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The lipid polyester composition of *Arabidopsis thaliana* and *Brassica napus* seeds

Isabel Molina, Gustavo Bonaventure [1], John Ohlrogge, Mike Pollard *

Department of Plant Biology, Michigan State University, Wilson Road, East Lansing, MI 48824-1312, USA

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Abstract

Mature seeds of *Arabidopsis thaliana* and *Brassica napus* contain a complex mixture of aliphatic monomers derived from the non-extractable lipid polyesters deposited by various seed tissues. Methods of polyester depolymerization of solvent-extracted seeds and analysis of aliphatic monomers were compared. Sodium methoxide-catalyzed depolymerization, followed by GC analysis of the acetylated monomers, was developed for routine quantitative analysis suitable for 0.5 g seed samples. In *Arabidopsis* seeds, the major C16 and C18 monomers identified included ω-hydroxy fatty acids and α,ω-dicarboxylic acids derived from palmitate, oleate and linoleate, and 9,10,18-trihydroxyoctadecenoic acid. Among monomers which can collectively be considered likely to be derived from suberin, docosan-1-ol, docosane-1,22-diol, 22-hydroxydocosanoic acid, 24-hydroxytetracosanoic acid, tetracosane-1,24-dioic acid and ferulic acid were the major species. Compared to *Arabidopsis*, *Brassica* seeds showed a roughly similar proportion of monomer classes, with the exception that alkan-1ols were 3-fold higher. Also, there were much less C24 aliphatic species and significant amounts of C14–C16 alkan-1ols, including *iso*- and *anteiso*-methyl branched compounds. Dissection and analysis of mature *Brassica* seeds showed that the trihydroxy C18:1 fatty acid was found mainly in the embryo, while ferulate, fatty alcohols and C22 and C24 species were specific to the seed coat plus endosperm.

Keywords: *Arabidopsis thaliana*; *Brassica napus*; *Brassicaceae*; Seeds; Lipid polyesters; Cutin; Suberin; Aliphatic monomers

1. Introduction

Plants synthesize two distinct types of insoluble polymers derived from fatty acids: cutin and suberin (collectively called lipid polyesters). Cutin is the structural component of the plant cuticle, the outermost layer of aerial organs of higher plants (Kolattukudy, 1980a,b; Kolattukudy and Espelie, 1985). Waxes embedded in the cutin make the cuticle an efficient barrier against desiccation and gas exchange (Riederer and Schreiber, 2001). The cuticle constitutes the immediate contact zone between the plant and its environment and can function as a barrier to protect against pathogen attack. It controls the diffusion of molecules into plant tissues and plays a role in maintaining the separation of organs during organogenesis. While the cuticle lies on the outer face of the primary cell wall, suberin is located between the inner face of the primary cell wall and the plasma membrane (Kolattukudy, 1980a). Typically, suberin acts as a barrier to control the movement of water and solutes, and to contribute to the strength of the cell wall (Nawrath, 2002). Suberin is typically found in outer bark, and in the epidermis and endodermis of roots. It is also deposited as a wound response by injured plant cells (Kolattukudy, 2001). Suberized cells also occur in other plant tissues, such as bundle sheaths of grasses, in the chalazal region of seed coats (Espelie et al., 1980), at the boundary between the plant and its secretory organs (Thompson et al., 1979), as well as in fibers of cotton (Yatsu et al., 1983; Schmutz et al., 1996).

Cutin polyester is typically composed of esterified hydroxy- and polyhydroxy-C16 and C18 fatty acids

* Corresponding author. Tel. +1 517 355 5237; fax: +1 517 353 1926.
E-mail address: pollard9@msu.edu (M. Pollard).
[1] Present address: Department of Plant Molecular Biology, University of Lausanne, Lausanne, CH-1015, Switzerland.

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(Holloway, 1984; Kolattukudy, 1980a,b; Heredia, 2003). In C16-rich cutins, 16-hydroxy- and 10,16-dihydroxy-palmitic acids are usually dominant, while in C18-rich cutins 9,10,18-trihydroxystearic acid and 9,10-epoxy-18-hydroxy-stearic acid monomers and the corresponding octadecenoic acids are common. In addition, glycerol has been found esterified to cutin aliphatic monomers (Graça et al., 2002) while minor amounts of hydroxycinnamic acids and carbohydrates have been reported as structural components of cutin (Kolattukudy, 1977; Fang et al., 2001). Suberin, on the other hand, contains both aliphatic and aromatic monomers (Holloway, 1984; Bernards et al., 1995; Kolattukudy, 2001; Bernards, 2002). The aliphatic polymer is composed mainly of C16 to C28 ω-hydroxy fatty acids and C16 to C26 α,ω-dioic acids, the latter of which are diagnostic for suberin. There is little mid-chain oxygen functionality. A characteristic feature is the presence of monobasic monomers of very long chain fatty acids and alcohols (C20 to C32, with C22 and C24 being the most common). The aromatic network is a hydroxycinnamate-derived polymer, primarily comprised of ferulic acid, N-feruloyltyramine, cinnamic acid, *p*-coumaric acid or caffeic acid (Bernards et al., 1995). Glycerol is another major compound of this polyester, constituting up to 20% by weight of suberin in oak, cotton and potato (Moire et al., 1999; Graça and Pereira, 2000a,b). The current model describes suberin as a hydroxycinnamic acid-monolignol polyphenolic domain embedded in the primary cell wall and covalently linked to a glycerol-based polyaliphatic domain (Bernards, 2002).

The chemistry of cutin, which varies both with species and organ analyzed (Espelie et al., 1979; Kolattukudy and Espelie, 1985), has been studied largely in leaves and fruits (Martin and Juniper, 1970; Kolattukudy, 1980b). Less is known about the composition of polyesters associated with seeds, in part because a reliable protocol for their analysis has not been developed. The seed coat plays an essential role in seed survival by providing mechanical and chemical protection, acting as a barrier to gas and water exchange, and maintaining seed dormancy (Boesewinkel and Bouman, 1995). The maternally derived epidermal tissues in the seed (i.e. the seed coat) are capable of forming a cuticle, which is not necessarily on the outer surface of the organ (Martin and Juniper, 1970). A cuticle, which may originate from the ovule, can also develop between the seed coat and the remains of the nucellus or endosperm. Early in the development of citrus seeds, cuticle-free channels to the embryo sac exist at the chalazal region and are later sealed with suberin polymers (Espelie et al., 1980). This has also been observed in wheat grains (Zee and O'Brien, 1970) and barley seeds (Cochrane, 1983). Suberized cell walls have also been described in the epidermis of cotton seeds (Ryser, 1992). The basic cellular layers that form the seed coat in *Brassicaceae*, which includes the genera *Arabidopsis* and *Brassica*, are similar among species in this family (Moise et al., 2005). Although several cytological studies have been performed (Van Caeseele et al., 1981, 1982; Beeckman et al., 2000), no evidence of cuticles and/or suberized cell walls in the mature seeds of *Brassica* species were reported. Such features may have been overlooked.

Plant lipid polyesters are poorly understood at the structural, biosynthetic and genetic levels. However, the molecular genetic tools available for *Arabidopsis thaliana* should change this picture. Both forward and reverse genetic screens require robust chemical analyses to complement assays of functional properties such as cuticle permeability, organ fusion phenotypes, or pathogen susceptibility. Such analyses have only recently been published (Bonaventure et al., 2004; Xiao et al., 2004; Franke et al., 2005). However, a reliable method for seed polyester analysis is currently lacking. In this work, we report the development of a quantitative method to analyze the polyester monomer content and composition in whole seeds of *A. thaliana* and *Brassica napus*.

2. Results and discussion

2.1. Monomer analysis methods – introduction

The analysis of cutin and suberin monomer composition and content requires a depolymerization step to cleave ester bonds. Typically, this is achieved by one of four methods; saponification, acid-catalyzed transmethylation, base-catalyzed transmethylation, or hydrogenolysis (Holloway, 1984; Kolattukudy, 2001). Analysis of the extracted monomers by GC or GC–MS is usually undertaken after derivatization to produce TMSi ethers and esters, since they give very diagnostic mass spectra. Saponification and transmethylation will also cleave amides, producing fatty acids or their methyl esters, respectively. In choosing between the various methods, there is a trade-off between ease and reliability of the assay, and the loss or overlap of specific components. Our aim was to produce a robust method that could be used routinely for GC analysis of total seed polyesters. Apart from instrument availability, one reason for using a GC method for a quantitative screen is that the FID detector offers a very linear response over a very wide mass range and a simple theoretical correction factor when compared to total ion current quantification by GC–MS. Previously we used hydrogenolysis in conjunction with deuteriolysis (Walton and Kolattukudy, 1972) to analyze the polyesters present in the epidermal layer of Arabidopsis leaf and stem (Bonaventure et al., 2004). A drawback of this method is that it requires GC–MS analysis to distinguish the degree of deuteriation of fatty polyols, in order to make assignments of structure. For example a 1,ω-diol hydrogenolysis product may be derived from 1,ω-diol, ω-hydroxy fatty acid and/or 1,ω-dicarboxylate monomers. The isotopomer analysis may introduce errors especially if it is conducted on weak molecular ion multiplet peaks. This is particularly problematic for lower abundance fatty polyol products. In our hands *O*-TMSi esters