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Developmental Regulation of Cell Interactions in the *Arabidopsis fiddlehead-1* Mutant: A Role for the Epidermal Cell Wall and Cuticle

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Although the plant epidermis serves primarily a protective role, during plant development some epidermal cells specialize, becoming competent to interact not only with pollen but also with other epidermal cells. In the former case, these interactions mediate recognition, germination, and pollen growth responses and, in the latter case, result in interorgan fusions which, most commonly, alter floral architecture in ways that are thought to promote reproductive success. In either case, all of the initial signaling events must take place across the cell wall and cuticle. In *Arabidopsis,* mutation of the *FIDDLEHEAD* gene alters the shoot epidermis such that all epidermal cells become competent to participate in both types of interactions. In *fdh-1* mutants, epidermal cells manifest not only a contact-mediated fusion response but also interact with pollen. Since carpel epidermal derivatives manifest both of these properties, we postulated that *fdh-1* epidermal cells were ectopically expressing a carpel-like program. In this report we demonstrate that manifestation of the *fdh-1* phenotype does not require the product of the *AGAMOUS* gene, indicating that the phenotype is either independent of the carpel development program or that *fdh-1* mutations activate a carpel-specific developmental program downstream of the *AG* gene. Furthermore, we demonstrate that plants bearing mutations in the *fdh-1* gene show significant changes in cell wall and cuticular permeability. Biochemical analyses of the lipid composition of the crude cell wall fraction reveal that *fdh-1* cell walls differ from wild-type and manifest significant changes in high-molecular-weight lipid peaks. These results suggest that cell wall and cuticular permeability may be important determinants in developmental signaling between interacting cells and implicate lipids as important factors in modulating the selectivity of the permeability barrier presented by the epidermal cell wall and cuticle. © 1997 Academic Press

INTRODUCTION

In plants only a few examples have been described where morphogenetic signals are known to be exclusively transduced across the outer epidermal cell wall and cuticle. These include early stages in pathogen–host interactions (Ryan and Farmer 1991), pollen–stigma interactions (Knox 1984; Pruitt and Hülskamp, 1994a,b), and carpel fusion (Siegel and Verbeke 1989; Verbeke 1992; Verbeke and Walker 1986). In all these cases, the transduction of signals between participants occurs via an extracytoplasmic route, across the cuticle, the cell wall, and the plasma membrane.

Based on numerous estimates using a variety of techniques, cell walls of dicotyledonous plants are thought to permit free diffusion of molecules having a diameter between 6.6 and 8.6 nm (Baron-Epel *et al.,* 1988). In addition to molecular size, hydrophobicity and charge properties also influence the permeability of the wall to a given molecule (Wyatt and Carpita 1993; Roberts, 1990). The state of wall hydration also influences molecular transport across the wall because the size of the microvoids will increase as the cell wall approaches full hydration (Berlyn, 1969). For molecules partitioning to or translocating across the cell wall therefore, both the molecular composition and the architecture of the matrix can determine whether a molecule

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is excluded from passage across the wall and can affect the rate at which a particular molecule transits the wall. Such molecular discrimination represents at least one potential control point for signal transduction between interacting cells.

An additional transport barrier present on epidermal cells is the cuticle. This outer covering is a complex of cutin, polysaccharide microfibrils, and waxes (Kerstiens, 1996) and is thought to be the true limiting barrier which regulates epidermal permeability (Baur *et al.*, 1996; Kerstiens, 1996). The cuticle is thought to be functionally important not only as a permeability barrier but also serves to limit water loss, provides a mechanical barrier, and acts as a medium for plant signals perceived by insects and microbes as well as a reflective surface for attenuating radiation (Kerstiens, 1996).

A large number of mutants deficient in cuticular waxes have been identified in a variety of species (Koornneef *et al.*, 1989; Lemieux, 1996; Post-Beittenmiller, 1996). Interestingly, in *Arabidopsis* some of these wax-deficient mutants are also conditionally sterile. In *cer1, cer3,* and *cer6* mutant *Arabidopsis* plants cellular interactions important for reproduction are disrupted such that pollen fails to respond and does not hydrate when placed in direct contact with the stigmatic papillar cell (Hülskamp *et al.*, 1995; Preuss *et al.*, 1993). Two of these three *CER* genes have recently been cloned (Aarts *et al.*, 1995; Hannoufa *et al.*, 1996) and although the precise function of the *CER3* gene is not clear, both *CER1* and *CER3* are thought to be components of lipid biosynthetic pathways (Aarts *et al.*, 1995; Hannoufa *et al.*, 1996; Jenks *et al.*, 1995; Koornneef *et al.*, 1989; Lemieux, 1996; McNevin *et al.*, 1993; Negruk *et al.*, 1996; Xia *et al.*, 1996).

During floral ontogeny epidermal cells not only specialize to interact with pollen but, in many flowering plants, also interact with each other (Cusick, 1966). In many species these types of epidermal interactions give rise to supplementary structures and tissues that aid in the pollination process either by altering floral architecture or by giving rise to tissues such as those comprising the transmitting tract of the carpel. Although our understanding of this developmental process remains limited it is clear from studies done on *Catharanthus roseus* that for adhesion to occur cells must contact and undergo a reciprocal recognition reaction similar to that seen in pollen–stigma interactions. In *C. roseus* it has been shown that the morphogenetic factors promoting these developmental responses are small water-soluble molecules (Siegel and Verbeke, 1989) and that these factors are exchanged across the cuticles and cell walls of laterally contacting epidermal cells after the carpel primordia grow into contact (Siegel and Verbeke, 1989; Verbeke 1992). Although all contacting cell surfaces adhere and form a suture, some cells along the fusion suture also undergo redifferentiation, acquiring a nonepidermal fate (Walker, 1975a,b).

In *Arabidopsis* a class of mutants has been described that manifests a contact-mediated fusion response (Lolle *et al.*, 1992) similar mechanistically to carpel fusion in *C. roseus*. In these mutants, however, epidermal cells remain morphologically distinct along the fusion suture and do not redifferentiate in response to contact (Lolle *et al.*, 1992). In addition to manifesting the fusion response *fdh-1* epidermal cells can also interact with pollen (Lolle and Cheung, 1993). This interaction mimics that seen on the wild-type stigma in that it is a species-limited response; only pollen from closely related species will hydrate, germinate, and produce a pollen tube (Lolle and Cheung, 1993). In this report we describe experiments designed to test whether *fdh-1* shoot epidermal cells ectopically express a carpel-like developmental program and explore biological characteristics distinguishing *fdh-1* epidermal cells from wild-type. Based on our findings, several conclusions can be drawn. First, the phenotype of the *fdh-1* mutant is not dependent on the expression of the *AGAMOUS* gene and therefore does not require the ectopic expression of the entire carpel program. Second, *fdh-1* mutants exhibit a dramatic modification of the epidermal permeability barrier. Third, the only striking and detectable biochemical difference between *fdh-1* and wild-type plants can be resolved in the lipid compositional profiles of crude cell wall fractions. These findings implicate cell wall permeability as a potentially important regulatory component in epidermal cell–cell signaling and support the notion that cuticular lipids play an important role in modulating not only pollen hydration but also epidermally mediated organ fusion.

MATERIALS AND METHODS

Plants and Plant Growth Conditions

Plants were maintained under either a long day (16 hr day/8 hr night) or a 24-hr light regime and were illuminated with a mix of fluorescent and incandescent lights (100–175 micromoles m^{-2} sec^{-1} at pot level). Plants were grown in a 9:3:1 vermiculite:soil:sand mixture and plants were watered every 3–5 days as needed with a nutrient solution containing 4 g of fertilizer (Peters 20-20-20) per liter or in CustomBlen Plus (Griffin Greenhouse Supplier) and watered as needed with distilled H_2O. The ambient temperature was maintained at 20°C (±4°C). Plants used for biochemical analyses were grown on ProMix BX under continuous fluorescent illumination (60–70 mmole m^{-2} sec^{-1}) at 23°C and 70% relative humidity. Leaves were collected from plants in bolting or early flowering stages 25 days after planting. Wild-type plants were obtained from nonsegregating sibling populations (line 440) and *fdh-1* mutant plants and their wild-type siblings from segregating populations (line 447). Leaves which showed signs of senescence or extreme deformation were not used in these analyses.

Plants harboring the *agamous* (*ag*) mutation were obtained from V. Irish (Yale University). Double mutants were generated by crossing plants heterozygous for the *ag* mutation with plants either heterozygous or homozygous for the *fdh-1* mutation. Lines producing hydration-defective pollen, *cer1-2211, cer3-2186,* and *cer6-2654,* were generated in our laboratory and have been described previously (Hülskamp *et al.*, 1995).

Pollen Growth Assays

Pollen from wild-type or mutant plants was applied to plant surfaces (rosette and cauline leaves or floral buds) and analyzed by either light or scanning electron microscopy (SEM). Pollen was applied to rosette leaves from wild-type, *fdh-1*, and *ag fdh-1* double mutants, the leaves were isolated, and the pedicel was embedded in 0.5% agarose on a glass microscope slide. To determine the time required for hydration, observations were made continuously for the first 15 min and then every 10 min thereafter for up to 1 hr. Video images were captured using a Nikon Microphot-FXA light microscope equipped with an Optronics VI-470 camera. For samples processed for SEM, pollen was applied to the plant surface and the plant returned to the growth chamber. Approximately 24 hr after pollen application tissue samples were collected, fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid), and prepared for SEM as previously described (Lolle *et al.*, 1992).

Chlorophyll Extraction and Quantitation

Three- to six-week-old plants were collected, the roots removed, and the fresh weight measured and recorded for each sample. Whole plants, leaves, or inflorescences were then carefully immersed in 30 ml of 80% ethanol at room temperature (RT) and the containers wrapped in foil to protect the samples from light. One hundred-microliter aliquots of supernatant were removed at 10-min intervals (up to 60 min) and then after 120 min following immersion. Chlorophyll content was determined by measuring absorption spectra at 664 and 647 nm (Hiscox and Israelstam, 1979). The micromolar concentration of total chlorophyll per gram of fresh weight of tissue was calculated using the equation Total micromoles chlorophyll = $7.93(A_{664}) + 19.53(A_{647})$.

Protein Analysis

Intact whole leaves were removed from 3- to 4-week-old mutant and wild-type plants and placed in 24-well sterile cell culture dishes on ice containing 400 μl of nanopure water, Tris-buffered saline (TBS: 50 m*M* Tris, pH 7.5, 150 m*M* NaCl), or Tris-buffered saline with Tween (TTBS: 50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.05% Tween 20). Samples were incubated for approximately 45 min on ice. Wash solutions were gently pipetted over the tissue every 10 min. Care was exercised so as not to damage the leaf tissues during manipulations. Washes were collected and stored at −20°C. In some samples, a protease inhibitor PMSF (phenylmethylsulfonyl fluoride) was added to the washes at a final concentration of 4 m*M*.

Bradford assays (Bio-Rad) were performed on a fraction of the wash solutions and total protein content determined by extrapolation from a standardized curve. Volumes corresponding to a fixed fresh weight of tissue (total wash volume per gram of tissue) were loaded on precast 4–15% gradient SDS–acrylamide gels (obtained from Bio-Rad) and electrophoresed using standard Tris–glycine buffer. Protein bands were visualized by silver staining gels using a Bio-Rad silver staining kit according to the manufacturers' instructions. Proteins were also transblotted onto nitrocellulose filters (Micron Separations Inc., NitroBind, 0.45 μm) and treated with one of nine different DIG-labeled lectins (AAA, ACA, ConA, DSA, GNA, PHA-L, PNA, RCA 120, WGA: obtained from Boehringer Mannheim) according to the manufacturers' instructions.

Cytochemistry and Lectin Treatments of Tissue Sections

Dewaxed 8-μm tissue sections were treated according to the following protocols. Periodic acid hydrolysis and Schiff (PAS) staining for carbohydrate was carried out as described by Berlyn and Miksche (1976). Lipid stains included Sudan III, Fat Red 7B (Sigma), and Fluoral Yellow 088 (Sigma). For Sudan III staining, hydrated sections were pretreated in 10% chromic acid for 10 min and stained for 1 hr at RT in a solution containing 0.7% Sudan III (w/v) in pure ethylene glycol (Berlyn and Miksche, 1976). Staining with Fat Red 7B and Fluoral Yellow 088 was carried out as described by Brundrett *et al.* (1991). Napthol Yellow S was used as a general stain for protein (Berlyn and Miksche, 1976). Ruthenium Red and the hydroxlyamine–ferric chloride reactions were used to test for the presence of pectin-like substances (Jensen, 1962). Samples stained with Fluoral Yellow 088 or Napthol Yellow S were viewed with a Leitz (Diaplan) fluorescence microscope equipped with a I 2/3 optical cube.

For lectin binding analysis, dewaxed tissue sections were rehydrated through a graded ethanol series to water and then equilibrated in TBS. Sections were then incubated in blocking reagent (0.5 g blocking reagent from Boehringer Mannheim in 100 ml TBS) for a minimum of 30 min at RT or left overnight at 4°C. Tissue sections were washed twice in TBS and once in Buffer 1 (TBS plus 1 m*M* $MnCl_2$, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$). Sections were then incubated with one of nine different digoxigenin (DIG)-conjugated lectins (AAA, ACA, ConA, DSA, GNA, PHA-L, PNA, RCA 120, WGA; obtained from Boehringer Mannheim) in Buffer 1 for 1 hr at RT (lectins were used at the manufacturer's recommended concentrations). Sections were then washed three times for 10 min each in TBS followed by incubation in anti-DIG alkaline phosphatase Fab fragments for 1 hr at RT. To visualize lectin–anti-DIG binding sites, a staining solution containing 375 μg/ml nitroblue tetrazolium and 187.5 μg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in Buffer 2 (Tris–HCl 100 m*M*, pH 9.5, 50 m*M* $MgCl_2$, 100 m*M* NaCl) was used. Slides were immersed in the staining solution and left to develop for approximately 10 min at RT. Staining reactions were stopped by rinsing slides in distilled water.

Determination of Monosaccharide Composition

Leaf samples were frozen in liquid nitrogen and ground to a course powder. Half of the pulverized leaf material from each batch was extracted twice with 80% ethanol for 30 min at 85°C. The crude cell wall material was then divided into four samples per batch and dried *in vacuo*. Hydrolysis of the crude cell wall material and quantitation of neutral monosaccharides via gas chromatography of alditol acetates was carried out as described previously (Reiter *et al.*, 1993).

Determination of Fatty Acid Composition

Part of the leaf material collected from the plants was fractionated into ethanol-soluble and ethanol-insoluble material. The following fresh weight amounts were used: wild-type (line 440), 250 mg, and *fdh-1* (line 447), 210 mg. The leaf powder was extracted in scintillation vials with 10 ml of 80% ethanol each for 20 min at 80°C. Following centrifugation, three equal amounts from each supernatant were transferred to screw cap vials and marked as the ethanol-soluble material. The contents of each of these vials was

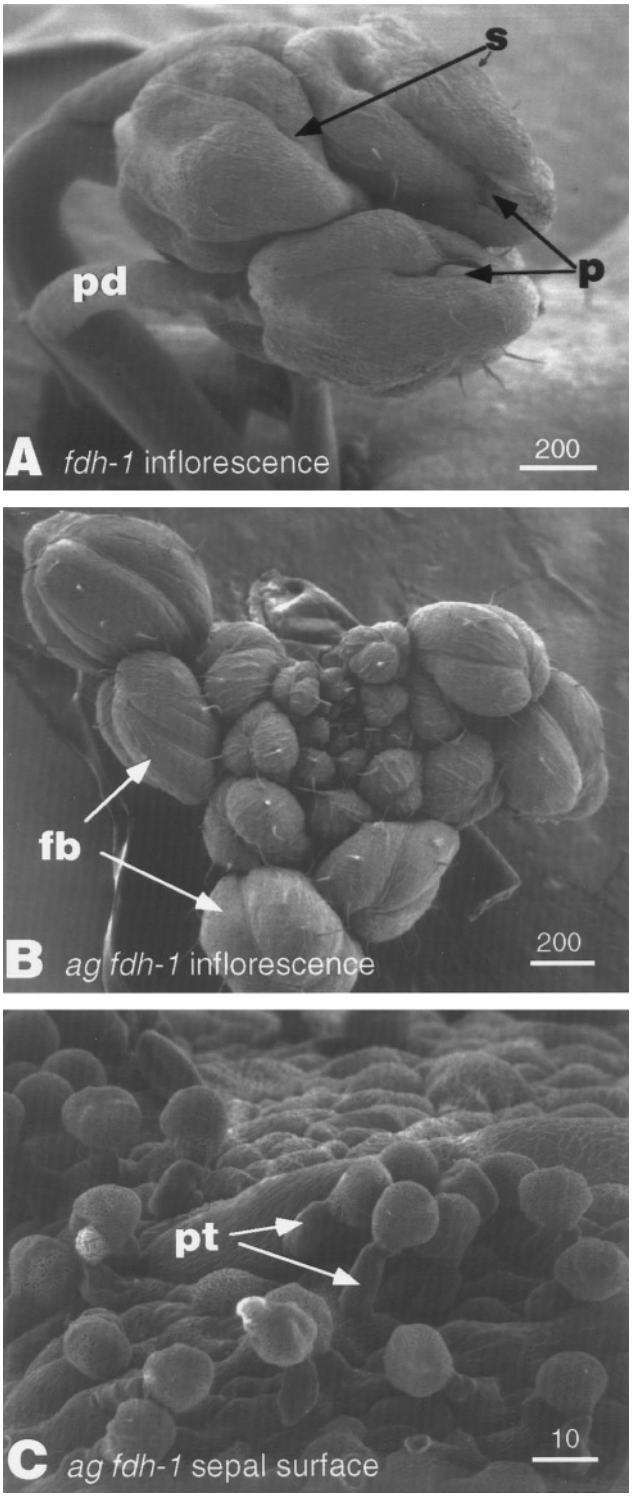

FIG. 1. Scanning electron micrographs showing organ fusion on a *fdh-1* (A) and *ag fdh-1* double mutant (B) and pollen growth (C) on an *ag fdh-1* double mutant. (A) Floral buds adhere causing curving of pedicels (pd) in *fdh-1* mutants and prevent self-pollination by trapping stamens within the fused sepals (s). Pistil growth, although similarly perturbed, is not as severely affected and as flowers mature, protrusion of the entrapped pistil (p) is usually observed. (B) In *ag fdh-1* double mutants floral buds (fb) fuse in a manner analogous to that seen in *fdh-1* mutants. Due to the lack of a pistil, the floral buds retain a more blunted appearance. (C) Wild-type pollen when applied to vegetative *ag fdh-1* surfaces as well as floral organs such as sepals will hydrate and grow pollen tubes (pt). Scale bars indicate magnification in micrometers.

dried in a vacuum evaporator at 30°C for 90 min. Pelleted material from the above ethanol extractions was reextracted with 10 ml of 80% ethanol each for 20 min at 80°C and the supernatant discarded. The residue (crude cell wall material) was washed twice with 10 ml of 80% ethanol at room temperature, transferred into three screw cap vials per type of residue, and dried down via lyophilization. Derivatization of all samples to fatty acid methyl esters (FAMEs) used the protocol of Miquel and Browse (1992). FAMEs were analyzed in splitless mode on a Hewlett–Packard 5890 Series II Plus gas chromatograph equipped with a flame ionization detector. Separation was done on a 0.75-mm ID glass column (30 m in length) coated with SP2330 (Supelco). Inlet and detector temperatures were set to 240° and 250°C, respectively. The oven temperature program was as follows: 100°C for 1 min, 30°C/min temperature ramp to 160°C followed by a 10°C/min ramp to 240°C; the inlet pressure was 20 kPa during the first 5 min of the run followed by a 10 kPa/min ramp to 40 kPa. After 2 min at 40 kPa, the pressure was increased to 100 kPa at 200 kPa/min and held at 100 kPa until the end of the run. Detector responses were calibrated using a rapeseed oil standard (Supelco). The detector response for the cell wall-specific lipid at RT 8.92 was assumed to be identical to that of C24:0.

RESULTS

Analysis of ag fdh-1 Fusion Phenotype and Pollen Hydration

Floral induction is not required for either organ fusion or pollen growth since *fdh-1* plants maintained under short-day growth conditions still manifest both characteristics (Lolle *et al.,* 1992; Lolle and Cheung, 1993). However, in these experiments the plants used eventually flowered and therefore were capable of expressing a carpel developmental program. To determine whether genetically ablating the normal pathway of carpel development would affect the *fdh-1* mutant phenotype, *fdh-1* plants were crossed to plants heterozygous for the *ag* mutation. In plants homozygous for the *ag* and *fdh-1* mutations, both late ontogenetic fusion and ectopic pollen growth still took place indicating that the phenotypes are additive (Fig. 1).

On wild-type *Arabidopsis* stigmas, self-pollen grains hydrate rapidly, swelling to approximately twice their diameter within approximately 5–10 min (Hülskamp *et al.,* 1995; Preuss *et al.,* 1993; Pruitt and Hülskamp, 1994). The hydration reaction is specific and requires recognition events between the stigma and the resident pollen grain. A number of *cer* mutants which are defective in various aspects of lipid metabolism also produce pollen defective in the hydration

response (Hülskamp *et al.*, 1995; Preuss *et al.*, 1993). When placed on receptive stigmatic surfaces pollen from these mutants fails to hydrate. Copollination with wild-type pollen, however, can facilitate hydration and rescue the mutant pollen, as can increasing the relative humidity. Presumably, enhanced cell wall permeability might also alleviate this hydration barrier if the barrier to pollen hydration is simply due to an impedance in water transfer from sporophytic tissue to the pollen grain. When rates of wild-type pollen hydration were tested using intact *fdh-1* leaves mounted on microscope slides, hydration rates were found to be comparable to those seen on wild-type papillar cells. As shown in Fig. 2, hydration was complete within 5–10 min. However, when pollen from hydration-defective *cer* mutants was applied, mutant pollen did not hydrate even after prolonged contact with the *fdh-1* leaf surface (Figs. 2B–2D).

To test whether the presence of the *ag* mutation had any effect on the hydration reaction, *ag fdh-1* double mutants were tested with wild-type pollen. As was the case for *fdh-1* plants, hydration was complete with 5–10 min. Similarly, hydration-defective *cer* pollen did not hydrate on double mutant leaf surfaces even after prolonged contact (data not shown).

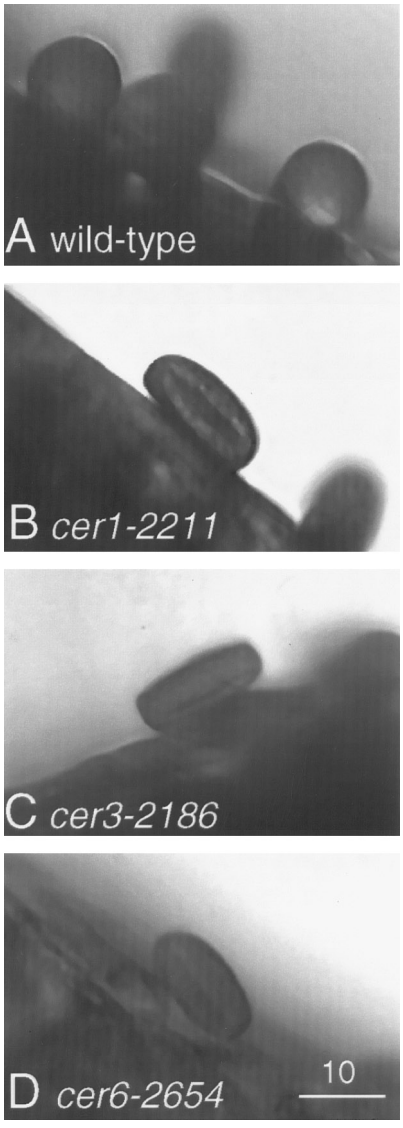

FIG. 2. Video capture images of wild-type (A) and hydration-defective (B–D) pollen derived from *cer1, cer3,* and *cer6* mutants on *fdh-1* rosette leaf surfaces. As shown in A, wild-type pollen hydrates and becomes spherical in appearance within 5–10 min after transfer to the leaf surface. Hydration-defective pollen (B–D) does not hydrate and retains its elliptical shape even after prolonged exposure to the leaf surface. Scale bar indicates magnification in micrometers.

Analysis of Cell Wall Sieving Properties

Previously we observed that *fdh-1* tissues showed greater permeability to chemical fixatives than wild-type tissues and tended to leach chlorophyll more rapidly when immersed in alcohol (Lolle and Cheung, 1993). These findings suggested that cell wall properties (the cell wall defined here as including the cuticle) might differ in mutant plants. To test this possibility, cell wall sieving characteristics were compared between wild-type and *fdh-1* plants.

Chlorophyll a and b are relatively small molecules having molecular weights of 892 and 906, respectively, and can be readily extracted from plants by immersing tissues in acetone or ethanol (Hiscox and Israelstam, 1979). We took advantage of the ease with which chlorophyll content can be quantified spectrally and tested the relative rates of chlorophyll diffusion by immersing intact shoots in 80% ethanol and then measuring the relative increase in chlorophyll concentration in the supernatant at fixed time intervals. Results from these experiments are shown in Fig. 3. As indicated by the graphed data, chlorophylls diffuse out of *fdh-1* tissues rapidly and equilibrate within approximately the first 30 min following immersion. Wild-type samples failed to reach equilibrium after 120 min.

Protein Profiles of Leaf Surface Washes

Do mutant cell walls also manifest permeability changes to larger molecules? To address this question surface washes of intact *fdh-1* leaves were analyzed for protein content and compared to washes prepared from wild-type leaves. In each case, leaves were bathed in solutions of H_20, TBS, and TTBS, aliquots of each solution electrophoresed on SDS–polyacrylamide gels, and extracted protein species visualized by silver staining. As shown in Fig. 4 several different protein bands were evident, although multiple bands common to both *fdh-1* and wild-type were also detected. Although the protein profiles observed varied from trial to trial, a consistent trend was observed. More protein bands were typically visible in washes prepared from *fdh-1* tissues and a number of proteins ap-

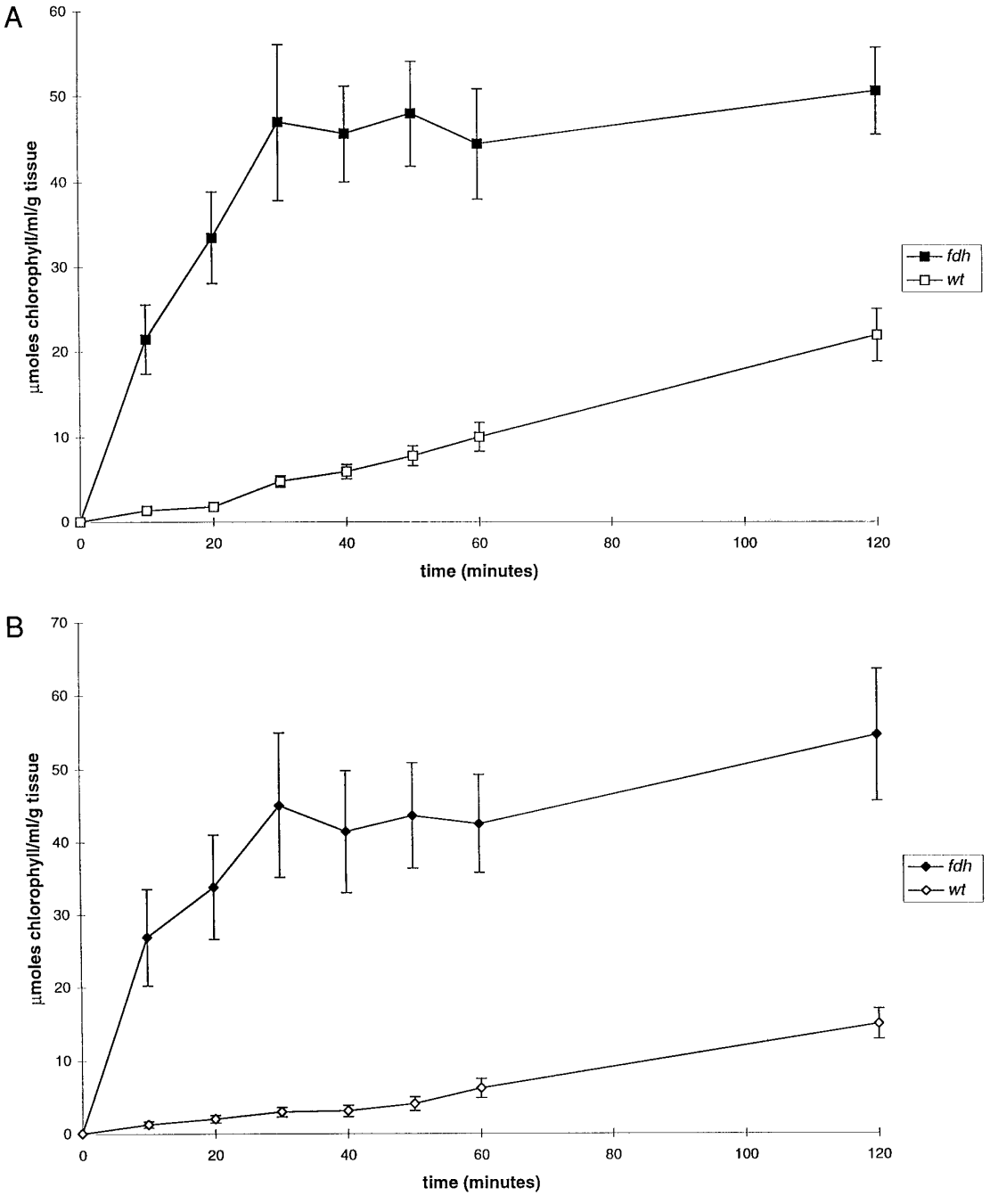

FIG. 3. Graphs showing amount of chlorophyll extracted as a function of time from intact leaves (A) and inflorescences (B) immersed in 80% ethanol. As shown in both A and B, chlorophyll diffuses rapidly out of *fdh-1* tissues relative to wild-type, reaching equilibrium within the first 30 min following immersion. The differences observed at every time point are statistically significant at $P = 0.01$ for both leaf and inflorescence tissues. Error bars indicate the standard error of the mean.

peared to be present in significantly greater quantity in the *fdh-1* leaf washes. No protein species unique to either wild-type or *fdh-1* samples could be unequivocally resolved using this method.

Cytochemical Profile

Mutant and wild-type tissue was treated with a variety of biological stains to test for differences in lipid, carbohy-

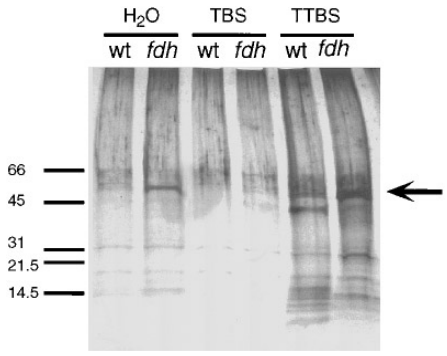

FIG. 4. SDS–PAGE of total protein obtained from leaf washes of wild-type and *fdh-1* tissues. Proteins were visualized by silver staining. Treatment of *fdh-1* leaf surfaces with water liberated multiple proteins, including a prominent species of approximately 55 kDa (arrow). No protein bands unique to *fdh-1* tissues were detected using this method. Molecular weights of protein standards are shown in kDa.

drate, and protein staining. In light of the measurable changes in *fdh-1* wall permeability, we were primarily interested in whether a cuticle was present and if the cuticle distribution was altered on *fdh-1* epidermal surfaces. Three different lipid stains were used to stain for cuticle: Fat Red 7B, Sudan III, and Fluoral Yellow 088. In both *fdh-1* and wild-type samples, sectioned material stained positively for the presence of a cuticle irrespective of the stain employed. Cuticle was detected on all organs as well as between fused organs, as shown in Fig. 5A, consistent with previous TEM observations (Lolle *et al.*, 1992). Furthermore, cuticle was deposited in a gradient on maturing *fdh-1* organs reflecting the expected maturation profile for that organ (data not shown). No feature of the lipid staining profile distinguished *fdh-1* tissues from wild-type.

The PAS reaction was used as a general stain for carbohydrates. With the exception of strong PAS staining between fused sepals and petals in some *fdh-1* flowers (see Fig. 5C), no outstanding differences were observed when comparing wild-type and *fdh-1* tissues. Only the transmitting tract of wild-type and *fdh-1* pistils gave similarly intense staining using this reaction (Fig. 5B).

No obvious enrichment or alteration in protein accumulation was detected using Napthol Yellow staining procedures on *fdh-1* and wild-type tissues.

Lectin Binding

Our cytochemical findings indicated that differences could be detected between *fdh-1* and wild-type samples with respect to carbohydrate staining profiles in some samples. Since some loss of soluble material during fixation is expected aliquots of *fdh-1* and wild-type leaf washes were dotted onto nitrocellulose filters and challenged with nine different DIG-labeled lectins to determine if any soluble glycoconjugates were elevated or uniquely expressed in *fdh-1* mutants. Of the nine lectins used only the AAA lectin, which detects α (1–6)-linked fucose residues in complex N-glycan structures, bound preferentially to washes from *fdh-1* leaf surfaces (data not shown). Further analysis of these washes on Western blots, however, revealed that a high-molecular-weight smear (greater than 95 kDa) cross-reacted with the AAA lectin in both *fdh-1* and wild-type samples. In samples washed with TBS, only *fdh-1* showed this smear, while wild-type and *fdh-1* leaf samples that had been bathed in TTBS both had cross-reactive material. In each case, the volume of wash solution electrophoresed had been adjusted such that the same net wet weight of tissue was compared. On tissue sections, AAA lectin bound to the epidermal surfaces of *fdh-1* vegetative tissues, as well as tissues lining the transmitting tract of the pistil. In wild-type samples, however, only the cells lining the transmitting tract of the pistil bound AAA lectin. With the remaining eight lectins binding was not exclusive to *fdh-1* leaf washes, although the relative intensity of lectin binding protein bands on Western blots was always more pronounced than that in wild-type samples.

Biochemical Analysis of the Cell Wall and Cuticle

Using two different techniques we were able to identify measurable differences in the cell wall sieving properties of *fdh-1* plants. Our chlorophyll diffusion assays and surface washes indicated that permeability to a broad size range of molecules is enhanced. In order to determine whether any detectable biochemical changes accompany these alterations in cell wall properties, a biochemical analysis of the neutral monosaccharide and lipid composition of cell wall fractions was undertaken. As shown in Fig. 6A, no significant differences could be resolved in neutral monosaccharide composition of wild-type and *fdh-1* cell walls. However, substantial differences in the lipid profiles of the crude cell wall fractions from wild-type and *fdh-1* mutants were found. As shown in Fig. 6B, the long chain fatty acids C20:0 and C22:0 shared elevated abundances in *fdh*-derived walls compared to wild-type, while lower molecular weight lipids were underrepresented in *fdh*-derived walls. A fatty acid with a retention time beyond C24:0 was more abundant in *fdh*-derived cell wall material than in cell wall material from phenotypically wild-type plants (Fig. 6B). This compound represented the most abundant lipid released from cell walls upon methanolysis, while its abundance in ethanol-extractable membrane lipids was very low (data not shown). For this reason this compound was tentatively termed ''cell wall-specific lipid'' (CWL). Its electron-impact mass spectrum fragmentation patterns were typical of fatty acids but its identity could not be established from comparisons with database entries.

DISCUSSION

Developmental signaling in which diffusible factors mediate a response typically requires the exchange of signal

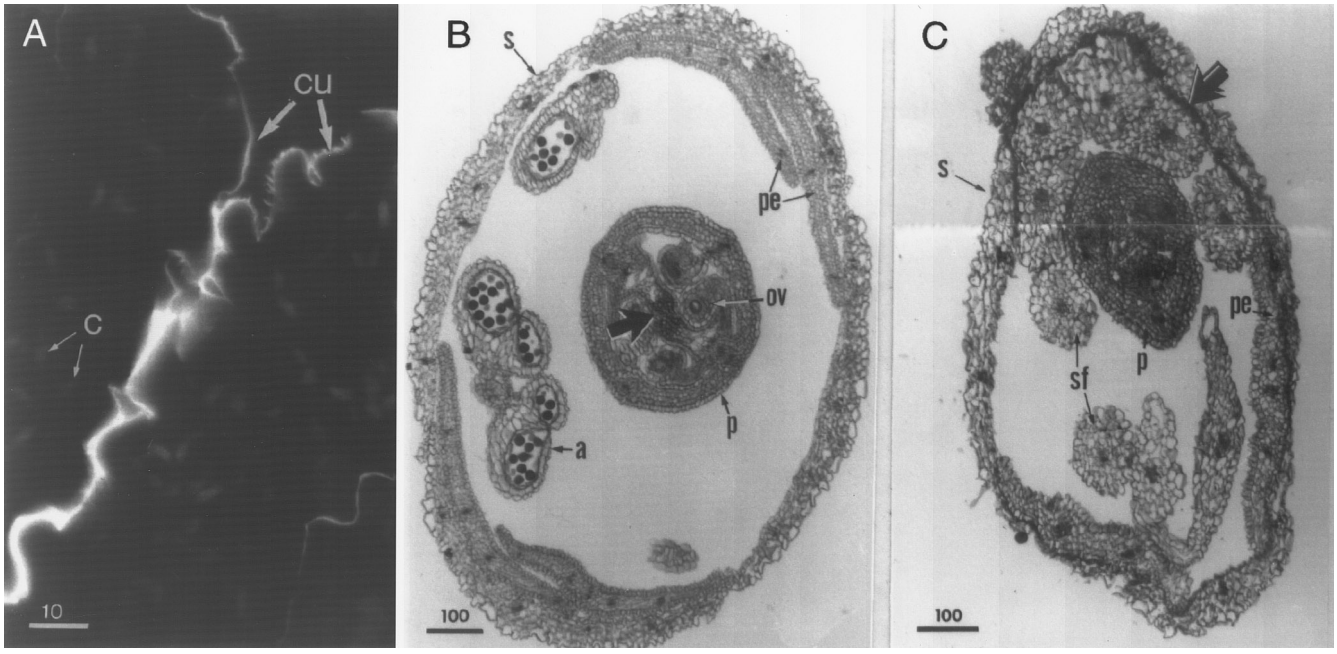

FIG. 5. Fluorescent (A) and light micrographs (B and C) of *fdh-1* floral buds stained for lipid (A) and carbohydrate (B and C). (A) Cross section through a sepal–sepal fusion suture formed by two neighboring buds showing epidermal cuticle (cu) staining with the fluorescent stain Fluoral Yellow 088. Note the enhanced staining in the fusion suture itself. c, chloroplasts. (B) Cross section through an unopened wild-type floral bud stained for carbohydrates using the PAS reaction. All organs including sepals (s), petals (pe), anthers (a), and the central pistil (p) show some staining. The most reactive material, however, is found in the transmitting tract (large arrow) separating the two ovule (ov) containing locules. (C) Cross section through a mature *fdh-1* floral bud stained for carbohydrate using the PAS reaction. Fusion has occurred between all floral whorls including the sepals (s), petals (pe), stamens, and pistil (p). A darkly staining material is most prominent at the fusion suture (large arrow) between the outermost sepals and the inner organ whorls, including stamen filaments (sf). Scale bars indicate magnifications in micrometers.

molecules between interacting cells either directly via cytoplasmic connections or indirectly via an extracytoplasmic route. In transiting the plant cell wall, a given molecule may interact with the components of the wall matrix resulting in its exclusion from, binding to, or translocation across the wall compartment. Size, charge, and hydrophobic properties of the signaling molecule will influence not only its localization but also the conformation and biological activity. As such the cell wall represents one potential control point for developmental signaling between interacting cells and may be important in regulating cell interactions in plants. In the case of the epidermis, an additional barrier exists which poses further constraints on signaling processes. That barrier is the lipid-rich epidermal cuticle.

During normal development, the only cases in which the cell interactions are known to take place exclusively across cell walls involve epidermal derivatives. These epidermal cell interactions fall into two classes; those which take place between the male gametophyte and the sporophyte during pollination and those which take place between epidermal cell pairs on the same plant during ontogenetic fusion. Both types of interactions involve the exchange of signals across the cell wall and cuticle of the epidermis. During the initial stages of the pollination process, stigmatic papillar cells interact with compatible pollen across the cell wall and pellicle (the proteinacious outer cuticle) (Pruitt and Hülskamp, 1994 a,b). Work by Hülskamp *et al.* (1995) and Preuss *et al.* (1993) has shown that, in *Arabidopsis,* papillar cells respond to contact by releasing water to the pollen grain and that long chain lipids sequestered in the exine of the pollen grain potentiate this release. In the case where epidermal cells interact to give rise to fused structures such as the multiloculate carpel, diffusible morphogenetic factors transit the outer walls and cuticles of epidermal cells lining the incipient fusion suture. In *C. roseus,* where carpel fusion has been extensively studied (Verbeke, 1992), impermeable barriers placed between prefusion carpels prevent fusion and all of the subsequent cellular changes that accompany this response (Walker, 1978a,b). Although the molecular identity of these morphogens has not been determined it is known that these factors are small water soluble molecules (Siegel and Verbeke, 1989; Verbeke, 1992).

In both pollen–stigma interactions and organ fusion, reciprocal recognition reactions and the associated signaling events must take place across the cell wall and cuticle. Our

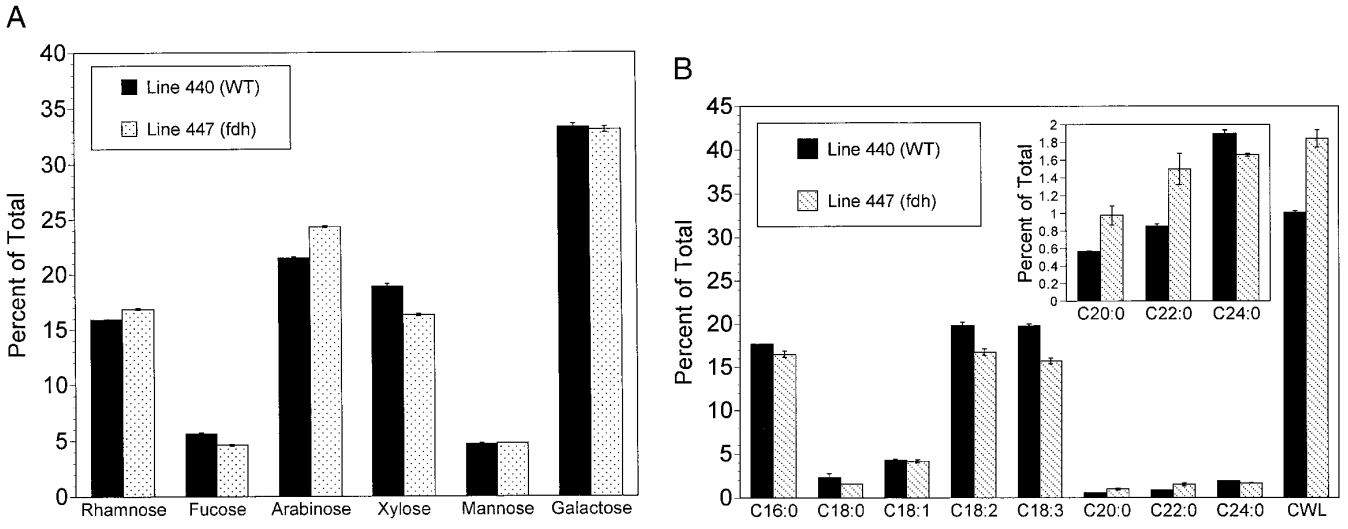

FIG. 6. Histograms showing monosaccharide (A) and lipid (B) composition values for cell wall material derived from wild-type and *fdh-1* leaf tissue. No significant differences were detected in the neutral sugar compositions between wild-type and *fdh-1* cell walls. A significant difference (defined as a 1.25-fold difference in percent of total), however, was detected in the lipid composition of the crude cell wall fractions derived from wild-type and *fdh-1* leaves. An increase in C20:0 and C22:0 (shown in the inset) was detected in *fdh-1* crude cell wall fractions, as was a pronounced increase in a high-molecular-weight lipid peak marked CWL.

analysis of the *fdh-1* mutant suggests that these two types of cell interactions are mechanistically related and depend on changes in cell wall properties that make permissive both types of interactions. In *fdh-1* mutant plants epidermal cell walls and cuticles are more permeable than wild-type walls as determined by two different criteria: chlorophyll diffusional rates out of intact tissues and protein extractability. Our chlorophyll extraction experiments demonstrate that the altered sieving properties of *fdh-1* cell walls and cuticles facilitate rapid diffusion of small molecules. Our leaf wash experiments suggest that this enhanced permeability is not restricted to small molecules but also affects larger molecules such as proteins although the rate at which these molecules transit the wall has not been determined. If small molecules analogous to the diffusible factors detected in *C. roseus* carpels are also involved in the *fdh-1* fusion process, then based on our findings we can conclude that the *fdh-1* epidermal cell wall and cuticle provides a suitable matrix through which this diffusion can take place and at relatively rapid rates; both prerequisites for this process to occur. Similar changes in cell wall sieving properties may also occur in carpel epidermal cells in *C. roseus* and these changes may play an important role in facilitating this cell–cell interaction and subsequent developmental response.

No protein bands were detected which were unique to *fdh-1* leaf washes. Similarly, in our lectin binding assays, no glycoconjugates could be detected which distinguished *fdh-1* from wild-type in any unique way. Together these data suggest that *fdh-1* plants do not produce any relatively abundant proteins or glycoconjugates that are not also found in wild-type plants. On tissue sections these AAA lectin binding sites localized to vegetative tissues as well as the transmitting tract in *fdh-1* plants, while they showed a more restricted expression profile in wild-type tissues, being localized only to the transmitting tract. This difference in localization could easily be ascribed to changes in *fdh-1* wall properties (including changes in the cuticle) that in some way facilitate partitioning of these molecules to the surface in tissues other than the transmitting tract. Alternatively, these molecules may be synthesized ectopically in *fdh-1* plants and hence be present on epidermal cell surfaces other than those found in wild-type.

Double mutant lines harboring mutations in both the *AG* and *FDH* genes have an additive phenotype. Neither fusion events nor pollen interactions are measurably perturbed in the *ag fdh-1* genetic background. Furthermore, when tested for changes in permeability using our chlorophyll diffusion assay similarly elevated rates of diffusion can be detected in *ag fdh-1* double mutants (Lolle *et al.*, unpublished results). As such, it seems evident that the phenotype of *fdh-1* mutants is not dependent upon the product of the *AGAMOUS* gene. It remains a possibility that the *fdh-1* mutant phenotype is due to the ectopic expression of a part of the carpel development program which is not dependent upon *AGAMOUS* expression for its function. Our previous experiments describing species specificity in the pollen response (Lolle and Cheung 1993) and the experiments described here testing hydration-defective pollen derived from *cer1*, *cer3*, and *cer6* mutants on *fdh-1* leaf surfaces support the notion that *fdh-1* epidermal cells mimic the response profile of normal *Arabidopsis* stigmatic papillar cells. In addition to

preserving species specificity, *fdh-1* as well as *ag fdh-1* epidermal cells offer an intact hydration barrier despite the measurable changes in permeability that exist. If the factors promoting pollen recognition and hydration are normally exclusively expressed on stigmatic papillar cells this would strongly argue that mutations in the *FDH-1* gene result in the ectopic expression of these factors throughout the *Arabidopsis* epidermis. Alternatively, it is possible that all of the factors required for both types of cell interactions are ubiquitously expressed by most if not all shoot epidermal cells and that under normal circumstances the permeability barrier offered by the epidermal wall and cuticle prevents these interactions from taking place. Localized changes in the permeability properties of the outer epidermal cell wall and cuticle, such as presumably occur in carpels and on stigmatic papillar cells, would then permit these types of interactions. In either case it is clear that the permeability properties of the epidermal cell wall and cuticle are greatly altered in the *fdh-1* mutant plants and this suggests that these changes may play a key role in facilitating interactions between epidermal cells.

How might the regulation of these types of epidermal interactions be achieved? Since the only detectable differences between wild-type and *fdh-1* mutant plants are manifested in cell wall and cuticular properties and, in particular, in the lipid composition of the crude cell wall fraction, we suggest that the selective modification of the cell wall/cuticle lipid composition offers one mechanism for regulating these developmental responses. Studies on plant cuticles indicate that cuticular waxes constitute the main barrier to transport (Baur *et al.*, 1996). Furthermore, three *Arabidopsis* mutants defective in some aspect of wax production (*wax1*, *cer10*, and *cer13*) are also known to manifest organ fusion (Jenks *et al.*, 1996). Our results do not imply, however, that the signals and factors regulating epidermal fusion and pollen recognition and growth are one and the same. Rather, these findings only support the notion that modifying the permeability barrier promotes a permissive condition allowing the orchestration of subsequent developmental events. In the case of pollen hydration it is clear from *in vitro* studies that pollen development, once triggered by hydration, can proceed relatively autonomously. It is not known whether epidermally mediated organ fusion is similar in this regard. Based on studies on *C. roseus*, the response of cells to contact is variable and that variability is dependent upon the developmental age of the participating cells. More mature cells do not redifferentiate in response to contact along the fusion suture but rather retain their epidermal fate and simply respond by adhering to one another. It may be that additional signals are exchanged between immature cells through the extracytoplasmic medium of the cell wall, promoting redifferentiation. Alternatively, immature cells may respond differently than more mature cells to the same morphogenetic factors.

In maize a class of mutants has been described which manifests epidermally mediated organ fusion (Becraft *et al.*, 1996). Characterization of the fusion process indicates that striking changes occur in epidermal morphology and that cells trapped at the fusion suture appear to proliferate. Although the authors do not mention whether pollen growth is supported on the mutant epidermis, the phenotypic features described suggest that the fusion process as it occurs in the *crinkly4* mutant is distinct from that seen in *fdh-1* mutants. Furthermore, sequence analysis of the wild-type copy of the affected gene reveals homology with mammalian tumor necrosis factor receptors. As such, it seems likely that the mechanism by which fusion is achieved in *crinkly4* is distinct from that seen in *fdh-1* mutants.

In summary, our data suggest that changes in cell wall/cuticle properties offer one mechanism for regulating epidermal cell interactions. It is not clear, however, if subtle and perhaps currently undetectable changes in cell wall properties would favor conditions where one type of interaction is permitted while the other is blocked. In the hope of elucidating how fusion is regulated and to determine whether the two types of interactions can be expressed independently of one another we have undertaken a comprehensive genetic analysis of the organ fusion pathway. By identifying additional mutants and ultimately by isolating the affected genes we hope to gain a better understanding of the regulation of epidermal cell interactions.

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