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

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Abstract The cuticle coats the aerial organs of land plants and is composed of a cutin matrix embedded and overlayed 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.

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 pathways: 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 ximosesubunit 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
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 Lemoine (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. Takui 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 μE m−2 s−1 of photosynthetically active radiation). Ten-day-old seedlings were transplanted to soil (Sunshine Mix 5, SunGro, Sesa 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/YREI (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′-CACGTGAGATGAGCCACCTCTTTCTCTT-3′) and PRT6-R9 (5′-AAGAACCATTCCCTCCCCAGA-3′) were designed to amplify a 1165 bp cDNA fragment of PRT6 and CER3-FOR (5′-GGAGCTCCTGGATGTCCA-3′) and CER3-REV (5′-GGTTGCGATTCTTTCACCT-3′) were designed to amplify a 498 bp cDNA fragment of CER3. The GAPC constitutive control was amplified using primers GAPC-p1 (5′-TCAGACTCGAGAAAGGTACACTTCG-3′) and GAPC-p2 (5′-GATCAAGTCGACCACACCGG-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,N-bis(trimethylsilyl)triﬂuoroacetamide with 1% trimethylchlorosilane (Pierce, Rockford, IL), derivatized at 80 °C under continuous light conditions (100 μE m−2 s−1 of photosynthetically active radiation). Ten-day-old seedlings were transplanted to soil (Sunshine Mix 5, SunGro, Sesa Beach, Alberta, Canada) and grown at 22 °C under long-day conditions (16-h-light/8-h-dark cycle). Wax samples were evaporated to dryness, dissolved in 50 μl of N,N-bis(trimethylsilyl)-triﬂuoroacetamide 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.

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 deﬁned leaves were dried over silica, weighed, 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 triﬂuoroacetamide (Macherey–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 ~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
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 At5g02310/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 (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. 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, cer3-1, and cer3-4 plants showing glaucous or glossy phenotypes.](image)

![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.](image)
We therefore examined CER3 cer3-4 mutations do not result in complete loss of function. wax2 likely to cer3 contributed to the lack of recognition that cer3-4 and cer3-3 were considerably lower than the wild type (Fig. 3), in agreement.

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

<table>
<thead>
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<th>cer3 allele</th>
<th>Alternate name</th>
<th>Ecotype</th>
<th>Mutagen</th>
<th>Description of mutation</th>
<th>Reference</th>
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<td>Ws</td>
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<td></td>
<td>Insertion in promoter; partial</td>
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<tr>
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<td>T-DNA</td>
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<td>[36]</td>
</tr>
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<td>EMS</td>
<td>Nonsense, Trp to stop at amino acid 533; partial</td>
<td>This study</td>
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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 monoxygenases [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, thehigher 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 At15g02310 previously described to be the CER3. In support of this notion, Garzón et al. [34] provide unequivocal evidence that At15g02310 codes for PRT6, a ubiquitin ligase of the N-end rule pathway of protein degradation.

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