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Partial depolymerization of genetically modified potato tuber periderm reveals intermolecular linkages in suberin polyester

José Graça [a,*], Vanessa Cabral [a], Sara Santos [a], Pedro Lamosa [b], Olga Serra [c], Marisa Molinas [c], Lukas Schreiber [d], Friedrich Kauder [e], Rochus Franke [d]

[a] Instituto Superior de Agronomia, Centro de Estudos Florestais, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal
[b] ITQB-UNL – Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República-EAN, 2780-157 Oeiras, Portugal
[c] Laboratori del Suro, Department de Biologia, Universitat de Girona, E-17071 Girona, Spain
[d] Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany
[e] Solana Research GmbH, Eichenallee 9, D-24340 Windeby, Germany

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ABSTRACT

Suberin is a biopolyester found in specialized plant tissues, both internal and external, with key frontier physiological functions. The information gathered so far from its monomer and oligomer composition, and in situ studies made by solid state techniques, haven't solved the enigma of how the suberin polyester is assembled as a macromolecule. To investigate how monomers are linked in suberin, we analyzed oligomer fragments solubilized by the partial depolymerization of suberin from potato (*Solanum tuberosum*) tuber periderms. The structure of the suberin oligomers, namely which monomers they included, and the type and frequency of the inter-monomer ester linkages, was assessed by ESI-MS/MS and high resolution NMR analysis. The analyzed potato periderms included the one from wild type (cv. Desirée) and from plants where suberin-biosynthesis genes were downregulated in chain elongation (*StKCS6*), ω-hydroxylation (*CYP86A33*) and feruloylation (*FHT*).

Two building blocks were identified as possible key structures in the macromolecular development of the potato periderm suberin: glycerol – α,ω-diacid – glycerol, as the core of a continuous suberin aliphatic polyester; and glycerol – ω-hydroxyacid – ferulic acid, anchoring this polyaliphatic matrix at its periphery to the vicinal polyaromatics, through linking to ferulic acid. The silencing of the *StKCS6* gene led to non-significant alterations in suberin structure, showing the relatively minor role of the very-long chain (>C28) fatty acids in potato suberin composition. The silencing of *CYP86A33* gene impaired significantly suberin production and disrupted the biosynthesis of acylglycerol structures, proving the relevance of the latter and thus of the glycerol – α,ω-diacid – glycerol unit for the typical suberin lamellar organization. The silencing of the *FHT* gene led to a lower frequency of ferulate linkages in suberin polyester but to more polyphenolic guaiacyl units as seen by FTIR analyses in the intact polymer.

1. Introduction

Suberin is a lipid polyester found in plants' barrier tissues, where it plays a number of key roles including confinement and insulation from the surroundings, control of water exchanges and protection against biotic and abiotic aggressions (Lendzian, 2006). In plants with secondary growth, suberized cells pack in successive layers, building the periderm (Fig. 1A). Periderms cover aerial stems, as part of trees' outer bark, but also underground stems, forming the skin of tubers. In potato (*Solanum tuberosum*), the periderm differentiation keeps evolving after harvesting for 10–15 days, giving rise to the "mature skin", which is essential to protect the tubers against biotic attacks and abiotic stresses (Neubauer et al., 2013). Potatoes are one of the most important global food crops and its post-harvest conservation poses a major challenge, with periderm deficiencies responsible for significant economic losses worldwide (FAO, 2001).

The comprehension of suberin as macromolecule, and therefore how it plays its significant physiological roles, is still very limited. The study of suberin has been hampered by its insolubility, monomer complexity and interpenetration with the other cell wall polymers, namely polyaromatics and polysaccharides, which together build up suberized cell walls. What is known is that suberin is a polymeric glycerolipid assembled from long-chain

* Corresponding author at: Instituto Superior de Agronomia, Edifício de Engenharia Florestal, 1349-017 Lisboa, Portugal.
E-mail address: jograca@isa.ulisboa.pt (J. Graça).

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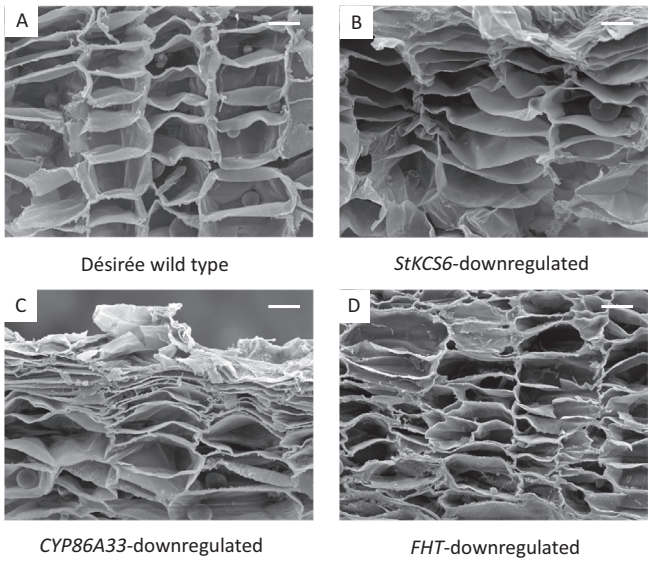

Fig. 1. Enzymatically isolated periderms of (A) wild type potatoes (cv. Desirée), and genetically engineered periderms of (B) *StKCS6*-RNAi, (C) *CYP86A33*-RNAi and (D) *FHT*-RNAi potatoes; cross sections as observed by SEM, white bar 20 μm.

α,ω-bifunctional fatty acids, namely ω-hydroxyacids and α,ω-diacids, reticulated with glycerol (Graça and Santos, 2007). Small amounts of aromatic monomers, in particular ferulic acid, are also covalently linked in the suberin polyester (Graça, 2010; Riley and Kolattukudy, 1975). The relation of ferulic acid with the polyaromatics and the nature of the latter remain controversial, with argued similarities with lignin (Bernards and Razem, 2001; Lapierre et al., 1996; Marques and Pereira, 2013). Thus, for clarity in the terminology of this study, we refer to suberin as the aliphatic polyester. In suberized cell walls, suberin is also associated with significant amounts of soluble lipids, the "waxes", which in potato can amount to 20% of the periderm weight and be mostly responsible for its low permeability (Schreiber et al., 2005).

The suberin composition was assessed in a number of plants after its complete depolymerization, showing tissue and species-derived variability in the monomer structure, regarding chain-length and degree of mid-chain oxidation (Holloway, 1983; Kolattukudy et al., 1975). The intact suberin was studied by solid-state (CPMAS) NMR, in potato periderm, either natural or wound-induced (Bernards et al., 1995; Serra et al., 2014; Stark and Garbow, 1992), as well as in other suberized plant materials, like in *Quercus suber* cork (Gil et al., 1997; Neto et al., 1995). These NMR results showed that suberin included methylene chains with different dynamic behavior, the shorter with the highest mobility and the longer with the lowest, together with the spatial separation of the aliphatic and aromatic domains (Gil et al., 1997; Yan and Stark, 1998).

Conceivable as "molecule sequencing" another approach for the study of the suberin macromolecule has been its partial depolymerization, by chemical or enzymatic methods, and the structural analysis of the ensuing soluble oligomers by mass spectrometry (Graça and Pereira, 1997; Rocha et al., 2001; Wang et al., 2010). In this way dimeric and trimeric esters, comprising as residues glycerol, long-chain ω-hydroxyacids and α,ω-diacids, and ferulic acid, were found in potato periderm (Graça and Pereira, 2000), and cork suberins (Graça and Santos, 2006a). The partial degradation of wound potato suberin by hydrolysis also released oligomers including triacylglycerols, a trimeric aliphatic ester, aromatic acids bridged by an aliphatic ether (Wang et al., 2010), and ferulic acid esterified to aliphatics (Arrieta-Baez and Stark, 2006). The insight brought by the oligomer structures solubilized from suberin led to a working model for the suberin macromolecule (Graça and Santos, 2007).

A further approach to the comprehension of the suberin macromolecule can be given by the identification and manipulation of genes and proteins presumably involved in the biosynthesis of its monomers and their ester assembling (Serra et al., 2014). Lists of candidate genes for suberin biosynthesis were drawn from the analysis of cork differentiating tissue (Soler et al., 2007) and *Arabidopsis* mutants (Franke et al., 2012). The relevant role of Cytochrome P450 oxigenases (*CYP*s) in the oxygenation of fatty acid precursors both at mid and end-chain, leading to the epoxy-acids, ω-hydroxyacids and α,ω-diacids characteristic of suberin was proved in molecular genetic approaches (Pinot and Beisson, 2011). Other genes encoding enzymes with functions directed to the main suberin structural groups were found as part of the suberization process, including GPAT acyltransferases, which promote the esterification of long-chain aliphatic acids to glycerol, feruloyl transferases (such as FHT), able to esterify ferulic acid to the ω-hydroxyacids, and 3-ketoacyl-CoA synthases (KCS), promoting chain elongation (Beisson et al., 2012; Ranathunge et al., 2011). Results of solid state NMR analyses combined with data from complete depolymerization of genetically engineered periderms allowed several observations. The analysis of *StKCS6*-RNAi potato periderms shows that a modest reduction in chain elongation capacity beyond C28 produces a periderm similar to wild type in suberin composition, in mechanical performance and in water permeability (Serra et al., 2014, 2009a). On the other hand, C*YP86A33*-RNAi periderms showing a reduced capacity of terminal hydroxylation and crosslinking, have a greater fraction of mobile alkyl chains (Serra et al., 2014), lose the typical lamellar structure and have enhanced water permeability (Serra et al., 2009b). Moreover, downregulation of ferulate ester formation in *FHT*-RNAi periderms remodels the periderm with more flexible aliphatic chains but also increases the aromatic constituents that are resistant to transesterification; this modification attenuates cooperative motions at the junctures between hydroxyfatty acid units and produces a periderm that is mechanically compromised (Serra et al., 2014) and highly permeable to water (Serra et al., 2010).

In the present work we analyzed the wild type (cv. Desirée) and the genetically modified *StKCS6*-RNAi, *CYP86A33*-RNAi and *FHT*-RNAi potato periderms (Fig. 1) by partial depolymerization. The suberin oligomers obtained were analyzed by ESI-MS/MS and high-resolution solution-state NMR, including one dimensional (^{1}H, ^{13}C APT) and two-dimensional correlation techniques (COSY, HSQC and HMBC). The ESI-MS analysis showed how the monomers were sequentially linked and the NMR analysis allowed the identification of all types of inter-monomer suberin linkages and their relative frequency in the different potato periderms. The results fostered the discussion of how the suberin macromolecule is assembled in suberized cell walls and the role of some genes in its biosynthesis. A contribution to a better understanding of how suberin forms and develops in potato periderm, in order to eventually improve it through gene manipulation for enhanced post-harvest conservation, is intended.

2. Results and discussion

2.1. The partial depolymerization of suberin: oligomer yields

The partial depolymerization of the potato periderms' suberin was done applying a mild methanolysis catalyzed by an insoluble strong base, $Ca(OH)_2$. The reaction time and mass proportions of periderm material and calcium hydroxide were optimized to maximize the yield of oligomers, taking into account the number of monomer residues they included and its representativeness of