

The *CER3* wax biosynthetic gene from *Arabidopsis thaliana* is allelic to *WAX2/YRE/FLP1*

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Received 23 May 2007; revised 11 June 2007; accepted 12 June 2007

Available online 3 July 2007

Edited by Ulf-Ingo Flügge

Abstract The cuticle coats the aerial organs of land plants and is composed of a cutin matrix embedded and overlaid with waxes. The *Arabidopsis CER3* gene is important for cuticular wax biosynthesis and was reported to correspond to *At5g02310* encoding an E3 ubiquitin ligase. Here, we demonstrate that *CER3* is not *At5g02310* and instead corresponds to *WAX2/YRE/FLP1* (*At5g57800*), a gene of unknown function required for wax biosynthesis. *CER3* protein has also been implicated in cutin production because strong *cer3* alleles display organ fusions. Leaf cutin analysis of two *cer3* alleles did not reveal significant differences in cutin load or composition, indicating that *CER3* has no major role in leaf cutin formation.

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Keywords: *CER3*; *WAX2/YRE/FLP1*; Cuticular wax; Cutin; *Arabidopsis*

1. Introduction

Aerial surfaces of vascular plants and some bryophytes are coated with a lipid barrier called the cuticle that protects them against environmental stresses such as drought and pathogen attack. The outermost layer of the cuticle consists of a film of epicuticular waxes with wax crystals often protruding from this surface. Beneath this outer layer and overlaying the cell wall is the region composed of cutin polyester matrix embedded with intracuticular waxes. Details of cutin polyester assembly from fatty acid monomers are largely unknown. Similarly, the molecular mechanisms by which cuticular waxes are synthesized in epidermal cells and then deposited on the surfaces of plants are poorly understood.

The first step in wax biosynthesis is the elongation of saturated C16 and C18 fatty acyl-CoAs, produced in the plastid, to generate very-long-chain fatty acyl-CoAs 20–34 carbons in length. These elongated fatty acyl-CoA chains, formed on the endoplasmic reticulum, are then converted to the various chemical classes of waxes by one of two wax biosynthetic path-

ways: a decarbonylation pathway and an acyl reduction pathway [1]. The decarbonylation pathway is initiated by the production of aldehydes from fatty acyl-CoA precursors followed by decarbonylation to yield the odd-chain alkanes. A proportion of the alkanes is then converted to secondary alcohols and ketones. In the acyl reduction pathway, primary alcohols are formed by the reduction of fatty acyl-CoAs followed by the generation of alkyl esters through the condensation of primary alcohols with fatty acids.

Genetic approaches have proven invaluable in isolating genes involved in wax production. Wax-deficient mutants are often readily detectable by their glossy appearance compared with the whitish appearance of wild-type plants. Such mutants are known in a number of plant species, including barley (*Hordeum vulgare*), maize (*Zea mays*), and *Arabidopsis thaliana* [2]. The molecular identification of wax-related genes has been achieved mainly in *Arabidopsis* and maize using either forward or reverse genetic approaches. Some of these genes encode biosynthetic enzymes. *CER6*, *KCS1*, *GL8A*, *GL8B*, and *CER10* are components of the fatty acid elongase and generate very-long-chain fatty acid precursors for wax biosynthesis [3–7]. *CER1*, *GL1*, and *WAX2/YRE1/FLP1* contain motifs characteristic of a class of integral membrane enzymes, while *CER2* has motifs that are present in a large family of coenzyme A-dependent acyltransferases [8–14]. Although these motifs are suggestive of metabolic roles, the biochemical activities of these proteins are presently unknown. The only enzyme with known activity subsequent of fatty acid elongation is *CER4*, a fatty acyl-CoA reductase responsible for the production of primary alcohols [15]. Several cloned genes encode regulatory proteins. *WIN1/SHN1* are AP2/EREBP-type transcription factors that when overexpressed cause an increase in cuticular wax levels [16,17], while *CER7* is a core exosome subunit that controls the gene expression levels of *WAX2/YRE/FLP1* [18]. *CER5* encodes a plasma membrane-localized ABC transporter that is required for transport of wax to the cuticle [19]. Further molecular and biochemical characterization of these genes/proteins and identification of additional genes is critical for elucidating the wax biosynthetic pathways, and will provide tools for the controlled genetic manipulation of the plant cuticle.

Cer3 mutants of *Arabidopsis* have low stem wax loads compared to wild-type plants due to dramatically reduced levels of aldehydes, alkanes, secondary alcohols, and ketones [20]. The *CER3* (*At5g02310*) gene was previously reported to encode a 795 amino acid protein that is homologous to α -type E3 ubiquitin ligases [21]. Here, we provide evidence that this report was in error and that *At5g02310* is not the *CER3* gene. Using

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cer3 mutants we identified the *CER3* gene and show that it is allelic to *WAX2/YRE/FLP1*, a gene of unknown function required for wax biosynthesis and cuticle formation. Garzón et al. [34] have recently demonstrated that *At5g02310* encodes an E3 ubiquitin ligase of the N-end rule pathway designated PROTEOLYSIS 6 (PRT6).

2. Materials and methods

2.1. Plant material and growth conditions

T-DNA insertional line SALK_004079 (Col-0 ecotype), and *cer3-1* (*Ler* ecotype) were obtained from the *Arabidopsis* Biological Resource Center (www.arabidopsis.org). *Cer3-2* (*Ws* ecotype) and *cer3-3* (originally called *cer21*, *Ws* ecotype) were obtained from Dr. Bertrand Lemieux (York University, Toronto, Ontario, Canada), *cer3-4* (Col-0 ecotype) was obtained from Dr. Peter McCourt (University of Toronto, Toronto, Ontario, Canada), and *yre* (*cer3-6*, Col-0 ecotype) was obtained from Dr. Takuji Wada (Plant Science Centre, RIKEN, Kanagawa, Japan). Seeds were stratified for 3–4 days at 4 °C before germination on minimal medium agar plates [39] at 20 °C under continuous light conditions (100 $\mu\text{E m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation). Ten-day-old seedlings were transplanted to soil (Sunshine Mix 5, SunGro, Seba Beach, Alberta, Canada) and grown at 22 °C under long-day conditions (16-h-light/8-h-dark cycle).

2.2. Semi-quantitative RT-PCR analysis

For analysis of the transcription profiles of *PRT6* (*At5g02310*) and *CER3/WAX2/YRE1* (*At5g57800*) by RT-PCR, total RNA was extracted from 6-week-old stems using a guanidine-HCl and phenol/chloroform extraction procedure [22], and reverse transcribed using 1 μg of total RNA template, oligo(dT)₁₈, and Superscript II reverse transcriptase (Invitrogen). One microliter of a 1:1 diluted RT reaction was used as template in a 20 μl polymerase chain reaction with gene-specific primers. PRT6-F2 (5'-CACGTCGACATGGAGACCAACTCTTCTCTT-3') and PRT6-R9 (5'-AGCAAACCATCTCCCCAGA-3') were designed to amplify a 1165 bp cDNA fragment of *PRT6* and CER3-FOR (5'-GAGGCTCCTGTGAGTTCCA-3') and CER3-REV (5'-GCTTGCATCTCCTTTCACCT-3') were designed to amplify a 498 bp cDNA fragment of *CER3*. The *GAPC* constitutive control was amplified using primers GAPC-p1 (5'-TCAGACTCGAGAAAGCTGCTAC-3') and GAPC-p2 (5'-GATCAAGTCGACCACACGG-3'), which amplifies a 245 bp cDNA fragment.

2.3. Wax extraction and analysis

Six-week-old stems were immersed in chloroform for 30 s to remove epic- and intra-cuticular waxes. Wax samples were evaporated to dryness under a stream of nitrogen, dissolved in 50 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Pierce, Rockford, IL), derivatized at 80 °C for 90 min and analyzed by gas-liquid chromatography as described in [19]. Quantification of compounds was based on flame ionization detector peak areas, which were converted to mass units by comparison with an internal standard, 17:1 methylester.

2.4. Analysis of residual-bound lipids (cutin)

The details of this procedure have been described [23,24]. Briefly, plants were grown until flowering, 15–20 mature leaves were harvested and their areas measured by scanning. Soluble lipids were removed from samples by extensive extraction in methanol:chloroform (1:1, v/v), and the delipidated leaves were dried over silica, weighed and stored, or used directly for analysis. Following depolymerization of the residual-bound lipids using 1 N methanolic-HCl (Supelco), the hydrophobic monomers were extracted in hexane containing 10 μg dotriacontane (Sigma–Aldrich) as internal standard. The organic phase was evaporated, derivatized with 20 μl bis-(*N,N*-trimethylsilyl)-tri-fluoroacetamide (Machery–Nagel) and 20 μl pyridine and separated and identified by GC–MS using a gas chromatograph 6890N equipped with a quadrupole mass selective detector 5973N (Agilent Technologies, Boeblingen, Germany). Quantitative determination was carried out with the same GC system equipped with a flame ionization detector.

3. Results

3.1. *At5g02310* is part of a much larger gene called *PRT6* (*At5g02300*)

The *CER3* gene was reported to encode a 795 amino acid protein (*At5g02310*) with homology to α -type E3 ubiquitin ligases [21]. This class of E3 ligase is involved in N-end rule mediated degradation of proteins by the proteasome [25,26]. We thus became interested in *CER3* as a potential regulator of wax biosynthesis through the specific degradation of proteins controlling wax biosynthesis. This type of E3 ligase is present in all eukaryotes thus far examined and in all cases it is a large protein of approximately 2000 amino acids. Studies of yeast *UBR1* identified five domains that are critical for function [26], also present in *UBR1* homologs from other organisms (Fig. 1A). The only exception seemed to be *Arabidopsis* where *CER3* (*At5g02310*) encoded a polypeptide comprised of only domains IV and V. We searched the *Arabidopsis* genome and found that the gene encoding domains I to III (*At5g02300*) was immediately upstream of *CER3*.

The proximity of *At5g02300* and *At5g02310* suggested that this was in fact one gene encoding a \sim 2000 a.a. protein, as is the case in other organisms. The predicted coding regions were only 50 bp from the annotated STOP codon of *At5g02300* to the annotated START codon of *At5g02310* (Fig. 1B). Inspection of EST databases revealed an EST (GenBank accession AV529153) that spanned the predicted reading frames of the two genes, an indication that a longer transcript is produced from a single gene. Based on this EST, we reannotated the

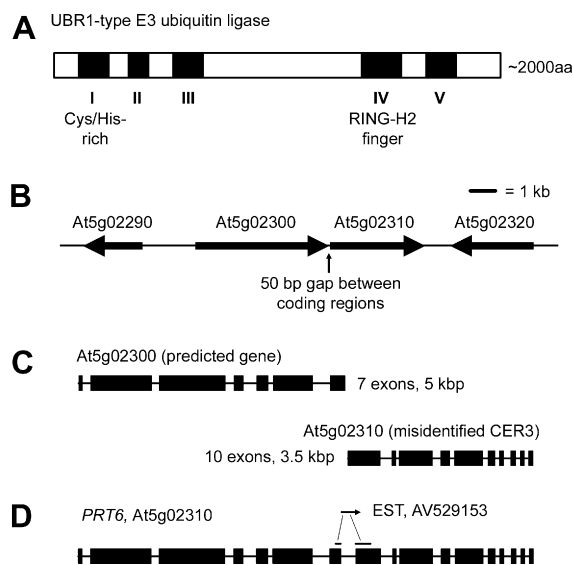


Fig. 1. *At5g02300* and *At5g02310* comprise a single gene called *PRT6*. (A) Schematic representation of UBR1-type (or α -type) E3 ubiquitin ligase, which is involved in N-end rule mediated degradation of proteins by the proteasome. The five domains that are critical for yeast *UBR1* function [26] are depicted by black boxes. (B) The originally annotated genomic region surrounding *At5g02300* and *At5g02310*, with individual genes represented by arrows. *At5g02310* was erroneously reported to encode *CER3*. (C) Exon-intron structure of the originally annotated *At5g02300* and *At5g02310* genes. Black boxes represent exons. (D) The corrected *PRT6* (*At5g02310*) exon-intron gene structure, with an EST (GenBank accession AV529153) that spanned the predicted reading frames of the two genes shown as an arrow above, which provided evidence that the *PRT6* gene is transcribed into a single long mRNA.

exon/intron structure of this portion of the gene, which consists of 17 exons and 16 introns spanning an 8500 bp region (Fig. 1C and D). The predicted cDNA has an open reading frame of 6006 bp including the STOP codon, coding for a 2001 a.a. protein. This is in agreement with other independent annotations of the exon/intron structure of this gene performed without the bias of *At5g02310* being a separate gene (e.g. SALK database). We renamed this *At5g02300/At5g02310* gene *PRT6* as designated by Garzón et al. [34]. There are no other homologs of this gene present in the *Arabidopsis* genome.

3.2. *PRT6* is not involved in cuticular wax biosynthesis

We sequenced the entire *PRT6* gene in two independent *cer3* alleles in an attempt to find the lesions causing the loss of function. We examined the *PRT6* genomic sequence in *cer3-1* (*Ler*), generated by EMS mutagenesis [27], and in *cer3-4*, an additional EMS-induced mutation identified in the Col-0 ecotype. No sequence changes compared to the respective wild-type sequences were found. Even more surprisingly, we failed to find a T-DNA insertion downstream of the *PRT6* gene that was reported to be present in the *cer3-2* allele [21]. There were also no changes in the transcript levels of *PRT6* in any of these mutants (data not shown).

These results suggested that the *cer3* alleles that we were working with were either distinct from the original *cer3* alleles

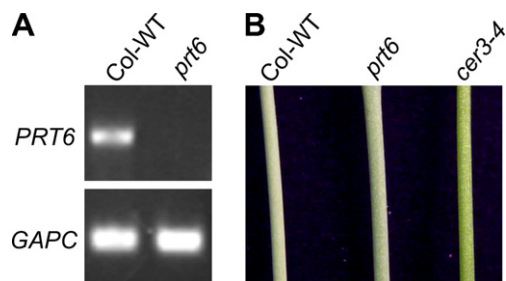


Fig. 2. A *prt6* (*At5g02310*) loss of function mutation does not result in a visible alteration in cuticular wax load. (A) Top, semi-quantitative RT-PCR of steady state *PRT6* (*At5g02310*) mRNA levels in a *prt6* mutant (SALK_004079 T-DNA insertion line) compared to wild-type (*Col-WT*). Bottom, expression of *GAPC* as a loading control for the corresponding lanes in the top image. (B) Stems from 6-week-old wild-type, *prt6*, and *cer3-4* plants showing glaucous or glossy phenotypes.

(e.g. due to a potential seed mix up) or that the *PRT6* gene had been misidentified by Hannoufa et al. [21]. To test this, we obtained a SALK line (SALK_004079) with a T-DNA insertion in the third exon of the *PRT6* gene. No *PRT6* transcript was detected in this line (Fig. 2A) indicating that it is a loss of function *PRT6* mutant. However, no glossy phenotype was observed for this line (Fig. 2B). Quantification of the wax load also failed to reveal a difference between this mutant and wild-type plants (Fig. 3). We therefore, concluded that *PRT6* is not involved in cuticular wax production.

3.3. Cloning of the *CER3* gene

Based on evidence that *PRT6* is not a protein required for wax biosynthesis, and the discrepancy between the physical location of *PRT6* at the top of chromosome 5 and the genetic location of *cer3-1* mutation on the opposite end of chromosome 5 [27,28], we set out to identify the true *CER3* gene. We initially intended to exploit the T-DNA tagged mutant *cer3-2* to isolate the region flanking the T-DNA insertion in the *cer3-2* line by TAIL PCR or plasmid rescue. When our attempts using *cer3-2* line failed, we decided to try the *cer21* mutant which was reported to be allelic to *cer3* [29], despite being originally described as a separate locus. We first verified that *cer21* and *cer3* were allelic by complementation crosses and renamed this allele *cer3-3*. *Cer3-3* is a T-DNA insertion line in the *Ws* ecotype. Using plasmid rescue, we found that a T-DNA insertion in this line was present in *At5g57800*, a gene that is important for wax biosynthesis, already characterized by three independent groups that named it *WAX2/YRE/FLP1* [11–13]. The T-DNA in the *cer3-3* mutant was inserted in the promoter region of *At5g57800* (Fig. 4A). We also found a partial T-DNA insert in the promoter region of *At5g57800* in the *cer3-2* allele following PCR amplification of the region. Sequencing of *At5g57800* in *cer3-1* revealed a single base pair alteration that resulted in an alanine to threonine change at amino acid residue 408 of the predicted protein, whereas the *cer3-4* mutant had a single base pair change that caused a tryptophan to stop codon change at amino acid residue 533 producing a truncated protein. To avoid further confusion of gene nomenclature, we have renamed all of the alleles of the *At5g57800* gene with the prefix ‘*cer3*’ followed by an allele number as per convention in *Arabidopsis* genetics (Table 1 and Fig. 4A). *Cer3* was chosen as the name because it was the designation of the originally isolated mutant [27].

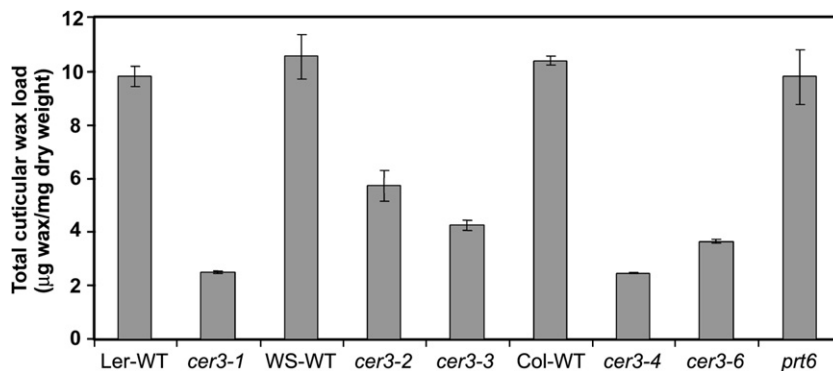


Fig. 3. Cuticular wax loads on stems of *cer3* and *prt6* mutants. Each bar represents the total cuticular wax loads on stems of various *cer3* mutants (Table 1) and the SALK_004079 allele of *prt6*. Each bar represents the mean of three independent analyses of wax extracts from three pooled individuals.

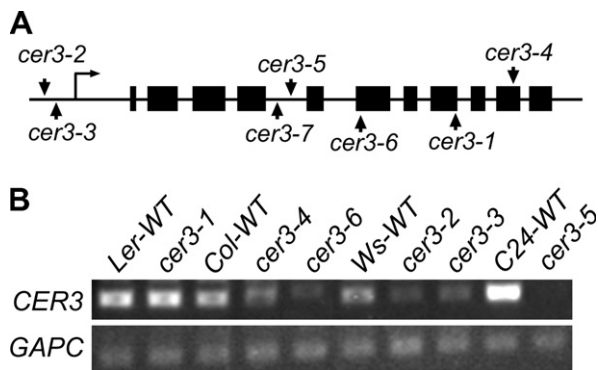


Fig. 4. Structure of the *CER3* gene (*At5g57800*) and transcript levels in *cer3* mutant lines. (A) Exon–intron structure of the *CER3* (*At5g57800*) gene. Black boxes represent exons and the bent arrow represents the transcription start site. *Cer3-2* and *cer3-3* are T-DNA insertions in the promoter of *CER3*, *cer3-5* and *cer3-7* are T-DNA insertions in the fourth intron, *cer3-6* is a T-DNA insertion in the sixth exon, and *cer3-1* and *cer3-4* are EMS-induced point mutations (Table 1). (B) Top, semi-quantitative RT-PCR of steady state *CER3* (*At5g57800*) mRNA levels in *cer3* mutants compared to the corresponding wild-type ecotypes. Bottom, expression of *GAPC* as a loading control for the corresponding lanes in the top image.

3.4. *CER3* expression, wax loads and cutin content of *cer3* mutant alleles

A distinctive feature of the *cer3-5* (*wax2*) and *cer3-6* (*yre*) alleles characterized by Chen et al. [12] and Kurata et al. [13] is the presence of fusions between aerial organs. The *cer3-1*, *cer3-2*, *cer3-3* and *cer3-4* alleles do not have this phenotype, which contributed to the lack of recognition that *cer3* was in fact allelic to *wax2*. This could be because *cer3-1*, *cer3-2*, *cer3-3* and *cer3-4* mutations do not result in complete loss of function. We therefore, examined *CER3* transcript levels in these different alleles (Fig. 4B). *Cer3-1* has wild-type levels of expression and *cer3-4* transcript levels are only moderately reduced. In addition, both predicted *CER3* proteins from these mutants have changes in the C-terminal regions due to single base-pair changes, suggesting that they may have retained partial function. *Cer3-2* and *cer3-3*, which contain T-DNA insertions in the promoter, have significantly reduced transcript levels, but are not transcriptional nulls also allowing for some residual *CER3* activity. Conversely, *cer3-5* (*wax2*; Fig. 4B) and *cer3-6* [13] are devoid of *CER3* transcripts.

To determine if variations in *CER3* transcript levels detected in *cer3* mutants affected levels or composition of cuticular wax present on their stems, we compared these parameters in different *cer3* alleles. The stem wax loads in all of the *cer3* mutants were considerably lower than the wild type (Fig. 3), in agreement with previous findings [13,20]. Their wax compositions were also quite similar to published data [13,20], exhibiting

major reductions in levels of aldehydes, alkanes, secondary alcohols, and ketones, and lower levels of C26 and C28 primary alcohols but much higher levels of C30 primary alcohols. The absence of organ fusions in the weaker *cer3* alleles was probably not due to major differences in cuticular wax accumulation or composition because *cer3-1* and *cer3-4* had the same level of total wax reduction as *cer3-6*, which is a null mutation.

The presence of organ fusions in some, but not all *cer3* mutants, may be due to differences in the cutin load or composition in the allelic series, since such fusions have been correlated with defects in cutin [30]. *CER3* had previously been suggested to be involved in cutin production and/or deposition [12], but detailed cutin analyses of *cer3* mutants have not yet been conducted. We therefore, examined the cutin content and cutin monomer composition in leaves of a strong allele *cer3-6* and a weaker allele, *cer3-4*, both in the Col-0 genetic background (Fig. 5). Our analysis did not reveal major differences in the load or the composition of cutin characteristic monomers, including unsubstituted fatty acids, ω -hydroxy fatty acids and α,ω -dicarboxylic acids, between the *cer3* alleles and the wild type. The only variation detected was that in the levels of 2-hydroxy acids, compounds previously identified as *Arabidopsis* leaf polyester constituents [23], but probably predominantly derived from sphingolipids [31]. The fact that the stronger *cer3-6* allele that exhibits organ fusions (Fig. 4) actually has lower 2-hydroxy acid content than the weaker *cer3-4* allele without organ fusions, suggests that above-normal levels of 2-hydroxy acids are not the underlying cause of organ fusions. More importantly, overall similar cutin loads and compositions of *cer3* mutants and the wild type demonstrate that *CER3* is not involved in cutin formation in leaves.

4. Discussion

In their 1996 paper, Hannoufa et al. [21] reported the identification of the *CER3* gene (GenBank accession X95962) and in a subsequent publication the same researchers [32] showed that the *cer3* mutation can be complemented with the corresponding gene. However, upon completion of the *Arabidopsis* genome sequence for chromosome 5 it became apparent that the chromosomal location of X95962 corresponding to the *At5g02310* gene does not match the previously determined location of the *cer3-1* mutation on the genetic map [27,28]. Our results presented in this paper resolve this ambiguity and provide conclusive evidence that *CER3* is not *At5g02310*.

The realization that *CER3* is not *At5g02310* immediately generates two questions: (1) What then is the role of *At5g02310* (*PRT6*), and (2) Which gene is the true *CER3* mutated in *cer3* wax deficient lines? *PRT6* exhibits high sequence

Table 1
Nomenclature and description of *cer3* alleles

| <i>cer3</i> allele | Alternate name | Ecotype | Mutagen | Description of mutation | Reference |
|--------------------|----------------|------------|---------|--|------------|
| <i>cer3-1</i> | | <i>Ler</i> | EMS | Missense, Ala to Thr at amino acid 408; partial | [27] |
| <i>cer3-2</i> | | <i>Ws</i> | T-DNA | Insertion in promoter; partial | [36] |
| <i>cer3-3</i> | <i>cer21</i> | <i>Ws</i> | T-DNA | Insertion in promoter; partial | [36] |
| <i>cer3-4</i> | | <i>Col</i> | EMS | Nonsense, Trp to stop at amino acid 533; partial | This study |
| <i>cer3-5</i> | <i>wax2</i> | <i>C24</i> | T-DNA | Insertion in fourth intron; null | [12] |
| <i>cer3-6</i> | <i>yre</i> | <i>Col</i> | T-DNA | Insertion in sixth exon; null | [13] |
| <i>cer3-7</i> | <i>flp1</i> | <i>Col</i> | T-DNA | Insertion in fourth intron; null | [11] |

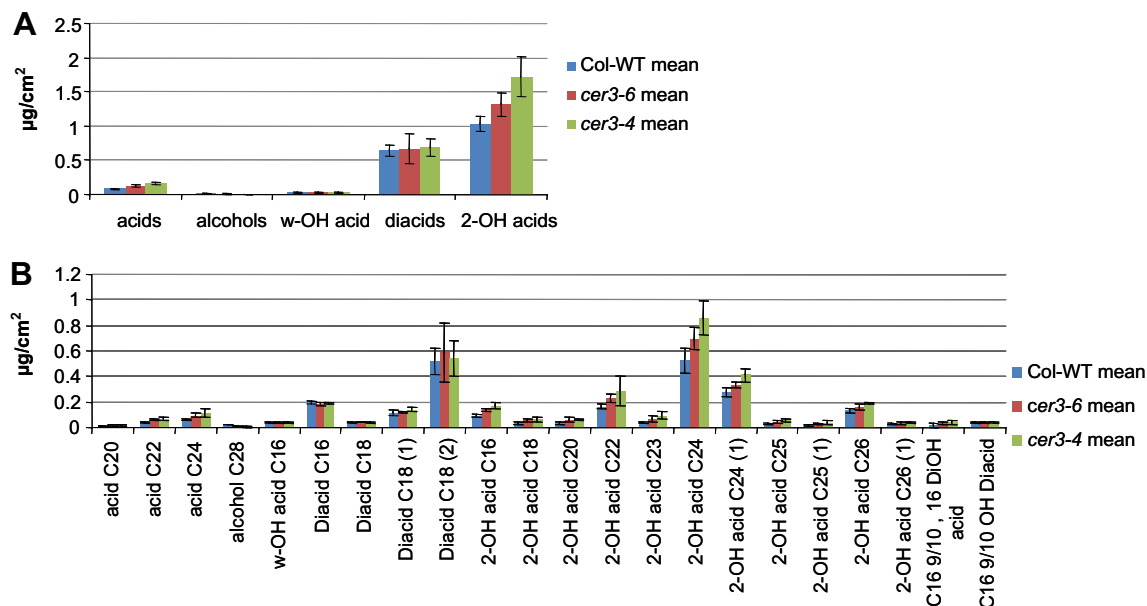


Fig. 5. Compositional analysis of residual bound lipids (cutin) in *cer3-4*, *cer3-6* and wild-type leaves. Aliphatic substance class composition (A) and monomer composition (B) in rosette leaves of 5-week-old *cer3-4*, *cer3-6* and wild-type plants. Leaves were totally extracted, transmethylated by 1 N methanolic-HCl and released monomers analyzed by GC. Absolute amounts of predominant compounds are given in $\mu\text{g cm}^{-2}$ as mean of four replicates and standard deviation (error bars). Acids were analyzed as methyl esters, hydroxyl groups were analyzed as trimethylsilyl esters. ω -OH acid, ω -hydroxyacid; diacid, α,ω -dicarboxylic acid; 2-OH acid, 2-hydroxyacid.

similarity along its entire length with the yeast *UBR1* known to encode an α -type E3 ubiquitin ligase required for substrate selection in the N-end rule pathway of protein degradation via proteasome [25,26]. In addition, the predicted PRT6 protein contains all five domains known to be critical for the *UBR1* function [26], suggesting that PRT6 is also a ubiquitin ligase. N-end rule refers to the amino terminal residue, the nature of which determines the half-life of a protein. In yeast, *UBR1* mediates ubiquitination of proteins with both hydrophobic and basic amino termini. This is not the case in plants, where several E3 ubiquitin ligases are associated with the N-end rule pathway, as shown for PRT1 which is involved in degradation of proteins with aromatic amino terminal residues [33] and now PRT6 which is specific for proteins with basic amino acids at their amino termini [34].

Cloning of the *CER3* gene revealed that it is identical to *WAX2/YRE/FLP1* [11–13], a gene required for cuticle formation in *Arabidopsis*. The predicted *CER3* protein has six putative transmembrane domains at the N-terminus and a large, soluble C-terminal domain [12]. The N-terminal domain also contains eight conserved histidines in a tripartite motif (HX3H, HX2HH, HX2HH), which is typical of a di-iron-binding site essential for catalytic activity in a large family of integral membrane enzymes such as fatty acyl desaturases, hydroxylases, and monooxygenases [35]. The N-terminal domain of *CER3* is highly related to that of *Arabidopsis CER1*, including conservation of the histidine residues. *Cer1* mutants exhibit increases in aldehyde levels and dramatic reductions in alkanes, secondary alcohols and ketones, suggesting a block in the conversion of aldehydes to alkanes [36]. It was proposed that the *CER1* protein is an aldehyde decarbonylase [8], but biochemical support for this is lacking. Thus, although the sequences of *CER3* and *CER1* suggest enzymatic roles in wax biosynthesis, the reactions carried out by these proteins are unknown.

Point mutation in and partial deletion within the soluble C-terminal domain of *CER3* severely compromises function indicating that it is important for wax production. This domain does not have strong similarity to any of the known sequences, and is divergent from the C-terminal domain of *CER1*. *CER3* and *CER1* have different roles in wax biosynthesis and this may be due to distinct activities of their C-terminal domains. A complete loss of function of *CER3* causes organ fusions [12,13]. Based on this phenotype, Chen et al. [12] speculated that *CER3* may be a dual function protein contributing not only to wax but also cutin production/deposition. Our analysis of leaf cutin in two different *cer3* lines did not identify any qualitative or quantitative changes in cutin-specific monomers. We therefore conclude that at least in leaves, *CER3* activity is not required for cutin biosynthesis. This may not be true throughout the plant because the *WDA1* protein from rice, related to *CER1* and *CER3*, was reported to be involved in cutin monomer formation in the anther [37], suggesting that *CER3* may have a similar role in specific tissues.

The only compounds detected at a higher concentration in *cer3* leaves are 2-hydroxy acids (Fig. 4). These fatty acids, especially the predominant C24 type, are characteristic of sphingolipids and are likely released during cutin depolymerization by transmethylation [31,38]. If that is the case, the higher levels of 2-hydroxy acids would indicate higher accumulation of sphingolipids in *cer3* mutants. Elevated levels on sphingolipids can be rationalized by a higher concentration of acyl-CoA precursors in the acyl-CoA pool in wax-deficient lines that can be used for biosynthesis of other types of lipids, including sphingolipids.

In summary, we have cloned the *CER3* gene (*At5g57800*) disrupted in the *cer3* wax-deficient mutants of *Arabidopsis* and demonstrate that it encodes a protein of unknown function identical to *WAX2/YRE/FLP1* required for cuticular

wax biosynthesis. The *CER3* gene is therefore distinct from the *At5g02310* previously described to be the *CER3*. In support of this notion, Garzón et al. [34] provide unequivocal evidence that *At5g02310* codes for PRT6, a ubiquitin ligase of the N-end rule pathway of protein degradation.

Acknowledgements: We thank the *Arabidopsis* Biological Resource Center at Ohio State University for the *cer3-1* mutant, Dr. Bertrand Lemieux for the *cer3-2* and *cer3-3* mutants, Dr. Peter McCourt for the *cer3-4* mutant, Dr. Matthew Jenks for the *wax2* (*cer3-5*) mutant, Dr. Takuji Wada for the *yre* (*cer3-6*) mutant, and the Salk Institute for Genomic Analysis Laboratory for providing the T-DNA insertion mutant SALK_004079. We also thank Prof. Andreas Bachmair for sharing his results prior to publication and Dr. Shelley Hepworth for assistance with figures and for critical reading of the manuscript. Funding by the Natural Sciences and Engineering Research Council of Canada to L.K. is gratefully acknowledged.

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