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The identification of cutin synthase: formation of the plant polyester cutin

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Abstract

A hydrophobic cuticle consisting of waxes and the polyester cutin covers the aerial epidermis of all land plants, providing essential protection from desiccation and other stresses. We have determined the enzymatic basis of cutin polymerization through characterization of a tomato extracellular acyltransferase, CD1, and its substrate, 2-mono(10,16-dihydroxyhexadecanoyl)glycerol (2-MHG). CD1 has *in vitro* polyester synthesis activity and is required for cutin accumulation *in vivo*, indicating that it is a cutin synthase.

Fossil evidence suggests that evolution of a hydrophobic cuticle was essential for terrestrial colonization by plants, ~400 million years ago¹. As the primary interface between the plants and their above-ground environment, the cuticle is critically important in limiting water loss and plays additional key roles in defense against pests and pathogens, as well as establishing organ boundaries during development². The cuticle consists of an insoluble polyester of

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Author Contributions

T.H.Y. purified recombinant protein, performed chemical analysis of surface soluble lipids and acyltransferase assays. L.B.B.M. performed gene and protein expression experiments and cutin analysis. H.M.V. and M.H.C. synthesized 2-MHG. T.I. identified and performed initial characterization and rough mapping of the mutant. Y.E. and L.Z. performed fine genetic mapping experiments and constructed the transgenic complementation vector. A.J.M. performed the tissue specific gene expression experiments. G.J.B. conducted light microscopy experiments. L.B.B.M. and D.S.D. performed immunolocalization experiments. T.H.Y., L.B.B.M. and J.K.C.R. designed the study, analyzed the data and wrote the paper. T.H.Y. and L.B.B.M. contributed equally to the study.

Competing financial interests

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hydroxy fatty acids, known as cutin, which is covered and infiltrated with a variety of waxes. While the generic composition of the cutin polymer is known, the mechanism and site of cutin polymerization have remained long standing questions^{3,4}.

Cutin is typically exceptionally abundant in the fruit cuticles of tomato (*Solanum lycopersicum*); however we previously identified several tomato mutants with dramatic deficiencies in cutin⁵. One of these, *cutin deficient 1 (cd1)*, has approximately 5–10% levels of fruit cutin compared with the wild type (M82) genotype, an extremely thin cuticle and increased sensitivity to water loss and pathogen susceptibility (Fig. 1a, b; ref. 5). Fine mapping of the *cd1* mutation revealed it to lie within a five-exon gene (*CD1*; Supplementary Results, Supplementary Fig. 1) that is predicted to encode a member of the GDSL motif lipase/hydrolase (GDSL) family of proteins (Supplementary Fig. 2). GDSLs collectively exhibit diverse functions and substrate specificities⁶ and a broad taxonomic distribution, including prokaryotes and eukaryotes. In plants they are present as large gene families⁷ and, based on their expression patterns, it has been speculated that GDSLs may play a role in cuticle biosynthesis^{8–12}.

The *cd1* mutant has a point mutation introducing a stop codon upstream of two of the three predicted catalytic amino acid residues (Supplementary Fig. 1b, Supplementary Fig. 2). In the mutant, *cd1* transcript levels are reduced (Supplementary Fig. 3a) but the CD1 protein was not detected (Supplementary Fig. 3c,d), indicating that it is a null mutant. Complementation of the *cd1* mutant with the wild type gene driven by the constitutive Cauliflower Mosaic Virus 35S promoter rescued the phenotype (Supplementary Fig. 4a,b), confirming that the mutation in *CD1* is responsible for the cutin deficiency.

An analysis of the spatial distribution of CD1 proteins or transcripts showed that expression is highest in expanding organs, which require rapid cuticle synthesis to accommodate growth¹³, but is undetectable in roots, which have no cuticle (Supplementary Fig. 3b–d). Additionally, we used laser-capture microdissection of various pericarp tissues from young fruit to show that *CD1* transcript levels are highest in the outer and inner epidermal cell layers (Supplementary Fig. 5; ref. 9), both of which are responsible for cuticle synthesis⁹. Thus, *CD1* expression parallels spatial and temporal patterns of cuticle deposition at several levels.

Immunolocalization of CD1 in M82 fruits indicates that the protein is almost exclusively localized in the cuticle (Fig. 1c, Supplementary Fig. 6). More specifically, labeling density follows the contour of the cuticle over both the periclinal and anticlinal cell walls (Fig. 1d). This localization pattern suggests a role for CD1 late in the cutin biosynthetic pathway, leading us to investigate whether CD1 is directly involved in cutin polymerization.

Several enzymes have been shown through analysis of *Arabidopsis thaliana* mutants to be required for formation of the cutin polymer, including glycerol phosphate acyltransferase enzymes (GPATs)⁴. Recently, biochemical characterization of GPAT4 and GPAT6 showed them to possess both glycerol-3-phosphate acyltransferase activity specific to the *sn*-2 position, and phosphatase activity¹⁴. This may indicate a structural role for 2-monoacylglycerol esters (2-MAGs) in the cutin polymer, as these were identified in small

quantities in the products of partially depolymerized cutin¹⁵. Alternatively, the 2-MAG products of GPAT4 and GPAT6 may act primarily as acyl donors for the polymerization reaction. If this is true, and CD1 is indeed a cutin polymerase, we would expect that 2-MAGs would accumulate as free lipids in the surface tissues of the *cd1* mutant fruit, but not in the M82 wild type genotype.

Soluble surface lipids, collectively termed cuticular waxes, can readily be extracted from plants by brief immersion of intact organs in organic solvents¹⁶. In tomato fruits, these waxes consist primarily of a mixture of high melting-point alkanes and triterpenoids, while the cutin, a polyester of principally 10,16-dihydroxyhexadecanoic acid, is insoluble under these conditions. Although soluble 2-MAGs can be found in the waxes associated with suberin, they are not observed in cuticular waxes¹⁷. GC-MS analysis identified the 2-MAG species 2-mono(10,16-dihydroxyhexadecanoyl)glycerol (2-MHG, 1) in soluble surface lipids from *cd1* fruits at the rapidly expanding stage, when CD1 is normally most highly expressed (Supplementary Fig. 3c), but not in equivalent extracts from M82 fruit (Fig. 2). While chromatographic resolution was incomplete, the coincident single ion chromatograms of diagnostic fragments clearly show the specific accumulation of 2-MHG in the mutant (Fig. 2a). An additional, later-eluting trace peak of these ions likely corresponds to the thermodynamically favored 1-mono(10,16-dihydroxyhexadecanoyl)glycerol (1-MHG) isomer. The identity of the larger of the two peaks as representing the 2-isomer is confirmed by its earlier elution and the absence of the $M-103 = 547$ ion produced by α -cleavage between the 2- and 3- carbon in 1-MHG (Fig. 2b; ref. 15). Despite the clear accumulation of 2-MHG in the *cd1* mutant and not M82, the amount detected was relatively low (on the order of $0.1 \mu\text{g}\cdot\text{cm}^{-2}$, based on comparison to wax compound abundance), possibly due to feedback regulation of the upstream biosynthetic pathway or the relatively polar nature of 2-MHG compared with other surface soluble lipids.

We propose a model for cutin polymerization wherein CD1 transfers the hydroxyacyl group of 2-MHG to either another molecule of 2-MHG, or the growing cutin polymer itself (Fig. 3a). Experiments involving partial depolymerization of tomato cutin have identified oligomers primarily consisting of directly coupled 10,16-dihydroxyhexadecanoic acid monomers^{18,19}. This, combined with the observation that glycerol is quantitatively a minor component of tomato cutin¹⁵, suggests that the principal linkage in tomato cutin is between the carboxylic acid and hydroxyl groups of 10,16-dihydroxyhexadecanoic acid. The detection of small amounts of 2-MHG in the cutin polymer¹⁵ may therefore reflect the presence of 2-MHG 'primers' remaining in the polymer. The presence of polymerized 1-MHG could be a consequence of spontaneous acyl migration accelerated by the alkaline conditions used for *in vitro* depolymerization. To test our hypothesis that CD1 acts as an acyltransferase, we purified recombinant tomato CD1 protein following expression in *Nicotiana benthamiana* (Supplementary Fig. 7). Racemic 2-MHG was synthesized in six steps from monobenzyl-protected decane-1,10-diol (Supplementary Methods, Supplementary Scheme 1), and used as a substrate for *in vitro* polymerization assays. Lipid products of the assay were extracted with ethyl acetate and analyzed by MALDI-TOF mass spectrometry. A major series of ions separated by $m/z = 270.2$ was observed, consistent with the expected masses of sodium and potassium adducts of polyester oligomers with a glycerol

end group and up to seven 10,16-dihydroxyhexadecanoyl monomers (Fig. 3b). A control assay was performed using the S32A variant of CD1, prepared by site-directed mutagenesis. As expected, mutation of the conserved catalytic serine of CD1 to an alanine eliminated acyltransferase activity (Supplementary Fig. 8).

In vivo ester synthesis via transesterification of acyl glycerol by a lipase-like enzyme is not without precedent. For example, in animals, the extracellular acylation of cholesterol by transesterification of lecithin is catalyzed by lecithin cholesterol acyltransferase (LCAT). In the absence of cholesterol as an acyl acceptor, LCAT has acyl esterase activity²⁰. The unique feature necessary for this transesterification reaction is the action of the enzyme at the lipid-aqueous interface of high density lipoproteins. Here, cholesterol concentrations are high enough to favor the resolution of the acyl-enzyme intermediate by transesterification rather than hydrolysis. We propose that CD1 acts through a similar mechanism at the interface between the aqueous environment of the plant cell wall and the lipid phase of the nascent cuticle. Thermodynamically, the aqueous solubility of 2-MHG and insolubility of the polyester product would further drive the reaction towards polyester synthesis.

In vitro incorporation of fatty acids into the cutin polymer by crude plant enzyme preparations was first reported more than thirty years ago²¹. Moreover, recent molecular genetic characterization of cutin polymer synthesis has identified several intracellular acyltransferases that are involved in biosynthesis of presumed cutin precursors^{4,22}. However, the molecular basis of cutin polymerization following secretion of the precursors into the cell wall has remained a mystery. Here we show that CD1 is an extracellular enzyme that localizes in the developing cuticle and is required for cutin biosynthesis. We detected accumulation of 2-MHG, the corresponding 2-MAG of the major cutin monomer of tomato, in the cutin deficient *cd1* mutant and showed that recombinant CD1 catalyzes the successive transesterification of 2-MHG to yield polyester oligomers *in vitro*. Taken together, these results lead us to propose that CD1 is the principal catalyst of cutin polymerization and that the polymerization process is extracellular, at the site of cuticle deposition. Furthermore, a survey of protein sequences reveals *CD1* homologs in widely diverse species (Supplementary Fig. 9), and it has been reported that silencing the expression of two *Arabidopsis thaliana* homologs of CD1 resulted in phenotypes similar to other cutin deficient mutants²³, suggesting an evolutionarily conserved and ubiquitous mechanism of cutin biosynthesis in land plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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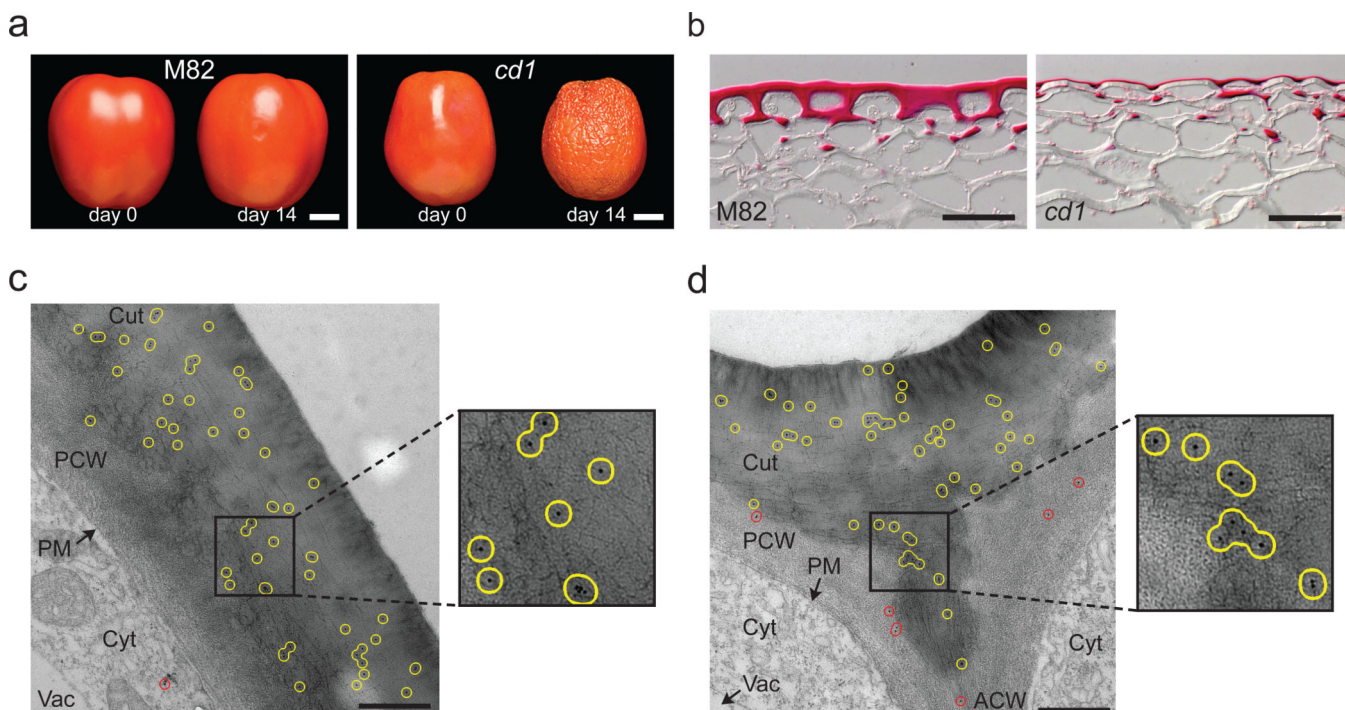


Figure 1. CD1 is a GDSL-motif lipase/hydrolase family protein that localizes to the nascent cuticle

(a) Fruits of the M82 wild type tomato cultivar and the *cd1* mutant on the day of harvest at the fully ripe stage and 14 days after storage at room temperature. Scale bars = 1 cm. **(b)** Light microscopy showing the cuticle of M82 and *cd1* ripe fruit stained with Sudan Red 7b. Scale bars = 50 μ m. **(c–d)** TEM immunolocalization of CD1 in the cuticle of 15 days post-anthesis (DPA) M82 fruits, over the periclinal cell wall of an epidermal cell **(c)** and in the anticlinal peg of cuticle between two adjacent epidermal cells **(d)**. Scale bars = 500 nm. Cut, cuticle; Cyt, cytoplasm; ACW, anticlinal cell wall; PCW, periclinal cell wall; PM, plasma membrane; Vac, vacuole. Gold particles are highlighted with yellow circles in the cuticle or red circles elsewhere, and magnified areas of each image are shown in the right panels.

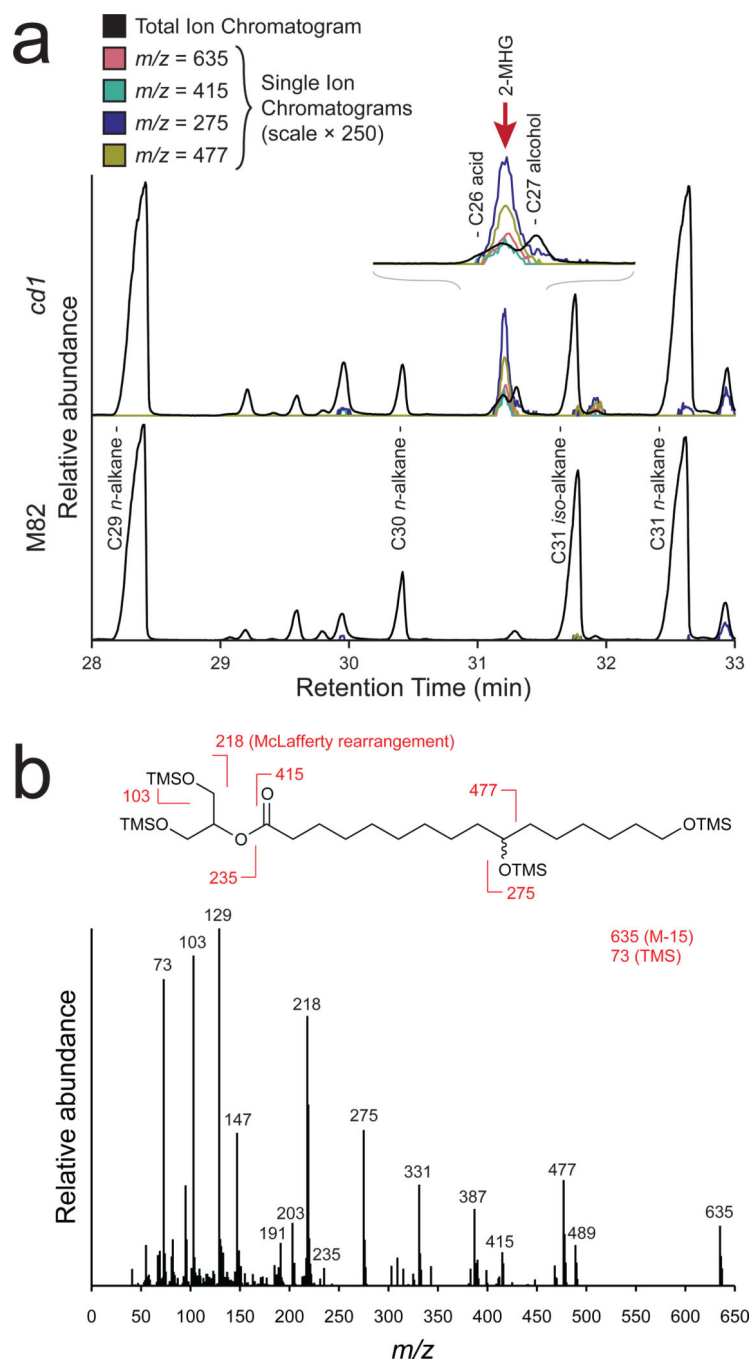


Figure 2. The identification of 2-MHG in the soluble surface lipids of *cdl* fruit
(a) GC-MS chromatograms of the *cdl* mutant and M82 wild type TMS-derivatized extracts. The total ion chromatograms and several single ion chromatograms corresponding to characteristic fragments of 2-MHG are shown. Inset is an enlargement of the region surrounding the 2-MHG peak. For reference, several of the wax compounds common to both mutant and wild type are labeled. **(b)** Mass spectrum from the 2-MHG peak found in the *cdl* extract, with interpretation of the spectrum inset. The spectrum of synthetic 2-MHG is

shown in Supplementary Fig. 10. For more detailed discussion of the spectrum of 2-MHG, see ref. 15.

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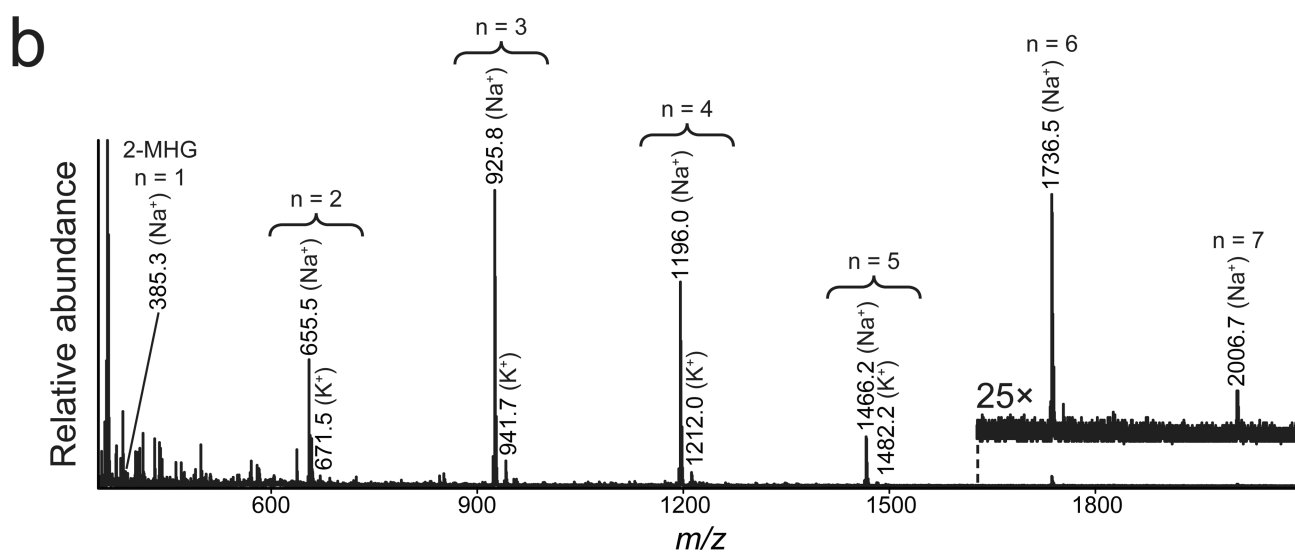
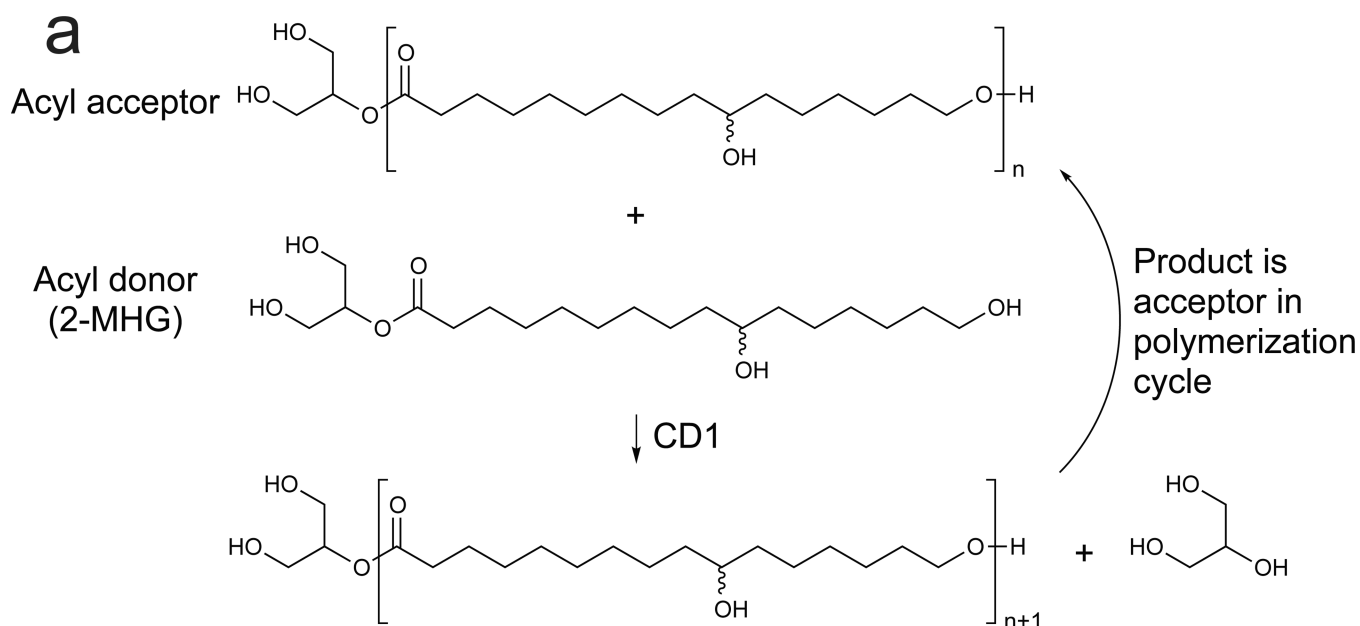


Figure 3. Acyltransferase activity of CD1

(a) Proposed model for CD1 catalyzed cutin biosynthesis by transfer of the hydroxyacyl group from 2-MHG to the growing polymer. For simplicity, ester linkage via the primary hydroxyl is shown, although linkage via the secondary hydroxyl can also occur in cutin¹⁸.

(b) MALDI-TOF positive ion spectra of lipid products from *in vitro* assays with 2-MHG substrate and purified recombinant CD1 enzyme.