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Analysis of aliphatic waxes associated with root periderm or exodermis from eleven plant species

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ABSTRACT

Aliphatic waxes can be found in association with suberized tissues, including roots. Non-polar lipids were isolated by rapid solvent extraction of mature regions of intact roots from eleven angiosperms, including both monocots and dicots. The majority of roots analyzed were taproots or tuberous taproots that had undergone secondary growth and thus were covered by a suberized periderm. The exceptions therein were maize (*Zea mays* L.) and rice (*Oryza sativa* L.), which present a suberized exodermis. The analysis herein focused on aliphatic waxes, with particular emphasis on alkyl hydroxycinnamates (AHCs). AHCs were widely distributed, absent from only one species, were found in both aerial and subterranean portions of tuberous taproots, and were associated with the fibrous roots of both maize and rice. Most species also contained monoacylglycerols, fatty alcohols and/or free fatty acids. Carrot (*Daucus carrota* L.) was the outlier, containing only free fatty acids, sterols, and polyacetylenes as identified components. Sterols were the only ubiquitous component across all roots analyzed. Monoacylglycerols of ω-hydroxy fatty acids were present in maize and rice root waxes. For species within the Brassiceae, wax compositions varied between subspecies or varieties and between aerial and subterranean portions of taproots. In addition, reduced forms of photo-oxidation products of ω-hydroxy oleate and its corresponding dicarboxylic acid (10,18-dihydroxy-octadec-8-enoate, 9,18-dihydroxy-octadec-10-enoate and 9-hydroxyoctadec-10-ene-1,18-dioate) were identified as naturally occurring suberin monomers in rutabaga (*Brassica napus* subsp. *rapifera* Metzg.) periderm tissues.

1. Introduction

Compositions of taproot surface lipids ("root waxes") were first reported by Espelie et al. (1980). The methodology employed allowed identification and quantification of free fatty acids, fatty alcohols, and alkanes and their mid-chain keto or hydroxy derivatives. Later studies in *Arabidopsis* provided evidence for additional root wax classes, namely alkyl hydroxycinnamates (AHCs), sterols and monoacylglycerols (MAGs) (Li et al., 2007; Molina et al., 2009). Plant AHCs are typically ferulic, coumaric, or caffeic acids esterified to saturated fatty alcohols with carbon chain lengths ranging from C16 to C32. They have been reported to occur in several higher plant species (García-Argáez et al., 1999; Gutiérrez Suárez et al., 2004; Schmutz et al., 1994; Yunoki et al., 2004). Typically they are found associated with suberized tissues, like the periderm of roots or stems (tree bark) (Bernards and Lewis, 1992; Freire et al., 2007; Kawanishi et al., 1990; Schreiber et al., 2005; Sun et al., 2006) and green cotton fibers (Schmutz et al., 1994). There is one report of alkyl coumarates associated with cutinized tissues (Santos et al., 2007).

Recent literature has described the identification of several enzymes required for root wax biosynthesis. These include enzymes also involved in suberin polymer synthesis. A glycerol-3-phosphate sn-2 acyltransferase (GPAT5; At3g11430) catalyzes the synthesis of lysophosphatidic acid, a precursor for both aliphatic suberin and root-wax associated MAGs (Beisson et al., 2007; Li et al., 2007; Yang et al., 2010, 2012). A specific HXXXD-motif/BAHD acyltransferase is responsible for the synthesis of ferulate esters in both the suberin polymer and periderm waxes of potato tubers (Serra et al., 2010). Whereas alkyl ferulates are the sole class of AHCs of potato periderm waxes, *Arabidopsis* taproot periderm waxes contain alkyl coumarates, alkyl caffeates, and small amounts of alkyl ferulates. A related HXXXD-motif/BAHD acyltransferase (At5g63560) in *Arabidopsis* was shown to be specific for the synthesis of alkyl caffeates in the periderm of taproots (Kosma et al., 2012). Three fatty acyl-CoA reductases

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(AtFAR1/4/5) are required for AHC synthesis, presumably by providing fatty alcohols as acyl acceptors (Domergue et al., 2010; Vishwanath et al., 2013).

Since several suberin biosynthetic enzymes are required for root wax synthesis (Kosma et al., 2012; Li et al., 2007; Serra et al., 2010), it can be surmised that at least some of these waxes are synthesized in suberizing tissues (e.g. the periderm). However, any temporal overlap between suberin and wax synthesis has yet to be directly demonstrated. Root wax extraction kinetics with *Arabidopsis* have shown that alkanes are immediately extracted (<10 s), whereas very-long-chain free fatty acids, primary alcohols, and AHCs take about 120 s for complete extraction (Li et al., 2007). Alkane extraction is thus suggestive of a superficial localization as would be found for cuticular waxes, whereas the other root wax components could have a deeper localization. Whether this implies an intracellular, extracellular or even a suberin lamellae-associated localization remains unclear. Periderm cells actively synthesizing suberin will have the molecular machinery to transport lipids to the cell wall, so it is reasonable to propose an extracellular site for the deposition of root waxes. Localization is likely related to function. Suberization as a wound response is well established and studied in potato tubers (Bernards and Lewis, 1992; Boher et al., 2013; Schreiber et al., 2005). Mature regions of roots that undergo secondary growth are covered by a suberized periderm that is considered a protective barrier against the environment. These root surfaces represent an important interface for interactions between plants and their environment in the rhizosphere. Collectively or individually, the root wax compounds may contribute to generic barrier- or permeability-associated properties of suberized tissues but, exact biological functions remain uncertain.

In this study, eleven plant species were analyzed for their root wax compositions. A particular emphasis has been the occurrence and diversity of AHCs. These are widely distributed throughout the higher plant kingdom. Aerial and subterranean portions of tuberous taproots were analyzed individually and it was found that wax and suberin content and composition vary substantially with position. For two species from the Brassiceae (rutabaga and rapeseed, *Brassica napus*; radish and daikon, *Raphanus sativus* L.), pairs of subspecies or varieties showed significant variability in composition. Also identified were reduced forms of photo-oxidation products of oleate-derived suberin monomers as native components of periderm suberin of rutabaga, with a distribution along the root axis consistent with degree of exposure to light.

2. Results and discussion

In the first section, it was confirmed that the root surface tissues are suberized. As some taproots have substantial above ground portions, analysis of the distribution of both root waxes and suberin monomers with position along the vertical axis in rutabaga was carried out. During this analysis novel suberin monomers were identified, which are reported in Section 2.3. Section 2.4 provides a description of lipid root classes across all the species analyzed, with Section 2.5 providing details for AHCs. Finally, in Section 2.6, information is provided on intraspecific variability. In discussing the details of composition below, a complete data set is available in Supplemental File [1] when data are not explicitly presented in the Tables or Figures.

2.1. Plant species analyzed and confirmation of suberization of their root periderm or exodermis

Plant species utilized in this study (Fig. S1) were selected from 6 plant families and represent species of agricultural and genomic interest differing in root morphology (Fig. S2; tuberous versus non-tuberous, fibrous versus taproot, etc.), abiotic stress tolerance and type of suberized tissue (periderm versus exodermis). Maize and rice were selected because they represent species with roots that do not undergo secondary growth (including periderm formation) but do possess a suberized exodermis (Hose et al., 2001; Schreiber et al., 2005). Nine plant species described in this study were grown in a greenhouse, two [*Arabidopsis* and salt cress (*Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick ecotype Yukon)] in a growth chamber, with the intent of minimizing any stress responses, although at this time it is not known how growth conditions and pathogens may affect root wax compositions. Only *Arabidopsis* and salt cress were not commercial cultivars. It is unclear how domestication of the 9 other species may affect compositions.

In parallel with chemical analyses, it was important to ascertain if each of the species possessed a suberized periderm, or exodermis in the case of maize and rice. Root periderm sections were analyzed via Transmission Electron Microscopy (TEM). Substantial variability was evident in the number and thickness of light and dark bands, termed lamellae, across species and between aerial versus subterranean portions. However, the TEM analyses were aimed at confirming suberization in the form of lamellae rather than extensively documenting inherent variability. Sections were taken from different areas of the root and from 2 to 3 biological replicates for each species. The micrographs presented in Figs. 1 and 2 are representative of what was observed in these samples. The micrographs clearly demonstrated that all species possessed the lamellar structures typical of suberized tissues and that both above-(aerial) and below-(subterranean) ground taproot portions possessed suberin lamellae (Figs. 1 and 2).

2.2. Heterogeneity of suberin and root wax compositions along the vertical axis of taproots

Some plant species develop tuberous taproots with substantial simultaneous growth above and below the soil-line; for example, rutabaga, radish (*R. sativus* L. var. *sativus* cv. French Breakfast) and daikon (*R. sativus* L. var. *niger* J. Kern. cv. Mino Early). Aerial portions are exposed to drier environments and more sunlight. Subterranean portions grow and develop in darker, more humid environments with greater exposure to rhizosphere-inhabiting microorganisms. Given these contrasting environments, it was to determine if wax and suberin compositions and periderm cell wall ultrastructure differed substantially between aerial and subterranean regions of the taproot periderm. Rutabaga was selected for these more comprehensive analyses.

TEM analysis demonstrated the presence of lamellae in aerial, interfacial and subterranean portions of the rutabaga periderm (Fig. 2). Although subterranean lamellae appear more disorganized than aerial lamellae, it is difficult to make precise interpretations as sectioning technique can influence the appearance of lamellae. Nonetheless, suberin lamellae were present in both the aerial and subterranean periderm of rutabaga with approximately 3 cell files of suberized periderm in each respective portion of the tuber.

In presenting tabulations of root wax composition, two general points should be made explicit. First, wax loads expressed on a mass per unit surface area basis do not imply a uniform distribution across the root surface layers. Second, different root wax classes may have distinctly different transverse or lateral localizations within the extracted surface tissue layers. Analysis of the waxes extracted by rapid immersion of intact rutabaga taproots demonstrated a wax composition similar to that reported for *Arabidopsis* (Kosma et al., 2012; Li et al., 2007). However, the total wax load (expressed on a unit surface area basis) of the aerial portion of the rutabaga tuber (5.7 ± 1 μg cm^{-2}, $n = 4$) was substantially