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Quantitative analysis of glycerol in dicarboxylic acid-rich cutins provides insights into Arabidopsis cutin structure

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ABSTRACT

Cutin is an extracellular lipid polymer that contributes to protective cuticle barrier functions against biotic and abiotic stresses in land plants. Glycerol has been reported as a component of cutin, contributing up to 14% by weight of total released monomers. Previous studies using partial hydrolysis of cuticle-enriched preparations established the presence of oligomers with glycerol-aliphatic ester links. Furthermore, glycerol-3-phosphate 2-*O*-acyltransferases (*sn*-2-GPATs) are essential for cutin biosynthesis. However, precise roles of glycerol in cutin assembly and structure remain uncertain. Here, a stable isotope-dilution assay was developed for the quantitative analysis of glycerol by GC/MS of triacetin with simultaneous determination of aliphatic monomers. To provide clues about the role of glycerol in dicarboxylic acid (DCA)-rich cutins, this methodology was applied to compare wild-type (WT) Arabidopsis cutin with a series of mutants that are defective in cutin synthesis. The molar ratio of glycerol to total DCAs in WT cutins was 2:1. Even when allowing for a small additional contribution from hydroxy fatty acids, this is a substantially higher glycerol to aliphatic monomer ratio than previously reported for any cutin. Glycerol content was strongly reduced in both stem and leaf cutin from all Arabidopsis mutants analyzed (*gpat4*/*gpat8*, *att1-2* and *lacs2-3*). In addition, the molar reduction of glycerol was proportional to the molar reduction of total DCAs. These results suggest "glycerol-DCA-glycerol" may be the dominant motif in DCA-rich cutins. The ramifications and caveats for this hypothesis are presented.

1. Introduction

Cutin is one of the two major extracellular lipid polyesters in higher plants. As the structural framework of plant cuticles, it is deposited at the outer surface of the primary cell wall. Together with the embedded and subtended waxes, this hydrophobic polymer covers the aerial surface of all land plants, and particularly leaf and stem epidermis, and therefore is one of the most abundant lipid polymers in nature. Cutin contributes to protective cuticle barrier functions against pathogen invasion and the control of gas, water and solutes fluxes, and plays a role in preventing organ fusion. Suberin, the other major lipid polyester, is deposited at the inner surface of the primary cell wall. It is most well known as a major constituent of tree bark, but is found in a variety of internal and external tissues and is also formed as a response to wounding, stress and abscission. Both cutin and suberin are fatty acid- and glycerol-derived polymers that are insoluble in water and organic solvents. Glycerol (**6**) (Fig. 1) was detected as a component of cork suberin in the late nineteenth century (Kügler, 1884), but it was identified as a component of cutin only recently (Graça et al., 2002). The fatty acids released through depolymerization can include simple fatty acids, ω-hydroxy fatty acids (ω-OHFAs) and α,ω-dicarboxylic acids (DCAs). Although C16 and C18 hydroxy fatty acids are usually the dominant cutin monomers (Kolattukudy, 1981), DCAs, originally considered as distinctive suberin monomers (Matzke and Riederer, 1991), have also been identified as major components in Arabidopsis stem and leaf cutins (Bonaventure et al., 2004; Franke et al., 2005), as well as *Brassica napus* leaf cutin (Bonaventure et al., 2004; Chen et al., 2011). The DCA-rich cutin is essential for the formation of stomatal ledges (Li et al., 2007a). Representative cutin monomers found in Arabidopsis are shown in Fig. 1 as **1**–**6**. Despite its well-documented monomer composition, the exact three-dimensional structure of cutin remains elusive.

The insoluble nature of cutin and suberin, and their integration

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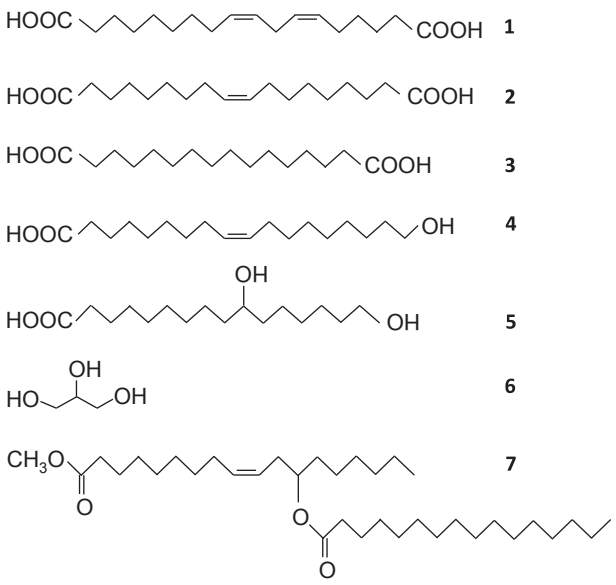

Fig. 1. Structures of the chemical entities described in text, including common Arabidopsis cutin monomers (**1**–**6**) and a synthesized lipid compound 12-palmitoyl-ricinoleate (**7**). Monomers **1**–**6** are analyzed by GC as methyl ester (for COOH groups) and acetyl ester (for OH groups) derivatives. Thus, for example, **4** is analyzed as methyl 18-acetyloxyoctadec-9-*cis*-enoate (methyl 18-acetyloxyoleate), and **6** as glycerol triacetate (triacetin). Collectively, monomers such as **1**–**3** are described as α,ω-dicarboxylic acids (DCAs), and monomers such as **4** and **5** as ω-hydroxy fatty acids (ω-OHFAs). Dihydroxypalmitates occur as a series of positional isomers, the major 10,16-isomer (**5**) being shown.

into the plant cell wall, makes structure determination of these polymers challenging. Through mild partial hydrolysis, monoacylglycerols have been identified from the isolated plant cuticles (Graça et al., 2002) and suberin enriched tissues (Graça and Pereira, 1997, 1999, 2000a; Graça and Santos, 2006). Glycerol-containing trimers, including those with DCA:ω-OHFA:glycerol stoichiometries of 1:0:2, 2:0:1, and 1:1:1, have also been identified from suberin (Graça and Pereira, 2000b; Graça and Santos, 2006). Such oligomer analyses define monomer bonding connectivity but do not produce a complete structural accounting. NMR spectroscopy is an alternative approach to providing a broad structural overview of cutin and suberin preparations, but details can be hard to finesse (Deshmukh et al., 2003; Fang et al., 2001; Stark et al., 2000; Yan and Stark, 2000). In this regard, genetic studies, and mutant analyses in particular, can be advantageous in allowing some inferences about overall polymer structure. For instance, CYP86B1 is a cytochrome P450 essential for the formation of C22:0 and C24:0 DCAs in suberin. Analysis of seed suberin composition in *cyp86b1* mutant established a significant molar portion (≥60%) of C22:0 and C24:0 fatty acids, challenging the traditional view of an extensive aliphatic domain in suberin (Molina et al., 2009). In the same study, the putative role of ester-linked ferulate in suberin lamellae structure was questioned using the *asft* mutant. Lack of this acyltransferase resulted in almost complete loss of suberin-associated ferulate but did not alter suberin lamellae ultrastructure.

In the past decade, significant progress has been made in cutin research using the model plant Arabidopsis. Enzyme families required for cutin biosynthesis have been identified. These include acyl-activating enzymes of LACS family, acyl-oxidizing enzymes of CYP86A subfamily, and acyltransferases of *sn*-2-GPAT family. Each of the three enzyme classes plays an essential role in the provision of cutin acylglycerol monomers, although some details of the process remain obscure (Pollard et al., 2008; Beisson et al., 2012). In particular, *LACS2* (Bessire et al., 2007; Schnurr et al., 2004), *CYP86A2* (or *ATT1*) (Molina et al., 2008; Xiao et al., 2004), and *sn*-2-*GPATs* 4 and 8 (Li et al., 2007a; Yang et al., 2010; Yang et al., 2012) are clearly important for Arabidopsis leaf and stem cutin deposition. Knocking out any of these genes individually, or as a pair (*gpat4*/*gpat8*), has led to large reductions in Arabidopsis cutin monomer loads, especially in the major monomer octadeca-6,9-diene-1,18-dioate (**1**) (C18:2 DCA). *sn*-2-GPATs play pivotal roles in providing glycerol (**6**) as a structural monomer for cutin through regiospecific *sn*-2 acylation of glycerol-3-phosphate (Yang et al., 2010, 2012). Depending on the monomer composition, putative three dimensional cutin structures could be a linear polyester chain, a dendrimeric structure or a heavily cross-linked domain (Pollard et al., 2008).

Since ω-OHFAs represent less than 20% of total aliphatics of Arabidopsis stem and leaf cutins, and DCAs are the major constituents, Arabidopsis stem and leaf cutins are referred to as DCA-rich cutins thereafter. The reduced compositional complexity of these particular cutins, as well as the availability of mutants that are blocked at different steps of cutin synthesis, offer an opportunity to probe the relationship of cutin aliphatic monomer to glycerol. In this regard, the stoichiometry of DCA-rich cutin monomers allows some inferences to be made about cutin structure. Although glycerol has been detected as an Arabidopsis leaf cutin component (Franke et al., 2005), its quantitative measurement has yet to be made. A major unanswered question is whether glycerol (**6**) is central for cross-linking aliphatics to form cutin structure(s) or whether its main function is as a carrier of fatty acyl groups for cutin assembly (Pollard et al., 2008). To address these issues, a stable isotope dilution GC/MS method was developed for quantitative analysis of glycerol (**6**) with simultaneous determination of aliphatic monomers. In addition, Arabidopsis mutants were utilized to explore the possible structural correlation between glycerol and fatty acid monomers. The molar ratio of glycerol:DCAs was 2:1 in both wild-type stem and leaf cutins. Even when allowing for a modest additional contribution from ω-OHFA, this is a substantially higher glycerol to aliphatic monomer ratio than previously reported for any cutin. Analyzing Arabidopsis mutants defective in genes/enzymes that are essential for DCA-rich cutin accumulation showed that the reduction of glycerol (**6**) content remains proportional to the reduction of DCAs. These findings provide new insights for the role of glycerol (**6**) in DCA-rich cutin structure. Glycerol-DCA-glycerol is proposed as the basic structural motif, while an extensive aliphatic domain, that is, a simple acylglycerol copolymer, is likely not present. A large discrimination between transmethylation rates for acylglycerols versus simple primary and secondary aliphatic esters is also reported, a discrimination that may benefit future cutin monomer analyses.

2. Results

2.1. Overview of analytical methods

For their phytochemical screen Graça et al. prepared leaf and fruit cuticles by digestion with cellulase and pectinase, depolymerized the preparations by NaOMe-catalyzed transmethylation, evaporated the total methanolysate to dryness, and analyzed the silylated monomer products by GC (Graça et al., 2002). 1,12-Dodecanediol was used as an internal standard for glycerol quantification, but as this molecule shares limited structural similarity and physical properties to glycerol (**6**), it is not ideal. Our initial glycerol (**6**) analysis attempts using 1,2-alkanediol as internal standard were less consistent and produced lower glycerol (**6**) levels, suggesting that they could not account for all glycerol (**6**) losses. The low boiling points of compounds such as glyceraldehyde and 1,2-propanediol excluded them as internal standards for glycerol (**6**) quantification (Graça and Pereira, 2000c). In studies with

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