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Phytochemistry 66 (2005) 771–780
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Wax constituents on the inflorescence stems of double *eceriferum* mutants in Arabidopsis reveal complex gene interactions

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Received 2 September 2004; received in revised form 9 November 2004

Abstract

To shed new light on gene involvement in plant cuticular-wax production, 11 *eceriferum* (*cer*) mutants of Arabidopsis having dramatic alterations in wax composition of inflorescence stems were used to create 14 double *cer* mutants each with two homozygous recessive *cer* loci. A comprehensive analysis of stem waxes on these double mutants revealed unexpected *CER* gene interactions and new ideas about individual *CER* gene functions. Five of the 14 double *cer* mutants produced significantly more total wax than one of their respective *cer* parents, indicating from a genetic standpoint a partial bypassing (or complementation) of one *cer* mutation by the other. Eight of the 14 double *cer* mutants had alkane amounts lower than both respective *cer* parents, suggesting that most of these *CER* gene products play a major additive role in alkane synthesis. Other results suggested that some *CER* genes function in more than one step of the wax pathway, including those associated with sequential steps in acyl-CoA elongation. Surprisingly, complete epistasis was not observed for any of the *cer* gene combinations tested. Significant overlap or redundancy of genetic operations thus appears to be a central feature of wax metabolism. Future studies of *CER* gene product function, as well as the utilization of *CER* genes for crop improvement, must now account for the complex gene interactions described here.

Keywords: Arabidopsis; Cruciferae; Biochemical-genetic; Double mutant; Cuticular wax; *Eceriferum*

1. Introduction

Arabidopsis thaliana has a typical plant cuticular wax composition, and previous studies show how Arabidopsis provides a model system for elucidating gene control over plant wax synthesis (Jenks et al., 2002). All but one of the typical *eceriferum* mutants in Arabidopsis (*cer1–cer24*) were identified in mutagenized populations based on reductions in the visible glaucousness of the inflorescence stems. Over 81% of the constituents on wild-type stems are *n*-alkanes and their metabolites secondary alcohols and ketones, with nearly all *cer* mutants most deficient in these constituents. Long-chain acyl-coenzymeA (CoA), the initial wax precursors, arise by elongation of CoA activated C_{16} and C_{18} acyl-chains by consecutive addition of two-carbon units, with previous reports suggesting that each elongation step is performed by a separate microsomal elongase complex (Liu and Post-Beittenmiller, 1995; Kunst and Samuels, 2002). Acyl-CoAs serve as direct precursors for synthesis of free fatty acids, primary alcohols (Vioque and

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doi:10.1016/j.phytochem.2005.02.001

Kolattukudy, 1997) and esters (Kolattukudy, 1967). The major flux in the wax pathway however occurs with the conversion of the very long chain acyl-CoAs to aldehydes by an acyl-CoA reductase (Vioque and Kolattukudy, 1997), and then aldehyde conversion to alkanes by an aldehyde decarbonylase (Schneider-Belhaddad and Kolattukudy, 2000). A large part of the alkane pool is converted to secondary alcohols, which are then converted to ketones by two separate oxidases, respectively (Kolattukudy et al., 1973). Interestingly, none of the single *cer* mutants are completely absent in any wax constituent class even though many have null *cer* alleles (Jenks et al., 1995, 1996; Rashotte et al., 2001, 2004), and four of the six known wax gene sequences appear to encode metabolic proteins (Jenks et al., 2002; Chen et al., 2003). Whether *cer* mutants are able to maintain a low flux in all branches of the wax pathway by utilizing redundant enzymatic, secretory, or regulatory mechanisms, compensation by related enzymes, or whether multiple distinct wax production pathways exist in Arabidopsis, is still unknown.

Progress in understanding gene involvement in wax metabolism has been hindered by difficulties in accurately describing wax gene product functions based on their amino acid sequence homologies with known proteins (Jenks et al., 2002). Moreover, most *CER* genes are known only by chemical mutagenesis alleles, and so the cloning of these genes and subsequent elucidation of their product functions will require many years for completion. To advance our understanding of the genetic operations governing wax metabolism in the absence of these more detailed molecular-genetic studies, 11 Arabidopsis *cer* mutants having dramatic wax compositional changes and affecting both early and late steps in the wax metabolic pathway of the inflorescence stem were used to create 14 double *cer* mutants having two recessive *cer* loci in homozygosity. The stem wax constituents on these double mutants are used here to provide the first analysis of *cer* gene interactions, and to shed new light on the genetic framework underlying *CER* gene control over cuticular wax production.

2. Experimental

2.1. Plant material

Arabidopsis thaliana (L.) Heynhold ecotype Landsberg *erecta* (*Ler*), and respective isogenic *cer* mutants, were grown in the horticulture growth chambers at Purdue University under constant light (125 μmol m^{-2} s^{-1}), temperature (22 °C day/19 °C night) and average relative humidity (47 ± 14% day/59 ± 18% night). Wassilewskija (WS) wild types, and respective isogenic *cer* mutants, were grown in growth chambers at the University of Arizona (22 °C, 16-h photoperiod, ~240 μmol m^{-2} s^{-1}, 75–90% RH). Data was obtained using aspirated temperature and humidity thermistors and logged every 5 min by Priva Precision VI software (Priva Computers, Inc., Vineland Station, Ontario). The single *cer* mutants in the WS background (*cer1-cer4*) were produced using T-DNA insertion mutagenesis as described in McNevin et al. (1993), whereas all Ler derived *cer* mutants (*cer6–cer9*, *cer13*, *cer19*) were produced using chemical mutagenesis as described in Koornneef et al. (1989). Two single recessive loci *cer* mutants from the same isogenic background were hybridized to create F_1 plants (all F_1 showed full complementation of the wild-type glaucous stem), and these were self-pollinated to produce an F_2 population. The single cer mutants were selected to represent early and late steps in the putative wax metabolic pathway, and combined in a way that would best allow the testing for possible epistatic relationships. To find double mutants, 25–40 plants with a *cer* phenotype from each F_2 generation (expected 9:7 segregation, wild-type:*cer*) were examined using gas-chromatography to select putative doubles having wax composition changed from both *cer* parents. Plants selected from the F_2 that showed clear wax compositional deviation from both *cer* parental lines were backcrossed to both *cer* parents and at least 15 plants scored from each for visible stem wax phenotype. In two cases where the putative double mutants could not be identified with confidence based on wax composition, approximately 20 F_2 plants were individually crossed to each *cer* parent and the F_1 backcross population (of at least 15 plants from each *cer* parental backcross) scored for presence or lack of visible wax deposition on the stem. Putative doubles producing only glossy (bright green with reduced visible wax) stem phenotypes in backcross populations made to both original *cer* parents verified their subsequent designation as a double mutant.

2.2. Chemical analysis of cuticular waxes

Waxes were collected from mid-flowering stems of 30–33-day-old plants derived from the *Ler* ecotype, and 25-day-old plants derived from more early-maturing WS ecotype. Stem samples were inserted into a standard glass vial 20 and 12 ml GC grade hexane approximately added. The tissues were agitated for 30 s and the solvent decanted off into new vials. Tissues and vials were given a 1 s rinse with hexane approximately 2 ml and then the solution decanted into the sample vial. The hexane-soluble cuticular wax extracts were evaporated to dryness under a N_2 stream and the dried residue prepared for gas chromatography by derivatization using BSTFA [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide] (Jenks et al., 1995). Derivatization was for 15 min at 100 °C. After surplus BSTFA was evaporated under nitrogen, the sample was redissolved in hexane for

analysis with a Hewlett–Packard 5890 series II gas chromatograph (GC) equipped with a flame ionization detector (FID). The GC was equipped with a 12 m, 0.2 mm HP-1 capillary column with helium as the carrier gas. The GC was programmed with an initial temperature of 80 °C and increased at 15 °C min^{-1} to 260 °C, where the temperature remained unchanged for 10 min. The temperature was then increased at 5 °C min^{-1} to 320 °C, where the temperature was held for 15 min. Quantification was based on FID peak areas and the internal standard hexadecane. Specific correction factors were developed from external standards and applied to the peak areas of the free fatty acids, primary alcohols, and alkanes. For all other peaks, a factor of 1.03 was assigned (the average correction for 9 standards at comparable concentrations). The total amount of wax was expressed per unit stem surface area, calculated by measuring the length and width of stem segments. All values represent the average of three or four replicate plants, after verification in test-crosses and advancement to the F_3 generation. Selected subsamples were analyzed in a gas chromatograph-mass spectrometer (FinniganMAT/Thermospray Corporation, San Jose, CA). Wax amounts were analyzed using the Student *t* test with proportional (*p*) values included as needed.

3. Results

3.1. Total wax amount

Total wax amounts in the two ecotypes Wassilewskija (WS) and Landsberg *erecta* (Ler) was 1580 μg dm^{-2} (Table 1) and 2576 μg dm^{-2} (Table 1), respectively. Ecotype Wassilewskija is the isogenic parent for *cer1, cer2, cer3*, and *cer4* and ecotype Landsberg *erecta* is the isogenic parent for *cer5, cer6, cer7, cer8, cer9, cer13*, and *cer19*. The average standard deviation (s.d.) for the total wax amounts, class amounts, and amount of individual constituents for all lines tested was 8.3% of the mean. Other important statistically significant differences are noted. In the single *cer* mutants, total wax on inflorescence stems ranged from 123 μg dm^{-2} (7.8% of WS) in *cer1* to 1548 μg dm^{-2} (98% of WS) in *cer4* (Table 1). *cer4* wax amount is equivalent to wild-type ($p = 0.83$), whereas all other reductions in total wax amount are statistically significant ($p \leqslant 0.05$). Only four of 14 new *cer* double mutants, *cer2 cer4*, *cer3 cer4*, *cer5 cer8*, and *cer5 cer9* (Table 1), had a total wax amount lower than the lowest respective *cer* parent ($p \leqslant 0.05$). Interestingly, five double *cer* mutants, *cer1 cer3*, *cer2 cer3*, *cer6 cer9*, *cer8 cer9*, and *cer8 cer19*, produced significantly more total waxes than one of the original *cer* parents

Table 1
Cuticular waxes on Wassilewskija (WS) and Landsberg (Ler) ecotype, and single and double mutants, expressed as amounts of each wax class and total wax (μg dm^{-2})

Genotype	Acid	Aldehyde	1-Alcohol	Alkane	2-Alcohol	Ketone	Ester	Total
WS	29.7	52.3	120	759	179	405	35.0	1580
cer1	21.2	32.3	39.4	13.3	5.0	6.8	5.1	123
cer2	17.6	2.7	182	15.0	4.8	5.9	13.2	241
cer3	11.9	10.9	238	229	43.1	133	27.0	693
cer4	20.6	150	12.6	842	157	358	8.1	1548
cer1 cer2	17.5	1.3	95.4	17.0	10.4	1.7	66.5	210
cer1 cer3	14.3	7.5	155.7	16.3	17.0	0.4	74.2	285
cer1 cer4	19.1	48.1	22.5	7.9	6.9	0.1	20.7	125
cer2 cer3	16.7	8.6	517	13.5	4.3	1.6	44.5	606
cer2 cer4	9.5	9.1	80.9	22.1	7.8	1.6	36.5	168
cer3 cer4	8.0	5.6	5.7	120.2	18.4	66.3	9.7	234
Ler	25.2	81.8	263	1271	154	694	86.7	2576
cer5	16.7	20.4	183	269	80.5	368	71.6	1009
cer6	43.3	42.8	272	23.1	9.3	4.5	53.7	449
cer7	15.9	13.8	241	220	23.3	156	26.0	696
cer8	519	64.1	208	514	65.4	323	77.5	1771
cer9	116	51.9	342	302	36.6	229	111	1188
cer13	20.0	16.0	220	403	44.2	258	39.4	1001
cer19	35.8	58.6	172	411	32.5	252	77.0	1040
cer5 cer8	106	38.6	89.1	40.3	15.5	68.0	79.6	437
cer5 cer9	66.0	12.5	171	21.1	20.8	87.5	99.1	478
cer6 cer9	629	45.2	128	9.7	4.9	3.9	99.0	920
cer7 cer8	146	21.7	333	111	24.3	84.4	38.0	758
cer8 cer9	1046	45.7	280	62.4	14.3	54.6	136	1638
cer8 cer13	239	17.4	278	218	29.2	152	48.0	981
cer8 cer19	416	82.6	177	550	48.6	314	71.6	1659
cer9 cer19	143	51.9	297	309	37.4	210	56.5	1105

1- and 2-alcohol, primary and secondary alcohol, respectively; ketone, C_{29} ketone.

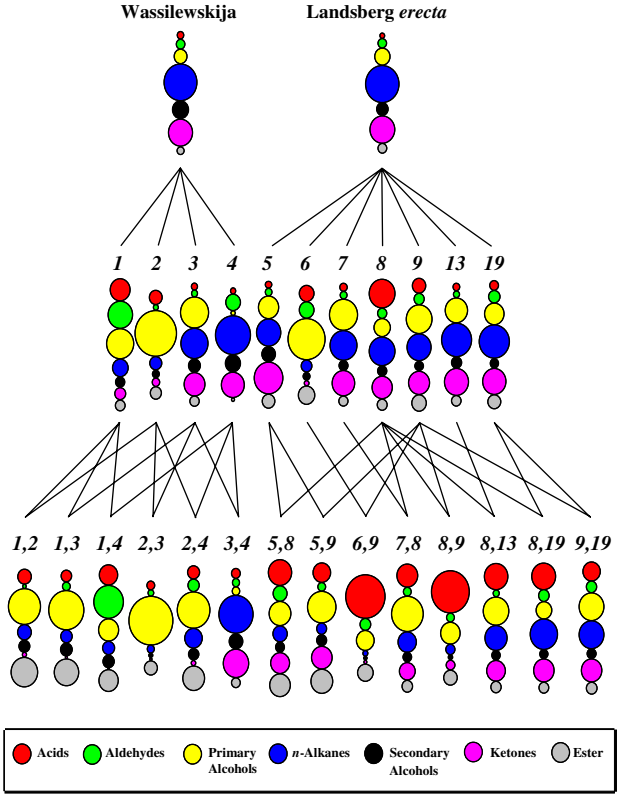

Fig. 1. Illustrative overview of major effects of single and double *cer* mutations on stem wax profiles. Oval size represents the proportion of each stem wax class relative to total amount per area of the specified wild-type or mutant. Ecotype Wassilewskija is the isogenic parent for *cer1*, *cer2*, *cer3*, and *cer4* and ecotype Landsberg *erecta* is the isogenic parent for *cer5*, *cer6*, *cer7*, *cer8*, *cer9*, *cer13*, and *cer19*, with the *cer* label omitted and double mutant loci separated by comma.

($p \leqslant 0.05$; Table 1). No double *cer* mutants produced more waxes than both respective *cer* parents (Table 1).

3.2. Wax class amounts

As one means to visualize the major relative changes in wax class pool sizes caused by single and double *cer* mutations, an illustrative diagram is presented wherein oval size represents the proportion of each stem wax class relative to total wax amount per area of the specified wild-type or mutant (Fig. 1). The general trend in all single mutants, except *cer4*, is a reduction in the alkane pool size and an increase in the ester, primary alcohol, and/or acid pools (Fig. 1). Double mutants show a trend toward further reduction in alkane proportions and further increases in the relative acid, primary alcohol, and ester pools. In all single and double mutants, the reduction in the secondary alcohol and ketone pools is directly proportional to the reduction in the alkane pool, except in single and double mutants containing *cer5* where secondary alcohol and ketone pools actually increase relative to the alkane pool (Fig. 1, Table 1).

The amount of alkanes on WS and Ler were 759 μg dm^{-2} (48% of total) and 1271 μg dm^{-2} (49% of total), respectively (Table 1). Except for *cer4*, all the single and double *cer* mutants showed a reduction in the amount of these alkanes relative to wild-type ranging from 7.9 μg dm^{-2} (1.0% of WS) in *cer1 cer4* and 9.7 μg dm^{-2} (0.8% of Ler) in *cer6 cer9* (Table 1) to 550 μg dm^{-2} (43.3% of Ler) in *cer8 cer 19* (Table 1). *cer4* alkanes were statistically equivalent to wild-type ($p = 0.15$; Table 1). Amounts of the alkane metabolites, the secondary alcohols and ketones, are reduced in all single and double *cer* mutants ($p \leqslant 0.05$; Table 1). Compared to wild-type, fatty acid amounts are reduced in all single and double mutants except *cer6*, *cer8*, *cer9*, *cer19*, *cer5 cer8*, *cer5 cer9*, *cer6 cer9*, *cer7 cer8*, *cer8 cer9*, *cer8 cer13*, *cer8 cer19*, and *cer9 cer19*, aldehyde amounts are reduced in all lines except *cer4, cer1 cer4*, and *cer8 cer19* ($p \leqslant 0.05$; Table 1), primary alcohol amounts are reduced in all lines except *cer2*, *cer3*, *cer6*, *cer9*, *cer1 cer3*, *cer2 cer3*, *cer7 cer8*, *cer8 cer9*, *cer8 cer 13*, and *cer9 cer19*, and, esters are reduced in all lines except *cer8*, *cer9*, *cer1 cer2*, *cer1 cer3*, *cer2 cer3*, *cer2 cer4*, *cer5 cer8*, *cer5 cer9*, *cer6 cer9*, and *cer8 cer9* ($p \leqslant 0.05$; Table 1).

Table 2
Cuticular wax free fatty acids on Wassilewskija (WS) and Landsberg (Ler) ecotype, and single and double mutants, expressed as amounts (μg dm^{-2})

Genotype	Acids							
	16	18	20	22	24	26	28	30
WS	3.4	2.8	1.2	0.4	1.4	11.8	7.0	1.8
cer1	2.4	2.3	0.0	0.9	2.0	3.1	2.5	8.0
cer2	2.4	2.0	0.0	0.0	0.4	9.9	2.9	0.0
cer3	1.9	1.9	0.3	0.5	0.7	1.7	4.9	0.0
cer4	1.9	1.7	0.3	0.0	0.8	5.0	6.7	4.2
cer1 cer2	0.1	0.0	0.0	0.1	9.3	8.1	0.0	0.0
cer1 cer3	0.4	0.0	2.2	0.0	10.9	0.8	0.0	0.0
cer1 cer4	0.1	0.0	0.3	0.0	5.6	11.4	0.0	1.7
cer2 cer3	3.4	4.1	1.3	0.0	1.2	4.0	2.8	0.0
cer2 cer4	0.1	0.0	2.6	0.0	0.0	6.9	0.0	0.0
cer3 cer4	2.9	2.6	0.7	0.0	0.0	0.0	1.8	0.0
Ler	3.2	1.4	0.7	0.3	2.6	2.6	11.5	3.0
cer5	1.9	0.7	0.5	0.4	3.9	3.4	5.8	0.1
cer6	3.9	1.7	1.4	1.1	7.6	16.7	10.8	0.1
cer7	3.4	1.0	0.4	0.3	2.1	1.7	6.3	0.6
cer8	2.9	1.4	1.5	1.6	18.4	32.6	200.3	260.1
cer9	2.5	1.3	0.7	0.8	36.8	40.1	21.5	12.7
cer13	3.7	1.0	0.5	0.3	2.5	1.5	6.5	3.9
cer19	3.2	1.2	0.7	0.7	7.0	5.9	12.8	4.2
cer5 cer8	19.7	13.6	4.6	2.4	14.1	11.9	26.7	13.2
cer5 cer9	3.5	1.6	1.1	1.9	19.9	26.0	10.6	1.4
cer6 cer9	3.4	1.4	2.0	18.6	528	64.8	10.5	0.7
cer7 cer8	11.3	4.0	2.3	1.7	11.3	9.5	25.7	79.7
cer8 cer9	3.8	2.1	2.0	6.4	220	246	393	173
cer8 cer13	5.1	2.8	2.1	1.0	13.9	16.2	57.3	141
cer8 cer19	3.5	2.2	1.6	2.0	21.5	30.8	164	191
cer9 cer19	3.4	1.8	0.9	1.6	54.8	48.2	22.9	9.6

Eight of the 14 double *cer* mutants had alkane amounts that were lower than both of the respective *cer* parents ($p \leqslant 0.05$; Table 1). *cer1 cer2* and *cer8 cer19* had alkane amounts that were equivalent to the highest alkane parent, while *cer1 cer3*, *cer2 cer3*, and *cer9 cer 19* alkane amounts were equivalent to that of the parent having the lowest alkane amount ($p > 0.05$; Table 1). Acid amounts in double mutants did not vary greatly from the single *cer* parents, except for *cer6 cer9, cer8 cer9*, and *cer9 cer19*, which showed a remarkable synergistic effect, having acid amounts that were 2500%, 4150% and 1650%, respectively, of wild-type amounts (Table 1). When *cer5*, *cer7*, and *cer13* were each combined with *cer8*, respectively, they reduced the increase in acids that was caused by the *cer8* mutation ($p \leqslant 0.05$; Table 1). As for aldehydes, it was noted that when *cer1*, *cer2*, and *cer3* were each combined in double mutants with *cer4*, respectively, each reduced the increase in aldehydes that resulted from the *cer4* mutation ($p \leqslant 0.05$; Table 1). Further, *cer5 cer9* showed much lower aldehydes than both respective *cer* parents ($p \leqslant 0.05$; Table 1). One double, *cer2 cer3* had a synergistic increase in primary alcohols producing more primary alcohols than the sum of primary alcohols on the single *cer2* and *cer3* mutants together ($p \leqslant 0.05$), and one double, *cer7 cer8*, had additive effects on primary alcohol amounts, producing more primary alcohols than either *cer* parent but less than the sum of both parents ($p \leqslant 0.05$). Interestingly, *cer3 cer4*, *cer5 cer8*, and *cer6 cer9* had primary alcohol amounts significantly lower than those of both parents, respectively ($p \leqslant 0.05$; Table 1). Four double *cer* mutants, *cer1 cer2*, *cer1 cer3*, *cer1 cer4*, and *cer2 cer4* had a synergistic increase in ester amounts relative to both *cer* parents, and one double mutant, *cer9 cer 19*, had ester amounts lower than both parents ($p \leqslant 0.05$; Table 1). Ester amounts of all other doubles were not significantly different from one or both of the parents ($p > 0.05$; Table 1).

3.3. Wax homologue chain length distributions

The most notable changes in acid chain length amount in the WS ecotype mutants occurred as an increase in the 24 carbon acids on *cer1 cer2*, *cer1 cer3*, and *cer1 cer4* (Table 3). In the Ler ecotype mutants, an increase in C_{20} to C_{30} acids on *cer8* was notable, with an especially large increase in the 30-carbon acid to

Table 3
Cuticular wax aldehydes on Wassilewskija (WS) and Landsberg (Ler) ecotype, and single and double mutants, expressed as amounts (μg dm^{-2})

Amount	Aldehydes			
	24	26	28	30
WS	0.4	2.5	6.3	43.1
cer1	0.0	0.0	1.7	30.6
cer2	0.0	1.1	1.6	0.0
cer3	0.0	1.3	4.1	5.5
cer4	0.0	3.8	6.0	140
cer1 cer2	0.0	1.0	0.0	0.4
cer1 cer3	0.0	2.1	0.7	4.6
cer1 cer4	0.0	3.8	0.0	44.3
cer1 cer3	0.0	2.1	5.0	1.5
cer2 cer4	0.0	6.3	2.3	0.5
cer3 cer4	0.0	0.0	4.8	0.7
Ler	1.4	6.8	19.5	54.1
cer5	1.1	7.6	5.4	6.3
cer6	4.4	26.8	10.0	1.5
cer7	0.9	4.5	3.4	5.0
cer8	1.5	6.7	21.0	34.9
cer9	1.1	12.4	23.9	14.5
cer13	0.8	2.8	3.8	8.6
cer19	5.8	13.5	26.5	12.7
cer5 cer8	1.3	31.7	3.0	2.6
cer5 cer9	0.9	6.1	2.3	3.1
cer6 cer9	15.4	23.8	4.5	1.5
cer7 cer8	1.2	13.3	2.8	4.4
cer8 cer9	1.2	13.5	22.3	8.7
cer8 cer13	1.0	2.9	4.1	9.4
cer8 cer19	2.1	7.6	28.6	44.4
cer9 cer19	2.1	14.2	24.8	10.9

Table 4
Cuticular wax primary alcohols on Wassilewskija (WS) and Landsberg (Ler) ecotype, and single and double mutants, expressed as amounts (μg dm^{-2})

Genotype	Primary alcohols			
	24	26	28	30
WS	3.1	28.9	55.4	32.3
cer1	0.8	7.6	12.3	18.8
cer2	0.7	125	55.1	1.7
cer3	1.0	19.9	43.9	173
cer4	0.1	1.7	1.9	8.8
cer1 cer2	0.7	42.8	49.3	2.5
cer1 cer3	1.4	14.1	65.6	74.5
cer1 cer4	2.1	3.3	16.6	0.5
cer2 cer3	1.6	277	234	4.8
cer2 cer4	0.5	44.2	35.6	0.6
cer3 cer4	0.0	0.0	0.0	5.7
Ler	7.1	56.7	137.6	61.2
cer5	3.7	47.9	106.5	24.7
cer6	20.7	201	49.1	1.7
cer7	3.3	38.2	72.5	127
cer8	9.3	58.6	121	18.8
cer9	5.8	118	197	21.3
cer13	2.8	24.3	62.8	130
cer19	16.0	57.2	85.1	14.1
cer5 cer8	4.1	33.0	45.0	7.1
cer5 cer9	3.8	84.0	68.6	14.2
cer6 cer9	36.1	82.8	8.1	1.1
cer7 cer8	6.5	68.4	112	146
cer8 cer9	7.2	139	129	4.6
cer8 cer13	4.3	36.7	83.9	153.1
cer8 cer19	9.4	54.7	95.5	17.0
cer9 cer19	9.2	113	156	19.1

Table 5
Cuticular wax alkanes on Wassilewskija (WS) and Landsberg (Ler) ecotype, and single and double mutants, expressed as amounts (μg dm^{-2})

Amount	Alkanes			
	25	27	29	31
WS	0.6	6.8	735	16.8
cer1	0.5	0.0	10.2	2.6
cer2	0.5	9.9	3.8	0.8
cer3	0.8	1.3	182	45.1
cer4	0.8	7.8	812	21.4
cer1 cer2	0.6	9.0	2.6	4.8
cer1 cer3	0.6	2.2	2.7	10.7
cer1 cer4	0.7	1.9	2.2	3.1
cer2 cer3	0.7	4.5	7.0	1.4
cer2 cer4	0.7	14.1	2.5	4.8
cer3 cer4	0.6	0.4	72.3	46.9
Ler	0.5	13.2	1196	61.3
cer5	0.3	4.0	228	36.2
cer6	0.2	14.3	5.5	3.0
cer7	0.5	2.2	211	7.0
cer8	0.7	15.1	490	8.0
cer9	1.2	12.5	280	8.3
cer13	0.5	3.3	371	27.6
cer19	1.7	33.0	348	28.7
cer5 cer8	0.6	3.0	33.6	3.1
cer5 cer9	0.3	1.1	15.8	4.0
cer6 cer9	3.5	1.9	2.2	2.2
cer7 cer8	0.6	2.2	91.2	17.2
cer8 cer9	1.1	8.3	51.1	1.9
cer8 cer13	0.3	2.6	202	12.9
cer8 cer19	1.1	18.2	513	17.3
cer9 cer19	0.8	13.9	285	9.5

Table 6
Cuticular wax secondary alcohols on Wassilewsklja (WS) and Landsberg (Ler) ecotype, and single and double mutants, expressed as amounts (μg dm^{-2})

Genotype	Secondary alcohols		
	27	29	31
WS	1.3	172	5.2
cer1	0.0	5.0	0.0
cer2	3.2	1.6	0.0
cer3	0.0	37.6	5.4
cer4	3.3	150	3.6
cer1 cer2	1.6	7.5	1.3
cer1 cer3	0.0	12.0	5.0
cer1 cer4	0.0	3.6	3.3
cer2 cer3	1.7	1.7	0.9
cer2 cer4	1.6	6.2	0.0
cer3 cer4	0.0	18.4	0.0
Ler	5.5	137	12.1
cer5	5.3	66.8	8.4
cer6	4.1	4.8	0.5
cer7	1.3	16.7	5.4
cer8	2.4	58.8	4.3
cer9	4.4	27.3	4.9
cer13	1.5	35.3	7.4
cer19	2.6	25.4	4.5
cer5 cer8	2.1	10.7	2.8
cer5 cer9	2.9	14.8	3.1
cer6 cer9	0.9	3.5	0.4
cer7 cer8	2.2	16.4	5.7
cer8 cer9	3.3	9.9	1.1
cer8 cer13	2.1	22.3	4.7
cer8 cer19	2.3	42.1	4.2
cer9 cer19	3.1	30.7	3.6

260.1 μg dm^{-2} (8570% increase). The 24 and 26 carbon acids on *cer9* increased to 36.8 μg dm^{-2} (1320% increase) and 40.1 μg dm^{-2} (1440% increase), all the acids on *cer 5 cer8* increased between 132% and 871% from Ler levels, the C $_{22}$, C_{24}, and C_{26} acids on *cer5 cer9* increased between 533% and 900% from Ler, the C_{22} and C_{24} acids on *cer6 cer9* increased between 6100% and 20,200% from Ler, most acids (especially the C_{30} acid) increased on *cer7 cer8* and *cer8 cer13*, and the C_{22} and longer acids increased on *cer8 cer9* between 2030% and 3250% from Ler levels (Table 2).

In the aldehyde class, the chain length distributions were affected primarily by large reductions in the C_{30} aldehyde (the longest quantifiable homologue) in all single and double mutants except *cer1, cer4, cer1 cer4, cer8*, and *cer8 cer19* that showed little or no change (Table 3). Notable elevations in aldehyde chains occurred in *cer1 cer4* showing elevated 26-carbon aldehydes, and *cer6, cer5 cer8, cer6 cer9, cer7 cer8, cer8 cer9*, and *cer9 cer19* that showed considerable elevation in either the 24- and/or 26-carbon aldehyde (Table 3).

For the primary alcohol constituents, an increase in the amount of shorter chain length C_{24} and/or C_{26} primary alcohols occurred in *cer2*, *cer1 cer2*, *cer2 cer3*, *cer2 cer4*, *cer6*, *cer8*, *cer9*, *cer 19*, *cer5 cer9*, *cer6 cer9*, *cer7 cer8*, *cer8 cer9*, *cer8 cer 19*, and *cer9 cer 19* (Table 4). Larger amounts of the longer chain-length primary alcohols occurred in *cer3*, *cer1 cer3*, *cer7, cer13*, and *cer8 cer13* (Table 4). The largest changes occurred in the 30-carbon alcohol on *cer3* whose amount increased to 173 μg dm^{-2} (436% increase), and the 26- and 28-carbon primary alcohols on *cer2 cer3* whose amounts increased to 277 μg dm^{-2} (858% increase) and 234 μg dm^{-2} (322% increase) (Table 4).

All single and double mutants showed reduced alkanes except *cer4*, and the most affected constituent was the 29-carbon homologue (Table 5). Relative alkane distributions on mutants exhibited little change except in *cer2*, *cer1 cer2*, *cer2 cer4*, *cer6*, *cer8*, and *cer19* that exhibited a slight shift to shorter alkanes (Table 5). Secondary alcohols are derived directly from alkanes, and showed similar reductions in chain length homologues in mutants, respectively (Table 6). All detectable ketones were 29 carbons, and ester chain lengths were not determined.

4. Discussion

The following discussion will focus on the most dramatic and sometimes unexpected effects of *CER*

gene interactions, and what these interactions reveal about the genetic framework underlying wax production by Arabidopsis inflorescence stems. Although previous studies have used double mutant analysis to examine interactions between genes involved in flower (Bowman et al., 1993, 2002; Scortecci et al., 2003), leaf (Serrano-Cartagena et al., 2002), and root development (Parker et al., 2000), stress signaling (Nibbe et al., 2002), and various carbohydrate and lipid metabolic pathways (Cano-Delgado et al., 2000; Niyogi et al., 2001; Thummel and Chory, 2002; Rylott et al., 2003), the double mutant analysis of *cer* gene interactions presented here is the first of its kind for plant wax synthesis, and among the most extensive for any plant metabolic network.

Five of the 14 double *cer* mutants, *cer1 cer3*, *cer2 cer3*, *cer6 cer9*, *cer8 cer9*, and *cer8 cer19*, produced significantly more total wax than at least one of their respective *cer* parents, indicating genetically a partial bypassing (or partial complementation) of one *cer* mutation by the other. Since total wax amounts on 10 of 14 double *cer* mutants were not less than both of the respective *cer* parents, a redirection of flux (or shunting) of wax precursors is the most common effect of these gene interactions. The most abundant wax class on Arabidopsis stems is alkanes, and alkanes (and alkane metabolites) are the major deficiency in all known *cer* mutants except *cer4*. In eight double *cer* mutants, alkane amounts were significantly lower than in both respective *cer* parents suggesting that the wild-type functions of these *CER* gene products overlap additively in alkane synthesis. As such, parallel alkane pathways are a distinct possibility. *cer* mutations that inhibit flux into the alkane branch typically cause shunting of substrates into other branches, the degree and direction of which being highly variable and specific to the *cer* mutation (see Fig. 2). Likewise, the influence of *cer* gene interactions on the shunting of precursors away from the alkane branch was also variable. For example, the *cer6 cer9*, *cer8 cer9*, and *cer9 cer19* double mutants showed a synergistic increase in the amount of acids, *cer2 cer3* had a synergistic increase in the amount of primary alcohols, *cer7 cer8* had an additive increase in primary alcohols, and *cer1 cer2*, *cer1 cer3*, *cer1 cer4*, and *cer2 cer4* had synergistic increases in esters. The differences in shunting caused by these gene mutations and their interactions reveal the presence of multiple operations controlling the distribution of precursor flux within the wax metabolic (and/or secretory) networks. In addition, no wax class was completely eliminated from the wax profile of any of 21 single *cer* mutants (Jenks et al., 1995; Rashotte et al., 2001) or any of the 14 new double *cer* mutants, even though *cer1*, *cer2*, *cer3*, and *cer6* occur as insertional knock-outs (Jenks et al., 2002; Jenks, unpublished). The inability to completely block substrate flux at any step in the wax pathway by single and double *cer* mutations provides additional evidence for significant redundancy in wax pathway operations.

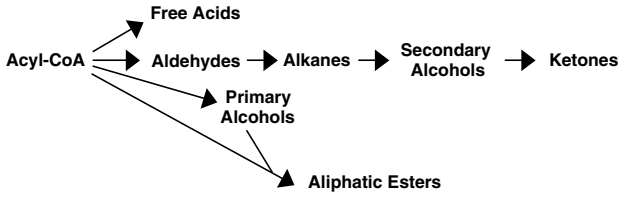

Fig. 2. Model cuticular wax metabolic pathway. Acyl-CoA wax precursors are lengthened by enzyme complexes called elongases, and then converted to either (1) free fatty acids by a putative acyl-CoA thioesterase, (2) to primary alcohols by a large acyl-CoA reductase, (3) to esters by condensation of acids with primary alcohols by a fatty acyl-CoA:fatty alcohol transacylase, or (4) to aldehydes by a small acyl-CoA reductase. Aldehydes can be converted to alkanes by a putative aldehyde decarbonylase, and then to secondary alcohols and ketones by two putative oxidases (from Kolattukudy, 1996).

The *cer1* mutant's alkane deficiency and the predicted CER1 protein sequence led Aarts et al. (1995) to suggest that *CER1* may encode a decarbonylase that converts aldehydes to alkanes, even though CER1 is not homologous to known decarbonylases, such as those in alga (Cheesbrough and Kolattukudy, 1984; Dennis and Kolattukudy, 1992). Further support for this came when elevated *CER1* expression by *WIN1* activation increased alkane production by Arabidopsis leaves (Broun et al., 2004). The *CER1* gene has high homology to *WAX2* (32% identity), however the *wax2* mutant's wax profile suggested that *WAX2* was required for acyl-CoA reduction to aldehydes rather than aldehyde decarbonylation to alkanes (Chen et al., 2003). Another important consideration is that *cer1* and *wax2* both significantly inhibit primary alcohol synthesis (Jenks et al., 1995; Chen et al., 2003). As such, a conclusion that *CER1* and *WAX2* encode simple decarbonylases seems unlikely. When the *cer1* was placed in the *cer2* and *cer3* backgrounds (mutants with primary alcohol amounts much higher than in wild-type), the primary alcohols on *cer1 cer2* and *cer1 cer3* were much lower than on the respective *cer2* and *cer3* parents. This new evidence thus contributes the argument that *CER1* is required for maximum primary alcohol production. Regardless of the exact mechanisms involved, these results show that the CER1 protein has some role to play in both aldehyde conversion to alkane, and acyl-CoA conversion to primary alcohol.

The *cer2*, *cer6*, *cer8*, *cer9*, and *cer19* mutations reduce wax chain length distributions suggesting that they inhibit acyl-CoA elongation (Jenks et al., 1995; Xia et al., 1997; Millar et al., 1999; Fiebig et al., 2000). *CER2* was predicted to encode a nuclear-local-

ized protein (Xia et al., 1997), whereas *CER6* encodes a β-keto acyl-CoA synthase that likely forms part of the acyl-CoA elongation complex (Millar et al., 1999; Fiebig et al., 2000). The *CER8*, *CER9*, and *CER19* gene sequences have yet to be identified. Based on wax chain length analysis, the *cer2*, *cer6*, and *cer9* mutations appear to inhibit C_{26} acyl-CoA elongation primarily (Jenks et al., 1995). However, the preceding C_{24} acyl-CoA elongation step is most suppressed in the *cer6 cer9* double mutant. These results reveal an interactive role for the CER6 and CER9 proteins in both the C_{24} and C_{26} acyl-CoA elongation reactions. Based on wax chain lengths, *cer8* inhibits C_{30} acyl-CoA elongation primarily (Jenks et al., 1995). We thus predicted that *cer9* would be epistatic to *cer8*. However, *cer8 cer9* instead displayed an intermediate effect on wax chain lengths, i.e., *cer8 cer9* primarily blocks C_{28} acyl-CoA elongation. These results indicate an interactive role for the CER8 and CER9 proteins in the C_{26}, C_{28}, and C_{30} acyl-CoA elongation reactions. In contrast, double mutants containing *cer2* provided no evidence that *cer2* was involved in elongation steps other than C_{26} elongation. *cer19* slightly inhibits the C_{28} elongation step (Jenks et al., 1995), leading us to speculate that like *cer9*, the *cer19* mutation would also be epistatic to *cer8*. Surprisingly, we observed that *cer8* was nearly epistatic to *cer19* (i.e., the *cer8 cer19* wax profile was very similar to *cer8*). A large build-up (or backup) of shorter acyl-CoA substrates in the elongation pool of *cer8 cer19* (potentially caused by *cer8*'s C_{30} elongation defect) apparently compensates for *cer19*'s subtle defect in acyl-CoA elongation. Regardless of the specific mechanisms involved, results here reveal numerous interacting factors associated with acyl-CoA elongation reactions, and provide evidence that individual genes function in more than one acyl-CoA chain elongation step. Interestingly, the *cer6 cer9* and *cer8 cer9* double mutants had among the lowest alkane amounts of any mutants, revealing a large additive interaction among *CER6, CER8*, and *CER9* in alkane synthesis. The molecular basis for the *CER6, CER8*, and *CER9* interactions in multiple elongation steps and alkane synthesis needs further exploration.

The longer wax chain lengths of *cer3*, *cer7* and *cer13* led to predictions that these mutations might suppress operations associated with the hydrolysis of fatty acyl-CoA, and thereby suppress the release of acyl chains from the elongation pool (Liu and Post-Beittenmiller, 1995; Jenks et al., 1995). Since aldehydes and their metabolites alkanes, secondary alcohols, and ketones (Fig. 2) are greatly reduced in the *cer3*, *cer7*, and *cer13* mutants, but primary alcohols are not (or little) reduced, these mutations apparently suppress acyl-CoA conversion to aldehydes used in the alkane pathway, but not acyl-CoA conversion to primary alcohols. When *cer7* and *cer13* were examined in the *cer8* background, they reduced the total free acids, aldehydes, and aldehyde metabolites to levels much below those of the *cer8* parent. Likewise, *cer3* generally lowered relative acid, aldehyde, and aldehyde metabolite amounts below those of the other parent in each respective double. These results lend support to previous speculation that *cer3*, *cer7*, and *cer13* inhibit reactions associated with hydrolysis of acyl-CoAs utilized for alkane but not primary alcohol production. Recent studies reveal that *CER3* encodes a protein with homology to ubiquitin-like E3 protein ligase (Hannoufa et al., 1996; Freiman and Tijan, 2003), and perhaps regulates wax synthesis by targeting for degradation specific wax metabolic or regulatory proteins (Jenks et al., 2002). It is unclear whether the CER7 and CER13 proteins impact similar functions as neither *CER7* nor *CER13* gene sequences have been identified.

The *cer4* mutation is predicted to block the conversion of acyl-CoAs to primary alcohols. Surprisingly, *cer1 cer4* and *cer2 cer4* produce significantly more primary alcohols than the *cer4* mutant. The *cer1 cer4* and *cer2 cer4* doubles also produce more esters than both *cer* parents, likely due to elevation in the amount of primary alcohols that serve as the precursors to esters. The *cer1* and *cer2* mutations thus partially bypass the *cer4* defect. Further studies are needed to reveal whether (and how) these interactions cause up-regulation of alternative primary alcohol synthetic pathways.

Mutation in the *CER5* gene appears to reduce alkanes, in part, by enhancing either the transfer or oxidative conversion of alkanes to secondary alcohols and ketones. The ratios of alkanes/secondary alcohols and alkanes/ketones in wild-type are 8.2 and 1.8, respectively, whereas these same ratios in *cer 5* were 3.3 and 0.7, respectively. These ratios appear highly conserved in Arabidopsis since no single or double *cer* mutant, except those containing *cer5*, have these ratios significantly changed from wild-type. Interestingly, the *cer5 cer8* and *cer5 cer9* double mutants showed even greater rates of conversion of alkanes to secondary alcohols and ketones than *cer5*, with the alkanes/secondary alcohols and alkanes/ketones ratios for *cer5 cer8* and *cer5 cer9* being 2.6 and 0.6, and 1.0 and 0.2, respectively. Instead of lessening the *cer5* effect, *cer8* and *cer9* interaction with *cer5* intensified it. A recent report that *CER5* may encode an ABC transporter (Pighin et al., 2003) lends support to the hypothesis that *CER5* may have a unique non-metabolic function in wax production. Whether the *CER8* and *CER9* genes (previously associated with lipid transfer/secretory functions (see Jenks et al., 1995), encode proteins that interact in the wax secretory pathway with CER5 is an intriguing possibility.

5. Conclusions

The lack of clear epistatic interactions between *cer* genes, the inability to reduce stem wax amounts in most (10 of 14) double *cer* mutants below those of both *cer* parents, the inability to completely block production of any class of stem waxes (or any pathway branch) in all 14 double *cer* mutants, and the many additive and sometimes synergistic effects on stem wax class and constituent amounts observed for these *cer* interactions, reveals a highly complex cuticular wax production pathway. To what degree the wax profiles of these double *cer* mutants were due to overlapping enzymatic, secretory, or regulatory functions is still uncertain. Nevertheless, findings presented here that much redundancy occurs in the wax production pathway is critically important if *CER* gene product functions are to be studied using in vitro or heterologous systems, or if these *CER* genes are to be used for crop improvement. This report has shed new light on the genetic framework underlying wax synthesis, and these new double *cer* mutants will provide a valuable resource for future biochemical and genomic studies to elucidate the role of *CER* genes in wax metabolism and secretion.

Acknowledgement

We thank Dr. Karl V. Wood of the Purdue Biochemistry Center for help in the mass spectral analysis.

References

Aarts, M.G.M., Keijzer, C.J., Pereira, A., 1995. Molecular characterization of the *CER1* gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell 7, 2115–2127.

Bowman, J.L., Eshed, Y., Baum, S.F., 2002. Establishment of polarity in angiosperm lateral rgans. Trends Genet. 18, 134–141.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M., Smyth, D.R., 1993. Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. Development 119, 721–743.

Broun, P., Poindexter, P., Osborne, E., Jiang, C.Z., Riechmann, J.L., 2004. *WIN1*, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. PNAS 101, 4706–4711.

Cano-Delgado, A.I., Metzlaff, K., Bevan, M.W., 2000. The *eli1* mutation reveals a link between cell expansion and secondary cell wall formation in *Arabidopsis thaliana*. Development 127, 3395–3405.

Cheesbrough, T.M., Kolattukudy, P.E., 1984. Alkane biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum sativum*. Proc. Natl. Acad. Sci. USA 81, 6613–6617.

Chen, X., Goodwin, S.M., Boroff, V.L., Liu, X., Jenks, M.A., 2003. Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell 15, 1170–1185.

Dennis, M., Kolattukudy, P.E., 1992. A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. Proc. Natl. Acad. Sci. USA 89, 5306–5310.

Fiebig, A., Mayfield, J.A., Miley, N.L., Chau, S., Fischer, R.L., Preuss, D., 2000. Alterations in *CER6*, a gene identical to *CUT1*, differentially affect long-chain lipid content on the surface of pollen and stems. Plant Cell 12, 2001–2008.

Freiman, R.N., Tijan, R., 2003. Regulating the regulators: lysine modifications make their mark. Cell 112, 11–17.

Hannoufa, A., Negruk, V., Eisner, G., Lemieux, B., 1996. The *CER3* gene *of Arabidopsis thaliana* is expressed in leaves, stems, roots, flowers and apical meristems. Plant J. 10, 459–467.

Jenks, M.A., Eigenbrode, S., Turtle, H.A., Feldmann, K.A., 1995. Leaf epicuticular waxes on the *eceriferum* mutants of Arabidopsis. Plant Physiol. 108, 369–377.

Jenks, M.A., Rashotte, A.M., Turtle, H.A., Feldmann, K.A., 1996. Mutants in *Arabidopsis thaliana* altered in epicuticular wax and leaf morphology. Plant Physiol. 110, 377–385.

Jenks, M.A., Eigenbrode, S., Lemeiux, B., 2002. Cuticular waxes of *Arabidopsis*. In: Somerville, C., Meyerowitz, E. (Eds.), The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD, USA. doi: 10.1199/tab.0016. Available from: http://www.aspb.org/publications/arabidopsis/.

Kolattukudy, P.E., 1967. Mechanisms of synthesis of waxy esters in broccoli (*Brassica oleracea*). Biochemistry 6, 2705–2717.

Kolattukudy, P.E., 1996. Biosynthetic pathways of cutin and waxes, and their sensitivity to environmental stresses. In: Kersteins, G. (Ed.), Plant Cuticles, an Integrated Functional Approach. BIOS Scientific Publishers Ltd., Oxford, pp. 83–108.

Kolattukudy, P.E., Buckner, J.S., Liu, T.Y.J., 1973. Biosynthesis of secondary alcohols and ketones from alkanes. Arch. Biochem. Biophys. 156, 613–620.

Koornneef, M., Hanhart, C.J., Thiel, F., 1989. A genetic and phenotypic description *of eceriferum* (*cer*) mutants *in Arabidopsis thaliana*. J. Hered. 80, 118–122.

Kunst, L., Samuels, A.L., 2002. Biosynthesis and secretion of plant cuticular wax. Prog. Lipid Res. 42, 51–80.

Liu, D., Post-Beittenmiller, D., 1995. Discovery of an epidermal stearoyl–acyl carrier protein thioesterase: its potential role in wax biosynthesis. J. Biol. Chem. 270, 16962–16969.

McNevin, J.P., Woodward, W., Hannoufa, A., Feldmann, K.A., Lemieux, B., 1993. Isolation and characterization of genome *eceriferum* (*cer*) mutants induced by T-DNA insertions in *Arabidopsis thaliana* 36, 610–618.

Millar, A.A., Clemens, S., Zachgo, S., Giblin, E.M., Taylor, D.C., Kunst, L., 1999. *CUT1*, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell 11, 825–838.

Nibbe, M., Hilpert, B., Wasternack, C., Miersch, O., Apel, K., 2002. Cell death and salicylate- and jasmonate-dependent stress responses in Arabidopsis are controlled by single *cet* genes. Planta 216, 120–128.

Niyogi, K.K., Shih, C., Chow, W.S., Pogson, B.J., DellaPenna, D., Bjorkman, O., 2001. Photoprotection in a zeaxanthin- and lutein-deficient double mutant of Arabidopsis. Photosyn. Res. 67, 139–145.

Parker, J.S., Cavell, A.C., Dolan, L., Roberts, K., Grierson, C.S., 2000. Genetic interactions during root hair morphogenesis in Arabidopsis. Plant Cell 12, 1961–1974.

Pighin, J.A., Balakshin, L., Western, T., Kunst, L., Samuels, L., 2003. *Cer5* encodes an ABC transporter involved in wax synthesis in Arabidopsis Abstract # 920. Plant Biology 2003 Program. American Society of Plant Biology, Rockville, MD.

Rashotte, A.M., Jenks, M.A., Feldmann, K.A., 2001. Cuticular waxes on *eceriferum* mutants of *Arabidopsis thaliana*. Phytochemistry 57, 115–123.

Rashotte, A.M., Jenks, M.A., Ross, A.S., Feldmann, K.A., 2004. Novel *eceriferum* mutants in *Arabidopsis thaliana*. Planta 219, 5–13.

Rylott, E.L., Rogers, C.A., Gilday, A.D., Edgell, T., Larson, T.R., Graham, I.A., 2003. Arabidopsis mutants in short- and medium-

chain acyl-CoA oxidase activities accumulate acyl-CoAs and reveal that fatty acid beta-oxidation is essential for embryo development. J. Biol. Chem. 278, 21370–21377.

Schneider-Belhaddad, F., Kolattukudy, P.E., 2000. Solubilization, partial purification, and characterization of a fatty aldehyde decarbonylase from a higher plant, *Pisum sativum*. Arch. Biochem. Biophys. 377, 341–349.

Scortecci, K., Michaels, S.D., Amasino, R.M., 2003. Genetic interactions between FLM and other flowering-time genes in *Arabidopsis thaliana*. Plant Mol. Biol. 5, 915–922.

Serrano-Cartagena, J., Candela, H., Robles, P., Ponce, M.R., Perez-Perez, J.M., Piqueras, P., Micol, J.L., 2002. Genetic analysis of *incurvata* mutants reveals three independent genetic operations at work in Arabidopsis leaf morphogenesis. Genetics 156, 1363–1377.

Thummel, C.S., Chory, J., 2002. Steroid signaling in plants and insects- common themes, different pathways. Gene Develop. 16, 3113–3129.

Vioque, J., Kolattukudy, P.E., 1997. Resolution and purification of an aldehyde-generating and an alcohol-generating fatty acyl-CoA reductase from pea leaves (*Pisum sativum* L.). Arch. Biochem. Biophys. 340, 64–72.

Xia, Y., Nikolau, B.J., Schnable, P.S., 1997. Developmental and hormonal regulation of the *Arabidopsis CER2* gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. Plant Physiol. 115, 925–937.