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

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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

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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-hydroxystearic 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 also were quantitatively problematic, giving variable response factors significantly lower than theoretical, probably because of injector decomposition. Thus, for this study, we preferred transmethylation with fatty acid methyl ester analysis over saponification and silylation of hydroxyl and carboxylate groups. For the GC analysis method we used acetylation, which provides a more stable derivative of hydroxyl groups; silylation was used for identification purposes in setting up the method.

Although base-catalyzed transmethylation will not destroy epoxy fatty acids, the subsequent acetylation will rapidly convert epoxides to their corresponding vicinal diol diacetates. This was an acceptable trade-off since in *Arabidopsis* 9,10,18-triol C18 fatty acid monomers are considerably more abundant than the 18-hydroxy-9,10-epoxy C18 fatty acid monomers, both of which give 9,10,18-triacetoxy-fatty acid methyl ester products. In practice transmethylation with sodium methoxide in methanol always produced variable amounts of saponified products, indicating water in the system, despite our attempts to thoroughly dry the extracted and finely ground seed residues. To combat this we used methyl acetate as a co-solvent at 15% volume (Christie, 1982). Any water in the system will produce NaOH from NaOMe. This NaOH will be rapidly removed by saponification of methyl acetate to produce sodium acetate and methanol. A small fraction of the free hydroxyl groups in the monomers released will be acetylated by the equilibrium transesterification reaction $ROH + MeOAc \leftrightarrow ROAc + MeOH$, but this does not matter, since for the routine analysis the transmethylated sample is fully acetylated prior to GC separation.

2.2. Characterization of monomer analysis methods

The whole mature *Arabidopsis* seed (about 18 μg dry weight) contains about 6–7 μg of lipid, mainly triacylglycerol (Li et al., 2006), but only about 45 ng of total polyester monomers. Finely ground seeds are quenched in hot isopropanol to inactivate any degradative enzymes. When this is followed by multiple extractions with chloroform–methanol mixtures almost all the soluble endogenous lipid is removed (>99.5%), but there are still some levels (15–20 ng/seed) of normal fatty acids and also sinapic acid released in the polyester analysis. This is reduced 10-fold when the extraction protocol also includes additional methanol and aqueous washes, as described in Section 4.2. These washes reduced the dry weight of the recovered seed residues from about 50% to 31% of the initial seed mass, yet gave similar or higher monomer recovery on a per seed basis. Triacylglycerol was recovered from these washes, indicating physical trapping as a dominant source of the excess normal fatty acids.

A 48 h time course was run for the NaOMe–MeOH–MeOAc reaction, with two internal standards, namely methyl heptadecanoate acting as a mass standard and ω-pentadecalactone acting as a control for both transmethylation and acetylation reactions (Fig. 1, upper panel). This showed that most of the polyester monomers were released in the first 30 min of the transmethylation reaction. The rapid depolymerization is expected (Holloway, 1984). However, with the exception of tetracosanoate, the normal fatty acids were transmethylated more slowly. We expect that most of the C16–C20 fatty acids (which represent 9.75 mol% of total monomers – Table 1) originate from “physically trapped” triacylglycerol. In support of this interpretation, we analyzed the *fae1* mutant line which is blocked in accumulation of C20 and C22 fatty acids in the seed triacylglycerols (Kunst et al., 1992; James et al., 1995). The effect of the *fae1* mutation on seed polyester monomers was minimal, with the exception that eicosenoate showed a 90% reduction (from 1.4 ± 0.4 to 0.12 ± 0.05 mol%) consistent with its origin from triacylglycerols. The C18 unsaturated fatty acids (5.5 ± 1.5 mol% in wild type) are therefore likely to be derived mainly from trapped triacylglycerols. The one fatty acid that is rapidly transmethylated is tetracosanoate, a behavior that groups it with other polyester monomers.

In addition to the time course, various amounts of the exhaustively extracted seed residue were transmethylated with the NaOMe–MeOH–MeOAc reagent under the standard conditions (60 °C for 2 h) and polyester monomer content determined. The response was linear over the 25–400 mg sample range tested (Fig. 1, lower panel). Particular attention was paid to the recovery of aromatic components. Spiking seed residues with methyl coumarate, ferulate or sinapate gave essentially quantitative recovery (>90%) of these components for the transmethylation–acetylation protocol. Spiked tyramine recovery was lower (ca. 30%) and not enhanced by additional extractions under alkaline conditions. Thus the lack of tyramine observed in seed residue depolymerizations, even when run with either basic and acidic transmethylations over extended periods to allow for complete amide bond cleavage, is taken as an indication that tyramine adducts are not part of the seed polyester matrix unless they have been cross-linked through phenol coupling reactions.

A comparison of recently published monomer compositions from Arabidopsis leaf, stem and cuticle preparations obtained by four depolymerization methods (Nawrath, 2006) shows that there is a substantial discrepancy over the presence of C22:0, C24:0, C24:1 and C26:0 2-hydroxy fatty acids reported in some studies (Franke et al., 2005; Kurdyukov et al., 2006), and their absence in others (Bonaventure et al., 2004; Xiao et al., 2004; Suh et al., 2005). We compared depolymerization by acid and base catalyzed transmethylation on the same batch of seed residues for 1 and 48 h of reaction, using only organic solvents or the combined organic and aqueous extractions to produce delipidated seed residues. The data set for the 48 h depolymerizations are given in Fig. S1. Both acid and base-catalyzed transmethylations when run for extended times give 2-hydroxy fatty acid methyl ester (FAME) products. However, the acid-catalyzed reaction is much faster, producing

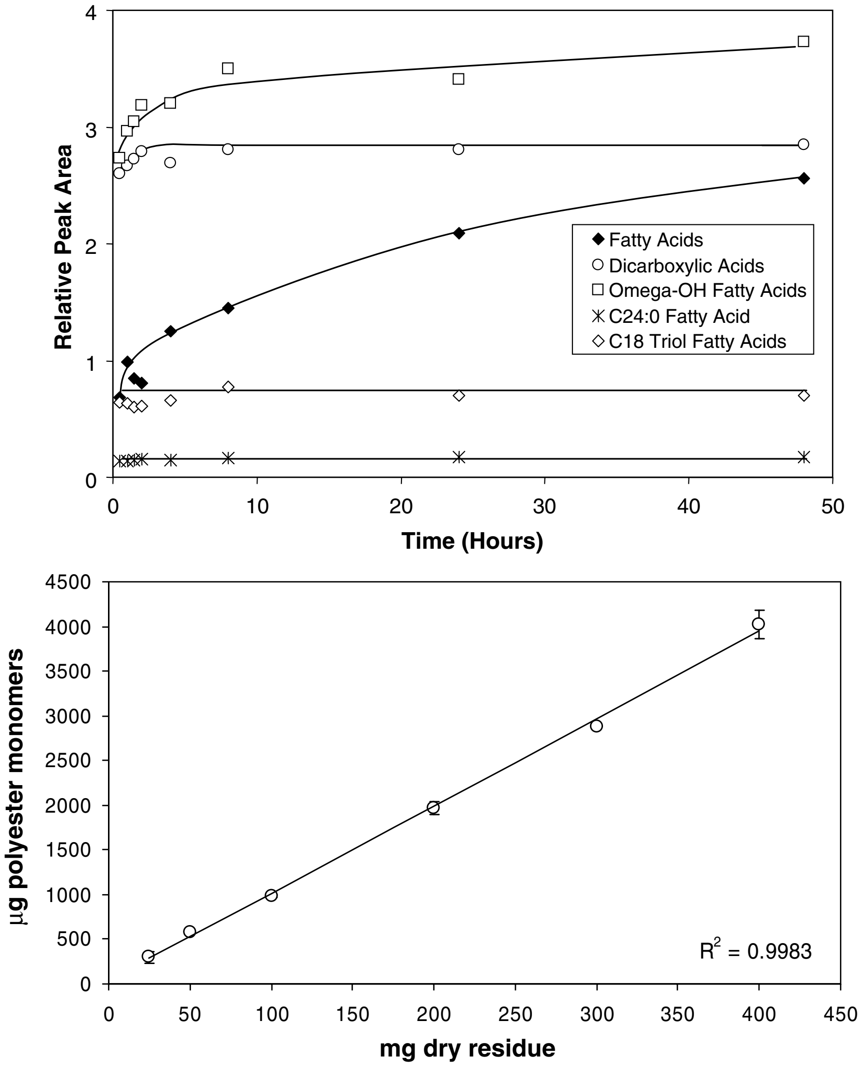

Fig. 1. Standardization of the NaOMe–MeOH–MeOAc transmethylation reaction for depolymerization of exhaustively extracted *Arabidopsis* seed residues. Products were analyzed by FID-GC after acetylation, with the TIC of peaks of interest normalized against the TIC peak area for the recovery of methyl heptadecanoate internal standard. The upper panel shows the time course for appearance of total normal fatty acid methyl esters, total α,ω–dicarboxylate dimethyl diesters, total ω-hydroxy fatty acid methyl esters, methyl tetracosanoate and methyl trihydroxyoctadecenoate products. The lower panel shows the amount of total monomers recovered when 25–400 mg of seed residues was added to the reagent and transmethylated for 2 h at 60 °C. Each data point is the average of duplicates.

observable 2-hydroxy FAMEs within the first hour, whereas the base-catalyzed reaction does not. At 48 h of transmethylation, the acid-catalyzed method produced 8.2 ± 1.23 mg of total monomers/g seed residue, of which 0.75 ± 0.08 mg/g (9.1%) was 2-hydroxy fatty acid, whereas the base catalyzed method produced 7.5 ± 0.84 mg/g of total monomers, of which 0.44 ± 0.1 mg/g (5.9%) was 2-hydroxy fatty acid. The monomer distribution between the two methods was similar (Fig. S1). The aqueous extractions did not remove significant 2-hydroxy fatty acids from the seed residues.

In setting up our method we have chosen depolymerization by NaOMe-catalyzed transmethylation reaction over a short time to minimize the contribution of the 2-hydroxy fatty acids. We suspect that the 2-hydroxy fatty acids and low levels of very long-chain fatty acids (e.g. C26:0, C26:1, C28:0, C28:1) are derived from the *N*-acyl groups of sphingolipids, since these acyl compositions are characteristic of sphingolipids (Ohnishi et al., 1983; Imai et al., 1995; Markham et al., 2006). Additional reasons for this conclusion are as follows. Firstly, if 2-hydroxy fatty acids were present in the insoluble matrix as *O*-acyl esters, then reaction with $LiAlH_4$ should produce 1,2-diol products. However, although hydrogenolysis of the seed residues with $LiAlH_4$ produces quantitative recovery of alcohol products corresponding to all other aliphatic components, 1,2-diols are absent. Hydrogenolysis will not cleave amide linkages, but instead reduce amides to amines, thus producing products which are not observed in the GC–MS analysis. Secondly, the slow kinetics of 2-hydroxy fatty acid transmethylation is consistent with their presence as amides, not esters. And thirdly, the bulk of sphingolipids in plants are not readily extractible in organic solvents (Sperling et al., 2005; Markham et al., 2006). These are the glycosylated inositol phosphoceramides, which may not be extracted in our protocol. Thus any 2-hydroxy fatty acids recovered by transmethylation will likely represent bulk seed sphingolipids. That is not to say that sphingolipids may not play a role in either the biogenesis of the cuticle, or the cuticle itself. However, leaf and stem, where cuticles can be isolated, seem a better system to investigate this possibility than seed.

Table 1
Monomer composition for the depolymerization of solvent-extracted wild type *Arabidopsis thaliana* seed residues by NaOMe-catalyzed transmethylation

#. Polyester monomer	Relative amount (mol%)
1. Octadecan-1-ol	1.5 ± 0.1
2. Eicosan-1-ol	1.5 ± 0.1
3. Docosan-1-ol	2.8 ± 0.25
4. Nonadecan-1-ol, branched	0.3 ± 0.05
5. Tricosan-1-ol, branched	0.35 ± 0.05
Total alkan-1-ols	*6.5 ± 0.45*
6. 16-Hydroxyhexadecanoic acid	1.75 ± 0.1
7. 18-Hydroxyoctadecadienoic acid	4.55 ± 0.6
8. 18-Hydroxyoctadecenoic acid	3.45 ± 0.25
9. 18-Hydroxyoctadecanoic acid	0.25 ± 0.05
10. 20-Hydroxyeicosanoic acid	0.65 ± 0.1
11. 22-Hydroxydocosanoic acid	4.2 ± 0.25
12. 22-Hydroxydocosanoic acid, branched	0.7 ± 0.05
13. 23-Hydroxytricosanoic acid	0.55 ± 0.05
14. 24-Hydroxytetracosanoic acid	12.6 ± 0.45
15. 24-Hydroxytetracosanoic acid, branched	0.4 ± 0.05
16. 25-Hydroxypentacosanoic acid	0.3 ± 0.05
17. 26-Hydroxyhexacosanoic acid	0.2 ± 0.05
Total ω-hydroxy fatty acids	*29.6 ± 1.95*
18. 1,16-Hexadecane dioic acid	1.8 ± 0.1
19. 1,18-Octadecadiene dioic acid	8.9 ± 0.75
20. 1,18-Octadecene dioic acid	3.4 ± 0.2
21. 1,18-Octadecane dioic acid	0.5 ± 0.05
22. 1,22-Docosane dioic acid	1.65 ± 0.1
23. 1,24-Tetracosane dioic acid	8.5 ± 0.4
Total 1,ω-Dicarboxylic acids	*24.75 ± 1.6*
24. 1,20-Eicosane diol	0.3 ± 0.05
25. 1,22-Docosane diol	2.4 ± 0.2
Total 1,ω-alkane diols	*2.7 ± 0.25*
26. Hexadecanoic acid	2.0 ± 0.25
27. Octadecanoic acid	0.35 ± 0.1
28. C18:1, C18:2, C18:3 acids	5.5 ± 1.5
29. Eicosanoic acid	0.5 ± 0.05
30. Eicosenoic acid	1.4 ± 0.4
31. Docosanoic acid	0.5 ± 0.05
32. Tetracosanoic acid	1.5 ± 0.1
33. Hexacosanoic acid	0.55 ± 0.05
34. Hexacosenoic acid	0.45 ± 0.1
35. Octacosenoic acid	0.15
36. Octacosenoic acid	0.3 ± 0.05
37. Dotriacontanoic acid	0.1
38. Dotriacontenoic acid	0.15
39. Tetratriacontenoic acid	0.15
Total fatty acids	*13.6 ± 2.9*
40. 2-Hydroxytetracosanoic acid	0.4 ± 0.15
41. 10,16-Dihydroxyhexadecanoic acid	0.55 ± 0.25
42. 9,10,18-Trihydroxyoctadecenoic acid	4.8 ± 0.85
Secondary hydroxy-containing species	*5.75 ± 1.25*
43. Ferulate	15.2 ± 1.3
44. Sinapate	1.4 ± 0.5
45. C29:1 Sterol (sitosterol?)	0.5 ± 0.05
Other	*17.1 ± 1.9*

Three extractions of bulked *Arabidopsis thaliana* seed (ecotype Col0) batches were performed and each seed residue was analyzed in triplicate, to give nine determinations, reported as the average ± SD. GC analyses were undertaken on acetyl derivatives. Peaks that were identified and that are at least 1% of the peak area of the greatest peak, 24-hydroxytetracosanoate, were summed to give 100 mol%. Unidentified peaks represented 18% of the identified peak by peak area. Numbers correspond to the peaks of the chromatogram in Fig. 2.

2.3. Monomer analysis methods – identification and quantification of monomers

The basis of the identification of the products released by sodium-methoxide catalyzed transmethylation was mass spectrometry of TMSi derivatives of the transmethylation products. These were compared to a set of standard compounds, data from mass spectroscopy libraries and the literature (Heller and Milne, 1978; Murphy, 1993; Bonaventure et al., 2004). Additional evidence of structure came from the retention times of homologous series when compared to known standards before and after TLC fractionation, and from catalytic hydrogenation of the transmethylated sample prior to silylation and GC–MS analysis. The results from transmethylation were also compared with the composition obtained from hydrogenolysis, and from analysis of the molecular ion clusters of mono-, di-, tri- and tetraol-products of deuterium incorporation after deuteriolysis. Close correspondence of the major components by hydrogenolysis/deuteriolysis and transmethylation indicates that they are present mainly if not exclusively as esters, not amides in the solvent-insoluble residue. Diagnostic MS ions for the major monomers obtained by transmethylation and silylation of *Arabidopsis* seed residues are given in supplement (Table S1). In addition, a presumed lignan and two diterpene acids were persistent minor components, tentatively identified as described in the supplement. One important criterion for ensuring good reproducibility is to harvest fully mature seed. The deposition of *Brassica* seed polyesters occurs fairly late in seed maturation and compositional changes occur right up to seed maturity. In particular, the deposition of 10,16-dihydroxypalmitate is a very late event. Presumably the same occurs in *Arabidopsis*.

Fig. 2 shows a typical chromatogram for the arabidopsis seed monomers from methanolysis followed by acetylation. The only significant peak overlap is of eicosan-1-ol with dimethyl octadecadiene-1,18-dioate. As a screening tool this is unlikely to cause a problem because octadecan-1-ol contributes almost the same mol% as eicosan-1-ol (Table 1), allowing an approximate subtraction of the combined eicosan-1-ol/18:2 dicarboxylate peak. An exact quantification can be determined by one of several methods, which include hydrogenation or TLC separation to give an accurate measurement of eicosan-1-ol versus octacosan-1-ol, or by the simple expedient of using TMSi derivatives, when they separate well.

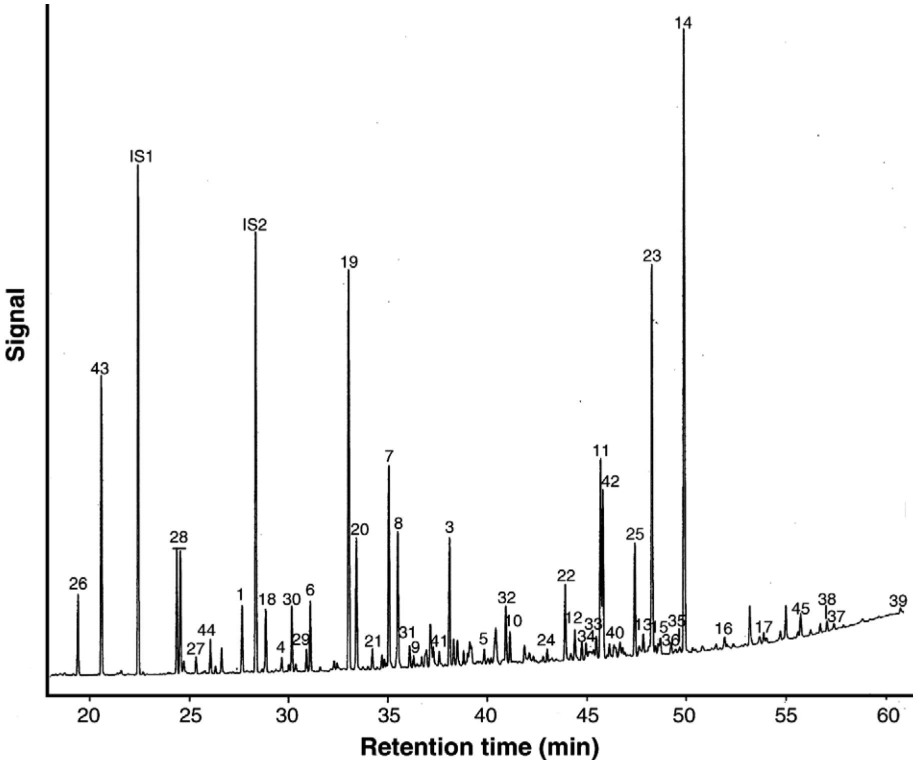

Fig. 2. Chromatogram for GC analysis (DB-5 column) of monomers released by depolymerization of solvent-extracted *Arabidopsis* seed residues with NaOMe-catalyzed transmethylation and then acetylation. The internal standards methyl heptadecanoate (IS1) and methyl 15-acetoxypentadecanoate (IS2) are indicated. The numbers on the peaks correspond to the monomers detailed in Table 1.

2.4. Identification of 9,10,18-trihydroxyoctadecenoate

Methyl trihydroxyoctadecenoate was identified as the major polyester monomer of mature *Brassica* embryos dissected from seeds (Section 2.8). It was therefore important to define its structure. The mass spectrum of the TMSi derivative from transmethylation showed a strong cleavage of the C(9)–C(10) bond with ionization towards the carboxyl end to give $m/z = 259$ ion (60%), and in the other direction a weaker $m/z = 301$ ion (5%). These ions define the C(9)–C(10) vicinal diol structure and place the double bond beyond C(11) (Eglinton and Hunneman, 1968). Cleavage at the C(10)–C(11) bond with ionization towards the carboxyl end gave a $m/z = 361$ ion (9%), which on further loss of TMSiOH produced the $m/z = 271$ ion (34%). Cleavage at the C(10)–C(11) bond is presumably driven by the proximal Δ12 double bond, which will produce the C(1)–C(10) fragment as the cation and the C(11)–C(18) fragment as an allylic-stabilized radical fragment. A small peak at $m/z = 545$ corresponding to (M-15) was also observed. After hydrogenation, the corresponding saturate had diagnostic $m/z = 259$ (100%) and 303 (30%) ions. Comparison of the retention times of the hydrogenated trihydroxy methyl ester with the products from apple peel cutin suggested the major isomer corresponds to that of apple peel cutin and therefore has the *erythro* configuration (Eglinton and Hunneman, 1968). Hydrogenolysis and deuterolysis produced octadecen-1,9,10,18-tetraol (Walton and Kolattukudy, 1972). Mass spectroscopy of the TMSi derivative showed cleavage of the C(9)–C(10) bond with ionization towards the carboxyl end to give a diagnostic $m/z = 303$ ion (8%), and in the other direction a weak $m/z = 301$ ion (2%). Cleavage at the C(10)–C(11) bond with ionization towards the carboxyl end gave a $m/z = 405$ ion (2%), which with further loss of TMSiOH gives the $m/z = 315$ ion (4%). On deuteriolysis the ions were 301, 305, 317 and 407, respectively, indicating that the precursor is indeed the trihydroxy fatty acid and not the 18-hydroxy-9,10-epoxy fatty acid and again placing the double bond at the distal end of the molecule. Since we have little of this material further experiments to define regio- and stereochemistry were not undertaken, but it is reasonable to assume that this main fatty acid polyol is derived from linoleate and therefore has a 12-*cis* double bond. As further evidence of this possibility we note that labeled linoleic acid has been shown to be a precursor of 9,10,18-trihydroxyoctadecenoic acid (Kolattukudy et al., 1973). Analysis of spectra for the minor C18 tetraol peak after hydrogenolysis and deuterolysis indicated that both 9,10,18-trihydroxyoctadecenoate and 9,10-dihydroxy-1,18-octadecene dicarboxylate were precursors.

2.5. Identification of branched-chain monomers

Iso- and *anteiso*-methyl-branched alkanes have occasionally been reported as constituents of epicuticular waxes (Kolattukudy, 1980b; Kolattukudy and Espelie, 1985), but such branched structures are rarely if ever observed for polyester monomers. In this work, branched-chain aliphatic monomers were identified as minor and sometimes significant components in seed polyesters. In *Brassica* seed there were substantially more branched-chain monomers present than in *Arabidopsis* seed, most notably as C14 (2.1 mol%) and C15 (4.9 mol%) saturated alkan-1-ols. After enrichment by preparative TLC the branched-chain primary alcohols were identified using GC–MS of their TMSi ethers. Their mass spectra were very similar to mass spectra for straight-chain primary fatty alcohols. Their retention times relative to straight-chain fatty alcohols were 13.6 and 14.75 for the C14 and C15 compounds, respectively. This compares to reductions of 0.37–0.44 and 0.27–0.31 ECL units, respectively, for *iso*- and *anteiso*-methyl-branches relative to straight-chain compounds on non-polar GC columns (Body, 1984); finally, the C15 component co-eluted with the *anteiso*- and not the *iso*-pentadecan-1-ol standard. Thus the compounds were identified as 12-methyltridecan-1-ol (*iso*-tetradecan-1-ol) and 12-methyltetradecan-1-ol (*anteiso*-pentadecan-1-ol). They presumably arise from primers for fatty acid synthesis derived from valine and isoleucine, respectively. *Arabidopsis* seed contained small amounts of C18, C19, C22 and C23 branched-chain alkan-1-ols (Table 1).

Turning to the ω-hydroxy fatty acid methyl esters, this fraction isolated by preparative TLC from *Brassica* seed transmethylation products did not contain branched-chain hydroxy fatty acid methyl esters although it did contain odd-chain hydroxy fatty acid methyl esters (Table S2). However, in *Arabidopsis* seeds the most abundant branched-chain compounds were identified by the mass spectra of their OTMSi-ether derivatives as methyl branched-chain hydroxy-docosanoate and hydroxy-tetracosanoate, respectively (0.7 and 0.4 mol%, Table 1). They ran before the corresponding straight-chain compounds by 0.55 and 0.60 ECL units, respectively, on the DB-5 column. The question arises as to the exact structure of these compounds. Chromatographic data describes the behavior of *iso*- and *anteiso*-methyl-branched chains, which cause reductions of 0.4 and 0.3 ECL units, respectively, compared to straight chains (Body, 1984). Also for comparison, on a non-polar column methyl 17-acetoxystearate elutes before methyl 18-acetoxystearate by 0.75 ECL units (Tulloch, 1964). As the compounds are of even-carbon we can rule out *anteiso*-branching derived from isoleucine precursor. Considering the branched-chain methyl hydroxy-docosanoate (the same reasoning applies to the tetracosanoate homolog) we have to distinguish between (i) 21-hydroxydocosanoate, (ii) other positional isomers that give secondary alcohols of the straight-chain compound, (iii) 21-hydroxy-20-methylhenicosanoate, and (iv) other positional isomers of 20-methylhenicosanoate containing a secondary hydroxyl group. There are no authentic hydroxy fatty acid standards available to test these possibilities.

When the very long-chain hydroxy fatty acids and dicarboxylic acid esters were fractionated by preparative TLC it became clear that each class of compounds contained a similar profile of minor components, which eluted in a similar order by GC (Table 2). The odd-chain and branched-chain components in the dicarboxylate fraction are at lower levels than for the ω-hydroxy fatty acids and thus would be under the GC integration thresholds set for the analysis given in Table 1. On preparative silica TLC, the branched-chain hydroxy fatty acids esters and dicarboxylate diesters each ran very slightly ahead of the corresponding straight-chain esters or diester. This is the expected chromatographic behavior on introducing a methyl group in the α-position to the ester or primary alcohol functional group. The mass spectra of straight-chain saturated dimethyl diesters was dominated by ions at $m/z = 98$ (100%), with additional diagnostic ions at $m/z = 74$ and 112, and a relatively abundant (M-31) ion. However, the branched-chain dicarboxylates, while retaining the $m/z = 74$, 112 and 98 ions, had as their base peak $m/z = 88$. The mass spectra are shown in Fig. S2. The $m/z = 88$ ion is the expected ion arising from McLafferty fragmentation of a 2-methyl FAME, and confirms the structures of the branched chain components as dimethyl 2-methyl-heneicosane-1,21-dioate and dimethyl 2-methyl-tricosane-1,23-dioate, respectively. The mass spectra of the branched-chain hydroxy-FAME TMS ethers were essentially identical with the spectra of the straight-chain compounds. If (ω-*n*) hydroxy groups were present they should give distinctive CHROTMSi fragments (e.g. $m/z = 117$ for the (ω-1)-hydroxy group), but no such distinctive fragments were observed.

Our identification of 21-hydroxy-20-methylheneicosanoate and 23-hydroxy-22-methyltricosanoate methyl esters, and 2-methyl-branched even carbon number dicarboxylates in *Arabidopsis* seed polyesters lacks stereochemical definition, as it is based solely on mass spectral and chromatographic R_f and R_t data. However, it is consistent with the presence of odd-chain and *iso*- and *anteiso*-methyl branching found in *Brassica* seed polyester alkan-1-ols. Although these novel compounds are minor components, they provide biochemical insight by showing that the oxidation systems producing ω-hydroxy and carboxylate functional groups can readily accommodate a 2-methyl group.

Table 2
Minor components of purified hydroxy fatty acid methyl ester and dimethyl dicarboxylate fractions isolated from *Arabidopsis thaliana* seed polyesters

Chain	ω-Hydroxy fatty acid	Dicarboxylic acid
C20	0.11	0.01
C21	0.01	nd
C22 branched	0.07	0.02
C22	0.39	0.17
C23	0.06	0.02
C24 branched	0.035	0.02
C24	1.0	1.0
C25	0.03	0.01

For each fraction these components are shown normalized to the mass of the straight-chain C24 compound (1.0).

2.6. Analysis of A. thaliana seeds

Table 1 shows the monomers released by transmethylation from *A. thaliana* (ecotype Col0) seed residues that had been extracted with organic and aqueous solvents. The identified components give a total of 8.6 ± 0.5 mg/g seed residue (26.1 ± 1.5 μmol/g seed residue). This value results in an estimate of 46 ng monomers/seed, which corresponds to about 12 μg monomers/cm^2 seed surface area. The mass per unit seed area does not represent an actual thickness of a specific polyester layer, since the location of the polyesters in the various seed tissues has not been determined and since other components may be present in such layers. However, the mass/area value is given to make comparisons with reported values for the *Arabidopsis* polyester monomers of about 1.5 μg/cm^2 for leaf and 3–9 μg/cm^2 for stem (Suh et al., 2005) and with *Brassica* seeds (see below). Only 18% of the total integrated peaks were unknown compounds, none of which was greater than 1%. Trace amounts of expected monomers not reported in Table 1 such as tetracosane-1,24-diol, 18-hydroxy-octadecanoic acid, and tricosane-1,23-dioic acid were observed. In addition, hydrogenation and deuterolysis studies suggested that there are trace amounts of both 9-hydroxy and 9,10-dihydroxy C18 1,18-dicarboxylic acids; the former may be derived from the epoxide. Previously, high dicarboxylic acid concentrations were generally considered to be indicative of suberin like polyesters. However, the presence of high proportions of C16 and C18 dicarboxylic acids in the leaf and stem epidermal layers (Bonaventure et al., 2004), and the presence of these in the isolated cuticles (Franke et al., 2005), suggest that this generalization is not appropriate for *Arabidopsis*. *Arabidopsis* seeds contain high amounts of 1,ω-bifunctional long-chain aliphatic compounds (primarily C22:0 and C24:0), to a total of 36.8 mol%, plus ferulate at 15 mol%. The co-occurrence of these monomers is indicative of suberin in the seeds. Branched- and odd-carbon components make up 1.5 mol% of the total. Normal fatty acids constitute 13.6 mol%, but as stated earlier much of this may be derived from physically trapped triacylglycerols. Also, the very long-chain saturated and monounsaturated fatty acids, and 2-hydroxytetracosanoate (total of 2.2 mol%) may be derived from *N*-acyl groups of residual sphingolipids, and specifically the polar, highly glycosylated inositolphosphoceramides, which we assume are incompletely extracted by the protocol.

2.7. Analysis of B. napus seeds

The total seed polyester composition of *B. napus* was determined. The major components are shown in Fig. 3, in comparison with *A. thaliana*. A full tabulation for *B. napus* seeds is found in Table S2. A canola variety of rapeseed, *Westar*, was used to minimize the C20 and C22 fatty acid contamination from the seed oil residues. The same classes of monomers found *Arabidopsis* are also present in the *Brassica* seeds, but there are several important quantitative and qualitative differences (Fig. S3). Firstly, *Brassica* has significantly more 1-alkanols than *Arabidopsis* (20.4 versus 6.5 mol%). These are gained mainly at the expense of dicarboxylic acids. Secondly, the *Brassica* seed has a much higher content of shorter chain length (⩽C16) aliphatics (40.0 versus 6.1 mol%), and a reduction

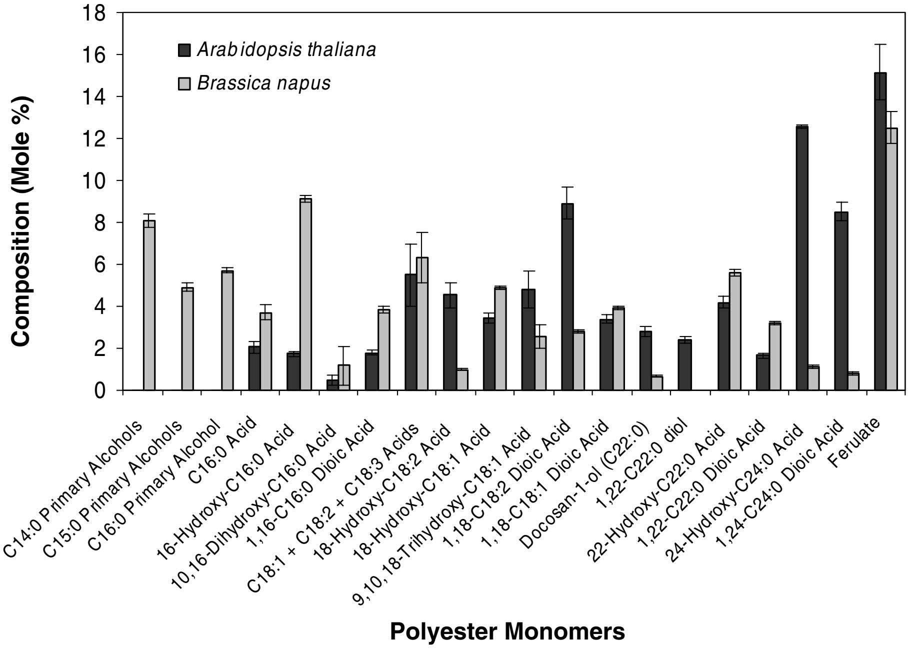

Fig. 3. Comparison of the major monomer composition after depolymerization of solvent-extracted *Arabidopsis thaliana* and *Brassica napus* seed residues by NaOMe-catalyzed transmethylation. Standard deviations for triplicate determinations are shown.

in C24 aliphatics (2.6 versus 22.9 mol%). In particular, the C14–C16 alkan-1-ols and 16-hydroxypalmitate are significant components of the depolymerized *Brassica* seeds when compared to *Arabidopsis* seeds, whereas 24-hydroxytetracosanoate and 1,24-tetracosane dioate are largely absent. Also, *Brassica* contains significantly more branched- and odd-carbon components than *Arabidopsis* (8.9 versus 3.2 mol% of the total). We expect that the considerable differences in monomer composition between *Arabidopsis* and *Brassica* seeds reflect genetics and seed size, but cannot rule out environment as a contributor to the variance. One of the reasons we conducted the analysis on *Brassica* seeds was to see if the polyester monomer compositions were similar enough between species to use *Brassica* instead of *Arabidopsis* in studies on seed development and seed tissue localization. Despite the species differences this appears to be a reasonable assumption.

For *Brassica* seeds, the identified components give a total of 1.94 ± 0.28 mg/g seed residue (6.8 ± 1.0 µmol/g seed residue). This value results in an estimate of 0.66 mg monomers/g seed, which, using the data set of Li et al. (2006) (*Brassica* seed wt. 4.2 mg, seed surface area 12 mm^2), gives an estimate of 23.1 µg monomers/cm^2 seed surface area. This is twice the value obtained for *Arabidopsis* and may be related to the fact that the seed coat is ~50 µm thick in *B. napus* and 20 µm thick in *Arabidopsis*.

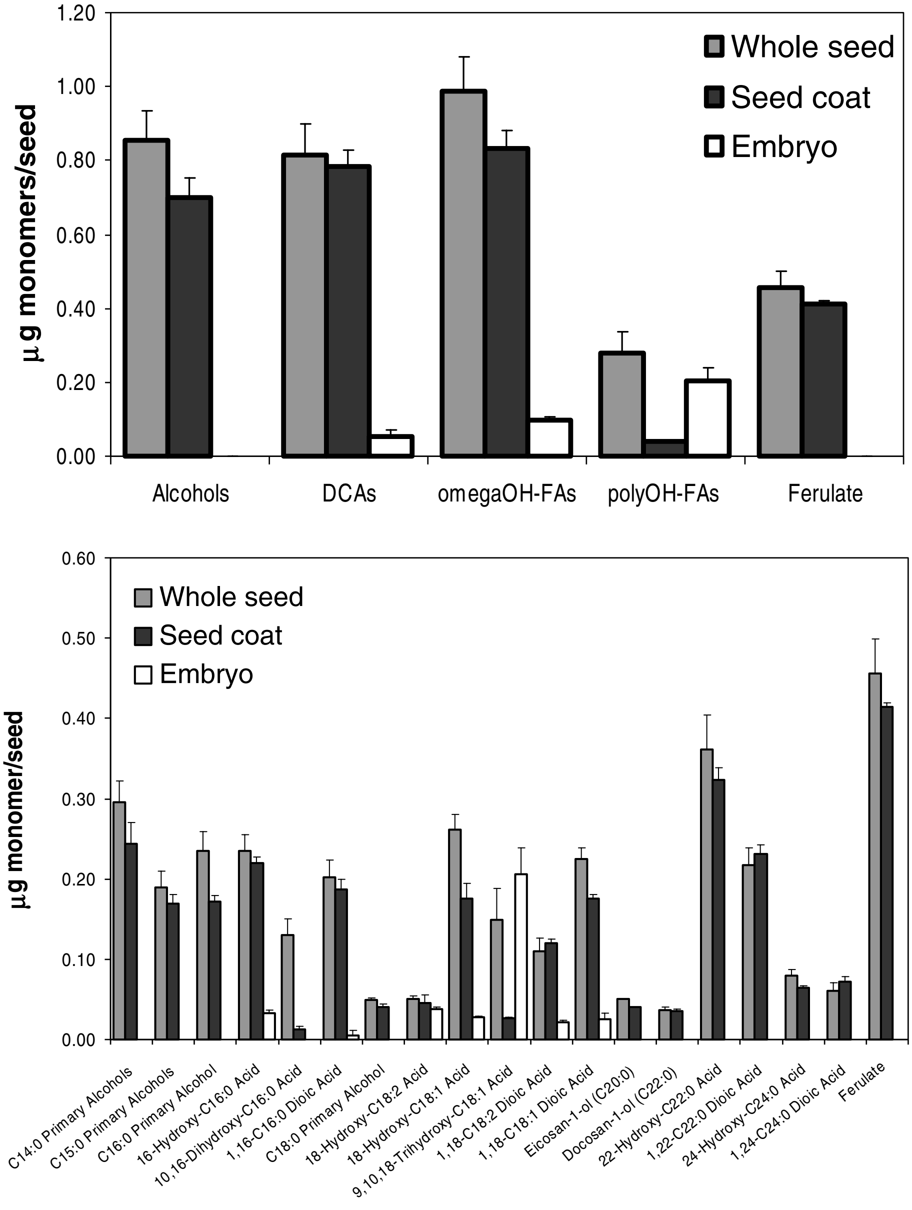

Fig. 4. The distribution of monomer classes in whole seed, seed coat and embryo of *Brassica napus* mature seeds (upper panel), and the distribution of individual monomers in whole seed, seed coat and embryo of *Brassica napus* mature seeds (lower panel). Standard deviations for triplicate determinations are shown.

2.8. Distribution of monomers between seed coat and embryo in B. napus seeds

Seeds are complex organs so it is expected that the polyesters may originate from a variety of distinct cell layers. To further investigate the specific location of polyester monomers within the seed, *B. napus* mature dry seeds were manually dissected into seed coats and embryos. The endosperm layer remained attached to the seed coats, as shown by the high content of the endosperm-specific ω7 monounsaturated fatty acids (Li et al., 2006) in extractable lipids from this fraction (data not shown). Fig. 4 (upper panel) shows the distribution of polyester monomer classes in seed coat/endosperm and embryo fractions in comparison to whole seeds. With the exception of the 9,10,18-trihydroxyoctadecenoic fatty acid, the composition of whole seeds reflects the composition of the seed coats (85% of total monomers). In embryos, the C18:1 trihydroxy fatty acid is dominant (about 60%) but C16:0, C18:1, and C18:2 ω-hydroxy fatty acids and α,ω-dicarboxylic acids are also present (Fig. 4, lower panel). Typical suberin monomers were not found in the embryo fraction, indicating no contamination by seed coat. 9,10,18-Trihydroxyoctadecenoic acid is considered an indicator of cutin, although the embryo "cutin" monomer composition differs significantly from the C18:2 dicarboxylic acid-rich polyester found in the epidermis of leaves and stems of *Arabidopsis* (Bonaventure et al., 2004; Franke et al., 2005) and in *B. napus* (data not shown).

Ferulate and C20–C24 saturated fatty alcohol and α,ω-bifunctional monomers, which are considered indicative of suberin especially when they occur together, are clearly specific to the seed coat (Fig. 4). If the C18:1 trihydroxy fatty acid was completely embryo-specific there might be about 10–15% contamination of the seed coat fraction by embryos. Visually inspected, there was very much less mass contamination, but surface layer cross-contamination remains a possibility. In this context, the cuticle found in grapefruit inner-seed coats was rich in C18 triol fatty acids (Espelie et al., 1980), suggesting that internal cuticles could have common features with "embryo-enriched" monomers reported here. However, as the mass of seed coat monomers dominates, any cross-contamination by the embryo cuticle will be very minor. Although C16:0 and C18:1 ω-hydroxy fatty acids and C16:0, C18:1 and C18:2 α,ω-dicarboxylic acids are found in the embryo, the dominant part of their masses are present in the seed coat.

Seed coat-enriched and embryo-enriched fractions obtained from *Arabidopsis* fractured mature seeds separated by floatation gave similar monomer distributions as for *B. napus* (data not shown) but the distinctions between the two fractions were not so clear, presumably because of greater cross-contamination. Based on analysis of *gpat5* mutant, which are suberin-associated acyltransferase mutants of *Arabidopsis* (data not shown), we believe that much of the C22 and C24 ω-hydroxy fatty acid and dicarboxylate deposition is associated with late stage suberization of the chalazal region. This is a conclusion that agrees with analyses of grapefruit seed polyesters (Espelie et al., 1980). High levels of α,ω-dicarboxylic acids have generally been considered indicative of suberin. However, the presence of high proportions of C16 and C18 dicarboxylic acids, and particularly of the unique octadecadiene-1,18-dioate in the leaf and stem epidermal layers of *Arabidopsis* (Bonaventure et al., 2004) and *B. napus* (data not shown), and the presence of these in the isolated Arabidopsis leaf cuticles (Franke et al., 2005), suggest that this generalization may not be appropriate for *Arabidopsis*. Thus, it is difficult to classify the remaining polyester monomers found in the seed coat as derived from suberin or cutin, or some intermediate or other form of polyester. Further work will require histochemical, electron microscopic and gene expression analyses to help classify polyester structures in the seed.

3. Conclusion

The genetics underlying plant polyester biosynthesis is largely unknown. However, the power of both forward and reverse genetic approaches in *Arabidopsis* allows for the identification of genes that affect the monomer composition of cutins and suberins. Such approaches first require identification and then facile but quantitative analysis of the polyester monomers. Such analyses of *Arabidopsis* cutins and suberins have only recently been reported (Bonaventure et al., 2004; Xiao et al., 2004; Franke et al., 2005). Genes have been identified through forward (Xiao et al., 2004; Kurdyukov et al., 2006) and reverse (Bonaventure et al., 2004) genetic screens. With catalogs of the genes involved in *Arabidopsis* lipid metabolism (Beisson et al., 2003) and of genes which are highly expressed in *Arabidopsis* stem epidermis, the site of cutin synthesis (Suh et al., 2005), it seems likely that reverse genetic approaches will accelerate. The juxtaposition of putative suberin and cutin monomers in the seed may allow for effective reverse genetic screening in a single sample for genes involved in either or both polyesters. The seeds of *Arabidopsis* contain the major polyester monomers found in the leaf and stem cuticle, namely 16-hydroxypalmitate, 10,16-dihydroxypalmitate, hexadecane-1,16-dioate, 18-hydroxyoleate, octadecene-1,18-dioate, 18-hydroxylinoleate and octadecadiene-1,18-dioate. In addition, there is a significant amount of 9,10,18-trihydroxyoctadecenoate, which is associated largely with the embryo, and a large proportion of monomers, including docosan-1-ol, docosane-1,22-diol, 22-hydroxydocosanoate, docosane-1,22-dioate, 24-hydroxytetracosanoate, tetracosane-1,24-dioate and ferulate, which collectively are characteristic of suberin and which are associated entirely with the seed coat. Sufficient seeds can be obtained from a single plant, to which are applied the solvent extraction/homogenization/drying steps then transmethylation, acetylation and GC analysis. The slow step is thorough solvent and aqueous washing to delipidate the seed. However, the aqueous washes might be omitted as it is not important to gather data on normal fatty acids and sinapate. A single GC analysis can be run with sample from less than 100 mg of seeds. Currently, we have successfully used the method to identify compositional changes in KO lines for three genes that are consistent with the loss of specific gene function, and are using it to screen additional KO mutant lines.

4. Experimental

4.1. Plant material

Wild-type *A. thaliana* (ecotypes Columbia and Wassilewskija-2) were grown on a mixture of soil:vermiculite:perlite (1:1:1 v/v/v) under white fluorescent light (80–100 $\mu E\ m^{-2}\ s^{-1}$) in a 18-h-light/6-h-dark photoperiod. The temperature was set at 20–22 °C and the relative humidity at 60–70%. Seeds were always stratified for 4 days at 4 °C. Seeds of *B. napus* cv Westar were planted in 30 cm plastic pots in a (2:1 v/v) mixture of soil:vermiculite and grown in an air-conditioned greenhouse under natural light supplemented with lamps to provide 18-h-light/6-h-dark photoperiod.

4.2. Sample delipidation of seeds

Whole seeds of *A. thaliana* or *B. napus* were ground in liquid N_2 using mortar and pestle, immersed in boiling isopropanol (25 ml/g fresh tissue) and heated for 10 min at 80 °C. After cooling, the tissue was finely ground with a Polytron and extracted for at least 4 h at room temperature by shaking at 300 rpm in *i*-PrOH. The extract was centrifuged (10 min at 800*g*) with the insoluble residue re-extracted by shaking overnight at 300 rpm with 25 ml/g *i*-PrOH. After centrifugation, the tissue was re-extracted with $CHCl_3$:MeOH (2:1 v/v) at room temperature by shaking for approximately 8 h. The residue was re-extracted with $CHCl_3$:MeOH (1:2 v/v) overnight and filtered through Whatman No. 1 filter paper. Additional steps were performed after air-drying the solvent extracted tissues and re-grinding the residue to achieve a smaller particle size. The residue was extracted successively with MeOH (30 min), H_2O (30 min), 2 M NaCl (1 h), H_2O (30 min), MeOH (30 min), (1:2 v/v) $CHCl_3$:MeOH (overnight) and (1:2 v/v) $CHCl_3$:MeOH (overnight). All extraction steps were performed in a shaker (300 rpm) at room temperature and the residue was separated from the solvent by centrifugation (10 min at 800*g*), except for the last step where the sample was filtered. The residue was air-dried and then placed under vacuum over anhydrous $CaCl_2$ until constant weight was reached. Residue yields after extraction, as percentage of initial seed weight, were: *A. thaliana* Col0-31%; WS-32%; *B. napus*-37.1%.

4.3. Methanolysis with sodium methoxide

Approximately 0.1 g of solvent-extracted *Arabidopsis* residue, dried to constant weight, was heated at 60 °C with periodic vortexing in MeOH (6 ml) containing 15% (v/v) methyl acetate and 6% (w/v) NaOMe. Methyl heptadecanoate (Sigma, USA) and ω-pentadecalactone (Fluka, Switzerland) were added as internal standards at 1 mg/g dry residue each. Different incubation times were applied for the time-course analysis (0.5–48 h), with 2 h selected as the reaction time for the optimized protocol. The reaction mixtures were acidified with glacial AcOH to pH 4–5, saline added and the FAMEs extracted with CH_2Cl_2 (10 ml). The organic phase was washed three times with dilute saline solution (0.5 M NaCl) and dried over anhydrous Na_2SO_4. The solvent was evaporated to dryness under N_2 and the product silylated to convert hydroxyl groups to their TMSi ethers or acetylated to produce the corresponding acetyl derivatives.

4.4. Additional depolymerization methods

The hydrogenolysis/deuterolysis protocol used for the analysis of *Arabidopsis* polyester monomers was described by Bonaventure et al. (2004). Methyl-heptadecanoate and ω-pentadecalactone were added as internal standards in the same amounts as indicated above. The polyol derivatives were silylated to convert free alcohols to their TMSi ethers. For acidic transmethylation several catalysts can be used, such as BF_3, HCl, or H_2SO_4 in methanol (Holloway, 1984; Christie, 2003). Based on the remarks of Christie (2003) we opted for a H_2SO_4–MeOH reagent. Dry seed residue (0.1 g) and the internal standards were heated at 80 °C with 4 ml of freshly prepared 5% (v/v) H_2SO_4 in MeOH plus 2 ml of toluene as a co-solvent for 48 h. To recover the FAMEs, 3 ml of 0.9% NaCl (w/v aq.) and CH_2Cl_2 (10 ml) were added, followed by centrifugation for 5 min at 800*g* to facilitate phase separation. The organic extract was washed several times with dilute saline, dried over dry sodium sulfate and evaporated under a stream of N_2.

4.5. Hydrogenation

To confirm structures, samples of polyester monomers obtained after methanolysis were hydrogenated by stirring in MeOH at room temperature for 2 h with platinum (IV) oxide catalyst in a hydrogen atmosphere at slightly greater than atmospheric pressure. This resulted in complete reduction of the double bonds. After addition of saline, the product was extracted into Et_2O with the later phase washed several times with dilute saline. Finally, the Et_2O solution was dried under nitrogen stream and the sample was silylated for GC–MS analysis.

4.6. Preparation of trimethylsilyl and acetyl derivatives

The products of hydrogenolysis or methanolysis were heated at 100 °C for 10 min in 0.1 ml pyridine and 0.1 ml

BSTFA (*N*,*O*-bis(trimethylsilyl)-trifluoroacetamide). After cooling, the solvent was evaporated under nitrogen and the product was dissolved in 0.5 ml heptane:toluene (1:1 v/v) for GC–MS analysis. To obtain the acetyl derivatives, the FAMEs were dissolved in 0.1 ml pyridine and 0.1 ml Ac_2O and incubated 1 h at 60 °C. The reagents were then removed under nitrogen gas and the dry samples were dissolved in 0.5 ml heptane:toluene (1:1 v/v) for GC or GC–MS analysis.

4.7. GC–MS and GC–FID analysis

GC (FID) analysis used a DB-5 capillary column (J&W Scientific, CA, USA; 30 m × 0.25 mm × 0.25 μm film thickness) with He carrier gas at 1.5 ml/min constant flow and temperature programmed from 140 °C to 310 °C at 3 °C/min, and then held for an additional 10 min at 310 °C. Samples were injected in split mode (30:1 ratio, 310 °C injector temperature) and peaks were quantified on the basis of their FID ion current. Peak areas (pA.sec) were converted to relative weights by applying FID theoretical correction factors; that is, by assuming the FID response is proportional to carbon mass for all carbons bonded to at least one H-atom (Christie, 1991). The S/N threshold was set as 1% of the major peak. Unknowns were estimated as their total peak area against known peaks, taking the total peak area of the latter as 100%. The retention time of fatty acid standards was determined under the same conditions and used to verify the predominant fatty acids. For GC–MS, a column of the same characteristics was used with He carrier gas at 2 ml/min and oven temperature programmed from 110 °C to 300 °C at 10 °C/min. Splitless injection was used and the mass spectrometer run in scan mode over 40–500 amu (electron impact ionization), with peaks quantified on the basis of their total ion current. For analysis of the molecular ion cluster, the detector was operated in single ion monitoring mode for ions (M-1) to (M+n+1), where M is the m/z value of the major natural isotopic abundance molecular ion and n represents the highest possible isotopic enrichment.

4.8. TAG separation and analysis

To analyze the lipid fractions eliminated in the additional washing steps, Arabidopsis seeds (3 g) were delipidated as described in Section 4.2. The solvent fractions from the additional washing steps were pooled and extracted with chloroform. The dried extract was dissolved in heptane and separated by TLC on K6 silica plates (Whatman, Clifton, PA). The plate was developed with 80:20:1 (v/v/v) hexane:Et_2O:AcOH, and lipids were detected by exposure to iodine vapors. Triolein (1 mg/ml) was used as standard.

4.9. Identification of branched alcohols

Approximately 20 mg of FAMEs prepared by NaOMe catalyzed transmethylation of *B. napus* seeds were separated by preparative TLC. The plate was developed with 80:20:1 (v/v/v) hexane:Et_2O:AcOH and the lipids were detected by spraying with 0.2% (w/v) 2′,7′-dichlorofluorescein/EtOH. Nine different bands were identified under UV light, individually eluted with $CHCl_3$, and dried under N_2 stream. The TMSi derivatives were analyzed by GC–MS. Methyl 13-methyltetradecanoate (*iso*-pentadecanoate) and methyl 12-methyltetradecanoate (*anteiso*-pentadecanoate) standards were purchased from Matreya Inc. and reduced to the corresponding fatty alcohols with $LiAlH_4$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.09.011.

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