substrate	ω-Hydroxylated fatty acid production, pmol·min^{-1} per mg of protein
Laurate (C12:0)	91 ± 26
Myristate (C14:0)	185 ± 53
Palmitate (C16:0)	320 ± 20
Oleate (C18:1)	283 ± 22
9,10-Epoxystearate	ND
Jasmonate	ND

Results are means with standard deviations; ND, not detectable.

Other members of the CYP94 family, CYP94A2 and CYP94A3, are selective for myristic acid (C14:0) and decanoic acid (C10:0), respectively (27, 28). The first member of the CYP86 family, CYP86A1 of *Arabidopsis*, hydroxylates fatty acids of various chain lengths and unsaturation (17). Our substrate-specificity experiments and analysis of the products released from LCR-mediated ω-hydroxylation of fatty acids indicate that LCR (CYP86A8) is similar to CYP86A1, but differs from CYP94A monooxygenases in its ability to metabolize 9,10-epoxystearate. The amino acid sequence of LCR shows 75%, 87%, and 84% similarity to CYP86A1, CYP86A2, and CYP86A7, respectively; this similarity suggests that these enzymes might also interact with a similar range of substrates. Whether the functions of the related fatty acid ω-hydroxylases in plants are similar to the function of LCR remains uncertain, because the consequences for the plant of loss of their function are not known. *LCR*, to the best of our knowledge the first cytochrome P450 ω-hydroxylase for which a mutant has been isolated, appears to encode a nonredundant function in the CYP86 gene family.

The Putative Role of *LCR* in Cutin Biosynthesis in the Epidermis. In plants, various types of hydroxylated fatty acids (HFAs) constitute monomer precursors for the biosynthesis of cutin in the epidermis. Cytochrome P450-dependent hydroxylation is a required step in the formation of C16 and C18 cutin monomers, which have been shown to form primary constituents of the cutin (29). The ω-hydroxyl group is used to form an ester bond with the carboxyl group of another monomer during the polymerization of cutin at the outer epidermal cell wall. The reiteration of this esterification may result in concatenated molecules (30, 31). Besides ω-hydroxyls, many cutin monomers were found to have from one to three in-chain hydroxyl groups, which may result from the cumulative activity of cytochrome P450s, lipoxygenases, and peroxygenases (22, 23). It is anticipated that the introduction of in-chain hydroxyls allows cross-linking of cutin monomers (29, 30); thus both ω-hydroxylation and in-chain hydroxylation are probably required for the formation of a continuous sheet of cutin on the outer surface of epidermal cells.

Recently, ectopic expression of the cutinase gene of *Fusarium solani* f. sp. *pisi* in transgenic *Arabidopsis* plants (31) was shown to impair cutin biosynthesis, and, strikingly, this was associated with the postgenital organ fusion phenotype similar to that of *lcr*. This similarity involves supporting pollen germination and growth by the leaf epidermis. Like most fusion mutants (18), cutinase-expressing transgenic plants exhibited higher cuticle permeability in chlorophyll leaching experiments; however, they were comparable to wild types with respect to the wax load (31), thus pinpointing the cutin as a target and supporting the idea that epicuticular waxes are not directly involved in ensuring that organs in maize and *Arabidopsis* grow independently and remain separate (6).

Taking these data into account, it is tempting to postulate that the cutin polymer acts as a barrier that mechanically isolates epidermis cells of adjoining organs, thus preventing fusion between their cell walls. However, to accommodate the earlier finding that epidermis with an abraded cuticle was not able to take part in grafts (32), one must also assume, in accord with this hypothesis, that the competence to form fusions is limited to particular (early) developmental stages. Although the biochemical function of the LCR protein strongly suggests that it is directly involved in biosynthesis of cutin monomers, further research focused on the epidermis of developing organs, with respect to *LCR* expression and cuticle composition, is required to confirm this role.

Does *LCR* Function Through Biosynthesis of Lipid-Derived Signals? The induction of pollen germination on the leaf epidermis, which was observed in *lcr* plants, is known to occur in several mutants of

Arabidopsis (18). Defects in the barrier function of the cuticle in these mutants may allow unknown lipophilic molecules to migrate through the interface between the epidermis cell and the pollen. The involvement of lipophilic molecules in provoking the specific adhesion of pollen to receptive stigmatic papilla cells and in inducing pollen germination was previously demonstrated (33, 34). Therefore, it is possible to explain the pollen induction phenotype of *lcr* in the context of the barrier hypothesis.

Several other features of the pleiotropic *lcr* phenotype also rescued by genetic complementation with the wild-type *LCR* gene—altered cell differentiation in the epidermis, reduced apical dominance, delayed senescence, and irregular shape of leaves—that occur independently of the organ fusions may be attributed to the effects of changes in lipid metabolism on developmentally important signaling pathways. The existence of HFA-dependent signaling in plant cells was proposed because various physiological effects have been documented as responses to the application of micromolar amounts of HFAs (35–38). Taking into account the broad substrate specificity of LCR and our lack of knowledge concerning the lipid-derived molecular signals involved in the development in plants, it is difficult to pinpoint pathways through which LCR-dependent fatty acid ω-hydroxylation might directly influence cell differentiation and development.

One might expect that the defect in fatty acid ω-hydroxylation in epidermis cells of the *lcr* mutant would increase the endogenous levels of nonesterified fatty acids and cutin monomers. Similar alterations may occur when ester bonds of the cutin matrix fail to form or when such linkages are subject to continual hydrolytic cleavage catalyzed by an esterase. Indeed, the abnormalities reported in the epidermis of transgenic *Arabidopsis* plants expressing a fungal cutinase gene are reminiscent of those in *lcr* and include the variation in cell size, the defects in cell differentiation, formation of multicellular protrusions, and the multilayered epidermis. Interestingly, free cutin monomers were not detectable in the chloroform-soluble surface waxes of the cutinase-expressing plants (31), supporting earlier data that HFAs are rapidly metabolized in the epidermis (39).

The involvement of HFAs as structural components of the protective cutin biopolymer in the outer cell wall of the epidermis, as constituents of membrane lipids (40, 41) and as biosynthetic precursors for biologically active compounds such as jasmonates, which display a wide range of hormone-like biological activities (42–44), suggests that a change in HFA metabolism could have far-reaching consequences for the plant; therefore, further research is needed to determine potential biological substrates of LCR. The regulation of response pathways in *lcr* may be elucidated, for instance, by using gene microarrays, thus allowing an assessment of the hypothesis that HFAs account for the complex phenotype of the mutant and play roles in establishing epidermal cell morphology and differentiation, plant architecture, and the timing of development.

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