Accumulation of Very-Long-Chain Fatty Acids in Membrane Glycerolipids Is Associated with Dramatic Alterations in Plant Morphology

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Transgenic Arabidopsis plants overexpressing the Arabidopsis FATTY ACID ELONGATION1 gene under the control of the 35S promoter from cauliflower mosaic virus accumulated very-long-chain fatty acids (VLCFAs) throughout the plant. In some transformants, C20 and C22 VLCFAs accounted for >30% of the total fatty acids, accumulating at the expense of C16 and C18 fatty acids. These C20 and C22 fatty acids were incorporated into all of the major membrane glycerolipid classes. Plants with a high VLCFA content displayed a dramatically altered morphology, which included the failure of flowering shoots to elongate, a modified spatial pattern of siliques, an altered floral phenotype, and a large accumulation of anthocyanins. In addition, these plants also exhibited a unique alteration of the chloroplast membrane structure. We discuss a possible role for VLCFAs in establishing the shape/curvature of the membranes, which in turn may affect the shape of the cell and ultimately that of the whole plant.

INTRODUCTION

The physical properties of membranes are largely determined by chain length, polarity, and the degree of unsaturation of fatty acids that comprise their lipids. The fact that each membrane in the cell consists of a characteristic set of lipid classes and that each class has a distinct fatty acyl composition suggests that the lipid structure/composition is important for membrane function (Ohlrogge and Browse, 1995). This notion is further supported by the observation that the lipid structure/composition of membranes is conserved throughout the plant kingdom. However, the exact relationship between lipid structure and membrane function is not well understood (Ohlrogge and Browse, 1995).

Common plant fatty acids, which are constituents of structural membrane glycerolipids, are C16 and C18 fatty acids with one to three cis double bonds. Fatty acids with chemical structures that differ significantly from this common theme are called unusual fatty acids (van de Loo et al., 1993). Examples of unusual fatty acids include very-long-chain fatty acids (VLCFAs; e.g., erucic [22:1]), medium-chain fatty acids (e.g., lauric [12:0]), hydroxylated fatty acids (e.g., ricinoleic [12OH-18:1]), and fatty acids with different positions of the double bond (e.g., petroselinic acid [18:1, Δ6]) (van de Loo et al., 1993). These fatty acids occur mainly in storage triacylglycerols (TAGs) of certain oilseed species but are excluded from polar glycerolipids and consequently from the membranes of cells. Presumably, the accumulation of unusual fatty acids in membrane lipids would perturb the integrity of the bilayer and have deleterious effects on the cell. Thus, plants have developed a process(es) to screen out unusual fatty acids from membrane lipids. Although the exact mechanism of this process is yet to be elucidated, there is evidence that phospholipases and acyltransferases contribute to the strong fatty acid bias observed between storage and membrane glycerolipids (Bafor et al., 1991, 1993; Frentzen, 1993; Stahl et al., 1995).

The strict censoring of the fatty acyl composition of membrane lipids has been highlighted recently in transgenic plants. Two unusual fatty acids, lauric and ricinoleic, require a single unique enzyme activity for their synthesis from primary lipid metabolism. Lauric acid biosynthesis requires a medium-chain acyl-acyl carrier protein thioesterase, whereas ricinoleic acid biosynthesis requires an 18:1 hydroxylase. Genes encoding these enzymes were isolated from California bay trees (UcFatB1; Voelker et al., 1992) and castor bean (FAH12; van de Loo et al., 1995), respectively. Expression of either one of these genes in transgenic plants under the control of the cauliflower mosaic virus 35S promoter resulted in the synthesis and incorporation of the unusual fatty acids in

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seed TAGs. In contrast, neither fatty acid accumulated to a detectable level in the vegetative parts of the plant (Eccleston et al., 1996; Broun and Somerville, 1997).

Subsequent experiments demonstrated that lauric acid was produced efficiently in leaves of 35S-UCFATB1 transgenic plants (Eccleston et al., 1996). However, it was excluded from membrane lipids and thought to be rapidly degraded by β-oxidation (Eccleston et al., 1996). This hypothesis was supported by studies with Escherichia coli. Whereas wild-type E. coli cells expressing UCFATB1 failed to accumulate 12:0, expression of UCFATB1 in E. coli mutants blocked in β-oxidation resulted in the accumulation of large quantities of 12:0 in the form of free fatty acids (Voelker and Davies, 1994). Analogous to the accumulation in plants, 12:0 did not accumulate in the membranes of these E. coli mutants, demonstrating that a general mechanism exists in cells to exclude unusual fatty acids from membrane lipids.

Similar to lauric and ricinoleic acids, VLCFAs are also present in seed TAGs of some plant species. However, in addition to seeds, VLCFAs are also synthesized in epidermal cells, in which they serve as the precursors of wax components (Post-Beittenmiller, 1996). VLCFAs also occur in sphingolipids of the plasma membrane (Lynch, 1993) and may therefore be present in all cells. VLCFAs are synthesized by a microsomal fatty acid elongation (FAE) system by the sequential addition of C2 moieties, which are derived from malonyl-CoA, to preexisting C18 fatty acids. Each cycle of FAE requires four enzymatic reactions: condensation, reduction, dehydration, and further reduction (Fehling and Mukherjee, 1991). Recently, James et al. (1995) isolated the FATTY ACID ELONGATION1 (FAE1) gene of Arabidopsis. This gene encodes a condensing enzyme, which is the first enzyme of the VLCFA biosynthetic pathway. FAE1 is seed specific and is involved in the synthesis of C20 and C22 fatty acids that are incorporated into seed TAGs (Kunst et al., 1992).

To examine the regulation of the VLCFA biosynthetic pathway, this seed-specific gene was ectopically expressed in the floral and vegetative tissues of Arabidopsis, in which significant amounts of C20 and C22 fatty acids are not found. Hence, the coding region of FAE1 was linked to the cauliflower mosaic virus 35S promoter, and the resulting binary vector construct (35S-FAE1) was transformed into Arabidopsis (Millar and Kunst, 1997). Surprisingly, and in contrast to transgenic plants expressing thioesterase and hydroxylase, the transgenic 35S-FAE1 plants were able to accumulate high levels of C20 and C22 VLCFAs in all tissues. This result implies that the other three enzyme activities of the pathway were present ubiquitously throughout the plant and that the VLCFA biosynthetic pathway was regulated through the expression of the condensing enzyme (Millar and Kunst, 1997). In this study, we present a detailed analysis of the effect of FAE1 expression on leaf lipid metabolism in these 35S-FAE1 transgenic plants. In addition, we describe the dramatic consequences that ectopic expression of FAE1 has on the membranes and morphology of the plant.

### RESULTS

**VLCFAs Can Constitute a Very High Proportion of the Total Fatty Acids in 35S-FAE1 Transgenic Arabidopsis**

In contrast to wild-type leaves, which do not contain significant levels of VLCFAs (<1% [w/w] of total fatty acids), transgenic Arabidopsis plants expressing 35S-FAE1 can accumulate high levels of these unusual fatty acids in their leaves (Millar and Kunst, 1997). To examine this finding more thoroughly, we determined a time course for the fatty acid composition of leaves from T4 seedlings of transgenic line 102. Our results show that the proportion of total VLCFAs, as a percentage of total fatty acids, increases with time (Table 1) and reaches almost 35% (w/w) of total fatty acids at senescence. In addition, analyses of the eight distinct VLCFAs present in the leaves of the 35S-FAE1 plants indicate that the proportion of saturated VLCFAs increases gradually over time and then accelerates at senescence. The proportion of unsaturated VLCFAs also increases gradually as the plant ages, but unlike the saturated VLCFAs, their proportion decreases at senescence. This is especially true for the polyunsaturated VLCFAs.

**VLCFAs Are Found in All of the Major Leaf Membrane Lipids**

In plant species that accumulate VLCFAs in seed TAGs, these fatty acids are selectively excluded or removed from seed membrane glycerolipids. We have analyzed wild-type Arabidopsis seeds and confirmed that only a small proportion (2 to 4%) of total seed VLCFAs occurs on structural membrane lipids (data not shown). Because transgenic 35S-FAE1 plants could accumulate VLCFAs in leaf tissues to >35% of total fatty acids and because Arabidopsis leaves do not contain significant quantities of storage lipids, we were interested in determining whether VLCFAs were being used for the synthesis of membrane lipids. To examine this possibility, individual lipids from 6-week-old Arabidopsis plants were isolated and their fatty acyl composition was determined (Table 2).

Six lipids were analyzed in detail. Four were phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidic acid (PA). Two were galactolipids: monogalactosyldiacylglycerol (MGD) and digalactosyldiacylglycerol (DGD). As expected, leaf glycerolipids extracted from wild-type plants contained detectable C20 and C22 fatty acids (Table 2). On the other hand, all phospholipid and galactolipid classes from the 35S-FAE1 transformants contained VLCFAs (Table 2).
Predominantly eukaryotic lipids, such as PC and PE, contained the highest proportions of VLCFAs, whereas MGD and DGD, which are made by both prokaryotic and eukaryotic pathways, had lower proportions of VLCFAs. Higher proportions of VLCFAs were found in DGD than in MGD, suggesting that a larger fraction of the DGD precursor originates from the eukaryotic pathway. This is also evident from the lower proportions of the plastid-specific 16:3 acyl group in DGD when compared with MGD. PG, which presumably is synthesized exclusively in the plastid, accumulated the lowest amounts of VLCFAs. Thus, it appears that the proportion of VLCFAs found in each lipid class depends on the portion of that lipid made by the eukaryotic pathway. To our knowledge, high levels of C20 and C22 VLCFAs have not been reported previously on these membrane glycerolipids. However, the presence of VLCFAs did not have an effect on the overall lipid composition of the leaf, because the proportions of the lipid classes present in the 6-week-old 35S–FAE1 plants were not significantly different from those of the wild-type plants (Table 2).

The proportions of VLCFAs reported for individual lipids (Table 2) were lower than those measured in the fatty acid analyses of whole leaves (Table 1). There are several possible reasons for this discrepancy. First, not all lipid classes are reported in Table 2. Preliminary analyses showed that neutral lipid classes, although minor components of total leaf lipids, contained significant proportions of VLCFAs. Second, as described below, plants with higher levels of VLCFAs have retarded growth. This may be important because a large number of leaves from many different plants were required for analyses of the fatty acid composition of individual lipid classes. Although a range of plants was used, the values shown in Table 2 are likely to be biased toward leaves with lower proportions of VLCFAs, because such leaves are generally larger and thus contribute more to the total mass of the sample. On the other hand, leaves with higher proportions of VLCFAs are smaller and contribute less. In contrast to the data presented in Table 2, analyses of fatty acid compositions of whole leaves shown in Table 1 were made with individual plants, so this bias does not exist.

### Polyunsaturated VLCFAs Are Found Preferentially on Plastidic Lipids

Examination of the VLCFA composition of MGD and DGD, which are lipids found exclusively in the plastid, showed that they contain primarily polyunsaturated VLCFAs. For example, in 6-week-old 35S–FAE1 plants, of the total C20 and C22 unsaturated fatty acids on MGD and DGD, 11% are monounsaturated, 29% are diunsaturated, and 60% are triunsaturated fatty acids. In contrast, in lipids of the eukaryotic pathway, such as PC and PE, 49% of C20 and C22 fatty acids are monounsaturated, 36% are diunsaturated, and 15% are triunsaturated. Because the majority of polyunsaturated VLCFAs synthesized in 35S–FAE1 plants accumulate on MGD and DGD, it seems likely that monounsaturated VLCFAs enter the plastid and are then desaturated by plastidic desaturases.

### The Increased Proportion of VLCFAs in Leaves during Senescence Can Be Explained by a Higher Proportion of Phospholipids

To further explore the observed increase in VLCFA content in senescing leaves of 35S–FAE1 plants, we compared the fatty acyl composition of individual lipids and free fatty acids of 10-week-old (Table 2, 35S–FAE1 [10]) and 6-week-old (Table 2, 35S–FAE1 [6]) transgenic Arabidopsis plants. We found that the proportions of VLCFAs in each lipid class and in the free fatty acid pool (data not shown) were not significantly different in these two age groups. The major difference was a higher proportion of the phospholipids PC and PE in 10-week-old plants. For example, in 6-week-old plants, PC and PE, which contain the highest amounts of

| Table 1. Time Course of Fatty Acid Composition of Transgenic (35S–FAE1) Leaves^a |
|-------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Plant Age (Weeks)             | 20:0     | 20:1     | 20:2     | 20:3     | 22:0     | 22:1     | 22:2     | 22:3     | Total VLCFAs |
| 3                             | 0.7      | 1.2      | 1.2      | 1.2      | —        | 0.1      | —        | —        | 4.4 ± 0.6  |
| 4                             | 1.5      | 1.2      | 1.3      | 1.4      | 0.7      | 0.6      | 0.5      | 0.4      | 7.6 ± 0.8  |
| 5                             | 3.2      | 1.6      | 1.4      | 1.4      | 1.5      | 1.4      | 0.8      | 1.2      | 12.4 ± 1.6 |
| 6                             | 3.5      | 2.2      | 1.6      | 1.3      | 1.4      | 1.5      | 0.7      | 1.5      | 13.6 ± 1.2 |
| 7                             | 4.0      | 2.8      | 1.3      | 1.0      | 2.0      | 2.9      | 1.6      | 2.6      | 18.1 ± 1.4 |
| 8                             | 5.3      | 4.2      | 1.5      | 1.3      | 2.6      | 5.6      | 2.7      | 2.7      | 25.9 ± 3.3 |
| 9                             | 5.7      | 5.1      | 1.8      | 0.8      | 3.4      | 7.7      | 3.0      | 2.9      | 30.2 ± 2.4 |
| 10                            | 7.5      | 4.9      | 1.7      | 0.8      | 4.4      | 8.6      | 3.3      | 2.7      | 33.9 ± 2.5 |
| Senesced                      | 14.3     | 3.4      | 0.2      | —        | 8.1      | 7.8      | 0.9      | 0.1      | 34.9 ± 2.9 |

^a Each value represents the fatty acid percentage (w/w) of total fatty acids. Each value is the mean of at least 15 measurements from different individual plants. Dashes indicate not detected. Data are from line 102.
VLCFAs, constituted only 14.4% (w/w) of total lipids, compared with 38.1% in 10-week-old plants (Table 2). On the other hand, the relative proportion of galactolipids, especially MGD, which contains the lowest levels of VLCFAs, decreased from 77.4% (w/w) of total lipids in 6-week-old plants to 52.4% (w/w) of total lipids in 10-week-old plants (Table 2). Thus, a possible explanation for the proportional increase in leaf VLCFA content during the later stages of plant development is a slower breakdown of phospholipids in comparison with galactolipids in senescing leaves. Consistent with this explanation is the disappearance of the polyunsaturated VLCFAs, which in this case may be acting as a marker for galactolipids because they are found primarily in MGD and DGD (Table 2).

### 35S-FAE1 Plants Accumulating High Levels of VLCFAs Have an Altered Morphology

When transforming Arabidopsis with the 35S–FAE1 construct, we produced 190 kanamycin-resistant lines accumulating varying amounts of VLCFAs (Millar and Kunst, 1997). We have shown previously that VLCFA levels correlate with FAE1 transcript levels, suggesting that the observed variation in VLCFA levels was due to the position of the transgene in the genome (Millar and Kunst, 1997). Detailed examination of 35S–FAE1 transformants demonstrated that plants could tolerate relatively high levels of VLCFAs in leaves (~10% [w/w] of total fatty acids in 6-week-old plants) without any visible, detrimental effect on their growth or development. However, in our population of 190 35S–FAE1 lines, we found six independent transformants with a very unusual morphology (Figures 1A and 1B). The morphological alterations were complex, but they were all distinct and reproducible, as described in detail below. Although the observed traits were present in all six lines, they varied in their severity from line to line. Fatty acid analyses showed that these six morphologically altered plants had the highest leaf VLCFA content of all the transgenic lines. Thus, it seemed likely that their unusual phenotype resulted from the expression of the 35S–FAE1 transgene and was possibly linked to the accumulation of VLCFAs.

### Table 2. Fatty Aycl Composition of Leaf Lipids from Wild-Type and 35S–FAE1 Plants

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Percentage of Total Lipids</th>
<th>C16&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C18&lt;sup&gt;b&lt;/sup&gt;</th>
<th>C20&lt;sup&gt;c&lt;/sup&gt;</th>
<th>C22&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Total VLCFAs</th>
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<td>MGD</td>
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<td></td>
<td></td>
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<tr>
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<td>30.5</td>
<td>67.5</td>
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<td></td>
<td></td>
</tr>
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<td>35S–FAE1(6)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.9</td>
<td>39.2</td>
<td>57.4</td>
<td>0.5</td>
<td>0.3</td>
<td>1.1</td>
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<td>0.6</td>
<td>0.6</td>
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<td>DGD</td>
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<td></td>
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<td>WT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.3</td>
<td>14.4</td>
<td>82.3</td>
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<td>26.7</td>
<td>58.4</td>
<td>2.8</td>
<td>0.9</td>
<td>2.3</td>
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<td>67.0</td>
<td>4.1</td>
<td>1.6</td>
<td>1.7</td>
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<td>PG</td>
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<tr>
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<td>7.0</td>
<td>2.8</td>
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<sup>a</sup>Each value represents the fatty acid percentage ([w/w] of total fatty acids) and is the mean of two independent measurements. Dashes indicate not detected.

<sup>b</sup>Each fatty acid chain length grouping represents the sum of four fatty acids, for example, C16 = 16:0, 16:1, 16:2, and 16:3.

<sup>c</sup>Six-week-old wild-type (WT) plants.

<sup>d</sup>Six-week-old 35S–FAE1 plants from line 60.

<sup>e</sup>Ten-week-old 35S–FAE1 plants from line 60.
To investigate this observation further, we followed the cosegregation of VLCFA content and plant morphology within one primary transformant, line 60, which contained VLCFAs but had a wild-type appearance. Segregation data of 131 T3 progeny of line 60 (Figure 2) showed that T3 plants fell into three distinct phenotypic classes: (1) biochemically and morphologically wild-type plants, (2) plants that accumulated VLCFAs in their leaves and had a wild-type morphology, and (3) plants that accumulated VLCFAs in their leaves but had an unusual morphological phenotype. The ratio of these three classes of plants was 32:62:37, respectively. This is approximately a 1:2:1 ratio ($\chi^2 = 0.57; P > 0.7$), suggesting that line 60 contained a single segregating transgene locus. Further genetic analysis of T4 progeny confirmed this hypothesis, showing that class 2 plants were hemizygous for the transgene (1:2:1 segregation in the T4 generation), whereas class 3 plants were homozygous for the transgene (all plants had an altered morphology). In addition, both class 2 and class 3 plants were kanamycin resistant, whereas the class 1 plants were kanamycin sensitive.

The levels of VLCFAs also segregated in the T3 progeny of line 60, with the morphologically unusual looking plants accumulating significantly higher levels of VLCFAs than the wild-type looking plants (Figure 2). This is consistent with data from the primary transformants in which the plants with the unusual morphology contained the highest levels of VLCFAs among the transgenic lines. Thus, it appears that a certain threshold level of VLCFA accumulation (or 35S–FAE1 expression) is necessary before plant development is disturbed.

**The Severity of the Unusual Morphological Phenotype Correlates with the Level of VLCFA Accumulation**

The variation in VLCFA levels among the homozygous progeny of line 60 (Figure 2) was evident from the phenotypes of the plants, with individual plants that had the highest VLCFA levels displaying the most severe phenotype (data not shown). To investigate further whether there is a correlation between the VLCFA levels present in vegetative tissues and the severity of the phenotype, we analyzed three independently generated transgenic lines, 60, 101, and 102, at 6 weeks of age. Two parameters were measured in each line: the levels of VLCFAs in their leaves and the height of the flowering shoot. We chose the latter because this is an easily measurable quantitative trait that indicates the severity of the phenotype. We found that there was a tight correlation between the levels of VLCFAs and the extent to which growth of the flowering shoot was stunted, with the line accumulating the highest levels of VLCFAs having the shortest shoots (Table 3). Thus, it seems that high levels of expression of the 35S–FAE1 transgene are deleterious to the plant.

This result raised the possibility that the expression of the transgene above a certain level may be lethal. To examine this possibility, we analyzed line 101 further. Line 101 was...
chosen because plants in this line displayed the unusual morphological phenotype as primary transformants carrying the transgene(s) in a hemizygous state, suggesting that 35S–\textit{FAE1} expression was very high. To determine whether a homozygous plant could be recovered and how severe its phenotype would be, we sowed seed from the primary transformant 101, and 261 T3 plants were obtained. Of those, 105 had a wild-type appearance, whereas 156 plants had the unusual morphology. None of the wild-type looking progeny contained VLCFAs in their leaves and were therefore true wild-type plants biochemically and morphologically. In contrast, all of the plants with the unusual morphology had high levels of VLCFAs in their leaves, as did the parental line. This result best fits the hypothesis that there is a single segregating transgene in line 101, with no plants homozygous for the transgene being recovered (2:1 ratio of unusual/wild-type; $\chi^2 = 5.28; P > 0.01$).

To confirm this hypothesis, we planted seed from 10 morphologically unusual T3 progeny on kanamycin selection media. In all cases, both kanamycin-resistant and kanamycin-sensitive plants were found among the T4 progeny, indicating that each parental T3 line was hemizygous for the transgene. The total number of resistant T4 plants compared with sensitive T4 progeny was 295 to 117, which again approximates a 2:1 ratio ($\chi^2 = 4.30; P > 0.01$). Thus, we were not able to recover any plants that were homozygous at this transgene locus. We believe that a possible reason for this is that the expression of the 35S–\textit{FAE1} transgene in line 101 is so high that it results in lethality.

Based on these data, it appears that the level of expression of the 35S–\textit{FAE1} transgene must fall within a specific and very narrow range to result in a viable plant with a visible morphological phenotype. This would explain why only a low number of primary transformants were recovered that exhibited this unusual morphology (six of 190).

### Ectopic Expression of \textit{FAE1} Causes Morphological Changes throughout the Plant

The most visible morphological alteration in the 35S–\textit{FAE1} Arabidopsis plants is the dramatically altered growth habit when compared with that of the wild type. As mentioned previously, plants with the highest VLCFA content in vegetative tissues exhibited severely stunted growth (Figure 1), a bushy appearance as a result of the failure of roots and floral shoots to elongate properly, and reduced apical dominance (Figure 3A). A possible consequence of the floral shoots failing to elongate is that the siliques develop in an abnormal spatial pattern, with all of them being clustered at the end of the shoot (Figure 3D). Rosette formation was also affected, resulting in a very disorganized asymmetrical leaf pattern and rumpled, slightly curled dark green leaves with petioles significantly shorter than those of the wild type (Figure 1B).

In addition, transgenic 35S–\textit{FAE1} plants had abnormal floral morphology (Figure 3C). The flowers were more round in comparison with those of the wild type (Figure 3B), and leaving only a small number of primary inflorescences.

### Table 3. Correlation of the Levels of VLCFAs with the Height of the Primary Inflorescence

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<thead>
<tr>
<th>Trait</th>
<th>Line 60 (Hemizygous)</th>
<th>Line 60 (Homozygous)</th>
<th>Line 101 (Hemizygous)</th>
<th>Line 102</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLCFA levels$^a$</td>
<td>9.9</td>
<td>12.5</td>
<td>10.4</td>
<td>12.0</td>
</tr>
<tr>
<td>Height of bolt (cm)</td>
<td>38.4 ± 4.7</td>
<td>5.3 ± 1.5</td>
<td>17.5 ± 3.4</td>
<td>11.1 ± 1.8</td>
</tr>
</tbody>
</table>

$^a$Levels are the total proportion of the C20 and C22 fatty acids (w/w) of the total fatty acids. Each value is the mean of at least 14 measurements. All plants were 6 weeks old.
the petals were shorter, so that the pistil protruded from the flower. Plants with the more severe phenotype also showed reduced fertility. However, five of the six primary transformants with the unusual morphological phenotype were able to set some seed. The only exception was the plant that had the most severe phenotype. This plant failed to bolt. The seeds from 35S–FAE1 plants (Figures 4F and 4G) have an altered morphology compared with that of wild-type seeds (Figure 4H), highlighting that developmental abnormalities are present throughout the plant.

The morphologically altered 35S–FAE1 plants had bright green stems, suggesting that they have a reduced wax load compared with wild-type plants, which is similar to some eceriferum mutants of Arabidopsis (McNevin et al., 1993). By using scanning electron microscopy, we confirmed that there was a reduction in the density of wax crystals on the stems of 35S–FAE1 plants. Furthermore, the shape of the crystals was altered, and they had a needlelike appearance rather than the characteristic platelike structure of wild-type plants (cf. Figures 4A and 4B). Scanning electron microscopy also showed that the cuticle of stems and siliques of 35S–FAE1 plants had become irregular and rough (cf. Figures 4C and 4D) and sparsely coated with small white globules (Figure 3C). Closer inspection of the globular structures under the scanning electron microscope revealed that they were protrusions of the cuticle (Figure 4E).

Gas chromatographic analysis demonstrated that the wax load on the stems of the morphologically altered 35S–FAE1 plants was reduced to ~50% of that of the wild type. The total wax load was 3650 (±551) µg/g dry weight on the 35S–FAE1 stems compared with 7106 (±1184) µg/g dry weight on wild-type stems. This reduced wax load was due to

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**Figure 3.** Morphological Alterations in 35S–FAE1 Plants Accumulating High Levels of VLCFAs.

(A) A 6-week-old 35S–FAE1 plant showing the lack of apical dominance. Instead of one dominant floral shoot, three or four shoots of similar height are present.

(B) A flowering shoot from wild-type Arabidopsis.

(C) A flowering shoot from 35S–FAE1 plants. Note the unusual floral morphology and white globular structures on the stems.

(D) Spatial arrangement of the siliques of 35S–FAE1 plants illustrating how they cluster at the end of the shoot.

(E) Eight-week-old 35S–FAE1 plants accumulating high levels of anthocyanins.

(F) New wild-type looking floral shoots emerging from 8-week-old 35S–FAE1 plants. These shoots do not originate from the rosette but rather from the junction of the inflorescences.

All of the plants are from line 60.
decreased levels of the C29 alkane (54% of that of the wild type), secondary alcohol (30% of that of the wild type), and ketone (37% of that of the wild type), which are three major wax components of Arabidopsis stems (cf. Figures 5A and 5B). In addition, levels of all primary alcohols were also reduced, reaching only ~50% of wild-type levels. In contrast, fatty acids and aldehydes of the 35S–FAE1 plants all appeared to be at wild-type levels. This alteration in proportions of wax components resulted in a chemical profile that is different from that of the wild-type plants (Figure 5). The presence of the FAE1 condensing enzyme, however, did not result in the synthesis of any novel wax compounds, although small amounts of C20 and C22 fatty acids were detectable, as was the C22 primary alcohol, whereas these molecules were not present in wax from wild-type plants (Figure 5). The described changes in wax synthesis/deposition are clearly associated with the 35S–FAE1 transformants exhibiting the unusual phenotype and were not present in 35S–FAE1 lines with a wild-type morphology.

Under our growth conditions, leaves of wild-type Arabidopsis usually begin to senesce after 6 to 7 weeks. However, leaves from the morphologically altered transgenic 35S–FAE1 plants typically did not start senescing until after 10 weeks of age. Even though siliques started drying out and seeds set, the stems and leaves remained green. The 35S–FAE1 plants also accumulated high levels of anthocyanins, so that the leaves turned bright purple (Figure 3E). The onset of the anthocyanin accumulation depended on the severity of the phenotype. Plants with a strong phenotype started turning purple at 4 to 5 weeks of age, whereas those with a less severe phenotype only began to accumulate anthocyanins at 8 or 9 weeks of age.

All of the independently isolated morphologically altered 35S–FAE1 plants approaching 6 to 8 weeks of age also con-

Figure 4. Scanning Electron Microscopy of Changes to the Surface Structures of 35S–FAE1 Transgenic Plants.

(A) Close-up of the density and shape of the epicuticular wax crystals on the stems of a 35S–FAE1 plant.
(B) Density of the wax bloom and shape of wax crystals on wild-type stems.
(C) Overview of the structure of a stem from a 35S–FAE1 plant.
(D) Stem from a wild-type plant.
(E) Close-up of a globular structure found on the stems of 35S–FAE1 plants.
(F) and (G) Shapes of seeds from 35S–FAE1 plants.
(H) Shape of a typical seed from a wild-type plant.

The 35S–FAE1 plants are all from line 60. Bars in (A) and (B) = 20 μm; bars in (C) to (E) = 100 μm; bars in (F) to (H) = 200 μm.
sistently produced new wild-type looking flowering shoots. The fertility, flower shape, and elongation of these shoots all appeared normal (Figure 3F). Furthermore, their wax profile and wax load (6208 ± 958 μg/g dry weight) were similar to those of wild-type shoots. Therefore, we decided to examine whether the VLCFA content in these revertant shoots was lower than in the unusual looking shoots. Analysis determined that the levels of VLCFAs in revertant shoots were more than ninefold lower (2.9% [w/w] of total fatty acids [average of 10 measurements]) than in the bolts with a mutant phenotype (27.4% [w/w] of total fatty acids [average of 10 measurements]). However, when seeds collected from the revertant shoots were planted, all resulted in plants with the unusual phenotype.

One aspect of plant development that was normal in 35S–FAE1 transformants was the growth rate of the plants. Transgenic seeds germinated as fast as wild-type seeds, cotyledons developed at the same rate, and their time to flowering was unaltered by the expression of the 35S–FAE1 transgene relative to the wild type (data not shown).

**The Morphologically Unusual 35S–FAE1 Plants Have Curved Thylakoid Membranes**

To determine the effects of changes in leaf membrane lipid composition at the cellular level, we examined thin leaf sections of the wild type and two independent 35S–FAE1 transgenic lines, 60 and 102, by using transmission electron microscopy. Figure 6 shows chloroplasts from the wild type and 35S–FAE1 transgenic line 60. Whereas the chloroplasts from the wild-type leaves display normal large grana stacks (Figures 6A and 6C), both grana and stroma thylakoid membranes in chloroplasts from the 35S–FAE1 leaves appear curved (Figures 6B and 6D). Examination of a large number of 35S–FAE1 chloroplasts established that the majority of grana in each chloroplast were structurally altered into curved stacks. Only occasionally in some chloroplasts could seemingly normal grana be found. On the other hand, distorted thylakoid membranes were never observed in wild-type chloroplasts or in 35S–FAE1 lines with a wild-type morphology. Our analyses also revealed that the degree of membrane curvature varied between chloroplasts in a cell, between cells, and especially between different transgenic plants. In general, transgenic plants with the more severe phenotype also seemed to have more pronounced perturbations of chloroplast ultrastructure.

**DISCUSSION**

**VLCFAs Are Not Perceived to Be Unusual Fatty Acids by the Leaf Glycerolipid Metabolism**

Because of their chain length, eicosenoic (20:1) and erucic (22:1) acids are considered unusual fatty acids (van der Loo et al., 1993). They accumulate to high levels in seeds of some plant species. In all of the species containing seed VLCFAs examined to date, lipid metabolism is regulated so
that VLCFAs are restricted almost exclusively to storage TAGs and are excluded from the membrane lipids that have structural functions, such as PC, PA, and PE (Taylor et al., 1991). Conversely, in the leaves of 35S–FAE1 plants, there appears to be no restriction of VLCFAs to storage lipids. Like common fatty acids, VLCFAs in leaf cells are distributed among all of the leaf lipid classes, with the only bias being toward lipids that are produced by the extraplastidic eukaryotic pathway, reflecting the extraplastidic location of FAE1. Thus, the mechanism(s) that operates to exclude VLCFAs from membrane glycerolipids of seeds is not efficient in the leaves of Arabidopsis plants.

The gradual increase in the proportion of VLCFAs in 35S–FAE1 plants between 6 and 10 weeks of age is probably associated with altered membrane lipid composition of senescing leaves rather than with the fact that the plant is unable to degrade VLCFAs. During senescence, chloroplasts are broken down, resulting in decreased levels of MGD and DGD. In contrast, the plasma membrane, which is primarily composed of phospholipids (mostly PC and PE), remains intact. Thus, the proportion of phospholipids, which contain the majority of VLCFAs, increases in leaf membranes as plants age. These data imply that the specificities of the enzymes involved in fatty acid degradation are broad enough to recognize VLCFAs. This observation, together with the finding that VLCFAs are present in all lipid classes, implies that many of the enzymes involved in leaf glycerolipid metabolism have relatively broad substrate specificities, allowing them to use C20 and C22 acyl chains or lipids containing these fatty acids. The major exceptions to this appear to be the specificities of the fatty acid desaturases, because the percentage of polyunsaturated VLCFAs is low compared with the polyunsaturated C18 and C16 fatty acids.

The observation that the percentage of polyunsaturated VLCFAs is low on the eukaryotic lipids PC and PE (Table 2) suggests that the microsomal desaturases have low activity for VLCFAs. This result is consistent with those of Spychalla et al. (1997), who found that the microsomal ω-3 desaturase exhibits low activity with C20 fatty acids. In contrast, the unsaturated VLCFAs that occur on the chloroplast lipids MGD and DGD are mainly polyunsaturated (Table 2), suggesting that chloroplast desaturases have a broad acyl chain–length specificity. These data, together with the fact that the jojoba condensing enzyme has very little activity on polyunsaturated C18 fatty acids (Lassner et al., 1996), would argue that polyunsaturated VLCFAs in 35S–FAE1 leaves arise mainly by the elongation of C18:1 to monounsaturated VLCFAs, followed by their import into the chloroplast, where they are desaturated.

Based on these analyses of the metabolism of VLCFAs in transgenic 35S–FAE1 plants, we conclude that VLCFAs are not recognized by the plant as unusual and that the mechanism(s) that eliminates lauric and ricinoleic acids from membrane glycerolipids (Eccleston et al., 1996; Broun and Somerville, 1997) does not act on C20 and C22 VLCFAs in the vegetative and floral parts of the plant. This raises the possibility that VLCFAs are present in membranes of all cells. For instance, Murata et al. (1984) found VLCFAs in phosphatidylserine, a ubiquitous but minor (<1%) membrane lipid of higher plants. Furthermore, sphingolipids contain VLCFAs. These lipids are structurally distinct from glycerolipids and are thought to be present in the plasma membrane of most if not all eukaryotic cells (Lynch, 1993). The requirement for VLCFAs in these two classes of lipids may be the reason why plant cells have not developed mechanisms to exclude VLCFAs from membrane lipids.

**Figure 6.** Chloroplasts from 35S–FAE1 Plants Have an Altered Thylakoid Membrane Shape.
(A) Transmission electron microscopy of a chloroplast from a wild-type rosette leaf.
(B) Transmission electron microscopy of a chloroplast from a 35S–FAE1 rosette leaf.
(C) Enlargement of the thylakoid stacks from a wild-type plant.
(D) Enlargement of the thylakoid stacks from a 35S–FAE1 plant illustrating the curvature that exists in these membranes.

The 35S–FAE1 plants are from line 60. Bars in (A) and (B) = 1 μm; bars in (C) and (D) = 0.1 μm.
High VLCFA Accumulation Results in an Altered Thylakoid Membrane Structure

It is well established that plants screen out unusual fatty acids, such as lauric and ricinoleic acids, from their membranes, presumably to maintain membrane integrity. However, direct biological evidence for a disruptive effect of unusual fatty acids on membrane structure is lacking (van de Loo et al., 1993). Here, we report that 35S–FAE1 transformants can accumulate VLCFAs to >30% (w/w) of the total fatty acid content of membrane glycerolipids. Associated with this VLCFA accumulation is an alteration of the ultrastructure of the thylakoid membranes, suggesting that the presence of VLCFAs may be causing the membranes to bend. Supporting this possibility is a recent isolation of a temperature-sensitive acetyl–CoA carboxylase mutant in yeast (Schneiter et al., 1996). In this mutant, reduced amounts of malonyl–CoA result in lower levels of C26:0 VLCFAs required for the synthesis of sphingolipids. This deficiency of VLCFAs is directly linked to a morphological defect, the separation of the inner and outer envelopes of the nuclear membranes (reviewed in Schneiter and Kohlwein, 1997). The authors hypothesize that VLCFAs are needed, directly or indirectly, to stabilize highly curved membranes around the nuclear pores, which are required for the proper assembly of the nuclear envelope. They argue that VLCFAs can promote curvature of lipid bilayers, because the extended hydrophobic tail requires interdigitation with the opposite membrane leaflet (Hui et al., 1984; Schneiter et al., 1996).

The results reported in this study are consistent with these findings. The curved thylakoid stacks from the chloroplasts of 35S–FAE1 plants suggest that thylakoid membrane lipids containing VLCFAs may be mimicking the structural role of sphingolipids. This is conceivable in view of recent findings by Lester et al. (1993) that yeast cells can only survive without sphingolipids by producing novel glycerolipids containing C26 fatty acids that structurally mimic them. Thus, our data support the hypothesis that VLCFA-containing membrane lipids can affect the curvature of membrane bilayers, which may be instrumental in determining the shape of cellular structures.

Can VLCFA Accumulation in Membranes Affect Plant Morphology?

Although it is apparent that lipid/fatty acid composition is essential for the correct functioning of the cell, alterations in fatty acid composition are rarely linked to morphological abnormalities in unicellular or multicellular organisms (Gibson et al., 1994). There is an Arabidopsis mutant in which elevated levels of 18:0 are associated with growth retardation. However, the overall body plan of this miniature plant remains normal (Lightner et al., 1994). Thus, the unusual 35S–FAE1 plants may be a unique example of where an alteration in fatty acid metabolism results in an alteration in the developmental pattern of a multicellular organism.

The unusual phenotype of the 35S–FAE1 plants is very complex, affecting the shape and morphology of every plant organ examined. Thus, it is likely that ectopic expression of FAE1 has caused an alteration in a fundamental process required for the normal morphological development of the plant. There are a number of possible causes for the observed phenotype. The most plausible is the direct effect of the incorporation of VLCFAs into the membrane lipids, resulting in alterations to their physical properties and leading to incorrect assembly of membrane bilayers. Furthermore, because VLCFAs accumulate to the highest levels in major plasma membrane lipids of 35S–FAE1 plants, PC and PE, it is conceivable that they could alter the curvature of the plasma membrane (similar to that of the thylakoid membranes), which in turn may affect the shape of the cell. For example, it has been shown in human erythrocytes that the fatty acid composition of PC can have a profound effect on its geometry (e.g., substitution of 18:1 or 18:2 with 20:4) and result in a completely new cell shape (Op den Kamp et al., 1985). Whether similar types of changes can occur in plant cells remains to be determined. It is generally thought that plant cell shape and size are governed by the mechanics of the rigid cell wall. However, the pattern of cellulose deposition within the cell wall is thought to be determined by the movement of cellulose synthase complex within the plasma membrane (Delmer and Amor, 1995). It is possible, then, that structural changes in the plasma membrane could interfere with the movement of cellulose synthase and result in an altered cell wall and ultimately changes in the shape of the cell.

Other Possible Causes for the Unusual Phenotype of 35S–FAE1 Plants

Besides VLCFA accumulation in cellular membranes, there are several other possible causes of the unusual phenotype associated with ectopic expression of FAE1. First, high levels of FAE1 expression could result in competition for substrates. For example, the depletion of malonyl-CoA from the metabolic pool, which is used by a number of metabolic pathways of the plant, may be the factor causing the phenotype. However, the pronounced accumulation in the 35S–FAE1 plants of anthocyanins, which require malonyl-CoA for their synthesis, would argue against malonyl-CoA depletion. Second, it could be that the high levels of the FAE1 protein per se are a problem. FAE1 is a condensing enzyme of the extraplastidic FAE pathway, and it is thought to operate in a complex with other proteins. Thus, the ectopic presence of FAE1 may result in competition with other non-seed condensing enzymes in forming active elongation complexes, resulting in the interference of other critical VLCFA pathways. However, the expression of another condensing enzyme, with high homology to FAE1, under the control of the 35S promoter did not result in transgenic plants with the unusual morphological phenotype (A.A. Millar and L. Kunst,
data not shown). A third factor causing the unusual phenotype could be the synthesis of novel compounds derived from the C20 and C22 VLCFAs. For instance, they could be used for the synthesis of eicosanoid-like products (Creelman et al., 1992), which in mammals are central hormonal signals involved in inflammatory responses and other physiological processes. However, to date, there is no evidence for the existence of C20 and C22 compounds of this nature in plants.

Conclusions

Analyses of many different mutants and transgenic plants have demonstrated that significant changes in membrane fatty acid composition, especially desaturation, can be made without obvious effects on plant morphology (Gibson et al., 1994). In contrast, a major change in the fatty acyl chain length of membrane glycerolipids dramatically alters plant morphology. Thus, this study links fatty acid biosynthesis to plant development, with membrane lipids performing structural functions not previously ascribed to them. In addition, this study highlights the potential deleterious effects of VLCFA accumulation in cellular membranes, emphasizing just how tightly regulated the expression of the condensing enzyme, which controls VLCFA biosynthesis (Millar and Kunst, 1997), needs to be.

METHODS

Plant Material

The construction of the 35S–FAE1 plasmid and its transformation into Arabidopsis thaliana have been described previously (Millar and Kunst, 1997). Plants were grown in Terra-lite Redi-earth (W.R. Grace and Co., Canada Ltd., Ajax, Ontario, Canada) at 21°C under continuous fluorescent illumination (100 to 120 \( \mu \text{E m}^{-2} \text{sec}^{-1} \)). For plants that were grown on agar plates and selected with kanamycin, see Katavic et al. (1994).

Fatty Acyl and Wax Analysis

For the determination of the fatty acid compositions, we prepared fatty acid methyl esters according to Browse et al. (1986b) by transmethylation in 1 N methanolic–HCl (Supelco Canada Ltd., Oakville, Ontario, Canada). Fatty acid methyl esters were then extracted in hexane and analyzed by gas-liquid chromatography (Kunst et al., 1992). The conditions used allowed the detection of very-long-chain fatty acids (VLCFAs) of up to C24 in length.

Components of cuticular waxes were determined on stems that had senesced and were completely dehydrated. Stems were dipped in a chloroform–methanol mixture (2:1) for 15 sec, and the extracted components were analyzed as described by McNevin et al. (1993), using an HP5890 gas chromatograph (Hewlett-Packard, Mississauga, Ontario, Canada). Quantitative analysis was based on the addition of a 17:0 internal standard. The peak identities were determined using known reference standards. Wax load was defined as the sum of the mass of C16-30 fatty acids, C24-30 aldehydes, C27-31 alkanes, C22-30 primary alcohols, C29 secondary alcohol, and C29 ketone.

Separation of Lipids

Rosette leaves (5 to 10 g) or seeds were frozen in liquid N\(_2\), and lipids were extracted according to Browse et al. (1986a). Individual lipid classes were separated by thin-layer chromatography on silica gel-coated plates (5250; J. T. Baker Inc., Phillipsburg, Nj.). The plates were first developed in ether–acetic acid (100:1 [v/v]), and neutral lipids were scraped from the plate and eluted with chloroform–methanol–ether (1:1:1 [v/v]). The plate was then developed in chloroform–methanol–acetic acid–water (100:40:20:10 [v/v]) to resolve the polar lipids. Polar lipids that ran together in this solvent system (phosphatidylglycerol [PG] with phosphatidylethanolamine [PE] and phosphatidylcholine [PC] with sulfolipid) were eluted with chloroform–methanol–water (1:2:0.8 [v/v]) and resolved on a separate plate by using chloroform–methanol–water (65:25:4 [v/v]). Finally, neutral lipids were separated on a new plate by using hexane–ether–acetic acid (70:30:1 [v/v]). Lipids were visualized by \( \text{I}_2 \) staining, identified by comparison with the RF values of reference standards, scraped from the plates, and transmethylated with 1 N methanolic–HCl (Supelco Canada Ltd.) at 80°C. The resulting methyl esters were then quantified by gas–liquid chromatography as outlined above.

Transmission Electron Microscopy

Rosette leaves and stems of 6-week-old plants were fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr, followed by a 1-hr incubation in 1% (v/v) osmium tetroxide in the same buffer. The specimens were dehydrated in a graded ethanol series and embedded in Spurr’s epoxy resin. Thin sections were made on RMC MT6000XL or Ultracut E (Reichert-Jung, Vienna, Austria) ultramicrotomes, stained with uranyl acetate and lead citrate, and examined in Zeiss 10A or 10C electron microscopes (Carl Zeiss, Oberkochen, Germany) at accelerating voltages of 60 or 80 kV.

Scanning Electron Microscopy

Stems for scanning electron microscopy were air dried, mounted on stubs, and coated with gold in a Nanotech SEMPrep2 sputter coater (Nanotech, Manchester, UK) before examination in a Cambridge model 250T scanning electron microscope (Leica, Cambridge, UK) at an accelerating voltage of 20 kV.

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