Cuticular Lipid Composition, Surface Structure, and Gene Expression in Arabidopsis Stem Epidermis[1][W]

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All vascular plants are protected from the environment by a cuticle, a lipophilic layer synthesized by epidermal cells and composed of a cutin polymer matrix and waxes. The mechanism by which epidermal cells accumulate and assemble cuticle components in rapidly expanding organs is largely unknown. We have begun to address this question by analyzing the lipid compositional variance, the surface micromorphology, and the transcriptome of epidermal cells in elongating Arabidopsis (*Arabidopsis thaliana*) stems. The rate of cell elongation is maximal near the apical meristem and decreases steeply toward the middle of the stem, where it is 10 times slower. During and after this elongation, the cuticular wax load and composition remain remarkably constant (32 $\mu g/cm^2$), indicating that the biosynthetic flux into waxes is closely matched to surface area expansion. By contrast, the load of polyester monomers per unit surface area decreases more than 2-fold from the upper (8 $\mu g/cm^2$) to the lower (3 $\mu g/cm^2$) portion of the stem, although the compositional variance is minor. To aid identification of proteins involved in the biosynthesis of waxes and cutin, we have isolated epidermal peels from Arabidopsis stems and determined transcript profiles in both rapidly expanding and nonexpanding cells. This transcriptome analysis was validated by the correct classification of known epidermis-specific genes. The 15% transcripts preferentially expressed in the epidermis were enriched in genes encoding proteins predicted to be membrane associated and involved in lipid metabolism. An analysis of the lipid-related subset is presented.

The plant cuticle is a continuous lipophilic layer covering the surface of all epidermal cell types (Esau, 1977) and is one of their distinctive characteristics (Holloway, 1982; Jeffree, 1996; Kunst et al., 2005). It forms a vital hydrophobic barrier over the aerial surfaces of land plants during primary stages of development, limiting nonstomatal water loss and gaseous exchanges, controlling the absorption of lipophilic compounds, providing mechanical strength and viscoelastic properties (Baker et al., 1982; Hoffmann-Benning and Kende, 1994; Riederer and Schreiber, 2001), preventing organ fusion during development (Lolle et al., 1998; Sieber et al., 2000), as well as protecting the plant from nonbiotic and biotic stressors from the environment (Schweizer et al., 1996). The cuticle is composed of cutin (Kolattukudy, 2001), a polymer of fatty acid derivatives, which abuts the cell wall and is embedded in and covered with a mixture of ubiquitous aliphatic compounds (mainly C24-C34 alkanes, alcohols, and ketones) called cuticular waxes (Kunst and Samuels, 2003), as well as minor, extremely diverse, compounds (Jetter, 2000; Jetter et al., 2002; Vermeer et al., 2003). Due to their close physical association, it is difficult to distinguish between the relative contribution of the cutin matrix and cuticular waxes to the physical properties and the biological functions of the cuticle. However, experimentally, the cutin and cuticular waxes are usually considered and analyzed separately, as the waxes are easily extracted in organic solvents while the cutin polymer remains insoluble.

The cutin matrix is composed mainly of C16 and C18 hydroxy and epoxy fatty acid monomers. Minor amounts of aromatic compounds are also present (Kolattukudy, 1980; Nawrath, 2002; Heredia, 2003). It has long been believed that cutin is formed by interesterification of fatty acids only, but it is now clear that glycerol is present and also esterified to the fatty acid monomers (Graça et al., 2002). The size and the exact structure of the polymer (dendrimeric, cross-linked, etc.) are still a matter of uncertainty. In Arabidopsis

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(*Arabidopsis thaliana*) epidermis, the major monomers released by polyester depolymerization of delipidated residues are not ω-hydroxy fatty acids, but dicarboxylic acids (Bonaventure et al., 2004). Thus it is not clear whether the rigid classification of cutin as highly enriched in ω-hydroxy fatty acids needs to be modified or whether the dicarboxylic acids represent a novel polyester domain.

Knowledge on the biosynthesis of cutin is based largely on a few early studies, mostly done with broad bean (*Vicia faba*) leaves. These studies indicated that cutin biosynthesis involves ω-hydroxylation followed by midchain hydroxylation and incorporation into the polymer (Kolattukudy and Walton, 1972), and that hydroxy fatty acids can be incorporated into cutin by a reaction requiring CoA and ATP (Croteau and Kolattukudy, 1974). Genetic approaches to cutin biosynthesis are relatively undeveloped. In the Arabidopsis *fatb-ko* line, where a disruption in an acyl-carrier protein (ACP) thioesterase gene results in reduced general availability of cytosolic palmitate (Bonaventure et al., 2003), there is an 80% loss of C16 monomers and a compensatory increase in C18 monomers in epidermal polyesters (Bonaventure et al., 2004). The first mutant demonstrated by chemical analysis to be specifically affected in cutin metabolism has been reported only very recently (Xiao et al., 2004). The ethyl methanesulfonate-induced mutant *att1* shows enhanced disease severity to a virulent strain of *Pseudomonas syringae*, a 70% decrease in cutin amount, a loose cuticle ultrastructure, and increased permeability to water vapor. *ATT1* (At4g00360) encodes a P450 monooxygenase belonging to the CYP86A family (Xiao et al., 2004) and has been demonstrated to catalyze the ω-hydroxylation of fatty acids (Duan and Schuler, 2005). ATT1 is thus probably responsible for the synthesis of ω-hydroxy fatty acid and/or dicarboxylic acid monomers found in Arabidopsis epidermal polyesters. In maize (*Zea mays*) leaves, a peroxygenase has been shown to be involved in the synthesis of C18 epoxy monomers of cutin (Lequeu et al., 2003). This enzyme, associated with a cytochrome P450 monooxygenase (Pinot et al., 1999) and a membrane-bound epoxide hydrolase, can catalyze in vitro the formation of these monomers (Blée and Schuber, 1993). The characterization of the Arabidopsis *lacs2* mutant indicates that LACS2, an epidermis-specific long-chain acyl-CoA synthetase, is involved in leaf cuticle formation and barrier function (Schnurr et al., 2004) and suggests the likely involvement of specific acyl-CoA pools in cutin biosynthesis. Whether the polymerization reactions of the cutin monomers occur inside or outside the epidermal cells, which enzymes are involved, and how cutin synthesis is coordinated with the deposition of waxes is still completely unknown and a major challenge in the study of plant epidermis.

Cuticular waxes of the Arabidopsis shoot are found both above the cutin matrix (epicuticular) as well as embedded in the cutin (intracuticular). The chemical compositions of the Arabidopsis stem and leaf waxes have been investigated in both wild-type and eceriferum (glossy) plant lines (Hannoufa et al., 1993; Jenks et al., 1995; Rashotte et al., 2001, 2004). The predominant wax components of Arabidopsis stems are C29 alkane, ketone, and secondary alcohols, together with smaller quantities of C28 primary alcohol and C30 aldehyde, while leaves have C31 alkane but lack the abundant ketones and secondary alcohols of the stem (for review, see Jenks et al., 2001). Stems also have much higher wax loads than leaves (more than 30 times higher in Arabidopsis ecotype Columbia [Col]-0). These saturated aliphatic wax components are synthesized as C16-C18 fatty acids in the plastid, followed by fatty acid elongation associated with the endoplasmic reticulum to form very-long-chain fatty acids (VLCFA; Post-Beittenmiller, 1996; Kunst and Samuels, 2003). The function of one of the condensing enzymes of the elongase complex was demonstrated by the *cer6* knockdown, which has a highly glossy stem phenotype and 7% of wild-type wax (Millar et al., 1999). Subsequent modifications occur to the VLCFAs, but the mechanisms and gene products involved are less well characterized. The mechanism of transport of waxes onto the epidermal surface is better understood as a result of the recent discovery of CER5, an ATP-binding cassette (ABC) transporter involved in the export of cuticular waxes to the stem surface (Pighin et al., 2004).

The cuticle is known to be synthesized at very early stages of embryo and organ development (Szczuka and Szczuka, 2003). In rapidly growing internodes of deepwater rice (*Oryza sativa*), it has been observed that the cuticle thickness remains constant (Hoffmann-Benning and Kende, 1994). However, whether the secretions of its cutin and wax components occur at the same time and whether cuticles have a constant composition during the dynamic process of rapid expansion of young organs has not been documented, although it is clearly an important question regarding the mechanism of cuticle formation. To investigate the timing of wax and cutin deposition, we have performed structural studies and lipid analyses on the epidermis of different parts of rapidly elongating Arabidopsis stems. In addition, to gain insights into the identity of the enzyme families and isoforms involved in the deposition of wax and cutin, we have undertaken a gene expression study of the epidermal cells of the stem, which is also the first genome-wide study of the transcriptome of the aerial plant epidermis. We report here on the results of these combined approaches and discuss the expression of a subset of genes involved in lipid metabolism in the light of the surface structure and the lipid composition of the stem cuticle.

RESULTS

The Length of Epidermal Cells Increases More Than 80-Fold during Stem Elongation

The bolting inflorescence stem of Arabidopsis provides an opportunity to study the biosynthesis of

cuticular lipids by the epidermis during cell expansion. When growth was measured over a 24-h period for 9- to 11-cm-long bolting stems, the total length of the stems increased by 3.7 cm ± 0.3 (mean ± SE, $n = 6$). The elongation of apical, but not basal, 0.5-cm segments was observed (Fig. 1). Most of the increase in total length (about 85%) occurred in the top 3-cm segment of the stem, 15% in the middle part, while no increase was detected below 7 cm from the top. The elongation rate was greater nearer to the apex and maximal in the first 0.5-cm portion. Given typical mean diameters of the stem segments, this elongation corresponds to an increase in epidermis surface area of 60 mm^2/24 h in the top 3 cm of the stem and 8 mm^2/24 h in the middle 3-cm segment.

Growth of epidermal cells was followed with cryo-scanning electron microscopy (SEM) and confocal scanning laser microscopy with propidium iodide staining of the cell wall. Near the shoot apex (Fig. 1A), the cells of the epidermis were nearly isodiametric, but they soon underwent anisotropic growth to elongate greatly in the axial dimension (Fig. 1B). At the base of a 10-cm stem, the cells were elongated and trichomes were seen (Figs. 1C and 2C). No trichomes were present in the middle and top segments (Fig. 2, A and B). When the cell dimensions were quantified using cryo-SEM, the length of the cells increased from 6 μm ± 0.3 (mean ± SE, $n = 24$) to 532 ± 44 μm (mean ± SE, $n = 14$), an increase of 87-fold. In contrast, cell widths did not increase significantly.

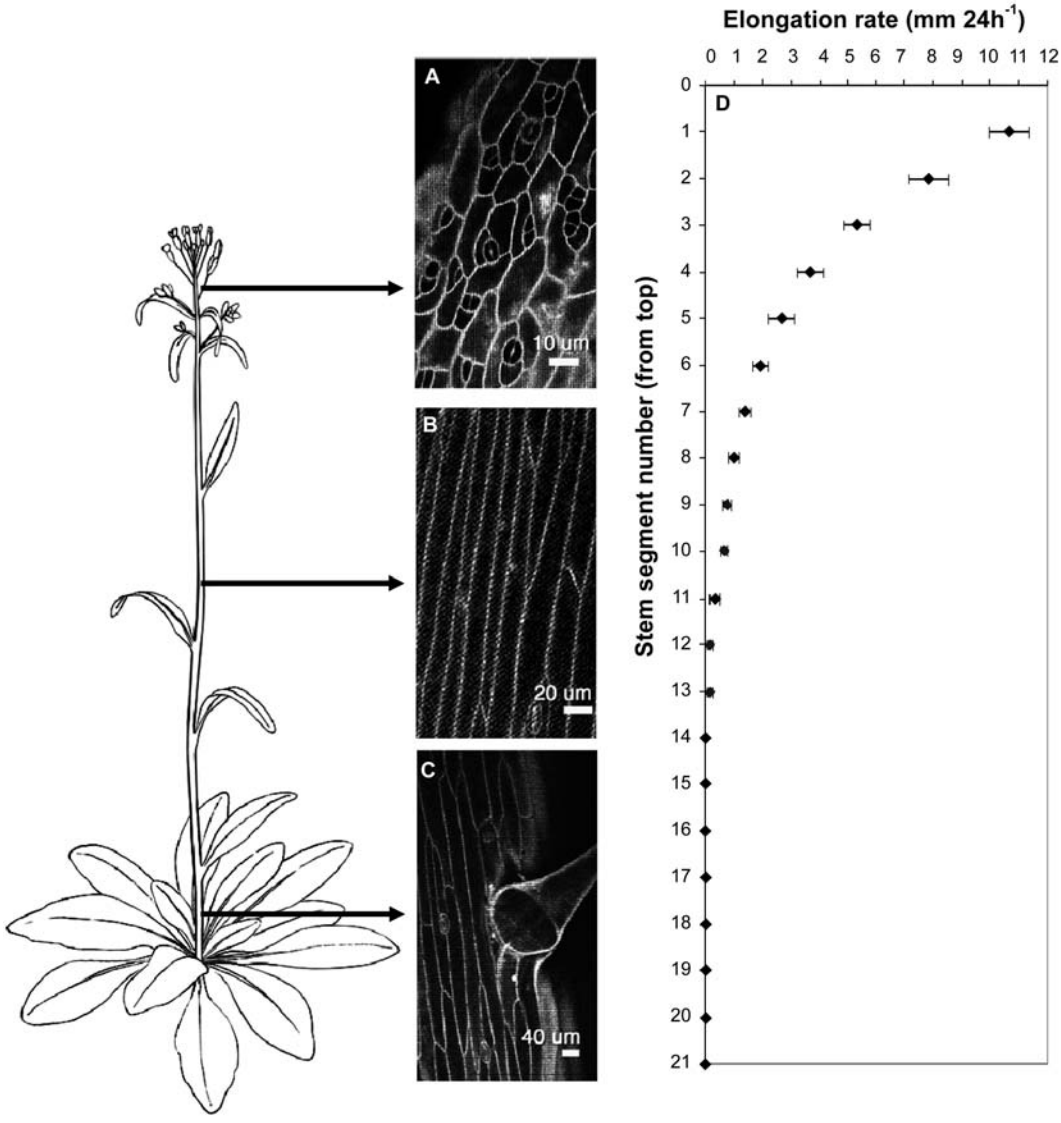

Figure 1. Size of Arabidopsis stem epidermal cells and stem elongation within 0.5-cm-long segments. A to C, Confocal scanning laser microscopy with propidium iodide staining of the cell wall of regions near the shoot apex (A), in the middle of the stem (B), and at the bottom of the stem (C). D, Thin marks were made with black ink along 9- to 11-cm stems, starting at the apex and defining 0.5-cm initial segments. The position of each mark relative to the base of the stem was measured after 24 h and elongation of each segment calculated. Values are means ± SE ($n = 6$).

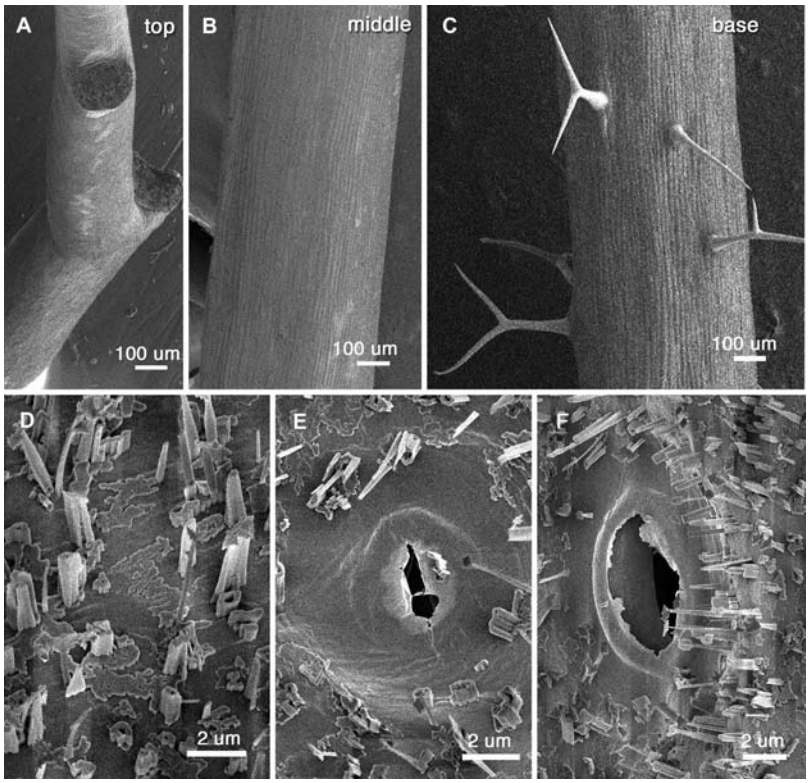

Figure 2. Cryo-SEM of developing inflorescence stem of Arabidopsis (Col-0). A to C, Low magnification view of top, within 1 cm of the apex (A), middle, between 4 to 5 cm from the apex (B), and base, between 8 to 9 cm (C). D to F, Higher magnification view of epicuticular crystals on the surface of the top (D), middle (E), and base (F).

The Load and Composition of Waxes Remain Constant along the Stem

To test whether the biosynthesis of cuticular waxes is coordinated with the rapid expansion of the stem epidermal cells, cryo-SEM and gas chromatography (GC) with mass spectroscopy (MS) and flame ionization detection (FID) were used. Cryo-SEM provided information about the epicuticular wax crystals, while the GC-FID and GC-MS provided quantitative wax load and composition information. Epicuticular wax crystals were found over the entire stem surface, both vertical rods, tubes, longitudinal bundles of rodlets, and horizontal, reticulate plates (Fig. 2, D–F). In the region of most rapid elongation, the cuticle showed striations and crystals distorted along the axis of elongation. The cuticle around stomata did not have epicuticular crystals, only a smooth epicuticular film (Fig. 2, E and F).

Qualitative and quantitative chemical analyses were performed to compare the cuticular wax in the top, middle, and basal 3-cm segments of the bolting inflorescence stem. The wax load, expressed on a per-unit-area basis, was constant between these segments of the stem (Fig. 3). The composition along the stem did not vary significantly (Fig. 4a), with C29 alkane, ketone, and secondary alcohols predominating, and the overall composition is similar to that reported previously (Hannoufa et al., 1993; Jenks et al., 1995; Rashotte et al., 2001). In addition, primary alcohols of chain lengths C26-C30 and alkyl esters were observed in all segments of the stem at very similar percentages. To increase the spatiotemporal resolution of our analysis and gain insights into the timing of wax deposition in the region of the stem elongating most rapidly, the top 3-cm segment of the stem was further divided into 1-cm portions and the waxes analyzed. No significant differences were found in the wax load or wax composition between the three portions of the top segment (data not shown).

Polyester Composition Is Constant along the Stem, But the Load Decreases at the Base

To quantify the polyester monomers in the stem, the method bypassing cuticle isolation we have reported

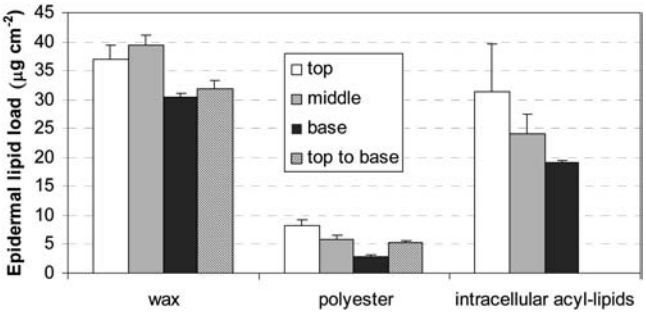

Figure 3. Epidermal loads of wax, polyester, and other acyl lipids along the stem. Fatty acid derivatives were analyzed using sections of whole stems for wax and polyesters and epidermal peels for other acyl lipids. The loads are expressed in micrograms of fatty acid derivatives per square centimeter of epidermis. Difference in polyester load between the middle (or top) and the base of stems was significant (Student's *t* test, $P < 0.05$). Other differences were not significant. Values are means ± SE ($n = 5$).

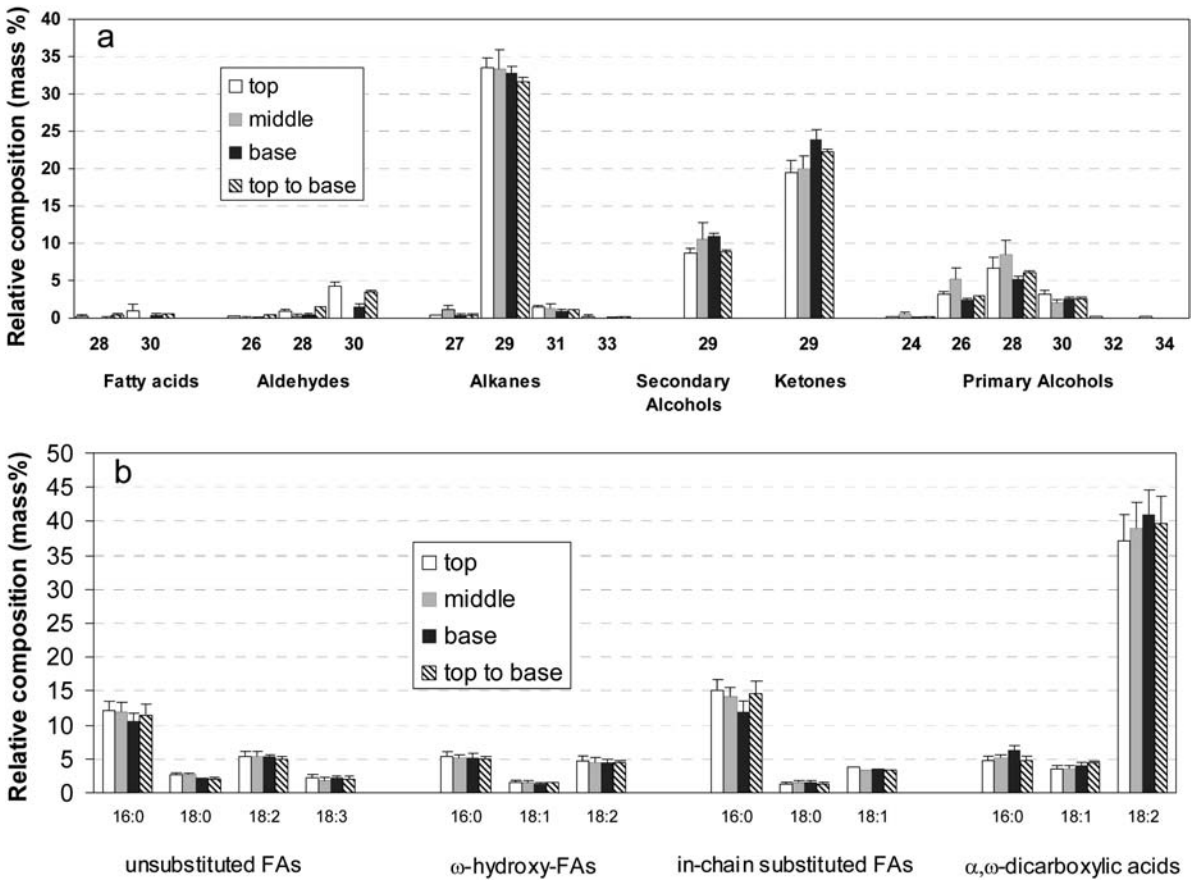

Figure 4. Relative composition of fatty acid derivatives in waxes and polyesters along the stem. Waxes (a) and polyesters (b) were analyzed using sections of whole stems. Values are means ± SE ($n = 5$).

earlier (Bonaventure et al., 2004) was used with minor modifications. Because our previous report has demonstrated that stem polyester monomers were located exclusively in the epidermis, analyses were done on whole-stem segments. The aliphatic monomer polyester composition in the 9- to 11-cm elongating stems was largely unsaturated aliphatic dicarboxylates and similar to the composition reported earlier for 20-cm stems of the same ecotype (Bonaventure et al., 2004; Fig. 4b). The variations in monomer composition observed in the three stem segments were minor. By contrast, the total load in aliphatic polyester monomers per surface area unit decreased substantially (about 2-fold) and significantly between the middle and lower portions of the stems, but not significantly between the upper and the middle portions (Fig. 3). Neither the load nor the composition of stem polyesters was significantly changed in the three stem segments by increasing the depolymerization time from 48 to 72 h (data not shown). This decrease in the amount of polyester monomers at the base of the stem could indicate a change in structure giving rise to a polymer less or not susceptible to depolymerization (e.g. cutan). A more refined analysis of the polyesters in portions of the apical segment as performed for waxes was hampered by the low amount of polyesters. Such an analysis may require the use of a plant species with larger stems.

In Stem Epidermis There Is a Considerable Flux of Lipids Exported onto the Surface

We also determined the fatty acid content of the intracellular acyl lipids (membrane and storage lipids) of epidermal peels. When compared to the amounts of fatty acid derivatives measured in waxes and polyesters, the intracellular lipids represented less than one-half of the total cell lipids (Fig. 3). The fatty acid composition of the stem epidermis was found to be similar to that of the whole stem (Supplemental Fig. 1). In all stem segments, the ratio of surface lipids to epidermal intracellular acyl lipids was about 3:2 in mass (Fig. 3). This means that the epidermal cells of the elongating stem top need to have machinery capable of exporting onto the surface more than one-half of the approximately 75 μg of fatty acids produced per square centimeter of epidermis. Given an average rate of expansion of 0.6 $cm^2/24$ h for the stem top, the accumulation of total fatty acids in the corresponding epidermis can be estimated to be around 1.9 $\mu g\ cm^{-2}\ h^{-1}$ and the net rate of export 1.1 $\mu g\ cm^{-2}\ h^{-1}$ (assuming that fatty acid accumulation commences when

cell elongation starts). This represents a considerable flux of hydrophobic compounds, possibly partially polymerized, that must go through the plasma membrane and the aqueous cell wall. Since this specialized machinery of synthesis and export of surface lipids is specific to the epidermis, it is reasonable to assume that most of the genes encoding the enzymes, transporters, and other proteins that are part of the machinery will be transcriptionally up-regulated in the epidermis compared to the other tissues of the stem. Expression of these genes could be stronger in the elongating top epidermis or restricted to it. The above rationale provided the basis for the microarray analysis described below.

Genome-Wide Microarray Analysis Identified about 1,900 Genes Up-Regulated in the Stem Epidermis

To investigate the mRNA levels of genes expressed in the epidermis of the apical and basal segments of the stems, we used the GeneChip Arabidopsis ATH1 Genome Array (Affymetrix) that represents 22,748 probe sets covering approximately 23,750 Arabidopsis genes (Redman et al., 2004). Epidermis manually peeled from stem segments (Fig. 5) was used as the source of RNAs. The small amounts of chlorophyll detected in the epidermis peels (on average 0.05 mg chlorophyll/g fresh weight versus 0.6 mg chlorophyll/g fresh weight in total stems), indicated that contamination by nonepidermal tissues was minor. In fact, the green pigmentation in the peels may arise largely from epidermal plastids, which in fluorescence microscopy show a low, but detectable, amount of red autofluorescence usually attributed to chlorophyll in chloroplasts (data not shown). Enrichment of epidermal mRNAs in the epidermal peels was confirmed by the epidermis-to-stem gene expression ratios measured for genes of known epidermal, extraepidermal, or ubiquitous expression (Table I). In all cases, the genes known to have epidermal expression by techniques other than microarrays were found to have a top epidermis-to-top stem mean gene expression ratio of 2.5 or above, while housekeeping genes had ratios between 1.6 and 0.7 and extraepidermal-expressed genes showed ratios of 1 or below. A global analysis of transcripts found to be up-regulated in the stem epidermis compared to the whole stem is presented below and in the supplemental data, with a particular emphasis on genes known or suspected of being involved in lipid metabolism.

Figure 5. Peeling of epidermis from Arabidopsis stems. A, Transverse section of stems viewed under a light microscope after staining with phloroglucinol. The epidermis is the transparent outermost single cell layer. B, Using manual dissection, epidermis can be isolated from the stem as a transparent film.

Transcripts from a total of about 13,000 genes were detected in the stem segments (Supplemental Table I), which is very close to the value of 60% of expressed genes in the stem reported recently (Ma et al., 2005). The Affymetrix Microarray Suite 5.0 (MAS 5.0) software identified more than 3,000 genes whose expression was increased in the epidermis compared to the whole stem (data not shown). Using some statistical parameters calculated by MAS 5.0 and a conservative cutoff value of 2.0 for the lower estimate of the epidermis-to-stem gene expression ratio (see ''Materials and Methods''), we identified about 1,900 genes up-regulated in the stem epidermis. This list breaks down into three subsets of about 600 each (Supplemental Table I): up-regulated in the epidermis of stem segments (top and base), in top segments alone (elongating), and in basal segments alone (nonelongating). These three subsets of transcripts (see Supplemental Table III for the identity) are likely to be enriched in genes related to various aspects of epidermal biology, such as trichome formation for the basal-only subset or cell elongation for the apical-only subset.

A list of the 40 most up-regulated, highly expressed transcripts in the apical-only subset is given in Table II as an example. Among these, several encode proteins belonging to expected categories, such as enzymes of cuticle biosynthesis or defense against pathogens. Interestingly, almost one-third of the proteins have putative regulatory functions, such as the seven putative protein kinases, a group highly represented in the epidermis (Fig. 6). This list is thus likely to yield good candidates for the cellular signaling pathways involved in the division, differentiation, or elongation of apical epidermal cells. Another important group representing about 40% of the total in Table II is proteins from families completely uncharacterized or whose function is so far unclear. The identification of these proteins as epidermis up-regulated should provide helpful clues in the determination of their function in the cell. Finally, the list contains a few proteins related to cell wall metabolism that could indicate the existence in the epidermis of a specific composition or structure of the external cell wall to which the cuticle may be anchored and through which surface lipids must be transported during cuticle formation.

The Epidermis Up-Regulated Set of Genes Is Enriched in Candidates Encoding Proteins Predicted to Be Membrane Associated, Extracellular, or Related to Stress/Stimulus or Lipid Metabolism

The subcellular location of the synthesis of waxes and cutin monomers and the mechanism of secretion are mostly unknown. However, due to the

Table I. *Genes with known preferential expression in epidermis or in vascular tissues were correctly identified by the microarray experiments using epidermal peels from stems*

List of genes whose preferential or specific site of expression is epidermis (top of the list) or vascular tissues (bottom) as shown by techniques other than microarrays. The organ investigated and the reference is given. For known housekeeping genes (middle), only a few randomly selected examples are given. Molecular function of the proteins encoded by the genes: *ACR4*, putative receptor kinase; *ATML1* and *PDF2*, homeodomain protein; *AtPIN1*, putative auxin efflux carrier protein; *AtPPT1* (=*cue1*), phosphoenolpyruvate/phosphate translocator; CER2, unknown protein; CER5, ABC transporter; *CBP20* and *CBP80*, nuclear cap binding protein; *CER6* (=*CUT1*) and *FDH (FIDDLEHEAD)*, β-ketoacyl-CoA synthase; *LACERATA*, fatty acid ω-hydroxylase; *LACS2*, long-chain acyl-CoA synthetase; *L23a*, 60S ribosomal protein; *PPX1* and *PPX2*, protein phosphatase; *UBQ1*, ubiquitin extension protein; *XCP1* and *XCP2*, cysteine peptidase; *WAX2* (=*YORE-YORE*), unknown.

Gene Locus	Gene Name	Expression Ratio[a]		Preferential Expression	Reference
		Top	Base		
				Epidermal	
At3g59420	*ACR4*	24.8	19.1	Epidermis (all organs)	Tanaka et al. (2002)
At4g21750	*ATML1*	6.2	5.5	L1 layer of shoot apex	Sessions et al. (1999)
At4g04890	*PDF2*	6.0	5.9	L1 layer of shoot apex	Abe et al. (2003)
At4g24510	*CER2*	4.1	6.0	Epidermis	Xia et al. (1997)
At2g26250	*FDH*	4.0	3.9	Epidermis	Yephremov et al. (1999); Pruitt et al. (2000)
At1g51500	*CER5*	3.5	3.1	Epidermis (stem)	Pighin et al. (2004)
At1g68530	*CER6*	3.1	3.1	Shoot epidermis	Millar et al. (1999)
At2g45970	*LACERATA*	3.1	2.2	Epidermis-specific	Wellesen et al. (2001)
At5g57800	*WAX2*	2.6	2.2	L1 layer of shoot apex	Chen et al. (2003); Kurata et al. (2003)
At1g49430	*LACS2*	2.5	1.2	Epidermis (leaf)	Schnurr et al. (2004)
				Ubiquitous	
At5g55260	*PPX2*	1.6	1.6	All tissues	Pujol et al. (2000)
At3g52590	*UBQ1*	1.1	1.6	Most tissues	Holtorf et al. (1995)
At4g26720	*PPX1*	1.0	1.2	All tissues	Pujol et al. (2000)
At3g18780	*Actin 2*	0.9	0.7	Vegetative tissues	An et al. (1996)
At2g13540	*CBP80*	0.8	0.8	Most tissues	Kmieciak et al. (2002)
At5g44200	*CBP20*	0.7	1.0	Most tissues	Kmieciak et al. (2002)
At1g49240	*Actin 8*	0.6	0.7	Vegetative tissues	An et al. (1996)
At3g55280	*L23a*	0.6	1.4	Most tissues	Volkov et al. (2003)
				Vascular	
At5g33320	*AtPPT1*	0.4	1.0	Stem vasculature	Knappe et al. (2003)
At1g73590	*AtPIN1*	0.2	0.2	Xylem and cambium	Gälweiler et al. (1998)
At4g35350	*XCP1*	0.1	0.5	Xylem	Funk et al. (2002)
At1g20850	*XCP2*	0.1	0.3	Xylem	Zhao et al. (2000)

[a]Mean ratio calculated from four epidermis-to-stem gene expression ratios, i.e. ratio of transcripts in epidermis versus transcripts in stems, determined by microarray experiments (Affymetrix ATH1 GeneChip) using the epidermis of a stem section (top or base) and the whole-stem section (top or base) as a reference.

hydrophobicity of these molecules, it is likely to involve membrane-associated enzymes and (at least in the case of waxes) transporters like CER5 (Pighin et al., 2004). Also, the mechanism by which waxes and cutin monomers, oligomers, or polymers reach their final destination through the cell wall is likely to involve extracellular proteins. Assembly of cutin monomers or oligomers might even take place outside the cell. For all these reasons, the epidermis up-regulated genes encoding proteins predicted to be (1) membrane associated, (2) secreted (especially extracellular), (3) related to transport, and/or (4) related to lipid metabolism are groups likely to contain strong candidates for the metabolism of surface lipids. A clear enrichment in the genes of three of four of these categories was in fact observed in the set up-regulated in the epidermis of the elongating top part of the stem, which is consistent with the lipid secretory function of epidermal cells. The enrichments observed in proteins predicted to be membrane associated, extracellular, secreted, and lipid related were between 1.5- and 3-fold (Fig. 6). A clear enrichment (1.5- to 2-fold) in proteins likely to play a role in the interaction between the plant epidermis and the environment, such as proteins involved in response to abiotic/biotic stimulus and response to stress, was also observed (Fig. 6).

A Subset of 85 Genes Related to Lipid Metabolism and Up-Regulated in the Epidermis Provided Strong Candidates for Wax and Cutin Synthesis

Among the 620 genes known or thought to be involved in acyl lipid metabolism in Arabidopsis (Beisson et al., 2003), a subset of about 85 was found to be up-regulated in the epidermis of the top stem (Table III). Among this subset are several genes previously identified in forward-genetics screens for mutants with altered wax (*WAX2*, *CER5*, *CER6*, etc.) as well as genes reported to be involved in cutin biosynthesis (*ATT1*, *LACS2*, *FATB*). This concordance of the microarray data with numerous results of forward genetics clearly validates this epidermal microarray approach as suitable

Table II. *List of the 40 most up-regulated highly expressed genes in top stem epidermis classified by functional category*

Putative Functional Category and Locus	Description of Encoded Protein	Expression Ratio[a] (Top)	Expression Ratio[a] (Base)
Lipid Metabolism			
At1g07720	β-Ketoacyl-CoA synthase family	9.9	2.7
At1g64400	Long-chain-acyl-CoA synthetase (LACS3)	9.4	1.7
At2g39400	Similar to lipase	13.4	4.8
Transport			
At5g40780	Putative Lys and His transporter	16.9	3.3
At3g55130	ABC transporter family	12.3	3.1
At5g55930	Oligopeptide transporter OPT family protein	8.2	9.0
Cell Wall Metabolism			
At4g30280	Putative xyloglucan:xyloglucosyl transferase	33.7	27.9
At4g02330	Similar to pectinesterase	29.7	211
At3g10720	Similar to pectinesterase	8.8	57.4
Disease Resistance			
At1g78860	Lectin family protein (mannose-binding)	33.8	19
At2g33050	Similar to disease resistance protein Hcr2	10.2	4.4
At1g75040	Pathogenesis-related protein 5 (PR-5)	10.0	1.0
Signal Transduction			
At1g21270	Wall-associated kinase 2	18.2	2.9
At5g20050	Protein kinase family protein	10.0	5.9
At1g11350	Ser-Thr kinase related	9.9	7.1
At1g21250	Wall-associated kinase 1	9.9	1.1
At2g13790	Leu-rich-repeat protein kinase family	9.3	3.4
At1g51805	Leu-rich-repeat protein kinase family	8.6	3.4
At4g31000	Calmodulin-binding protein	8.6	5.1
At4g23180	Ser-Thr kinase related	8.3	5.5
At2g01890	Putative purple acid phosphatase	8.3	1.8
At4g38550	Phospholipase-like protein	7.9	3.4
Transcription			
At4g31800	WRKY family transcription factor	11	3.8
At2g38470	WRKY family transcription factor	8.9	4.2
At2g02450	No apical meristem (NAM) family protein	8.2	2.8
Unknown			
At1g22890	Expressed protein	31.9	14.9
At1g78460	Expressed protein	25.2	9.2
At2g28570	Expressed protein	20.6	3.2
At3g28290	Integrin-related protein 14a	18.9	91.1
At5g20230	Plastocyanin-like domain-containing protein	14.9	9.1
At3g26200	Cytochrome P450-like	13.6	10.1
At2g05540	Gly-rich protein	11.2	80.2
At1g56150	Auxin-responsive family protein	10.5	6.1
At3g11600	Expressed protein	9.5	7.8
At5g05440	Expressed protein	9.2	2.3
At2g20950	Expressed protein	8.8	3.3
At4g36500	Expressed protein	7.9	3.4
At1g33600	Leu-rich-repeat family protein	7.9	8.4
At1g29430	Auxin-responsive family protein	7.8	9.6
At5g44020	Similar to soybean stem glycoprotein	7.4	36.2

[a]Mean ratio of epidermis-to-stem gene expression. Gene expressions were determined as in Table I. Among genes up-regulated in top stem epidermis, only those showing a signal intensity >1,000 in top epidermis are shown. Descriptions of proteins are from The Institute for Genomic Research, Munich Information Center for Protein Sequences, or Swiss-Prot.

for identification of additional gene candidates likely to be involved in wax and cutin biosynthesis.

For example, the new candidates for wax synthesis include specific members of multigenic families for which some members have already been previously shown to be involved in wax synthesis, e.g. the ketoacyl-CoA synthase (KCS) family. Figure 7 summarizes the level of gene expression and the epidermis-to-stem gene expression ratios found for the 20 members of the KCS family (out of 21) present on the ATH1 array. It can be clearly seen that there are five uncharacterized KCS genes showing an epidermal up-regulation higher

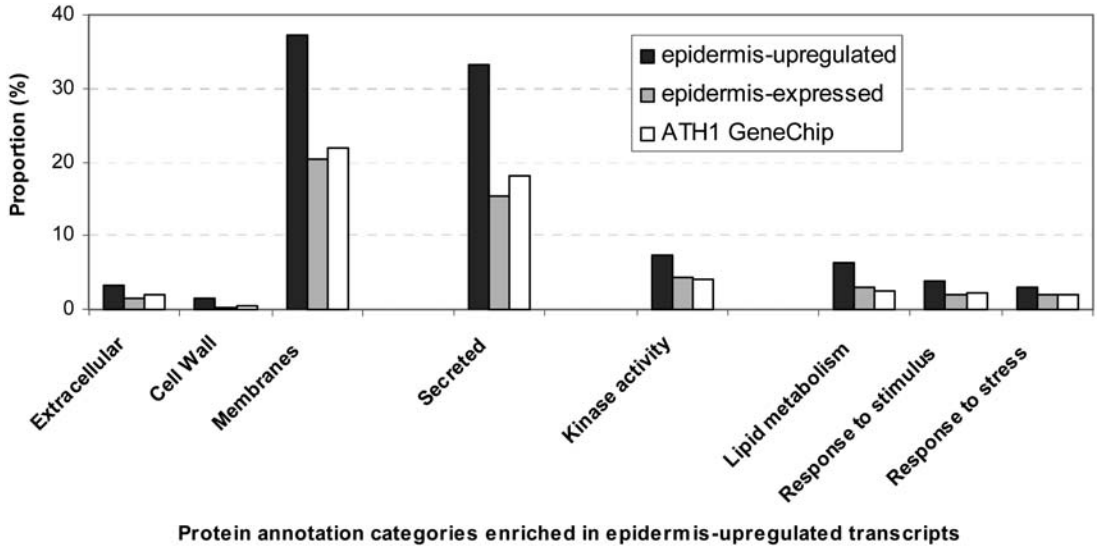

Figure 6. Transcripts up-regulated in stem epidermis are enriched in sequences encoding proteins from several functional categories. For each of three sets of transcript sequences (epidermis expressed, epidermis up-regulated, and present on the ATH1 GeneChip), the figure presents the percent of corresponding predicted proteins that are assigned to a selected functional category. Percentages refer to a functional category within each of four types of annotation. Functional categories extracellular, cell wall, and membranes refer to the type of annotation: cellular components from Gene Ontology (GO); secreted, targeting signals predicted by targetP software (http://www.cbs.dtu.dk/services/TargetP); kinase activity, molecular functions from GO; responses to stress/stimulus and lipid metabolism, biological processes from GO to which lipid metabolism was added. Functional categorization was done using annotations from The Arabidopsis Information Resource (http://www.arabidopsis.org) and the Arabidopsis Lipid Gene Database (Beisson et al., 2003). Expressed genes, called present in epidermis of stem tops and stem bottoms by Affymetrix MAS 5.0 software; upregulated genes, show an epidermis-to-stem gene expression ratio of $\geq$2.0 in stem tops and stem bases (90% confidence level). The complete charts showing all functional categories for each type of annotation are available from Supplemental Figure 2.

than the CER60 gene, which is known to affect wax synthesis. These genes are thus good candidates for the putative specific elongases thought to be responsible for each of the multiple elongation steps of wax synthesis. In addition, and as expected, the KCS FAE1 isoform (At4g34520) that is responsible for the elongation of fatty acids for storage lipids in seeds is not among the epidermis up-regulated KCS genes.

DISCUSSION

Using rapidly elongating Arabidopsis stems, we have performed measurements of the elongation of epidermal cells in conjunction with quantitative analyses of the cuticular lipids. In brief, the results indicate that polyester and waxes are deposited in the elongating apical part of the stem at a rate that allows the cuticle to keep a constant load and composition of surface lipids, despite the fast rate of elongation of the epidermal cells. A major metabolic function of these epidermal cells is extracellular lipid synthesis as illustrated by the fact that more lipid is transported out of the cell than remains in membrane glycerolipids (Fig. 3) and by the enriched expression of transcripts for many enzymes of lipid metabolism (Fig. 6). These isoforms preferentially expressed in the epidermis are thus strong candidates for roles in wax and cutin synthesis (Table III).

Deposition of Waxes

The wax analysis shows that the amounts of individual wax constituent do not vary significantly along the stem (Figs. 2–4) and within the apical stem segment (data not shown). The wax load on the total stem, determined in control experiments, confirms the findings for stem segments. These results for total stem wax match previous reports on the Col ecotype (Pighin et al., 2004), and are dramatically higher than the wax load of 0.75 $\mu g/cm^2$ on leaves (data not shown). The distribution of waxes on the surface of various stem segments is not likely to be influenced by diffusional transport within the cuticle because, clearly, the polyester components are not mobile and diffusion coefficients measured for fatty acids and alkanes in reconstituted cuticular waxes (Schreiber et al., 1996) imply that lateral wax diffusion will be a very slow process, less than 0.01 mm/d.

Because surface area expansion is greatest and unequal in the top segment (Fig. 1), the constant wax load and composition found both along the stem and within the top segment implies that the rate of net deposition of wax is strictly synchronized with epidermal cell expansion and largely limited to the top zone of the stem, i.e. early on during development. All wax constituents are therefore formed at similar rates and deposited at the same time in the expanding epidermal cells. Expression of all proteins involved

Table III. *Genes known or suspected of being involved in acyl lipid metabolism that are up-regulated in the epidermis of top stems*

Putative or Known Function of Protein	Locus Code	Ratio[a] (Top)	Ratio[a] (Base)	Signal[b] (Top)	Signal[b] (Base)
Acyl-ACP thioesterase FatB	At1g08510	2.4	1.4	9,644	6,072
Stearoyl-ACP desaturase	At5g16240	5.5	2.1	810	853
Acyl-CoA desaturase-like	At1g06350	3.1	41.3	11,859	1,360
Dihydroxyacetone phosphate reductase	At2g41540	5.1	11.3	3,889	787
Plastidial phosphatidic acid phosphatase	At2g01180	2.4	6.4	1,198	2,054
Choline kinase	At1g71697	4.2	2.9	770	1,011
Fatty acid alcohol oxidase	At3g23410	2.8	2.2	718	1,260
Monoacylglycerol lipase	At2g39400	13.4	4.8	4,426	5,573
Monoacylglycerol lipase	At2g47630	6	3.8	282	37
Monoacylglycerol lipase	At5g11650	4.1	1	535	934
Lipid acylhydrolase-like	At5g14930	2.7	4.2	236	337
Cytosolic homomeric acyl-CoA carboxylase	At1g36160	4.2	2.3	6,025	2,174
Ketoacyl-CoA synthase	At1g07720	9.9	2.7	2,866	2,121
Ketoacyl-CoA synthase	At5g04530	8.7	41.3	254	62
Ketoacyl-CoA synthase	At1g04220	8.1	3.6	6,599	1,867
Ketoacyl-CoA synthase	At2g15090	6.6	2.2	296	740
Ketoacyl-CoA synthase (CER60)	At1g25450	5.8	10.9	1,527	699
Ketoacyl-CoA synthase	At2g28630	5.5	7.4	3,857	2,420
Ketoacyl-CoA synthase	At5g43760	4.8	4.6	9,032	4,370
Ketoacyl-CoA synthase (KCS1)	At1g01120	4.7	4.2	21,884	11,052
Ketoacyl-CoA synthase (FIDDLEHEAD)	At2g26250	4	4	9,547	3,618
Ketoacyl-CoA synthase (CUT1=CER6)	At1g68530	3.1	2.8	19,397	13,537
Ketoacyl-CoA synthase	At2g16280	3.1	3.6	9,337	4,767
Ketoacyl-CoA reductase	At1g24470	5.6	3.5	1,219	854
Ketoacyl-CoA reductase	At1g67730	2.7	2.1	13,441	4,209
Fatty acyl-CoA reductase	At3g56700	31.8	2.3	491	4,471
Fatty acyl-CoA reductase (CER4)	At4g33790	2.6	4.7	14,665	5,669
WAX2 protein (=YORE-YORE)	At5g57800	2.7	2.2	25,019	19,380
CER1 protein	At1g02205	2.2	2.2	30,389	18,784
CER2 protein	At4g24510	4.1	6	8,305	2,540
Putative oxidoreductase (HOTHEAD)	At1g72970	4.1	4.3	1,888	296
Fatty acid ω-hydroxylase (CYP86A2=ATT1)	At4g00360	5.4	3.5	8,455	5,994
Fatty acid ω-hydroxylase (CYP86A8=LACERATA)	At2g45970	3.1	2.2	529	175
Fatty acid ω-hydroxylase (CYP86A7)	At1g63710	2.7	6.5	794	48
Fatty acid ω-hydroxylase (CYP86A4)	At1g01600	2.5	18.1	1,979	195
Fatty acid ω-hydroxylase (CYP94B3)	At3g48520	3.4	6.8	246	1,484
Acyl-activating enzyme (AAE16)	At3g23790	2.9	1.6	380	345
Long-chain acyl-CoA synthetase (LACS3)	At1g64400	9.5	1.7	5,753	3,511
Long-chain acyl-CoA synthetase (LACS1)	At2g47240	4	8.6	8,888	2,647
Long-chain acyl-CoA synthetase (LACS2)	At1g49430	2.5	1.2	2,521	398
Glycerol-3-P acyltransferase (GPAT8)	At4g00400	4	9.5	4,352	1,494
Glycerol-3-P acyltransferase (GPAT4)	At1g01610	3.5	12.3	6,183	1,568
Glycerol-3-P acyltransferase (GPAT2)	At1g02390	2.5	5.9	118	366
1-Acylglycerol-P acyltransferase (LPAT5)	At3g18850	2.3	2.2	6,123	1,214
Bifunctional wax ester synthase/DAGAT	At3g49210	7.8	3.1	805	304
Bifunctional wax ester synthase/DAGAT	At5g37300	4.2	16.6	6,431	1,258
Bifunctional wax ester synthase/DAGAT	At5g12420	3.6	11.2	123	537
Bifunctional wax ester synthase/DAGAT	At1g72110	2.9	12	1,960	83
Wax synthase	At3g51970	4.8	2.8	116	121
ABC transporter (WBC12=CER5)	At1g51500	3.5	3.1	14,194	4,110
ABC transporter (WBC1)	At2g39350	2.3	14.4	140	162
ABC transporter (WBC11)	At1g17840	3.6	4.2	10,137	4,644
ABC transporter (WBC18)	At3g55110	3.0	2.3	2,915	686
ABC transporter (WBC19)	At3g55130	12.3	3.1	3,172	4,884
Translocase	At1g72700	2.1	1.3	1,240	1,185
Translocase	At1g13210	6.9	4.1	1,810	1,815
Lipid transfer protein type 1 (LTP2)	At2g38530	19.5	24.5	904	827
Lipid transfer protein type 1 (LTP8)	At2g15050	4.3	3.4	18,270	10,531
Lipid transfer protein type 1 (LTP5)	At3g51600	2.8	3.6	28,335	19,619
Lipid transfer protein type 5	At1g27950	3.8	8.7	11,827	4,481

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Putative or Known Function of Protein	Locus Code	Ratio[a] (Top)	Ratio[a] (Base)	Signal[b] (Top)	Signal[b] (Base)
Lipid transfer protein type 5	At3g43720	3.2	4.8	13,127	6,167
Lipid transfer protein type 5	At1g55260	2.6	10.6	7,506	1,076
Lipid transfer protein type 5	At1g62790	2.5	1	4,199	2,033
Diacylglycerol kinase	At4g30340	2.9	1.4	1,929	704
DAD1-like acylhydrolase	At1g06800	4.6	6	297	203
DAD1-like acylhydrolase	At2g30550	4.1	0.8	2,585	1,462
Plastidial lipoxygenase	At1g72520	10.1	13.4	681	1,925
Plastidial lipoxygenase	At1g67560	2.0	1.8	2,333	1,541
Plastidial lipoxygenase	At1g17420	6.6	10	265	1,668
Cytosolic lipoxygenase	At1g55020	3.6	1.2	511	345
Oxo-phytodienoic acid reductase	At2g06050	5	1.9	2,015	5,873
Oxo-phytodienoic acid reductase	At1g76690	2.6	1.8	1,451	2,094
Oxo-phytodienoic acid reductase	At1g17990	2.3	1.4	1,801	3,781
Type II phosphoinositide 5-phosphatase	At1g05630	3.3	2.1	763	212
Patatin-like acyl-hydrolase	At3g63200	7.2	2	1,280	1,191
Patatin-like acyl-hydrolase	At3g54950	5.1	0.6	258	309
Phosphatidylinositol-4-kinase-γ	At1g26270	3.6	1.5	1,246	1,114
Phosphatidylinositol phosphate kinase type I/II A	At4g01190	3.2	1.8	195	140
Phosphatidylinositol phosphate kinase type III	At3g14270	3.5	2.1	1,181	1,452
Phosphatidylinositol phosphate kinase type III	At1g34260	2.5	1.2	498	607
PI-specific phospholipase C	At5g58670	3.3	1.2	417	1,018
Phospholipase D-γ	At4g11830	2.7	2.0	931	727
Phospholipase D-γ	At4g11850	2.2	2.5	109	216
Lysophospholipase	At1g64670	4.0	10.6	2,110	495
Lysophospholipase	At5g17780	2.0	1.7	141	92
PPT1-like thioesterase	At4g17480	10.4	0.9	200	12
PPT1-like thioesterase	At4g17440	36.9	184.4	136	518
PPT1-like thioesterase	At5g47330	8.8	1.5	838	973

[a]Mean epidermis-to-stem gene expression ratio obtained with the top or base of stems. Gene expressions were determined as in Table I. [b]Mean signal intensities are indicated to give an estimate of relative expression levels of the genes in the epidermis of top stems as well as in the epidermis of the base of stems.

in wax biosynthesis is likely synchronized and must be highly up-regulated during rapid epidermal cell expansion either transcriptionally or posttranscriptionally.

Constant wax loads on the lower segments of the stems might reflect a dynamic equilibrium between wax accumulation and wax loss by erosion or back transport (Jetter and Schaffer, 2001). But if wax turnover occurred, then it would certainly be at much slower rates than the accumulation in the top zone.

Deposition of the Polyester Matrix

The fact that the highest polyester loads were observed in the youngest part of the stem is consistent with previous observations that the major cutin synthesis occurs in young tissues (Kolattukudy, 1970). Palmitate applied exogenously to broad bean leaves was most highly incorporated into cutin in the youngest leaves, but incorporation was not detectable in the oldest leaves. Assuming that palmitate uptake was not limiting, this implies that cutin synthesis indeed occurs primarily in young organs. The 2-fold polyester reduction seen at the base of the Arabidopsis stems as compared to the middle of the stems (Fig. 3) is not likely to be caused by surface area expansion and dilution of the initial polyester load accumulated in the top zone (i.e. early during the elongation of epidermal cells). Indeed, there is very little or no more elongation in the stem bases (Fig. 1) and, in the zone where cell elongation is maximal (between the apex and the middle of the stems), the average polyester load does not vary significantly (Fig. 2). The extent to which the decrease in polyester load seen in the base is caused by a turnover of the components of the polyester matrix with a slow rate of resynthesis, or more likely by conversion of cutin (which can be depolymerized) to cutan (which cannot be depolymerized), remains to be determined. Monomers containing epoxy groups have been shown to be present only in young leaves in *Clivia miniata* (Jeffree, 1996) and could thus be involved in cutan formation in older organs. However, due to the difficulty of polyester analysis in Arabidopsis, as well as very low amounts, the putative Arabidopsis epoxy monomers (probably among the C18 in-chain substituted fatty acids in Fig. 4) were not fully identified. Dicarboxylic acids are major polyester monomers in Arabidopsis and no evidence was uncovered that indicated there is a significant difference in timing of their deposition as compared to ω-hydroxy fatty acids. These results are consistent with the idea that dicarboxylic acids are also part of the cutin polymer, or part

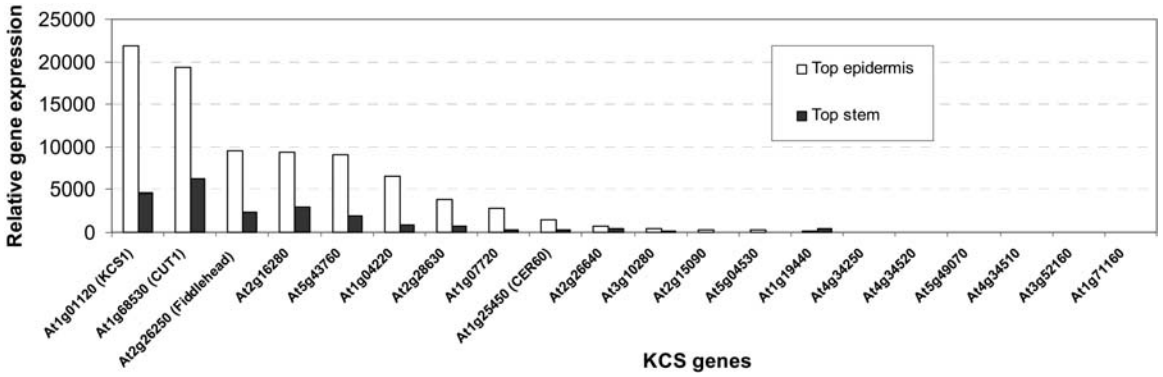

Figure 7. Relative gene expression levels of the members of the KCS gene family in epidermis of stem tops and in total stems.

of an epidermal polymer that is functionally closely related to cutin, and that together they form the matrix of the cuticle.

Wax and Polyester Loads

The wax load of the leaf is about 2 times lower than the polyester load: 0.75 $\mu g/cm^2$ (data not shown) versus 1.5 $\mu g/cm^2$ (this study). Since the average polyester load of the stem is 6 $\mu g/cm^2$, one would expect about 3 $\mu g/cm^2$ of waxes to be present on the stem surface (assuming the wax-to-polyester ratio of the leaf is the same). However, a much higher wax load was found in the stem: on average, 32 $\mu g/cm^2$ (this study). The difference is thus likely to come from the abundant epicuticular waxes of the stem, as evidenced by the wax crystals seen in the ultrastructure of the stem surface (Fig. 2), but not in that of the leaf surface (data not shown). It can therefore be estimated that epicuticular wax crystals represent about 90% of the total wax load in the stem. Finally, it should be stressed that the actual ratio of wax to matrix in the cuticle could be different from the wax-to-polyester ratio because the amount of cuticle lipid that cannot be depolymerized is unknown.

Gene Expression in Epidermal Cells

About 1,900 genes (15% of the genes detected in the stem) were identified as preferentially expressed in the epidermis of top and/or basal segments of stems. The majority of these genes (70%) had a transcript epidermis-to-stem ratio that fell in the 2.0 to 4.0 range, while 30% had a ratio greater than 4.0 (Supplemental Table II). This is consistent with what has been observed in maize for transcripts accumulating to at least 2-fold higher levels in epidermal cells than in vascular tissues (Nakazono et al., 2003). Moreover, more than 70% of the unique genes (or genes belonging to small gene families) identified as preferentially up-regulated in the maize epidermis had their best Arabidopsis hit (BLASTX search), showing also a clear up-regulation in the epidermis of Arabidopsis stems (data not shown). These include, for example, genes encoding the acyl-ACP thioesterase FatB or the cytosolic acetyl-CoA carboxylase (Table III). We have found that a subset of about 600 genes (about 5% of genes detected in stems) was specifically up-regulated in the epidermis of the elongating top segments (Supplemental Tables I and III). Interestingly, this is similar to the size of the subset of genes (672) that were found to be preferentially expressed in the epidermis of roots at a stage of longitudinal cell elongation and that represented about 6% of the genes detected in roots (Birnbaum et al., 2003). These observations and the correct classification of known epidermis-specific genes (Table I) suggest that many of the genes identified in this study as epidermis up-regulated are true hits and are thus likely to play a role in the biological processes occurring specifically in the epidermis, e.g. the synthesis of the cuticle.

The epidermal peel approach used here is based on the harvest of all epidermal cell types and thus transcripts specifically expressed in less abundant cell types, like guard cells, will be diluted by more abundant cell types. The candidate genes obtained here are therefore more likely to be genes expressed in all epidermal cells of a particular stem region. However, the example of the guard cell-specific MYB transcription factor At1g08810 (Cominelli et al., 2005) that is found to be 3.4-fold up-regulated in the epidermis in our dataset (Supplemental Table II) shows that cell type-specific proteins could also be included in the list of candidates. The genes that are up-regulated to the same extent in both the top and the basal epidermis are presumably related to functions or processes specific to the whole epidermis, whereas genes up-regulated only in the top or the basal epidermis are more likely to be involved, for example, in epidermal cell division, elongation, or cuticle formation for the top, and in trichome differentiation for the base.

To identify gene candidates for wax and cutin synthesis, we have focused mostly on the epidermis-to-stem gene expression ratio for the top stem. Even if there is a turnover of surface lipids in the epidermal cells of the nonelongating stem base, the rapid elongation and the high wax-polyester content observed in the stem top indicate that this machinery must be more

active in the epidermis of the top than in the epidermis of the base. In the case of transcriptional regulation, the transcripts of the corresponding genes are thus expected to be more abundant in the epidermis of the top segment than in the epidermis of the basal segment of the stem. However, it is striking that many genes involved in wax synthesis, such as the major elongase-condensing enzyme (CER6) that supplies alkane and alkane-derived (secondary alcohols and ketones) waxes, are still highly expressed or even up-regulated in the lower stem epidermis (Table III), where we would expect very low net flux into these components. It is possible that there is a turnover of the wax and/or cutin components in the nonelongating parts of the stems, requiring continued synthesis to maintain constant loads. An alternative hypothesis is that many of the genes involved in surface lipid metabolism could be expressed in the epidermis of all stem segments, but strongly controlled at the posttranscriptional level, depending on the elongation rate of the cell. This control might, for example, include a system that senses the wax-cutin loads to provide feedback to biosynthesis.

Lipid-related genes of Table III may not all be involved in surface lipid metabolism. Some are clearly related to cellular signaling, possibly playing a role in epidermal cell differentiation, elongation, and/or interaction with the environment. It is not likely that the signaling genes up-regulated in the epidermis are induced by wounding during the harvest of epidermal peels because peels were frozen in liquid nitrogen immediately and also several essential and well characterized genes of wound response (Delessert et al., 2004), such as At4g15440 (hydroperoxide lyase) and At5g42650 (allene oxide synthase), were not found to be significantly up-regulated in the epidermal peels as compared to the nonpeeled stem segments used as a reference (see Supplemental Table II).

Candidate Genes for Wax and Cutin Biosynthesis

Many reactions in the pathways for the synthesis of wax components remain obscure and some proteins identified by mapping of wax mutants cannot be assigned a clear molecular function (Kunst and Samuels, 2003). The only protein that has been demonstrated to play a specific role in cutin biosynthesis by a chemical analysis of cutin (*att1* Arabidopsis mutant) is a fatty acid hydroxylase (Xiao et al., 2004). Important enzymes, like putative acyltransferases, that might be responsible for the assembly of cutin polyester chains are clearly lacking in the current list of candidates (Yephremov and Schreiber, 2005), and therefore more gene candidate searches are needed. Analysis of cutin components is time consuming and not easy to use in a high-throughput screening of a forward-genetics approach, while identification of cutin mutants by indirect assays, such as organ fusion phenotype, chlorophyll leaching, or dye uptake, might yield other mutants not primarily affected in cutin synthesis. The analysis of the polyesters of insertion lines in selected candidate genes might therefore prove useful to discover members of large protein families or new proteins specifically involved in cutin as well as wax metabolism.

In addition to the KCS family (Fig. 7), another example of the utility of Table III for large gene families is provided by analysis of the expression of the subfamilies of putative lipid transfer proteins (LTPs) that have been suggested to be involved in wax-polyester monomer transport through the cell wall. In Arabidopsis, there are 72 putative LTPs that were classified into eight types based on the conserved Cys pattern (Beisson et al., 2003). Interestingly, all the LTPs up-regulated in the growing epidermis were found to be from type 1, the original group of LTPs shown to have in vitro lipid-binding properties, and from the large uncharacterized type 5 group. No LTP from the type 3 group was found, although this group is as large (11 genes represented on the ATH1 array) as the type 1 group. These results point toward type 1 and 5 groups as candidates for a function in cuticle synthesis, whereas the type 3 group might be related to other functions, such as signaling in the apoplast as suggested for one LTP of type 3 (Maldonado et al., 2002). Also potentially related to wax secretion, several ABC transporters from the same white-brown complex (WBC) subfamily as CER5 were up-regulated in the epidermis.

Our list of candidates for wax synthesis (Table III) includes uncharacterized members of the KCS family, such as At5g16280 or At2g43760, that have been recently identified as highly expressed in the aerial parts of 15-d-old expanding seedlings (Costaglioli et al., 2005). Our results thus confirm that they are likely to play an important role in wax synthesis. But other KCS genes, like At1g04220 or At1g07720, were also found to be clearly up-regulated in stem epidermis, although they are low expressed in 15-d-old seedlings. The same is true for LTP2 (At2g38530), which has a low global expression level when measured in seedlings, but is among the few epidermis up-regulated LTPs identified in the elongating stem. These genes exemplify the strength of our approach using only epidermis material in the discovery of gene candidates for cuticle synthesis. These examples could also indicate that, regarding candidate profiling for cuticle synthesis, it might be difficult to extend the results obtained on young seedlings to older plants and, conversely, because specific isoforms might be expressed in the shoot epidermis at different stages of development.

Concerning cutin biosynthesis, putative fatty acid hydroxylases and acyltransferases are proteins of special interest. The protein affected in the Arabidopsis *att1* cutin mutant (Xiao et al., 2004) is CYP86A2 (At4g00360 locus), a fatty acid hydroxylase belonging to the same cytochrome P450 monooxygenase subfamily as CYP86A8, which has also been implicated in cuticle formation in the Arabidopsis *lacerata* mutant (Wellesen et al., 2001). Not surprisingly, there are other members of the same subfamily that are also

up-regulated in the epidermis (Table III). The other Arabidopsis cytochrome P450 monooxygenase subfamily thought to encode fatty acid hydroxylases is the CYP94B family (Duan and Schuler, 2005). It is therefore likely that the CYP94B3 member listed in Table III is also related to oxidation of surface lipids or epidermal oxylipins. Since this gene is more expressed and up-regulated in the epidermis of the base of the stem than of the top of the stem, it is, for instance, a good candidate for the polyester modifications (e.g. cutan synthesis) that might occur at the base of the stem. Arabidopsis cytochrome P450 monooxygenases belonging to other subfamilies that are uncharacterized or involved in the oxidation of substrates not related to fatty acids were not listed in Table III.

The polyester synthases responsible for the assembly of cutin chains remain completely unknown and the acyltransferases listed in Table III are therefore potential candidates for this function. It is not likely that the epidermis up-regulated acyltransferases listed in Table III (1-acylglycerol-3-P acyltransferase [LPAT5] and glycerol-3-P acyltransferase [GPAT4], etc.) are involved in housekeeping membrane biogenesis because the cells of the tissues underlying the epidermis are also elongating and need to synthesize membrane lipids at about the same rate as epidermal cells. Thus, it is more likely that the acyltransferases of Table III are somehow involved in surface lipid synthesis. Indeed, LPAT activity was not detected for the recombinant LPAT5 (Kim et al., 2005), and no GPAT activity was found for the GPAT2 and GPAT3 isoforms (Zheng et al., 2003). Taken together, these observations raise the possibility that at least some of the GPAT acyltransferases isoforms, as well as LPAT5, act on intermediates of cutin and/or wax biosynthesis and are not involved in the synthesis of the glycerolipids composing the membranes or storage-oil bodies of the cell. Preliminary analysis of T-DNA insertion lines in some of these genes supports this hypothesis (data not shown).

CONCLUSION

The accumulation of cuticular lipids by the plant epidermis has been studied by a combined quantitative and qualitative analysis of the surface lipids and of the transcripts present in expanding epidermal cells during the elongation of Arabidopsis stems. The high loads and constant composition of polyesters and waxes found in the elongating top and middle zone of stems indicated that the synthesis and the secretion of these two components of the cuticle are highly coregulated to keep up with the pace of rapid epidermal cell expansion. Our microarray approach identified a subset of the genome (about 15% of the genes detected in the stem) that is preferentially expressed in the epidermis and provided a list of potential gene candidates for the machinery of assembly, secretion, and synthesis of surface lipids that can be tested by reverse-genetics approaches. This dataset should accelerate progress toward elucidation of the wax biosynthesis pathways and aid in unraveling the biosynthesis of cutin.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The wild-type ecotype Col-0 of Arabidopsis (*Arabidopsis thaliana*) was used for all experiments. Seeds were stratified for 3 to 4 d at 4°C and plants were grown on a mixture of soil:vermiculite:perlite (1:1:1) under white fluorescent light (80–100 μE m^{-2} s^{-1}) in an 18-h-light/6-h-dark photoperiod. The temperature was set at 20°C to 22°C and the relative humidity at 50% to 70%.

Stem Harvest and Stem Diameter Measurements

Each harvested 10- to 11-cm-long primary stem was cut into 3-cm-long segments (3 cm from the base of the stem, 3 cm from the base of the inflorescence, and 3 cm in the middle of the stem). The cauline leaves or siliques were cut off and the stem segments were kept in liquid nitrogen until use.

Diameters were measured using transverse hand sections of stems and light microscopy and, in addition, diameters of intact stems were measured with cryo-SEM. Typical diameters were around 0.6 mm for the basal and middle segments of the stems and around 0.4 mm for the apical segment. The surface areas of the segments were calculated using an average diameter for each segment and assuming cylinder geometry. In order to detect any significant departure from these standard diameters in the samples used for routine lipid analysis, the stem segments used were photocopied prior to chemical analysis and diameters were estimated from magnified copies.

Cryo-SEM

Segments from the apical 1 cm of stem were mounted onto cryo-SEM stubs with 25% dextran and plunged into liquid nitrogen. Frozen samples were transferred into an Emitech K1250 cryo-system, where water was sublimed for 30 min at −77°C and subsequently sputter coated with gold for 2.5 min at 35 mA. The coated samples were viewed with a Hitachi S4700 field emission SEM using an accelerating voltage of 2 kV and a working distance of 12 mm.

Propidium Iodide Staining and Confocal Laser Scanning Microscopy

Stem segments of Arabidopsis plants were immersed in a solution of propidium iodide (100 μg/mL; Sigma) and examined with a Radiance 2000 confocal laser-scanning microscope (Bio-Rad). The excitation wavelength was 568 nm with the emission filter set at 580 to 600 nm. All confocal images obtained were processed with ImageJ (http://rsb.info.nih.gov/ij) and Photoshop 5.0 (Adobe Systems) software.

Polyester Analysis

Thirty to 100 3-cm-long segments from 10- to 11-cm-long primary stems were used for each replicate. The analysis of the polyesters was conducted according to Bonaventure et al. (2004) with a slight modification (the use of centrifugation in 50-mL glass tubes at 800*g* for 20 min instead of filtration steps). Internal standards were methyl heptadecanoate and ω-pentadecalactone (Sigma). Polyesters from dried solvent-extracted residues of stems were depolymerized by hydrogenolysis with $LiAlH_4$ or methanolysis with $NaOCH_3$. The products recovered after hydrogenolysis were separated and quantified by GC as described previously (Bonaventure et al., 2004). For the products corresponding to several possible fatty acid derivatives, the proportions of diacids, diols, and ω-hydroxyl fatty acids were estimated based on the methanolysis data.

Wax Analysis

The cuticular waxes were extracted by immersing whole inflorescence stems or 3-cm-long segments of stems two times for 30 s into 5 mL of chloroform ($CHCl_3$) at room temperature. Both $CHCl_3$ solutions were combined

and *n*-tetracosane was added as internal standard. The solvent was removed under a gentle stream of nitrogen gas, and the remaining wax mixture was redissolved in 1 mL of $CHCl_3$ and stored at 4°C until used. The extracted area was determined by measuring the height and diameter of the stems assuming cylinder geometry.

Prior to GC analysis, chloroform was evaporated from the samples under a gentle stream of nitrogen gas while heating to 50°C. Then the wax mixtures were treated with bis-*N*,*N*-(trimethylsilyl) trifluoroacetamide (BSTFA; Sigma) in pyridine (30 min at 70°C) to transform all hydroxyl-containing compounds into the corresponding trimethylsilyl derivatives. The qualitative composition was studied with capillary GC (5890 N; column 30 m Hewlett-Packard-1, 0.32-mm i.d., film thickness = 0.1 μm; Agilent) with He carrier gas inlet pressure regulated for constant flow of 1.4 mL min^{-1} and a MS detector (5973 N; Agilent). GC was carried out with temperature-programmed injection in a 50°C oven, 2 min at 50°C, raised by 40°C min^{-1} to 200°C, held for 2 min at 200°C, raised by 3°C min^{-1} to 320°C, and held for 30 min at 320°C. The quantitative composition of the mixtures was studied using capillary GC with FID under the same GC conditions as above, but H_2 carrier gas inlet pressure regulated for constant flow of 2 mL min^{-1}. Single compounds were quantified against the internal standard by automatically integrating peak areas.

Isolation of Epidermal Peels

Epidermal peels were manually dissected as a thin transparent film using thin forceps under a dissecting microscope (Fig. 5). For each freshly cut 3-cm-long stem segment of 10- to 11-cm-long primary stems, peels were collected, immediately frozen in liquid nitrogen, and stored at −80°C for RNA isolation or fatty acid analysis.

Fatty Acid Analysis

Epidermal peels from about two to three stem segments were heated at 85°C for 1.5 h in 1 mL of methanol containing 5% H_2SO_4 (v/v) with triheptadecanoylglycerol and ω-hydroxy-pentadecanoic acid as controls. Alternatively, the epidermal peels were quenched 10 min in isopropanol at 85°C and the lipids were extracted with hexane (3:2 [v/v] hexane:isopropanol final) before transmethylation. Fatty acid methyl esters (FAMEs) were extracted two times with 2 mL hexane and the solvent was evaporated under nitrogen gas. FAMEs were redissolved in 100 μL pyridine; 100 μL of acetic anhydride was added and the mixture was vortexed and heated for 1 h at 60°C. After evaporation of the solvents under nitrogen gas, the FAMEs were redissolved in hexane and analyzed by GC with FID on a DB-23 capillary column (J&W Scientific) under conditions allowing the separation of regular and VLCFAs.

Gene Expression Analysis

Total RNAs were isolated from epidermal peels or whole-stem segments using the RNeasy kit (Qiagen). Double-stranded cDNA was synthesized from approximately 15 μg of total RNA by using a SuperScript double-stranded cDNA synthesis kit (Invitrogen) with oligo d(T) primer containing a T7 RNA polymerase promoter sequence at its 5′ end (GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGC-GG-(dT)24-3′; Genset). After synthesis of the second-strand cDNA, the cDNA was extracted with phenol-chloroform-isoamylalcohol, precipitated with ethanol, and resuspended in ribonuclease-free water. Labeled cRNA was generated from cDNA by in vitro transcription using a bioarray high-yield RNA transcript-labeling kit (Enzo Diagnostics) following the manufacturer's instructions and incorporating biotinylated CTP and UTP. After purification of biotin-labeled cRNA using an RNeasy column, 15 μg of the labeled cRNA were fragmented to a size of 35 to 200 bases by incubating at 94°C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate).

The fragmented cRNA was used for hybridization of Arabidopsis ATH1 gene chips (Affymetrix), which was performed in the Research Technology Support Facility at Michigan State University. Hybridization, washing, and detection of labeled cRNA were done as recommended by Affymetrix. Image acquisition and global data scaling were performed with Affymetrix MAS 5.0 software. The signal intensity values were scaled to a mean of 500 for each chip. The signal intensities indicated in the "Results" section are the mean from two biological replicates. The ratios of gene expression are the mean of four ratios of epidermis versus whole-stem segments calculated using multiple pairwise comparisons of epidermis versus stem. For each gene, a 90% confidence interval was calculated for the epidermis-to-stem ratio using a T-score and a degree of freedom of 3. Genes were considered as up-regulated in the epidermis if they were called present by the MAS 5.0 software in both biological replicates, and if the lower bound of the 90% confidence interval for the epidermis-to-stem gene expression ratio was ≥2.0. Indeed, for all epidermis-specific genes (Table I), the epidermis-to-stem ratio was found to lie in a 90% confidence interval whose lower bound was 2.0 or above. These results thus provided an empirical basis for the use of 2.0 as a cutoff value for the lower estimates of the ratios to identify epidermis-specific genes in our dataset. Other studies have indicated that robust gene expression changes can be consistently identified by selecting transcripts with a gene expression ratio >2.0 (mean ratio) for increases (Wodicka et al., 1997). Hence, a value of 2.0 for the lower estimate of the ratio is clearly conservative. This cutoff is also particularly conservative because the whole-stem segments used as a reference contain epidermis and the epidermis-to-stem ratios tend therefore to underestimate the up-regulation of transcripts in epidermis.

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