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Leaf Epicuticular Waxes of the *Eceriferum* Mutants in *Arabidopsis*[1]

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Wild-type *Arabidopsis* leaf epicuticular wax (EW) occurs as a smooth layer over the epidermal surface, whereas stem EW has a crystalline microstructure. Wild-type EW load was more than 10-fold lower on leaves than on stems. Compared with the EW on wild-type stems, EW on wild-type leaves had a much higher proportion of their total EW load in the form of alkanes and 1-alcohols; a large reduction in secondary alcohols, ketones, and esters; and a chain-length distribution for major EW classes that was skewed toward longer lengths. The *eceriferum* (*cer*) mutations often differentially affected leaf and stem EW chemical compositions. For example, the *cer2* mutant EW phenotype was expressed on the stem but not on the leaf. Compared to wild type, the amount of primary alcohols on *cer9* mutants was reduced on leaves but elevated on stems, whereas an opposite differential effect for primary alcohols was observed on *cer16* leaves and stems. Putative functions for *CER* gene products are discussed. The *CER4* and *CER6* gene products may be involved in fatty aldehyde reduction and C_{26} fatty acyl-coenzyme A elongation, respectively. *CER1*, *CER8*, *CER9*, and *CER16* gene products may be involved in EW substrate transfer. The *CER3* gene product may be involved in release of fatty acids from elongase complexes. *CER2* gene product may have regulatory functions.

EWs form the outermost layer of aerial plant organs. On leaf blades, they are thought to serve as a barrier against environmental stresses, including drought (Chatterton et al., 1975; Jordan et al., 1984), supra-optimal solar radiation (Reicosky and Hanover, 1978; Mulroy, 1979), freezing temperatures (Thomas and Barber, 1974), fungal pathogens (Hargreaves et al., 1982; Jenks et al., 1994), and phytophagous insects (Eigenbrode and Espelie, 1995). Leaf EW coatings have also been implicated in protection against mechanical damage (Eglinton and Hamilton, 1967) and anthropogenic stresses such as acid rain (Percy and Baker, 1990) and agricultural chemical sprays (Bukovac et al., 1979; Schreiber and Schonherr, 1992). Leaves are the major photosynthetic tissues in crop plants and often the first to be damaged by environmental stress. Recent success in the modification of plant lipids in transgenic plants (Ohlrogge, 1994) underscores the potential for developing crop plants with modified leaf EW and increased resistance to extreme environmental pressures.

Induced or naturally occurring mutations affecting plant EWs have been described for monocots, such as *Sorghum bicolor* (Jenks et al., 1992), *Hordeum vulgare* (Lundqvist and Lundqvist, 1988), and *Zea mays* (Bianchi et al., 1985), and dicots, such as *Pisum sativum* (Holloway et al., 1977) and *Brassica oleracea* (Eigenbrode et al., 1991a, 1991b). Recent studies have identified T-DNA-induced stem EW mutants in *Arabidopsis thaliana* (Feldmann, 1991; McNevin et al., 1993) that are allelic to previously identified ethyl methanesulfonate- and radiation-induced stem EW mutant loci (Koornneef et al., 1989), designated *cer*. These T-DNA-tagged *cer* genes will provide an effective method for the isolation of genes involved in EW biosynthesis.

Because leaf EW has been shown to play such an important role in insect (Eigenbrode and Espelie, 1995) and other plant stress resistance, we have initiated an analysis of the genetic components controlling EW biosynthesis on *Arabidopsis* leaves. The major leaf EW constituents of diverse plant species are similar and arise from a highly conserved EW biosynthetic pathway. We have selected *Arabidopsis* as a model plant system in which to dissect the molecular basis for leaf EW biosynthesis because of its favorable cultural and genetic characteristics.

Arabidopsis cer mutants were easily identified, since EW alterations were visible as a broad-band spectral shift in stem surface reflectance from glaucous white to glossy green (Koornneef et al., 1989). Wild-type leaf surfaces of *Arabidopsis* are glossy green, and previous mutagenesis studies have failed to identify leaf EW mutants. We suspected that *cer* mutations that affected EW on stems may also affect EW on leaves. To test this hypothesis, EW constituents on wild-type and *cer* leaves were analyzed. Presently, the exact role of *cer* genes in EW biosynthesis is unknown. Comparisons of EW constituent profiles on leaves and stems of wild type and *cer* were used to describe

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Abbreviations: ACP, acyl carrier protein; *cer*, *eceriferum* (wax bare); EW, epicuticular wax; FAS, fatty acid synthetase; Ler, Landsberg *erecta*; WS, Wassilewskija.

the putative functions of *CER* gene products in light of a proposed model for *Arabidopsis* EW biosynthesis.

MATERIALS AND METHODS

Plant Material

We used *Arabidopsis thaliana* ecotypes Wassilewskija (WS) and Landsberg *erecta* (Ler) along with near-isogenic chemical- and radiation-induced *cer* mutants from Koornneef et al. (1989) and T-DNA-induced *cer* mutants from McNevin et al. (1993). Plants were grown in a controlled environment chamber at 22°C and a 16-h photoperiod (approximately 240 μmol m^{-2} s^{-1}; 75–95% RH).

Microstructural Analysis

Leaf, stem, and silique tissues were air dried before sputter coating with gold-palladium using short, 20-s, repeated bursts to prevent melting of EW crystals. Specimens were examined using a scanning electron microscope at 12 kV. Three or more replicates from different plants were examined for each wild type and *cer* mutant.

Chemical Analysis

The hexane-soluble surface waxes (designated EW) were extracted from 15- and 25-d-old plants by immersing separate tissues in hexane for 30 s. Extracts were evaporated to dryness under a nitrogen stream and the dried residue was prepared for GC by derivatization using bis(trimethylsilyl)-acetimide (Hannoufa et al., 1993). Derivatization was at 75°C for 20 min. After surplus bis(trimethylsilyl)-acetimide was evaporated under a stream of nitrogen, the samples were redissolved in hexane for analysis with a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector. Selected subsamples were used for injection in a gas chromatograph-mass spectrometer to produce electron ionization-mass spectra. Both the gas chromatograph and gas chromatograph-mass spectrometer were equipped with a 12-m, 0.2-mm HP-1 capillary column with helium as the carrier gas. Both the gas chromatograph and gas chromatograph-mass spectrometer were programmed with an initial temperature of 80°C and increased at 15°C min^{-1} to 260°C, where the temperature was unchanged for 10 min. The temperature was increased at 5°C min^{-1} to 320°C, where the temperature was held for 15 min. Quantification was based on flame ionization detector peak areas and the internal standard hexadecane. In addition, specific correction factors developed from external standards were applied to the peak areas of alkyl fatty acids, 1-alcohols, and alkanes. For all other compounds, a factor of 1.09 was assigned (the average correction for 18 standards). The total amount of EW (EW load) was expressed per leaf, stem, or silique surface area. Tissue areas were determined using computer digitization. Wax esters were isolated by TLC and cleaved and derivatized with methanol:acetyl chloride (10:1, w/v) into fatty acid methyl esters and 1-alcohol products. The total amount of unknown surface chemical constituents was calculated from the cumulative peak areas for all unidentified peaks. Values represent the averages of at least three replicate plant extractions. One replicate equaled a bulk of either leaf, stem, or silique tissues from multiple plants.

Chemicals identified as EW constituents on *Arabidopsis* were abbreviated, respectively, as follows: tetradecanoic, hexadecanoic, octadecanoic, eicosanoic, docosanoic, tetracosanoic, hexacosanoic, octacosanoic, and triacontanoic acids as C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} free fatty acids; *n*-pentacosane, *n*-heptacosane, *n*-nonacosane, *n*-hentriacosane, and *n*-tritriacontane as C_{25}, C_{27}, C_{29}, C_{31}, and C_{33} alkanes; 1-tetracosanol, 1-hexacosanol, 1-octacosanol, 1-triacontanol, and 1-dotriacontanol as C_{24}, C_{26}, C_{28}, C_{30}, and C_{32} primary alcohols; tetracosanal, hexacosanal, octacosanal, and triacontanal as C_{24}, C_{26}, C_{28}, and C_{30} aldehydes; 13- and 14-heptacosanol, 14- and 15-nonacosanol, and 15- and 16-hentriacontanol as C_{27}, C_{29}, and C_{31} secondary alcohols; and 15-nonacosanone as C_{29} ketone.

RESULTS

Wild-Type Leaf EW

Abaxial and adaxial leaf blade surfaces of the wild-type WS appeared smooth (Fig. 1, A and B). By comparison, both stems (Fig. 1C) and siliques (data not shown) possessed microstructural waxes dominated by plates and tubes. Tubes were approximately 0.5 to 3.0 μm in height and 0.2 to 0.5 μm in width.

The total EW load on 15-d-old wild-type WS leaves (data not shown) was similar to the load on 25-d-old leaves (Table I). Wild-type WS and Ler leaves possessed 10 times and 25 times less EW, respectively, than the stems on d 25 (Table I). Stems were not present on d 15. The actual values in μg dm^{-2} for WS leaves and stems were 156.1 ± 9.2 (SE) and 1620 ± 16.9 (SE), respectively. The values for Ler leaves and stems were 97.2 ± 15.6 (SE) and 2410 ± 25.7 (SE), respectively. By comparison, the total EW load on 25-d wild-type WS siliques (data not shown) was slightly lower than on WS stems (Table I).

Chemical constituents on wild-type WS leaves changed only slightly between d 15 (data not shown) and d 25 (Fig. 2). Wild-type WS aldehydes decreased from 2.2% (data not shown) of total load to 1.5% (Table I), and primary alcohols (1-alcohols) increased from 10% (data not shown) to 24% (Table I) in this period.

The leaf 1-alcohols on 25-d-old wild-type WS and Ler were 24 and 15%, respectively, of the total leaf EW load (Table I). By comparison, stem 1-alcohols on 25-d-old wild-type WS and Ler were 6 and 5%, respectively, of the total stem EW load (Table I). The leaf blades of 25-d-old wild-type WS and Ler had secondary alcohols that were 0.3 and 0.1%, respectively, ketones that were 0.7 and 1.0%, respectively, and esters that were 0.1 and 0.2%, respectively, of the total leaf EW load (Table I). By comparison, the 25-d-old wild-type WS and Ler stem secondary alcohols were 11 and 13%, respectively, ketones were 24 and 29%, respectively, and esters were 3 and 0.7%, respectively, of the total stem EW load (Table I). On both wild-type WS and Ler, the amyrins (approximately 90% β-amyrin and 10% α-amyrin)

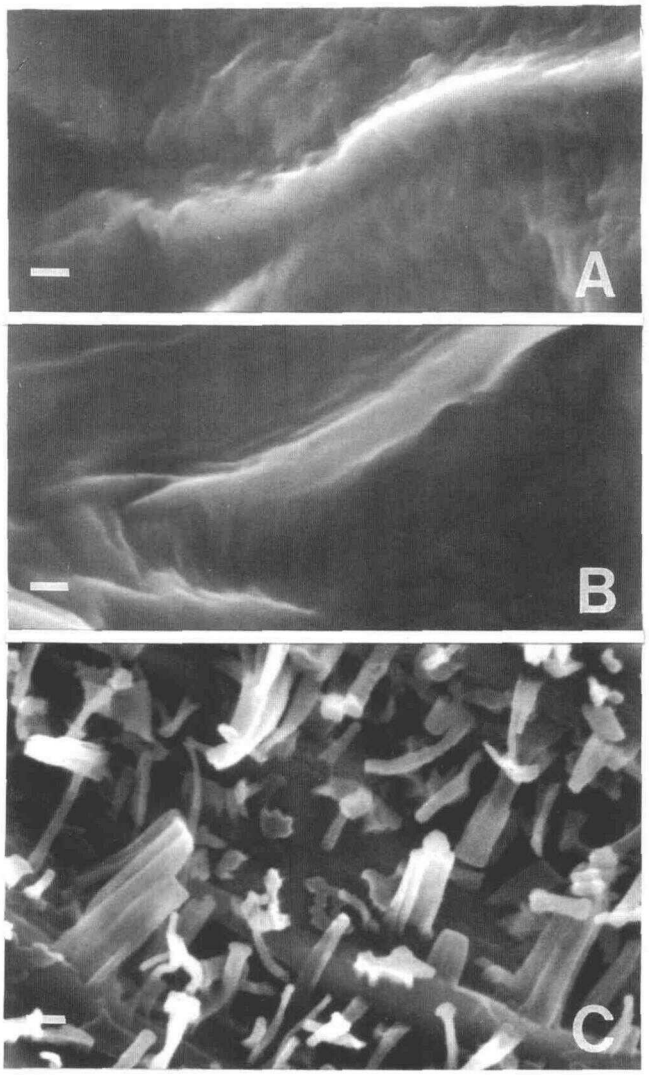

Figure 1. Wild-type WS microscopic surface morphology of air-dried specimens produced using scanning EM. A, Abaxial leaf surface. B, Adaxial leaf surface. C, Stem surface with short tube and plate EW microstructures. Bar = 1 μm.

and other triterpenoids were lower on the leaves than stems (Table I). Proportionally, 25-d-old wild-type WS silique EW constituents (data not shown) were similar to wild-type WS stem EW constituents (Fig. 2).

Wild-type WS and Ler leaves possessed longer chain-length homologs of the major EW classes than stems (Figs. 2 and 3). The 25-d-old wild-type WS and Ler leaves had C_{33} alkanes and C_{32} 1-alcohols that were not present in detectable amounts on stems (Figs. 2 and 3). The major leaf alkane was the C_{31} homolog, and the major stem alkane was C_{29} (Figs. 2 and 3). The major 1-alcohol was the C_{28} homolog in both leaves and stems (Figs. 2 and 3).

Leaf EWs of the *cer* Mutants

The abaxial and adaxial leaf blade surfaces of the eight *cer* lines presented were microscopically smooth because of the lack of EW crystals (data not shown) and were, therefore, identical with wild-type leaves. As in leaf EW of wild-type WS, leaf EW constituents on *cer1* through *cer4* changed little between d 15 (data not shown) and d 25 (Table I; Fig. 2). Silique EWs of 25-d-old *cer1* through *cer4* (data not shown) had EW constituents that were proportionally similar to their respective *cer* stems (Table I; Fig. 2).

All 16 *cer* lines examined had leaf EW loads that were nearly the same or lower than their respective wild types (Table I). The largest percentage reduction in leaf EW load on 25-d-old mutants from their respective wild types was 68% on *cer3* (Table I) and 48% on *cer6* (data not shown). The EWs were also reduced on stems of all *cer* mutants, except stems of *cer4* (Table I).

The same 16 *cer* mutants were screened at 25 d for alterations in the EW constituent profiles of the leaves. The proportions of unsaturated free fatty acids, even-chain-length alkanes, and odd-chain-length 1-alcohols were very low in both wild-type and *cer* leaf waxes; therefore, these constituents are not included in our report (except that they were used for quantification of total loads of alkanes and 1-alcohols). The leaf EW of *cer10*, *cer11*, *cer12*, *cer14*, *cer17*, *cer19*, and *cer20* had small reductions in the amount of individual EW constituent classes and are not discussed further. The leaf EW loads and chemical composition of *cer2* (Table I; Fig. 2) and *cer7* (data not shown) were essentially the same as their respective wild types, even though the stem EWs on these lines were altered (Fig. 2; data not shown).

Leaf EW homologs beyond 26 carbons in length were reduced on leaves of *cer6* (Fig. 3). The major leaf and stem alkane and 1-alcohol chain lengths on *cer6* were two carbons shorter than on wild type (Fig. 3). The stems of *cer2* had chain-length distributions that were nearly identical with *cer6* (Figs. 2 and 3).

Free fatty acids were 55% lower on leaves and 70% lower on stems of *cer3* than on the wild type (Table I). Aldehydes were 17% lower on leaves and 88% lower on stems of *cer3* than on the wild type (Table I). The major alkane on *cer3* leaves and major 1-alcohol on *cer3* stems were two carbons longer than in the wild type (Fig. 2). Stem alkanes but not leaf 1-alcohols of *cer3* were skewed toward longer lengths (Fig. 2). The EW profile on *cer7* stems was similar with that of *cer3* stems (data not shown).

The leaves and stems of *cer4* had greatly reduced levels of 1-alcohols with little change in other EW classes compared to the wild type (Table I; Fig. 2).

The *cer1* and *cer16* leaf EW had reduced alkanes compared to the wild types (Table I; Figs. 2 and 3). The *cer1* leaf 1-alcohols changed only slightly, whereas *cer16* leaves had increased 1-alcohols (Table I; Figs. 2 and 3). The *cer1* and *cer16* stems were decreased in both alkanes and 1-alcohols (Table I; Figs. 2 and 3).

On *cer9* leaves, free fatty acids beyond 26 carbons in length were not present (Fig. 3). The C_{26} fatty acid on *cer9* was 27 times higher than on the wild type (Fig. 3) Leaves of *cer9* also had large reductions in all alkane and 1-alcohol chain-length homologs (Table I; Fig. 3). In contrast, the stems of *cer9* had large reductions in alkanes but large increases in 1-alcohol amounts (Table I; Fig. 3).

Table I. *Leaf and stem EW on 25-d-old wild-type and cer mutants of Arabidopsis*

Values represent EW load in $\mu g\ dm^{-2}$ of leaf and stem area.

	Total Load[a]		Acids		Aldehydes		Alkanes		1-Alcohols		2-Alcohols		Ketones		Esters		Amyrins		Other Triterpenoids		Unknowns	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
WS[b]	156.0	1620	4.0	33.3	2.4	45.0	90.3	721	37.0	104	0.4	181.0	1.2	411.0	0.2	47.6	6.5	16.0	7.6	33.4	6.7	29.0
cer1	58.7	322	2.9	16.2	2.2	76.3	4.2	28	29.0	28	0.0	4.4	0.5	10.2	0.1	44.3	9.0	30.9	5.9	50.8	4.8	33.0
cer2	147.0	591	2.5	13.1	3.2	28.2	89.4	44	32.1	251	0.2	12.7	0.7	12.5	0.3	82.0	5.1	32.7	8.9	72.8	4.8	41.6
cer3	49.5	750	1.8	9.9	2.0	5.5	21.7	206	13.5	175	0.0	48.8	0.6	154.0	0.1	43.0	3.8	24.4	4.9	52.1	1.1	31.3
cer4	123.0	1670	1.4	29.7	2.6	117.0	99.9	759	2.6	20	0.1	177.0	0.7	430.0	1.5	6.2	3.0	28.5	7.7	58.5	3.4	42.8
Ler[c]	97.2	2410	2.9	30.9	1.7	76.8	59.4	1050	14.9	116	0.6	323.0	1.0	690.0	0.2	17.4	5.9	6.5	5.7	53.4	4.9	43.5
cer6	50.6	461	3.2	20.0	1.5	12.3	24.0	70	9.2	178	0.0	17.2	0.3	38.1	0.0	32.8	4.7	5.3	4.7	41.0	3.0	46.4
cer8	78.2	1291	11.2	195.0	1.5	85.9	35.4	371	8.6	108	0.1	101.0	0.4	279.0	3.2	55.7	4.7	4.0	7.1	44.9	6.2	46.9
cer9	89.8	913	63.0	47.3	1.1	18.1	6.8	232	3.8	186	0.0	70.7	0.1	211.0	1.0	67.4	2.5	4.3	6.1	43.4	5.4	32.5
cer16	64.5	227	6.0	19.2	3.3	37.6	8.7	24	24.0	32	0.0	1.7	0.6	3.6	6.4	28.7	4.9	18.9	5.1	32.6	6.0	28.9

[a] Represents the average amount of hexane-soluble surface wax in $\mu g\ dm^{-2}$ of three replicate leaf and stem surface areas. Values were calculated using an internal standard (hexacosane) and corrected peak areas from the gas chromatograph. [b] *WS is the parent of T-DNA-induced mutants.* [c] *Ler is the parent of ethyl methanesulfonate-induced mutants.*

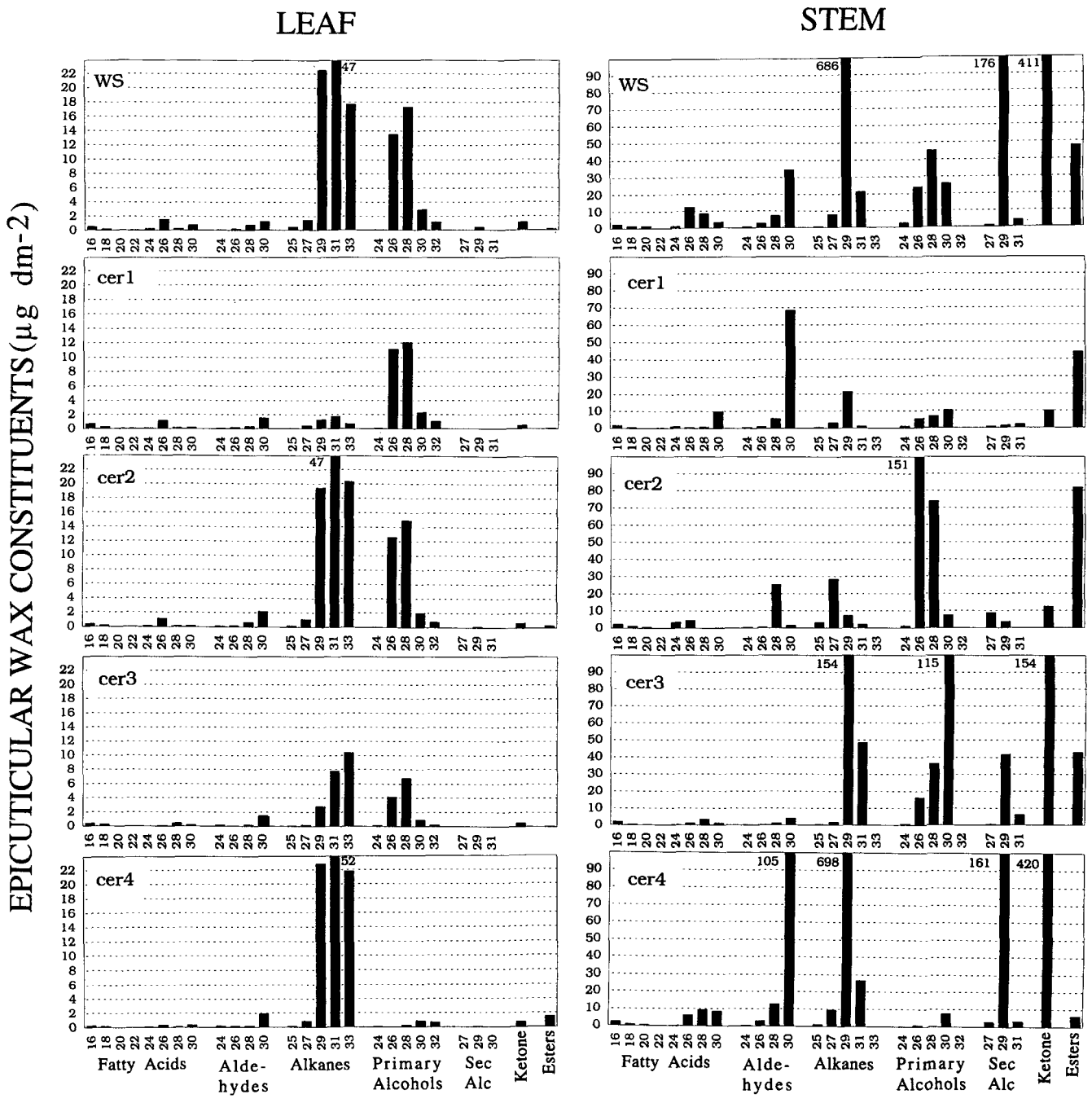

Figure 2. Leaf blade and stem EW chemistry of the 25-d-old wild-type WS and near-isogenic T-DNA-induced *cer* lines. Chemical classes and chain-length distributions are labeled on the horizontal axis for each near-isoline. The total EW amount for each chemical class in $\mu g\ dm^{-2}$ of surface area is labeled on the vertical axis for each line. Where the EW constituent amount was off the scale, a number designating the actual value is presented next to the bar. Sec Alc, Secondary alcohol.

The C_{30} acid on *cer8* leaves was slightly higher than on wild-type leaves but was increased by more than 250-fold on *cer8* stems over the wild type (Fig. 3). The total amount of other EW classes on *cer8* leaves and stems was lower than in the wild type (Table I; Fig. 3).

The amount of EW secondary alcohols and ketones on all *cer* mutants was reduced in direct proportion to the reduction of precursor alkanes below the wild type (Table I; Figs. 2 and 3).

The amount of EW esters increased on the leaves of *cer4*, *cer8*, *cer9*, and *cer16* over their corresponding wild types (Table I; Figs. 2 and 3). The amount of EW esters also increased on stems of *cer2*, *cer6*, *cer8*, *cer9*, and *cer16* (Table I; Figs. 2 and 3). In contrast, the amount of EW esters on *cer4* was reduced by 8-fold below that of the wild type (Table I; Fig. 2).

The amount of amyrins on the stems of *cer16* increased 3-fold above wild type (Table I). The amount of triterpe-

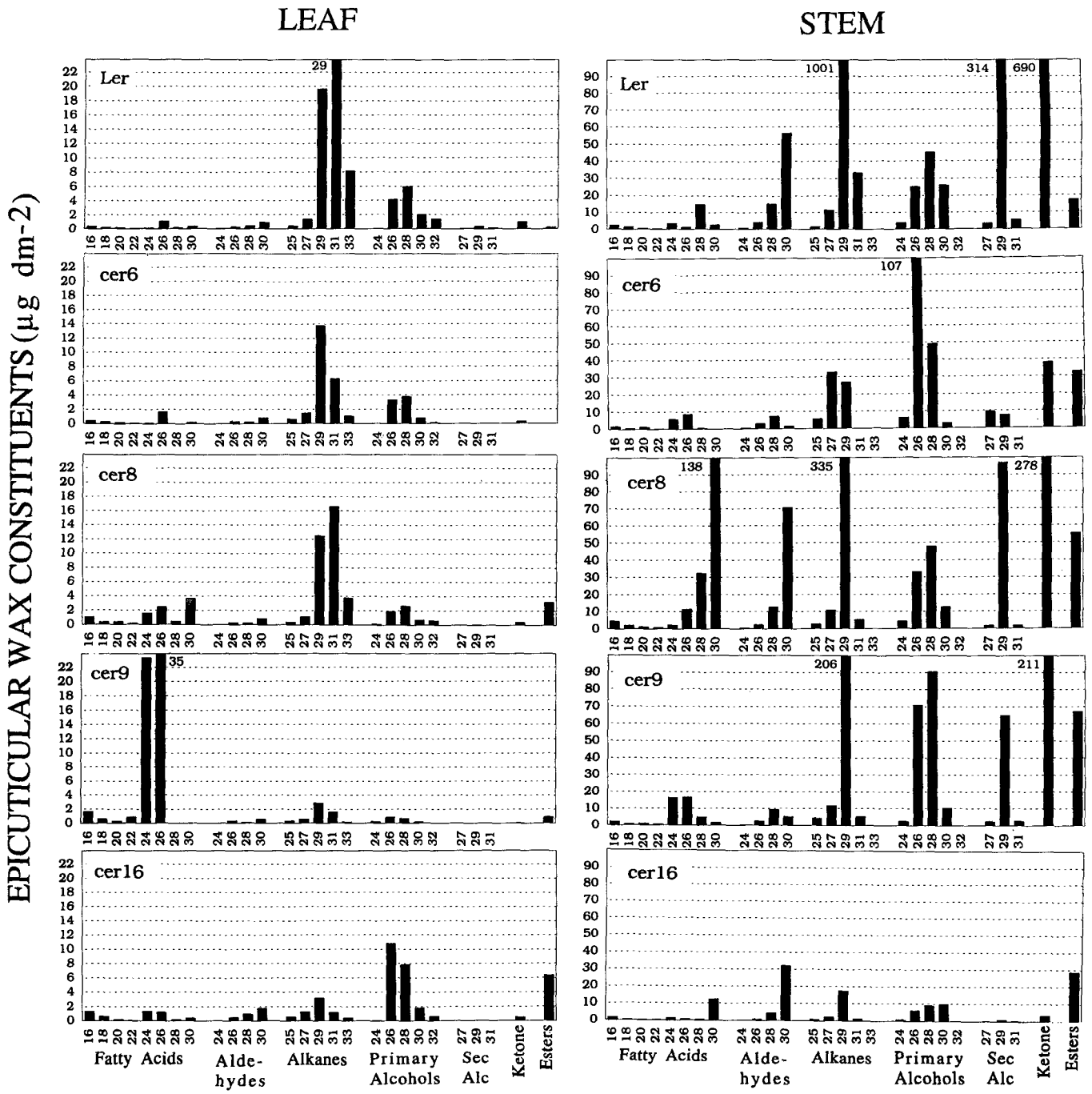

Figure 3. Leaf blade and stem EW chemistry of the 25-d-old wild-type Ler and near-isogenic *cer* lines. Labeling is as in Figure 2. Sec Alc, Secondary alcohol.

noids (terpenes) and unidentified peaks changed little on other *cer* leaves and stems (Table I).

DISCUSSION

Comparing our comprehensive description of leaf and stem EW chemical constituents on *A. thaliana* wild type and *cer* mutants with previous mutation analyses (von Wettstein-Knowles, 1982; Bianchi et al., 1985; Hannoufa et al., 1993) and radioactive labeling studies (Kolattukudy, 1975; von Wettstein-Knowles, 1982), we have developed a rudimentary model to explain the involvement of *CER* genes in leaf EW biosynthesis by *A. thaliana* (Fig. 4). Elucidation of the biochemical, secretory, and physiological function of leaf *CER* gene products will provide a basis for predicting which *CER* genes will be useful for genetic engineering to modify leaf surface waxes to increase environmental stress tolerance.

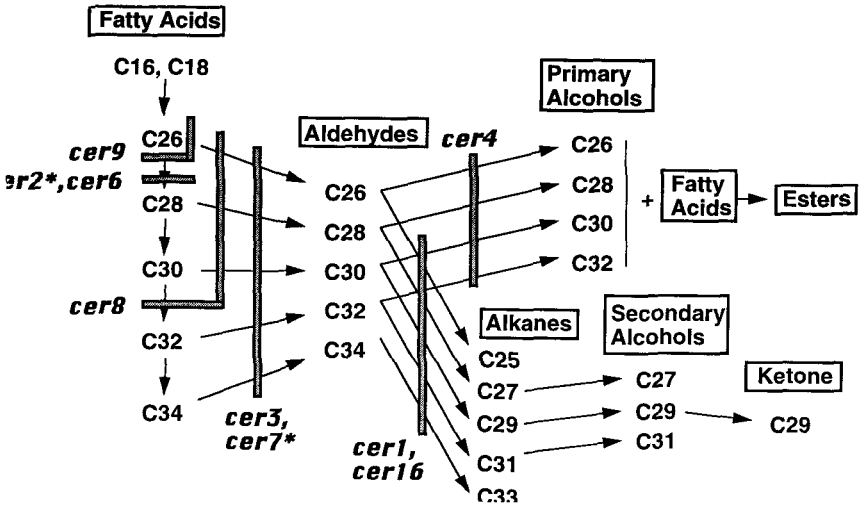

Figure 4. Model for substrate modification in the biosynthesis of leaf EW on *A. thaliana* showing putative lesions caused by *cer* mutations. Stem EW biosynthesis lacks the C_{32} 1-alcohol and the branch of the pathway beginning with the C_{34} fatty acid. The asterisks indicate *cer* mutants that have altered stem EW but not leaf EW.

Differences between the EW on leaves and stems of *Arabidopsis* suggest that EW biosynthesis is differentially regulated in these tissues. Compared with the EW on wild-type stems, EW on wild-type leaves had a much higher proportion of their total EW load in the form of alkanes and 1-alcohols; a large reduction in secondary alcohols, ketones, and esters; and a chain-length distribution for major EW classes that was skewed toward longer lengths. Wild-type stem surfaces were also glaucous and were covered by EW microstructures, whereas the wild-type and *cer* abaxial and adaxial leaf surfaces were glossy and lacked EW microstructures. Previous studies showed that the increase in glossiness on *cer* stems was correlated with a reduction in the density of optically refractive wax crystals (Koornneef et al., 1989).

Differential regulation in leaves and stems was also suggested by the finding that *cer2* and *cer7* gene mutations did not appear to affect leaf EW. The expression of these gene products appeared to be stem specific. Potentially, these genes (or gene products) may be involved in the regulation of events leading toward the production of stem-type EW during the developmental change from vegetative to reproductive growth.

Elongation of EW carbon chains is thought to occur in fatty acyl-CoA elongase complexes, which have similar function to the FAS complex (von Wettstein-Knowles, 1982; Bessoules et al., 1989). Fatty acyl-CoA reductase is thought to convert elongase products to aldehydes (Kolattukudy, 1971; Anderson et al., 1992). Aldehydes are thought to be either reduced by an aldehyde reductase to produce the 1-alcohols (Kolattukudy, 1971) or decarbonylated by an aldehyde decarbonylase to produce alkanes (Cheesbrough and Kolattukudy, 1984). In *Arabidopsis* wild type and *cer* mutants, differences in the chain-length distribution between leaves and stems of the major EW classes were probably related to differences in elongation by the leaf and stem elongation complexes. The presence of C_{33} alkanes on *Arabidopsis* leaves indicates that C_{32} fatty acid elongation to the C_{34} fatty acids must have occurred (since C_{34} acid is a precursor to the C_{33} alkane). Similar evidence for C_{32} elongation activity on stems was not present.

The fatty acyl-CoA reductase and aldehyde reductase in *Brassica* (Kolattukudy, 1971) and the aldehyde decarbonylase in *Pisum* apparently lacked chain-length specificity for their substrates (Bognar et al., 1984) and, therefore, modified fatty acyl precursors independently of chain length. On both leaves and stems of wild-type *Arabidopsis*, differences in the major chain lengths of alkanes and 1-alcohols suggested that different length aldehyde substrates were most efficiently utilized by the reductase and decarbonylase enzyme complexes. These differences may be due to chain-length specificity of the enzymes for their substrates and/or substrate transfer.

Previous mutation analyses (Baker, 1974; Bianchi et al., 1985; Hannoufa et al., 1993; Rich, 1994) and radioactive labeling studies (Kolattukudy and Brown, 1974; Cassagne and Lessire, 1978; Lessire, et al., 1985) suggested that each C_2 unit addition to the EW fatty acyl chain was accomplished by a unique chain-length-specific fatty acyl-CoA elongase complex rather than a single complex that uses various chain-length substrates such as FAS. McNevin et al. (1993) proposed that the *cer2* mutation may alter the *Arabidopsis* C_{26} elongase complex. We agree that the *cer2* mutation influences the C_{26} fatty acyl elongation step in stem EW biosynthesis. However, *cer2* does not affect C_{26} elongation in the leaf EW. Therefore, the *CER2* gene product may be a stem-specific regulator of the C_{26} elongase complex. The *cer6* mutation, by comparison, affects the C_{26} fatty acyl elongation step in both the leaf and stem. The *CER6* gene product could serve as a unit of the C_{26} elongase complex.

Pollard et al. (1991) implicated an acyl-ACP thioester hydrolase from bay laurel in the cleavage of acyl chains from ACP (ACP for FAS), terminating elongation to produce medium-chain-length fatty acids. Theoretically, a fatty acyl-CoA-specific thioester hydrolase would be required for release of long-chain fatty acids from the carrier protein CoA used in EW biosynthesis. We postulate that the reduced amount of free fatty acids and increased chain lengths in other EW classes on leaves and stems of *cer3* could be explained by inhibited hydrolysis of fatty acyl-CoA to free fatty acid and CoA. Such inhibition could

prevent both release of fatty acids and termination of elongation. Like fatty acids, aldehydes are also low relative to the other major EW constituent classes on *cer3*. The aldehydes are an important pool in the EW biosynthetic pathway, since they can be converted to both alkanes and 1-alcohols. The relatively low amount of surface aldehydes on *cer3* may reflect rapid conversion by aldehyde reductase and aldehyde decarbonylase of the aldehyde pool that was depleted by inhibited fatty acid release from the sites for elongation.

The inhibited production of 1-alcohols by *cer4* leaves and stems suggests that the *CER4* gene product may be involved in aldehyde reduction. This interpretation agrees with that of McNevin et al. (1993) and Hannoufa et al. (1993) regarding *CER4* gene product function in stem EW biosynthesis. In addition, we report that the *CER4* gene mutation has similar effects on both leaf and stem EW.

Other *cer* mutations altered multiple pools of EW constituents in a manner that may be explained by disruption of substrate-transfer functions. Evidence that lipid-transfer proteins may be involved in EW production was recently provided by Pyee et al. (1994), who identified a lipid-transfer protein in the EW layer of *Brassica*. McNevin et al. (1993) showed that the *cer1* mutation inhibited aldehyde conversion to alkanes on stems of 21-d-old plants and suggested that the *CER1* gene codes for a decarbonylase. However, our results showed a large reduction in stem 1-alcohols as well as alkanes on 25-d-old plants. McNevin et al. (1993) detected a proportional reduction in 1-alcohols on stems after 42 d but did not address this result when proposing the *CER1* gene product function. We suggest that the *CER1* gene product may be involved in a developmentally regulated transfer of aldehydes to both the decarbonylase and reductase. The *CER16* gene product may have a transfer function similar to that of *CER1* but may regulate 1-alcohol synthesis differently in leaves and stems. The *cer16* leaf 1-alcohols were much higher than wild-type 1-alcohols, whereas *cer1* leaf 1-alcohols were similar to those of the wild type.

The *cer9* mutation appeared to alter chain-length-specific transfer of fatty acyl chains through the EW biosynthetic pathway. Leaves of *cer9* lacked free fatty acids longer than 26 carbons and were reduced in all chain lengths of alkanes and 1-alcohols. Potentially, the *CER9* gene product may be involved in the transfer of C_{26} fatty acids to both the subsequent elongase complexes and the modifying enzymes further down the leaf EW biosynthetic pathway. In addition, whereas leaves of *cer9* had reduced 1-alcohols, stems of *cer9* had higher amounts of 1-alcohols. Therefore, the *CER9* gene product, like *CER16*, may be involved in tissue-specific transfer of EW substrates.

Like *cer9*, the *cer8* mutation also appeared to affect chain-length-specific transfer of fatty acyl chains to subsequent sites for modification in the EW biosynthetic pathway. The increased amount of fatty acids on *cer8* leaves (and especially stems) with a concomitant decrease in aldehydes and alkanes suggests that acyl chains were not being transferred from the elongase complex for conversion by fatty acyl-CoA reductase and aldehyde decarbonylase. Potentially, the *cer8* mutation alters a protein specifically involved in fatty acid transfer.

Complex *cer* phenotypes could also result from single mutations affecting transcription or other regulatory functions. Until evidence for this type of regulation is presented in association with plant EW production, speculation about such mechanisms will be premature.

Secondary alcohols and ketones on the surface of plants are presumed to be generated by a putative alkane hydroxylase and 15-secondary alcohol oxidase (Kolattukudy et al., 1973). Plant EW esters are thought to arise from transacylation by a putative acyl-CoA-fatty alcohol transacylase (Kolattukudy, 1970). None of the *cer* mutations appeared to specifically alter secondary alcohol, ketone, or ester production except by altering upstream elements in the biosynthetic pathway. For example, the reduction in *cer4* stem 1-alcohols may explain the reduction in *cer4* stem esters.

Since plant EW plays an important role in resistance to insects and other environmental stresses, understanding the function of *CER* gene products in EW production may lead to novel genetic approaches to crop improvement. Leaves are usually the first plant tissues to be damaged by environmental stress; therefore, the *CER* genes that are expressed in the leaf may be the most useful for overall crop improvement. Moreover, these leaf EW mutants can provide powerful tools for elucidating the role of specific plant EW chemical constituents on insect behavior and plant responses to a variety of other biotic and abiotic stresses.

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