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The Critical Requirement for Linolenic Acid Is Pollen Development, Not Photosynthesis, in an Arabidopsis Mutant

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The very high proportions of trienoic fatty acids found in chloroplast membranes of all higher plants suggest that these lipid structures might be essential for photosynthesis. We report here on the production of Arabidopsis triple mutants that contain negligible levels of trienoic fatty acids. Photosynthesis at 22°C was barely affected, and vegetative growth of the mutants was identical with that of the wild type, demonstrating that any requirement for trienoic acyl groups in membrane structure and function is relatively subtle. Although vegetative growth and development were unaffected, the triple mutants are male sterile and produce no seed under normal conditions. Comparisons of pollen development in wild-type and triple mutant flowers established that pollen grains in the mutant developed to the tricellular stage. Exogenous applications of α-linolenate or jasmonate restored fertility. Taken together, the results demonstrate that the critical role of trienoic acids in the life cycle of plants is as the precursor of oxylipin, a signaling compound that regulates final maturation processes and the release of pollen.

INTRODUCTION

The biophysical reactions of light harvesting and electron transport during photosynthesis take place in a uniquely constructed bilayer membrane, the thylakoid. In all photosynthetic eukaryotes, the complement of atypical glycerolipid molecules that form the foundation of this membrane are characterized by sugar headgroups and a very high level of unsaturation in the fatty acid chains, which compose the central portion of the thylakoid lamella bilayer. For example, monogalactosyldiacylglycerol, the major thylakoid lipid, typically contains >90% of α-linolenic acid (18:3) or a combination of 18:3 and hexadecatrienoic (16:3) acids, depending on the plant species (Jamieson and Reid, 1971). These very high levels of trienoic fatty acids are noteworthy because free radicals that are byproducts of the photosynthetic light reactions stimulate oxidation of polyunsaturated fatty acids. Because this oxidation might be expected to mediate against a high degree of unsaturation, it has been inferred that there is a strong selective advantage to having such high levels of trienoic fatty acids in the thylakoid. Therefore, it could be reasoned that these lipid structures must have some critical role in maintaining photosynthetic function.

To test this line of reasoning directly, we set out to develop Arabidopsis lines with reduced levels of 18:3 and 16:3 fatty acids. There are two distinct pathways in plant cells for the biosynthesis of glycerolipids and the associated production of polyunsaturated fatty acids (Roughan et al., 1980; Browse and Somerville, 1991). Both pathways are initiated by the synthesis of 16:0–acyl carrier protein (ACP) and 18:1–ACP by the combined action of a type II fatty acid synthase (Shimakata and Stumpf, 1982) and a soluble stearoyl–ACP desaturase (McKeon and Stumpf, 1982; Shanklin and Somerville, 1991) located in the chloroplasts or other plastids. The prokaryotic pathway (Roughan et al., 1980) located in the chloroplast inner envelope uses 18:1–ACP and 16:0–ACP for the sequential acylation of glycerol-3-phosphate and synthesis of glycerolipid components for the chloroplast membranes (Frentzen, 1993; Joyard et al., 1993). The eukaryotic pathway involves export of 16:0 and 18:1 fatty acids from the chloroplast to the endoplasmic reticulum and their incorporation into phosphatidylcholine and other phospholipids that are the principal structural lipids of all the membranes of the cell except for the chloroplast. In addition, the diacylglycerol moiety of phosphatidylcholine can be returned to the chloroplast envelope and used as a second source of precursors for the synthesis of chloroplast glycerolipids (Browse et al., 1986b).

In each pathway, further desaturation of 16:0 and 18:1 occurs only after these fatty acids have been incorporated into the major membrane lipids. Thus, most of the plant desaturases responsible for the synthesis of 18:3 and 16:3 are integral membrane proteins that use glycerolipids as substrates. With one exception (Schmidt et al., 1994), these membrane desaturases have not been solubilized and purified. Instead, much of our current understanding of desaturation in plants has come from

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the characterization of seven Arabidopsis mutants, each one deficient in the activity of a different membrane-bound desaturase (Browse and Somerville, 1991). In the absence of purified proteins, the mutants have also made it possible to clone genes that encode the desaturases, using map-based cloning and gene tagging techniques (Arondel et al., 1992; Okuley et al., 1994). In Arabidopsis, there are at least three desaturase enzymes that mediate the conversion of 18:2 and 16:2 acyl groups to 18:3 and 16:3. The fatty acid desaturation (FAD) genes *FAD7* and *FAD8* encode two chloroplast isozymes that can recognize as a substrate either 18:2 or 16:2 attached to any of the chloroplast glycerolipids (Browse et al., 1986a; McConn et al., 1994). The *FAD3* gene product, localized predominantly in the endoplasmic reticulum, uses 18:2 on phosphatidylcholine as its major substrate, although it is possible that it also acts on 18:2 groups of other phospholipids (Browse et al., 1993).

The operation of parallel pathways of glycerolipid desaturation complicates the task of eliminating trienoic fatty acids from the thylakoid membranes. For example, the *fad7-2 fad8* double mutants contain no 16:3 but ~17% 18:3 in their thylakoid membranes (McConn et al., 1994). The extrachloroplast membranes in leaves of the *fad3* mutant plants contain a considerable amount of 18:3, because the 18:2 lipid can be transferred to the chloroplast on the eukaryotic pathway, desaturated by the FAD7 and FAD8 enzymes, and then returned to the endoplasmic reticulum and to other extrachloroplast membranes (Browse et al., 1993).

In this study, our goal was to produce a *fad3-2 fad7-2 fad8* triple mutant line. We were surprised to find that although triple mutants contained <0.1% trienoic fatty acids, they were as vigorous as wild-type plants in growth and vegetative development. Obviously, trienoic fatty acids are not absolutely required as components of the thylakoid or other cell membranes. A totally unanticipated consequence of the lack of 18:3 and 16:3 lipids is the fact that the triple mutant plants are male sterile. We describe the production of tricellular but inviable pollen in the mutant and report chemical complementation of the male-sterile phenotype by linolenic acid and its octadecanoid derivative, jasmonic acid.

RESULTS

Production of Healthy Triple Mutant Plants

Earlier results indicated that the *fad3-1* and *fad7-1* mutations probably represent leaky alleles, each of which retains a small amount of the relevant desaturase activity (Browse et al., 1993; McConn et al., 1994). Consequently, we made crosses between *fad7-2* and *fad8* (McConn et al., 1994) and between *fad3-2* and *fad7-2*. The F_2 progeny from these crosses were screened by gas chromatographic analysis, and *fad7-2 fad8* and *fad3-2 fad7-2* double mutants were identified by their decreased trienoic fatty acid content of 17 and 20%, respectively. An F_1 plant derived from a cross between the two double mutants was allowed to self-pollinate, the resulting seed were germinated, and the leaf fatty acid compositions of individual F_2 progeny were examined for their content of trienoic fatty acids. Of 240 F_2 plants analyzed, 17 contained no detectable 16:3 or 18:3 (detection limit was ~0.1% of total), whereas the remaining plants exhibited a range in the proportion of total trienoics from 10 to 40%. Such a segregation pattern is a good fit to the Mendelian expectation ($\chi^2 = 0.284$, $P > 0.5$), indicating that the homozygous *fad3-2 fad7-2 fad8* progeny were not selected against during embryogenesis or seed germination. Indeed, visual comparison of wild-type and triple mutant plants (Figure 1) revealed no striking difference in vegetative growth and development. In later experiments, triple mutant plants were identified by fatty acid analysis from among the progeny of plants that contained just one wild-type allele at the *fad8* locus, that is, *fad3-2*(−/−) *fad7-2*(−/−) *fad8*(+/−).

Growth Rate and Photosynthesis Are Normal in the Triple Mutant Line

The remarkably normal vegetative phenotype of triple mutant plants indicated that metabolic processes, and in particular, photosynthesis, were not significantly compromised by the deficiency in trienoic fatty acids. To assess quantitatively the similarity of wild-type and *fad3-2 fad7-2 fad8* plants, the increases in shoot fresh weight were measured for plants growing under 140 μmol m^{-2} sec^{-1} continuous light at 22°C. As shown in Figure 2, there was no difference in the relative growth rates realized by samples of wild-type and *fad3-2 fad7-2 fad8* plants, indicating that, at least under our growth conditions, there is no overall effect of the trienoic acid deficiency.

Under some circumstances, photosynthesis may not be limiting for plant growth, so the lack of trienoic acids in chloroplast lipids might have effects on photosynthesis that were not reflected in the growth rate or appearance of the triple mutant plants. To investigate this possibility, we performed fluorescence analysis with wild-type and *fad3-2 fad7-2 fad8* plants. Two parameters commonly used to evaluate the overall capacity of photosynthetic processes are F_v/F_m, which assesses the potential quantum efficiency, and Φ_{II} (= $[F_m' - F_s]/F_m'$), which is correlated to the steady state quantum efficiency (Seaton and Walker, 1990; see Methods for definitions). The results shown in Table 1 indicate that under normal conditions, there is no significant difference in F_v/F_m between leaves of the wild type and *fad3-2 fad7-2 fad8*. The 5% reduction in Φ_{II} exhibited by leaves of *fad3-2 fad7-2 fad8* at 22°C (Table 1) was reflected in some but not all experiments. More detailed fluorescence analysis, including experiments that allow fluorescence quenching to be ascribed to different photochemical and nonphotochemical mechanisms (Krause and Weis, 1991), has consistently failed to detect any significant difference between wild-type and *fad3-2 fad7-2 fad8* plants measured at 25°C

Figure 1. Wild-Type and *fad3-2 fad7-2 fad8* Arabidopsis Plants.

Wild-type Arabidopsis (left) and the triple mutant (right) were grown for 4 weeks at 22°C under continuous illumination of 140 μmol m^{-2} sec^{-1}.

(J.-M. Routaboul, unpublished data), but preliminary results indicate that photosynthetic parameters show different responses to temperature in the wild type and the triple mutant.

Additional Lines with Very Low Levels of 16:3 and 18:3

The availability of the *fad7-1* allele (McConn et al., 1994) provided us with a means to generate lines with intermediate levels of trienoic fatty acids. Figure 3 shows the overall leaf fatty acid compositions of the single mutants and of various multiple mutants grown at 22°C. Each of the individual single mutants contained between 40 and 58% trienoic acids as compared with 61% in wild-type Arabidopsis. The double mutants *fad3-2 fad7-2* and *fad7-2 fad8* contained ~20% trienoic acids. This level represents the contribution of the remaining, functional desaturase: the *FAD8* gene product in the case of *fad3-2 fad7-2* or the *FAD3* gene product in the case of *fad7-2 fad8*. In contrast to the *fad3-2 fad7-2 fad8* line, the leaves of the *fad3-2 fad7-1 fad8* plant contained 5% trienoic acids (Figure 3). Because alleles at *fad7* are known to have a relatively precise gene–dosage effect (Browse et al., 1986a), we crossed the two triple mutant lines so that the resulting F_1 plants contained one copy of each of the *fad7* mutant alleles. Leaves of these *fad3-2 fad7-1/fad7-2 fad8* plants contained only 2% trienoic fatty acids. These supplementary genotypes provide important additional tools in studying the consequences of 18:3 deficiency.

The three genetic loci involved in generating the triple mutant are known to be the structural genes encoding three membrane-bound, ω-3 desaturases in Arabidopsis (Arondel et al., 1992; Iba et al., 1993; Yadav et al., 1993; Gibson et al., 1994). Our results indicate that these are the only quantitatively significant desaturases contributing to 16:2 and 18:2 desaturation in leaf tissues. Fatty acid analyses of other tissues (seed, roots, stems, and flowers) of *fad3-2 fad7-2 fad8* plants failed to provide evidence for any additional, tissue-specific 18:2 desaturase (data not shown).

Separation of the individual polar lipids in leaf extracts allowed a more detailed analysis of the lipid phenotypes of the triple mutant lines and provided the means to detect very low

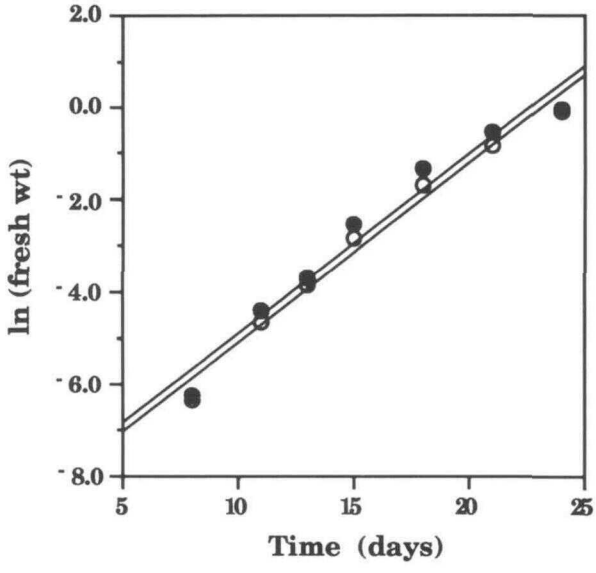

Figure 2. Relative Growth Rates of Wild-Type and *fad3-2 fad7-2 fad8* Arabidopsis Plants.

Plants were grown at 22°C under continuous illumination of 140 μmol m^{-2} sec^{-1}. At intervals between 8 and 24 days after sowing, samples of plants were harvested, and the fresh weight of the above-ground parts were measured. The relative growth rate (ω^{-1}) for the wild type (●) was 0.388 ± 0.025; for the mutant (○), it was 0.388 ± 0.033.

Table 1. Photosynthetic Fluorescence Parameters of Leaves from the Wild Type and Triple Mutants

Fluorescence Parameters	Wild Type	*fad3 fad7-2 fad8*
F_v/F_m	0.755 ± 0.010	0.763 ± 0.010
Φ_{II}	0.611 ± 0.016	0.580 ± 0.023

Plants were grown at 22°C and under continuous illumination of 140 μmol m^{-2} sec^{-1}. Fluorescence measurements were made at 22°C, as described in Methods. Values represent the average ± SD of 10 independent samples.

levels of 18:3 fatty acids as a consequence of the better signal-to-noise ratios during gas chromatography of fatty acid methyl esters derived from the purified lipids. The results shown in Table 2 confirm that the average content of 18:3 in leaf membrane lipids from *fad3-2 fad7-2 fad8* plants was <0.1%; no 16:3 was detected. The trace levels of 18:3 observed probably reflect a low level of competent enzyme activity resulting from one of the mutated *FAD* genes. However, it is not possible to exclude the possibility that an additional gene makes a very minor contribution to 18:2 desaturation in Arabidopsis leaf tissue. Both of the triple mutant lines exhibited a 20% decrease in the proportion of monogalactosyldiacylglycerol, the major chloroplast lipid. This decrease was associated with increases in the proportions of digalactosyldiacylglycerol and the extrachloroplast phospholipids. In *fad3-2 fad7-1 fad8* plants, the monogalactosyldiacylglycerol and digalactosyldiacylglycerol contained 12 and 9%, respectively, of 18:3 plus 16:3, whereas the other thylakoid membrane lipids, phosphatidylglycerol and sulfoquinovosyldiacylglycerol, contained <2% of these fatty acids. These observations indicate that the two galactolipids may be preferred substrates for the FAD7 desaturase and are in agreement with previous suggestions that the FAD8 desaturase has a substrate specificity that favors phosphatidylglycerol and sulfoquinovosyldiacylglycerol (McConn et al., 1994).

The Triple Mutant Is Male Sterile

The first generation of homozygous *fad3-2 fad7-2 fad8* plants did not set any seed, and flowers of the mutants retained their petals longer than did wild-type flowers (Figures 1 and 4A). A failure of the petals to senesce is typical of sterile flowers that do not experience the burst of ethylene production that normally follows fertilization and orchestrates flower maturation and senescence. To determine the nature of flower sterility, we performed reciprocal crosses between the wild type and triple mutants. Using anthers from wild-type flowers, it was always possible to pollinate the *fad3-2 fad7-2 fad8* plants and produce mature seed. In contrast, anthers from triple mutant flowers were unable to induce seed set on emasculated wild-type flowers. Closer examination of wild-type and *fad3-2 fad7-2 fad8* flowers (Figure 4B) revealed that the locules of the mutant anthers had not dehisced to deposit pollen on the stigmatic surface. However, manual disruption of the anther locules did not result in any seed set.

Eventually, the majority of *fad3-2 fad7-2 fad8* anthers did open, but separation of the stomium occurred later; the outward

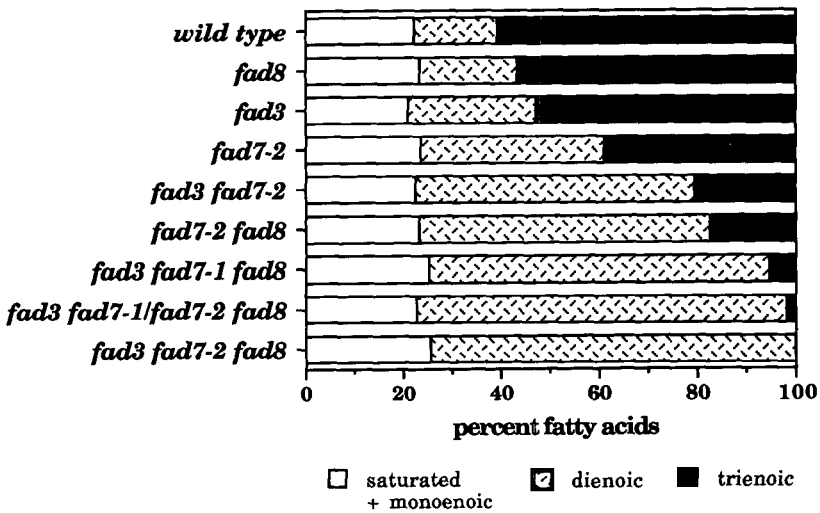

Figure 3. Overall Fatty Acid Compositions of Mutants Deficient in 18:3 and 16:3 Synthesis.

Plants were grown for 3 weeks at 22°C under continuous illumination of 140 μmol m^{-2} sec^{-1}. Leaf samples were derivatized using 2.5% H_2SO_4 in methanol, and the fatty acids were analyzed by gas chromatography. The data represent averages of 10 independent samples.

Table 2. Fatty Acid Composition of Arabidopsis Leaf Lipids from the Wild Type and Two Triple Mutants Grown at 22°C[a]

Glycerolipids	% of Total Polar Lipids	Fatty Acid Composition							
		16:0	16:1[b]	16:2	16:3	18:0	18:1	18:2	18:3
Monogalactosyldiacylglycerol									
Wild type	38.7	1.8	1.4	1.7	30.6	0.2	1.0	3.2	60.0
fad3-2 fad7-1 fad8	29.5	1.5	2.6	23.6	3.5	0.2	3.2	57.1	8.5
fad3-2 fad7-2 fad8	32.5	1.4	2.7	27.6	—[c]	0.2	3.4	64.6	0.1
Digalactosyldiacylglycerol									
Wild type	13.4	12.1	0.4	0.9	2.9	1.09	0.8	4.8	77.2
fad3-2 fad7-1 fad8	15.3	7.2	0.7	3.4	0.6	0.5	1.4	77.7	8.5
fad3-2 fad7-2 fad8	15.8	7.6	0.5	3.5	—	0.5	1.5	85.9	0.1
Phosphatidylglycerol									
Wild type	11.3	27.2	16.1	—	—	1.1	4.6	10.4	39.8
fad3-2 fad7-1 fad8	10.1	28.2	16.9	0.1	—	0.9	10.2	42.4	1.3
fad3-2 fad7-2 fad8	11.3	26.4	18.1	1.2	—	0.9	10.3	42.7	—
Sulfoquinovosyldiacylglycerol									
Wild type	3.5	34.4	0.3	—	—	2.9	2.9	16.3	41.6
fad3-2 fad7-1 fad8	2.7	31.4	0.8	—	—	2.6	5.6	57.8	1.7
fad3-2 fad7-2 fad8	3.9	30.8	1.0	2.1	—	1.8	5.5	57.3	0.1
Phosphatidylcholine									
Wild type	18.9	20.7	—	—	—	2.0	5.5	36.5	34.7
fad3-2 fad7-1 fad8	23.5	17.4	—	—	—	1.0	10.0	68.6	1.2
fad3-2 fad7-2 fad8	19.7	17.9	—	—	—	1.1	11.2	67.7	0.1
Phosphatidylethanolamine									
Wild type	10.9	29.0	—	—	—	2.1	2.3	38.5	27.2
fad3-2 fad7-1 fad8	13.4	25.1	—	—	—	1.3	4.0	66.8	0.8
fad3-2 fad7-2 fad8	12.2	25.8	—	—	—	1.3	5.6	65.4	0.1
Phosphatidylinositol									
Wild type	3.3	44.4	—	—	—	2.9	1.6	24.5	24.3
fad3-2 fad7-1 fad8	5.5	41.4	—	—	—	2.3	4.1	50.5	1.1
fad3-2 fad7-2 fad8	4.6	40.4	—	—	—	2.6	3.8	49.1	0.1

[a] Values represent the averages of three samples and are presented as mole percent.
[b] Sum of 16:1c and 16:1t fatty acids.
[c] Amounts were <0.1 mole percent.

bending of the locule walls, which leads to pollen release in the wild type, did not occur. Scanning electron microscopy (Figure 4C) showed the lack of proper dehiscence; however, it also revealed that pollen in the mutant anther appears morphologically normal. Our observation that triple mutant progeny are produced in a Mendelian ratio from either *fad3-2(+/−) fad7-2(+/−) fad8(+/−)* or *fad3-2(−/−) fad7-2(−/−) fad8(+/−)* parents indicates that it is the genotype of the maternal tissue (rather than those of the segregating, haploid microspores) that mediates the male-sterile phenotype and that very low levels of 18:3 are probably sufficient to ensure fertility.

Wild-Type and *fad3-2 fad7-2 fad8* Pollen Development

To examine possible differences in the development of wild-type and *fad3-2 fad7-2 fad8* pollen, young floral buds were fixed and cleared by a technique that permitted examination and optical sectioning of intact tissues (Herr, 1971). Cleared buds containing pollen at different, well-defined stages were studied, and the results indicated that pollen development in the mutant follows a course very similar to that of the wild type. Figures 5A and 5B show pollen mother cells undergoing the first division of meiosis to form haploid progeny. Following the second meiotic division, these cells become organized into tetrads of microspores encased in a callose wall (Figures 5C and 5D). The individual microspores are released by the action of a callase enzyme secreted by cells of the parental tissues (Figures 5E and 5F). The layer of specialized tapetal cells that provide many of the nutrients and other factors (including the extracellular callase) required for pollen development persists until late in development (Figures 5G and 5H). Soon before flower opening and the associated dehiscence of the wild-type anthers, the tapetum breaks down (Figures 5I and 5J) in a process that results in deposition of lipid-based sporopollenin on the exine of the mature pollen grains.

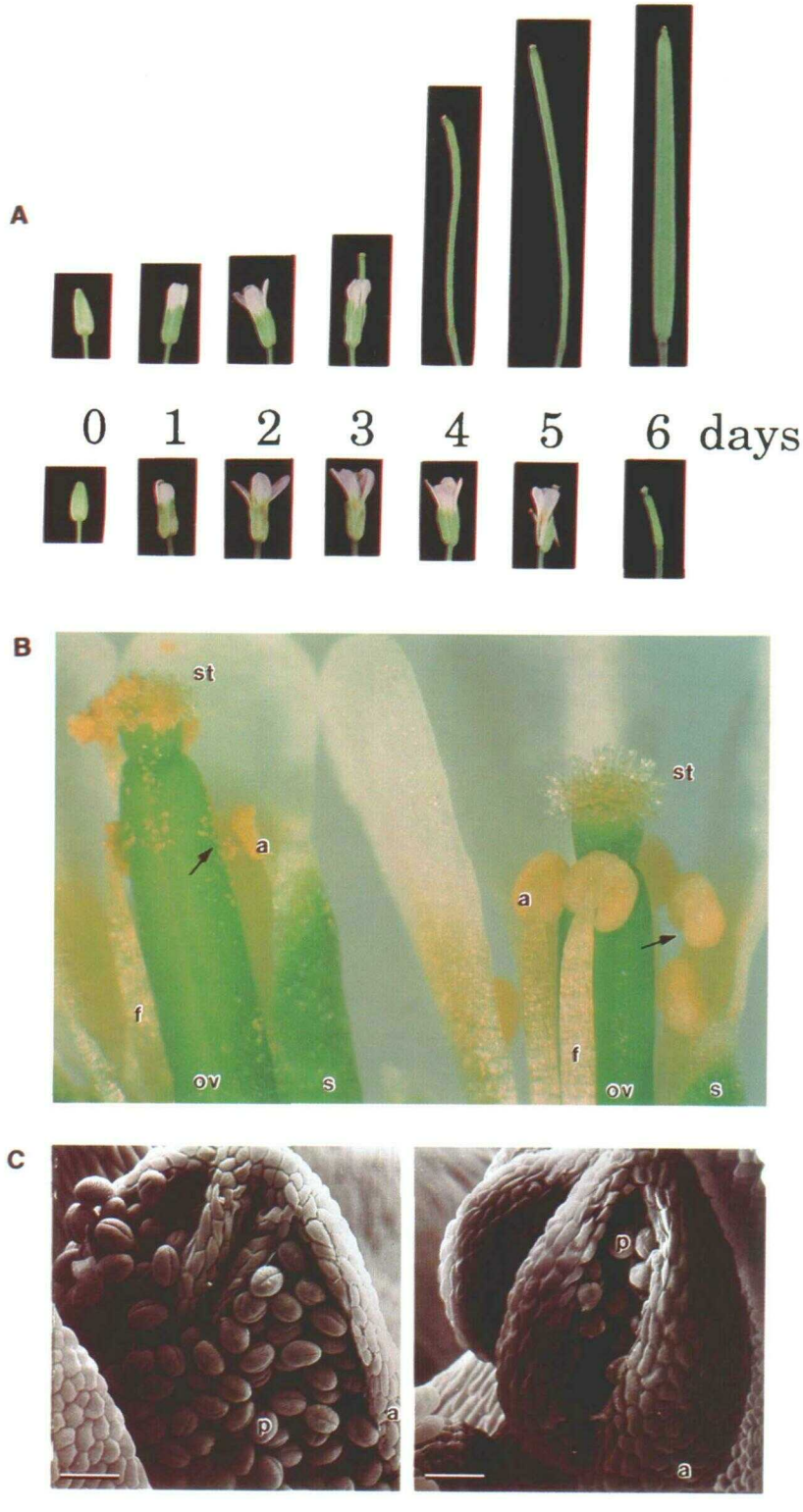

Figure 4. Morphology of Male Sterility in the *fad3-2 fad7-2 fad8* Triple Mutant.

(A) Time course of flower development in wild-type (top) and *fad3-2 fad7-2 fad8* (bottom) Arabidopsis.

(B) Wild-type (left) and triple mutant (right) flowers photographed 2 days after petals appeared at the tip of a bud. Anthers of the wild type had dehisced and deposited pollen on the stigma and style (left arrow). The *fad3-2 fad7-2 fad8* anthers had not yet dehisced (right arrow). a, anther; f, filament of the anther; ov, ovary; s, sepal; st, stigma.

(C) Scanning electron microscopy of a wild-type anther examined 2 days after petals appeared at the tip of a bud (left) and a *fad3-2 fad7-2 fad8* anther examined 4 days after petals appeared at the tip of a bud (right). a, anther; p, pollen. Bars = 50 μm.

The fluorochrome stain, 4',6-diamidino-2-phenylindole (DAPI), which binds specifically to double-stranded DNA, was used to stain pollen removed from anthers at stages immediately before or immediately after flower opening. The majority of pollen from both wild-type and *fad3-2 fad7-2 fad8* plants exhibited three fluorescent spots (Figures 6A and 6B) that correspond to one vegetative nucleus and two smaller generative nuclei (Stanley and Linskens, 1974). The presence of three nuclei indicates that the pollen had matured to the tricellular stage (Stanley and Linskens, 1974). Mithramycin, an alternative fluorochrome stain for DNA, provided similar results (data not shown).

To determine the viability of mature pollen, the contents of wild-type and *fad3-2 fad7-2 fad8* anthers were released into high molarity medium on microscope slides and double stained with fluorescein diacetate and propidium iodide. Fluorescein diacetate (a vital stain) is taken up by living cells and converted to impermeant fluorescein, which emits a green fluorescence under UV light (Heslop-Harrison and Heslop-Harrison, 1970). Conversely, propidium iodide is excluded from living cells but can label dead cells with a red-orange fluorescence under UV light (Regan and Moffatt, 1990). The results shown in Figures 6C and 6D demonstrate the very low viability of pollen from *fad3-2 fad7-2 fad8* plants relative to the wild type. Counting viable pollen (based on their fluorescence) in 23 microscope fields in four independent experiments indicated an average of 11% viable pollen from the *fad3-2 fad7-2 fad8* mutant as compared with 84% from the wild type.

Clearly, a significant proportion of the pollen from *fad3-2 fad7-2 fad8* plants is alive at maturity, but this pollen did not induce seed set when removed from anthers and applied to the stigmas of *fad3-2 fad7-2 fad8* or emasculated wild-type flowers. We therefore investigated germination of pollen from the wild type and *fad3-2 fad7-2 fad8* plants in vitro (Preuss et al., 1993). The analysis was carried out a minimum of three times in each of four separate experiments. More than 1000 pollen grains from wild-type plants viewed in 20 microscope fields showed an average germination of 82%, a figure in close agreement with the determination of average viability. In contrast, only eight germinated pollen grains were observed in 28 microscope fields (~1400 grains) of pollen from triple mutant plants (germination <0.6%). We consistently observed that pollen grains from the *fad3-2 fad7-2 fad8* plants that did germinate produced pollen tubes less than one-third the length of pollen tubes produced by wild-type pollen (Figures 6E and 6F).

Exogenous Linolenate Complements the Male-Sterile Phenotype

The observation that *fad3-2 fad7-1 fad8* plants were fully fertile despite having <5% 18:3 in their tissues suggested that the threshold requirement for 18:3 must be very low. Therefore, we treated wild-type and *fad3-2 fad7-2 fad8* plants during flowering by spraying them with a 0.1% (w/v) solution of the normal plant isomer of 18:3 (Δ9,12,15; all *cis*) as the sodium salt. Other plants were treated with sodium salts of either 18:2 or a second isomer of linolenate, γ18:3 (Δ6,9,12; all *cis*), as controls. Analysis of rosette leaves from triple mutants harvested at the end of the spraying treatment indicated that each of the exogenous fatty acids was taken up and incorporated into membrane glycerolipids to a level accounting for 5% of the total acyl groups. Plants treated with 18:2 or γ18:3 did not produce any seed. However, plants sprayed with Δ9,12,15–18:3 yielded, on average, >300 seeds per plant. When these seeds were germinated, all of the progeny plants exhibited the *fad3-2 fad7-2 fad8* fatty acid phenotype, indicating that the seeds could not be the result of fertilization by pollen from other plants.

In this experiment, relatively low levels of 18:3 derived from exogenous sources met the requirements for pollen maturation and release. However, the pollen on untreated *fad3-2 fad7-2 fad8* plants began to die shortly before the flower buds opened. To determine whether 18:3 penetrated the unopened buds, we measured the 18:3 content of anthers taken from untreated flowers and flowers that had been painted, at the bud stage, with Na18:3. In this experiment, we also measured the 18:3 contents of anthers from *fad3-2 fad7-1 fad8*, *fad3-2 fad7-1/fad7-2 fad8*, and wild-type plants and monitored the extent of seed set on the different plants. The data in Table 3 indicate that mutant genotypes in which the anthers contain only 1 to 2% 18:3 (compared with 44% for the wild type) are fertile. However, plants with a *fad3-2 fad7-1/fad7-2 fad8* genotype exhibited reduced seed set (~25% fewer siliques than on wild-type and *fad3-2 fad7-1 fad8* plants), suggesting that a level of 18:3 of 1% in the floral organs may represent an approximate lower limit for fertility. As expected, the infertile *fad3-2 fad7-2 fad8* flowers did not contain detectable 18:3. Painting flower buds with Na18:3 restored fertility; however, even though the outer sepals contained 14% 18:3, this fatty acid was not detected in the anthers of either unopened or opened flowers.

These findings clearly suggest that if 18:3 is required in the anther tissue, it is in amounts that are beyond the detection limit of the experiment. Possibly, 18:3 is not specifically required in the anther tissue, and the critical requirement for 18:3 is not likely to be as a structural component either of cell membranes or of the outer sporopollenin and tryphine layers of the mature pollen. Instead, the results suggest that 18:3 in other flower organs might be converted to a compound that regulates cellular functions in the anthers and thereby mediates maturation and release of viable pollen.

An Essential Role for Jasmonate in Pollen Development

Jasmonic acid and methyl jasmonate activate wound responses in plants (Farmer and Ryan, 1992; Gundlach et al., 1992) and have been postulated to perform roles in many other developmental and environmental response processes (Sembdner and Parthier, 1993). The structure and biosynthesis

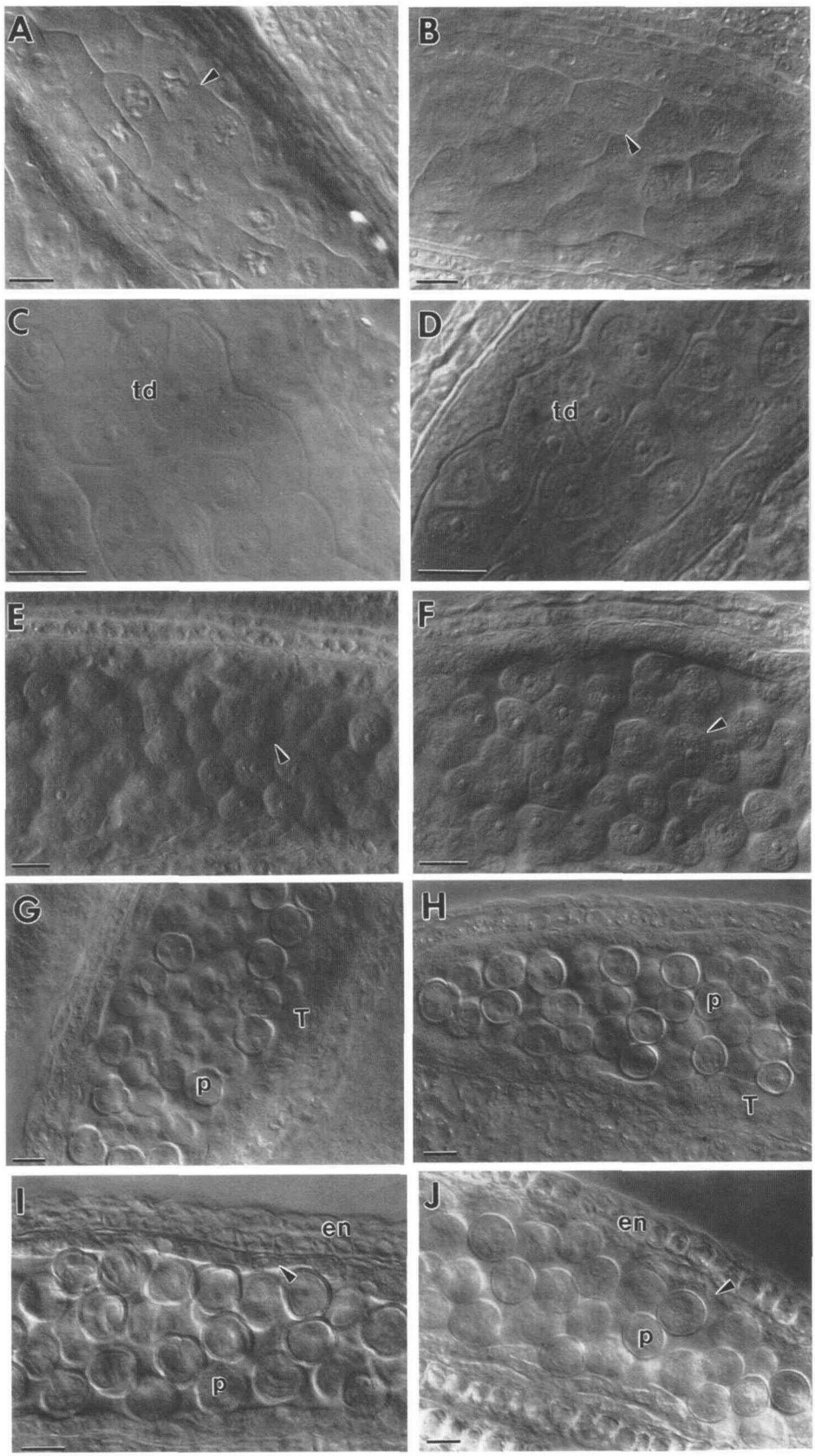

Figure 5. Stages of Pollen Development in Wild-Type and *fad3-2 fad7-2 fad8* Arabidopsis.

Table 3. Linolenic Acid Content of Floral Organs and Seed Set Observed in Arabidopsis Mutants and the Wild Type

Plant Genotype	Floral Organs				Seed Set
	Sepals	Petals	Carpels	Anthers	
Wild type	43.5 ± 1.9[a]	32.8 ± 1.1	36.7 ± 1.1	39.7 ± 0.9	+
fad3-2 fad7-1 fad8	4.9 ± 0.3	2.2 ± 0.3	3.0 ± 0.4	2.2 ± 0.3	+
fad3-2 fad7-1/fad7-2 fad8	2.8 ± 0.1	1.1 ± 0.3	1.7 ± 0.1	1.0 ± 0.1	±
fad3-2 fad7-2 fad8	<0.1	<0.1	<0.1	<0.1	-
fad3-2 fad7-2 fad8 after the application of Na18:3	14.3 ± 0.9	1.7 ± 0.4	1.5 ± 0.7	<0.1	+

[a] Data are mole percent of total fatty acids ± SE (n = 6). Plants were grown as described in Methods. Unopened flower buds on some *fad3-2 fad7-2 fad8* plants were painted with Na18:3 at the start of the dark period for 10 consecutive days. At the end of this time, the earliest treated buds had already formed fertile siliques. Open flowers above these siliques were sampled for analysis. (+), seed set occurred; (-), seed set did not occur.

of jasmonic acid have intrigued plant biologists because of parallels to eicosanoid second messengers that are central to inflammatory responses and other physiological processes in mammals (Creelman et al., 1992). Thus, jasmonic acid is synthesized from 18:3 (which is presumably released from membrane lipids by the action of a phospholipase A_2) by a pathway that is initiated by lipoxygenase. Cyclization and β-oxidation of the lipoxygenase product, 13(*S*)-hydroperoxylinolenic acid, result in jasmonic acid having a structure analogous in some respects to the prostaglandin E series of eicosanoids (Vick and Zimmerman, 1984). 13(*S*)-Hydroperoxylinolenic acid may also give rise to other compounds, including products derived from a hydroperoxide lyase reaction sequence: *trans*-3-hexenol, *trans*-2-hexenol, *cis*-3-hexen-1-ol, and traumatic acid (Croft et al., 1993).

To test whether jasmonic acid or other products of 18:3 metabolism could complement the male-sterile phenotype of *fad3-2 fad7-2 fad8* plants, we applied dilute solutions of jasmonic acid, *trans*-3-hexenol, *trans*-2-hexenal, *cis*-3-hexen-1-ol, and traumatic acid to flower buds. In this series of experiments, only plants treated with jasmonic acid produced seed, indicating that the production of viable pollen requires this oxylipin.

Currently available techniques that measured jasmonates require gram quantities of tissue (Creelman et al., 1992; Albrecht et al., 1993). For this reason, it was not practical to assay jasmonate levels in wild-type and mutant floral organs. However, in related experiments that measured jasmonate levels in leaf tissue of wounded plants by using the ELISA method (Albrecht et al., 1993), we found that *fad3-2 fad7-2 fad8* plants consistently produced levels of jasmonates that were close to the detection limit of the assay and <3% of levels produced by the corresponding wild-type controls (M. McConn and A. Conconi, unpublished data). These results are consistent with the proposal that the mutant plants contain very little or no jasmonate as a result of the deficiency in the substrate linolenate. Together with the results of the chemical complementation experiments, these findings provide strong evidence for a central role for jasmonates in the final stages of pollen development.

In subsequent experiments, the viability of pollen from jasmonic acid–treated mutant plants was measured using fluorescein diacetate/propidium iodide staining. The results of these experiments indicated that jasmonic acid restored the viability of *fad3-2 fad7-2 fad8* pollen to at least 80% of that of wild-type controls.

DISCUSSION

Because trienoic fatty acids are invariably abundant components in membranes of photosynthetic eukaryotes, it was by no means certain that Arabidopsis mutants lacking 16:3 and

Figure 5. (continued).

(A) and **(B)** Cleared anthers of wild-type and *fad3-2 fad7-2 fad8* Arabidopsis, respectively, at the haploid stage (arrowheads) in microspore development.
(C) and **(D)** Cleared anthers at the tetrad (td) stage for wild-type and *fad3-2 fad7-2 fad8* Arabidopsis, respectively.
(E) and **(F)** Released microspores (arrowheads) for wild-type and *fad3-2 fad7-2 fad8* Arabidopsis, respectively.
(G) and **(H)** Pollen (p) with intact tapetum (T) for wild-type and *fad3-2 fad7-2 fad8* Arabidopsis, respectively.
(I) and **(J)** Tapetal breakdown (arrowheads) in the anthers of wild-type and *fad3-2 fad7-2 fad8* Arabidopsis, respectively. en, endothecium; p, pollen. Bars = 10 μm in **(A)** to **(J)**.

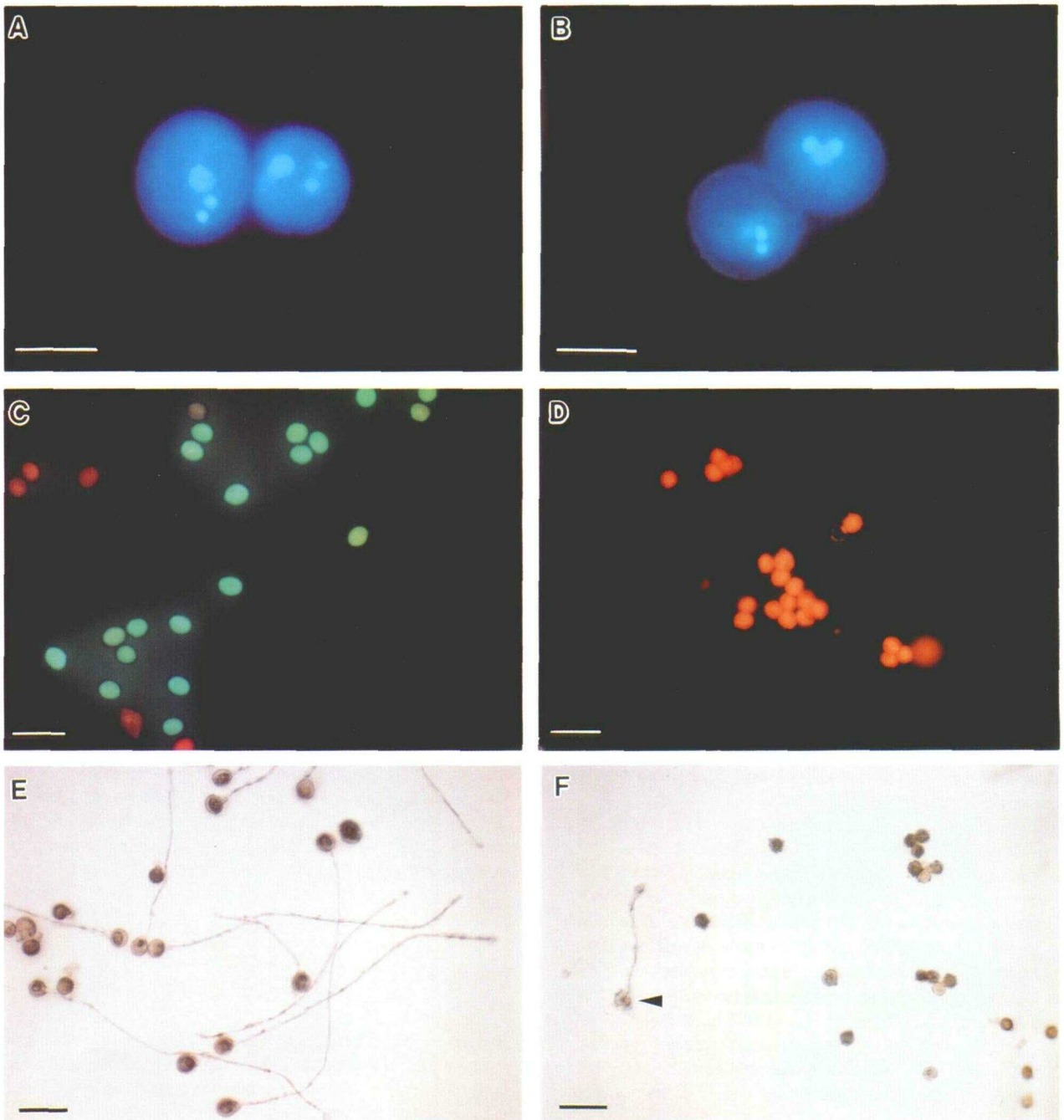

Figure 6. Maturation, Viability, and Germination of Pollen from Wild-Type and *fad3-2 fad7-2 fad8* Arabidopsis.

(A) and **(B)** Pollen from the wild type and *fad3-2 fad7-2 fad8*, respectively, stained with DAPI to show the one vegetative and two generative nuclei characteristics of mature, tricellular pollen of Arabidopsis. Bars = 10 μm.

(C) and **(D)** Wild-type and *fad3-2 fad7-2 fad8* pollen double-stained with fluorescein diacetate and propidium iodide to show viable (blue-green) and dead (red-orange) pollen grains. Bars = 50 μm.

(E) and **(F)** Wild-type and *fad3-2 fad7-2 fad8* pollen after 16 hr on germination medium. The only *fad3-2 fad7-2 fad8* pollen grain to have germinated (*arrowhead in* **[F]**) has a pollen tube that is approximately one-third the length of those produced by wild-type pollen grains. Bars = 50 μm.

18:3 would be viable. However, our characterization of growth and photosynthesis in *fad3-2 fad7-2 fad8* plants clearly indicates that these highly unsaturated fatty acids do not have any crucial role in maintaining photosynthesis in higher plant chloroplasts. This is not to say that trienoic fatty acids are irrelevant to photosynthetic function. Conservation of the high trienoic content of thylakoid membranes through the evolutionary time scale attests to their importance, but clearly their role is more subtle than expected. We have preliminary results that indicate that photosynthesis in triple mutant plants is reduced relative to that of the wild type at low temperatures (J.-M. Routaboul and J. Browse, unpublished data). Nevertheless, triple mutant plants grow well at temperatures as low as 6°C.

The only essential requirement for 18:3 in the plant life cycle appears to be as a substrate for the octadecanoid pathway, which produces signaling molecules such as jasmonic acid that are absolutely required to bring about the final stages of pollen development and anther dehiscence. Analysis of pollen development revealed no striking differences between wild-type Arabidopsis and the *fad3-2 fad7-2 fad8* triple mutant (Figure 5). Staining of mutant pollen with DAPI indicated that this pollen can mature to the tricellular stage. The lack of 18:3 and jasmonate appears to affect pollen development at a stage later than that of any of the previously described mutations that result in the production of aborted or dead pollen in Arabidopsis (Regan and Moffatt, 1990; Chaudhury, 1993). Our best estimates of the timing of jasmonate action suggest that jasmonic acid or Na18:3 must be applied 12 to 24 hr before flower opening to ensure seed set corresponding to the middle of stage 12, as defined by Smyth et al. (1990). This timing would be consistent with a malfunction or cessation of pollen cell function in the 24 hr immediately before flower opening and thus with the results shown in Figures 5 and 6.

The *fad3-2 fad7-2 fad8* triple mutant plants are profoundly male sterile. We have never recovered mature seed from untreated mutant plants. A concerted effort to recover seed from a group of 60 plants was unsuccessful. Based on the seed production of similar wild-type plants, the lack of seed formed in the *fad3-2 fad7-2 fad8* plants suggests that fertility in the triple mutant is $<10^{-5}$ that of the wild type. However, treatment of full-sized, unopened flower buds with jasmonic acid (or Na18:3) consistently produced mature healthy siliques containing 30 to 50 seeds. Clearly, jasmonic acid mediates a profound qualitative change in pollen fertility.

The male-sterile phenotype in the triple mutant is controlled by the genotype of the sporophytic tissue. This is true of the majority of male-sterile mutants, and the defects are often localized by direct or circumstantial evidence to processes occurring in the tapetum, the anther cell layer that bounds the locule and is intimately involved in all aspects of pollen microspore development (Chapman, 1987; Goldberg et al., 1993). It would seem reasonable to implicate the tapetum as the source of jasmonate, which might regulate final maturation processes in the pollen grains. It is certainly possible that in the wild type, the tapetum is the physiological source of jasmonate and that the pollen grains are the target. However, 18:3 applied to the flower buds can bring about seed set without significantly raising the 18:3 content of the anther tissues (Table 3). This observation strongly suggests that other floral tissues can be effective sources of octadecanoid derivatives to complement the male-sterile phenotype in the mutant. Indeed, the observation raises the possibility that in wild-type Arabidopsis, sepals and other organs of the flower are sources of jasmonate, with the tapetum being the target. In either case, transfer of jasmonate may occur by simple diffusion. This process could be assisted by the fact that the methyl ester of jasmonic acid (which is also normally present in plants) is volatile and could be transferred through the vapor phase within the closed bud. Alternatively, specific translocation of jasmonic acid or its derivatives in the vascular system might be involved.

A second component of male sterility in the triple mutant is the failure of the anther locules to dehisce correctly (Figures 4B and 4C). In some male-sterile mutants, the pollen is fully viable and the failure of pollination and fertilization is attributable solely to the fact that anther dehiscence does not occur (Dawson et al., 1993). Other male-sterile plants, in which pollen death results from selective destruction of the tapetum, are able to undergo anther dehiscence normally (Mariani et al., 1990). These examples indicate that the maturation of viable pollen is not directly linked to the complex processes that lead to dehiscence of the anther locules and release of the pollen (Keijzer, 1987). We cannot rule out the possibility that jasmonate affects dehiscence only through its actions in pollen maturation. However, in light of the findings of Mariani et al. (1990) and Dawson et al. (1993), it is more likely that jasmonate performs (at least) two separate signaling functions during flower development. One is to ensure the maturation of viable pollen. The second is to mediate the changes in cell wall structure of the stomium and in the cellular water relations within the endothecium that result in successful dehiscence of the anthers (Stanley and Linskens, 1974).

A role for jasmonate signaling in reproductive fertility has been inferred from characterization of *coi1* mutants of Arabidopsis, which are also male sterile. The *coi1* plants are resistant to the bacterial phytotoxin coronatine (whose structure is analogous in certain respects to that of jasmonic acid), and they fail to exhibit several typical responses when exposed to jasmonate (Feys et al., 1994). The suggested explanation for these observations is that *coi1* plants lack the jasmonate receptor, which also acts as the target for coronatine action. The characterization of the *fad3-2 fad7-2 fad8* mutant described here, and especially our ability to complement chemically the triple mutant's male-sterile phenotype with exogenous jasmonate, establishes essential roles for jasmonate in pollen maturation and release.

Although very many male-sterile mutants have been isolated and characterized at the genetic level, the *fad3-2 fad7-2 fad8* triple mutant becomes one of the few in which the defect has been defined at the biochemical level. The *fad3-2 fad7-2 fad8* mutant and the associated chemical complementation assay provide powerful tools to dissect further the signaling processes involved in pollen and anther development.

METHODS

Plant Material and Growth Conditions

The lines of *Arabidopsis thaliana* used here were descended from the Columbia wild type. The fatty acid desaturation (*fad*) mutant lines used, *fad7-1* (Browse et al., 1986a), *fad7-2*, *fad8* (McConn et al., 1994), and *fad3-2* (Browse et al., 1993), have been reported previously. Plants were grown on soil in controlled environment chambers at 22°C and continuous fluorescent illumination (140 μmol m^{-2} sec^{-1}) for 2 to 3 weeks, unless otherwise specified.

For measurement of growth rates, plants were grown at 22°C under continuous illumination of 140 μmol m^{-2} sec^{-1}. At intervals between 8 and 24 days after sowing, samples of plants were harvested and the fresh weight of the above-ground parts was measured. The relative growth rate (ω^{-1}) is the slope of the natural log of fresh weight versus the time in days. The error associated with ω^{-1} was calculated by $\omega^{-1} = s/(\Sigma(x - \bar{x})^2)^{0.5}$, where $s^2 = [\Sigma(y - \bar{y})^2 - (\Sigma(x - \bar{x})(y - \bar{y}))^2/\Sigma(x + \bar{x})^2]/(n - 2)$ and where $\bar{x}$ and $\bar{y}$ are the means of the time and the natural log of fresh weight, respectively.

Measurement of Fluorescence Parameters

Fluorescence measurements were made using a chlorophyll fluorometer (model PAM101; Heinz-Walz, Effeltrich, Germany). Leaves from plants grown on soil under continuous light (140 μmol m^{-2} sec^{-1}) at 22°C were dark-adapted for 1 hr at 25°C to allow relaxation of nonphotochemical quenching components. Leaves were placed under a modulated light beam with a photon flux density (PFD) of 0.025 μmol m^{-2} sec^{-1} to determine F_o, the minimal fluorescence level. This PFD was determined not to induce any significant variable fluorescence. The leaf was then pulsed with saturating light of 4500 μmol m^{-2} sec^{-1} to measure maximum dark fluorescence, F_m. The dark value of F_v/F_m was calculated from F_o and F_m, where $F_v/F_m = (F_m - F_o)/F_m$. The leaf was then exposed to actinic light with a PFD of 140 μmol m^{-2} sec^{-1} and allowed to reach steady state photosynthesis. The steady state value of fluorescence, F_s, was then measured, followed by a saturating pulse to determine F_m'. The steady state quantum efficiency (Φ_{II}) was then calculated as $\Phi_{II} = (F_m' - F_s)/F_m'$ (Genty et al., 1989).

Fatty Acid and Lipid Analysis

Overall fatty acid compositions of leaves, roots, and flower tissues were determined by gas chromatography after derivatization with 2.5% (v/v) H_2SO_4 in methanol (Miquel and Browse, 1992). Samples of leaf tissue were immersed rapidly in liquid nitrogen, ground to a fine powder, extracted, and analyzed for lipid composition (Miquel and Browse, 1992).

Microscopy

For scanning electron microscopy, mature flowers (open) were harvested and fixed overnight in 3% glutaraldehyde at 4°C. Samples were then washed three times for 10 min each in 0.1 M Pipes, pH 7.2, incubated in 2% (v/v) OsO_4 for 2 hr, and then dehydrated through a graded ethanol series (50, 60, 70, 80, 90, 95, 100% [v/v]). Once in 100% ethanol, the samples were transferred to a drying apparatus (model Samdri-PVT-3D; Tousimis, Rockville, MD) and critical point–dried in CO_2 (Boyde, 1978; Cohen, 1979). The specimens were then affixed to stubs with paraffin, coated with gold in argon with a sputtering device (model Hummer V; Technics, Alexandria, VA) (Echlin, 1981), and viewed on a scanning electron microscope (model S-570; Hitachi, Tokyo, Japan). Two independent preparations were examined.

For light microscopy, flowers were fixed in FPA50 (Sass, 1958) overnight at room temperature, dehydrated through a graded ethanol series as described above, and then cleared in Herr fluid (Herr, 1971) at room temperature. After 48 hr in Herr fluid, the flowers were dissected, immersed in fluid, and viewed on Raj slides (Herr, 1971). Both phase contrast and differential interference contrast optics were used to view clearings on an Austoplan microscope (Leitz, Wetzlar, Germany). Images were recorded on Kodak Technical Pan film and Ektachrome color slide film.

Double staining with fluorescein diacetate and propidium iodide was performed using the method of Regan and Moffatt (1990) with some alterations. A stock solution of 2 mg/mL fluorescein diacetate was made in acetone and added dropwise to 17% sucrose (w/v). Equal amounts of fluorescein diacetate and propidium iodide solutions were added to freshly isolated pollen. The pollen was transferred to a glass slide, covered with a coverslip, and viewed under UV light by using filter block I3 with excitation filters BP450-490, dichromatic mirror RKP510, and suppression filter LP520 (Leitz, Wetzlar, Germany). Staining for DNA with mithramycin or 4′,6-diamidino-2-phenylindole (DAPI) was carried out using the procedure of Coleman and Goff (1985). DAPI staining was viewed with UV light by using filter block A, with excitation filters BP340-380, dichromatic mirror RKP400, and suppression filter LP430 (Leitz).

Pollen Germination

Pollen was isolated from mature flowers by gently releasing them from the anther locules into 17% (w/v) sucrose. The liberated pollen was then placed onto plates of pollen germination medium, consisting of 17% (w/v) sucrose, 2 mM $CaCl_2$, 1.65 mM H_3BO_3, pH 7 (Preuss et al., 1993), and solidified with 6% (w/v) agar. Pollen was incubated for 12 hr at room temperature and then analyzed for pollen tube formation.

Chemical Treatments

Application of various fatty acid soaps obtained from Nuchek (Elysian, MN) was done by spraying or painting 0.1% (w/v) solutions directly on Arabidopsis flower buds (plants were ~4 weeks old and grown under continuous light [140 μmol m^{-2} sec^{-1} at 22°C]) and placing them in a 12-hr day/night light regime at 22°C with 140 μmol m^{-2} sec^{-1} at the beginning of a dark cycle. These plants were then sprayed at the beginning of the dark cycle for 10 consecutive days. The plants were then monitored for silique production. Mature siliques were harvested and allowed to desiccate for 1 week before planting. Leaves of the progeny were analyzed for their fatty acid composition by gas chromatography, as described above.

Other compounds were applied to the buds by painting or spraying. *cis*-3-Hexen-1-ol, *trans*-2-hexen-1-al, and *trans*-3-hexenol were applied as 0.1% solutions in 0.1% sodium 18:2. Jasmonic acid was applied at a concentration of 2 μmol/mL. A 1% stock solution of traumatic acid was solubilized with KOH to a pH of 7 to 7.5, diluted to a 0.1% solution in 0.1% sodium 18:2, and applied to flower buds.

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