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Review: What Nuclear Magnetic Resonance Can Tell Us About Protective Tissues

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Abstract

The epidermis and periderm protect plants from water and solute loss, pathogen invasion, and UV radiation. The cell walls of these protective tissues deposit the insoluble lipid biopolyesters cutin and suberin, respectively. These biopolymers interact in turn with polysaccharides, waxes and aromatic compounds to create complex assemblies that are not yet well defined at the molecular level. Non-destructive approaches must be tailored to the insoluble and noncrystalline character of these assemblies to establish the polymer and inter-component interactions needed to create functional barriers and structural supports. In the present mini-review, we illustrate the contribution of solid-state NMR methodology to compare the architecture of intact fruit cuticular polymers in wild-type and single-gene mutant tomatoes. We also show the potential of NMR-based metabolomics to identify the soluble metabolites that contribute to barrier formation in different varieties of potato tubers. Finally, we outline the challenges of these spectroscopic approaches, which include limited spectral resolution in solid state, differential swelling capabilities in solution, and incomplete dissolution in ionic liquids. Given the many genetically modified plants with altered suberin and cutin polymers that are now available, NMR nonetheless offers a promising tool to gain molecular insight into the complexity of these protective materials.

Keywords

cutin; suberin; cuticle; periderm; NMR; metabolomics

1. Introduction

The epidermal and peridermal coverings of plants serve both functional and structural roles, controlling the loss of water and solutes to the environment, restricting the entrance of pathogens, filtering harmful solar radiation, and offering mechanical support and stiffness [1]. The protective functions of these tissues are attributed principally to the insoluble polymers cutin and suberin, cross-linked polyesters with hydroxyfatty acid, glycerol, and phenylpropanoid constituents [2, 3] that are deposited in the outer non-cellular cuticle and

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the multilayered phellem tissue, respectively. It is necessary to understand the biosynthesis and transport of the precursors to the cell wall to shed light on the barrier functions of these materials. Furthermore, this insight can further assist in understanding the assembly of the monomers that form the polymer molecular structure and their intermolecular interactions with other cell wall constituents such as waxes, polysaccharides, and cutan [4, 5].

Degradative techniques based on the cleavage of ester bonds and subsequent identification of cutin and suberin monomeric constituents [6–9] have provided valuable compositional information but have well-known limitations: some molecular functionalities are rendered indistinguishable by depolymerization, part of the insoluble material remains intractable to further degradation without loss of chemical information [3]. Moreover, some soluble monomers remain unidentified, and the degradative methods destroy the polymeric architecture. Partial depolymerization to generate small soluble oligomers has allowed the identification of some linkages among monomers using either multidimensional solution-state NMR [10–13] or gas chromatography (reviewed in [7, 14]) in combination with mass spectroscopy (MS).

As an alternative approach, noninvasive solid-state nuclear magnetic resonance techniques offer structural information on intact plant polymers despite their insoluble and noncrystalline character. These NMR experiments have identified important functional groups, established cross-link sites, and revealed polymeric architecture for lime fruit cutin and potato, cork, and wound-induced suberin [15–18]. Moreover, solid-state NMR relaxation measurements have revealed the flexible aliphatic chains underlying the resiliency of the cutin-wax matrix [19] and the aliphatic suberin domain associated with a more rigid polysaccharide/aromatic cell wall matrix [20, 21] by two types of interactions [22]. These spectroscopic methods have also been used to monitor the formation of suberized cell walls induced by wounding in potato tissues [23–25]. In a complementary fashion, solid plant materials may be swelled in organic solvents, enhancing their molecular mobility and enabling the use of high-resolution magic angle spinning (HRMAS), an NMR technique that resolves overlapping NMR signals of similar molecular moieties, reveals covalent linkages, and defines the spatial proximity of polymer units [26–29].

In the present mini-review, we illustrate the usefulness of solid- and solution-state NMR to investigate two current issues for plant protective tissues: (i) changes in cutin molecular structure of genetically modified tomato fruits; and (ii) discrimination among up-regulated and down-regulated metabolites in the periderm of different potato varieties. We also briefly discuss the limitations of these approaches and how their reach may be extended in combination with other spectroscopic techniques. NMR is shown to be a potent analytical tool to broaden and deepen our understanding of protective barriers in genetically modified plant materials.

2. Solid-state NMR monitoring of tomato cutin chemical composition and cross-linking capacity

As noted above, the cutin biopolyester together with deposited waxes form a hydrophobic surface composite that functions as a protective membrane for terrestrial plants. Solid-state ^{13}C NMR using standard cross polarization (CP) and magic-angle spinning (MAS) methods offers a rapid method to identify various molecular structures present in the intact biopolyester [16, 30] and assess their flexibilities on several timescales [19, 31]. With ^{13}C direct polarization (DP) MAS, integrated NMR signal intensities have yielded quantitatively reliable ratios of carbon-containing chemical moieties of such heterogeneous plant polymers in red-ripe cultivated tomato fruits. These revealed a doubling of the hydrophilic-hydrophobic ratio $((\text{CHO}+\text{CH}_2\text{O})/(\text{CH}_2)_n)$ in cutin-deficient single-gene mutants that have

enhanced surface stiffness and compromised infection resistance [32]. Notable distinctions have also been made between the proportions of CHO vs. CH₂O carbons i.e., the prevalence of mid-chain hydroxyls and cross-links, of dewaxed cuticles (cutin) isolated at the 10 days post-anthesis developmental stage from the inner and outer epidermis of cultivated tomato fruit pericarp, respectively [33]. Illustrative measurements are shown in Fig. 1, which compares DPMAS ¹³C NMR spectra for enzymatically isolated and subsequently dewaxed cuticles of red-ripe cultivated wild-type (M82) and single-gene mutant (cd2) tomato fruits, [32] using a higher ¹H decoupling power to improve spectral discrimination of the CH₂O and CHO moieties. As a result, this made possible to assess the cross-linking capability of the cutin polyester at the red ripe stage. The peak intensity ratio of CHO branches (~72 ppm) to aliphatic fatty acid chains (~30 ppm) was used as an indicator of cross-linking capacity for the protective membranes. An enhancement of the (CHO/(CH₂)_n) ratio for the infection-prone cd2 mutant indicates a greater proportion of cross-linkable structural constituents.

3. NMR-based metabolic profiling of periderms from different potato varieties

Metabolomics, which combines analytical instrumental measurements and multivariate data analysis, is a powerful methodology for monitoring differences in metabolic pathways by measuring the concentrations of biochemical molecules in tissues that vary in phenotype, genotype, or developmental history [34]. Although less sensitive than mass spectrometry (MS), NMR spectroscopy offers a rapid, easily quantifiable, nondestructive, and unbiased way to develop a complete metabolic picture of plant (or animal) samples [35]. An NMR-based metabolomic study is illustrated in Fig. 2 for the tuber periderm of two white round potato varieties, Yukon Gold and Atlantic. The ¹H solution-state NMR spectra of polar extracts from native periderms of these two varieties are shown in Fig. 2A, where superimposed spectra of seven cv. Yukon Gold replicates and eight cv. Atlantic replicates, respectively, are seen to be highly reproducible. Visual comparison of the two sets of spectra reveals no significant differences between the two potato varieties. However, principal component analysis of the ¹H NMR spectra of native periderms from cv. Yukon Gold and cv. Atlantic samples clearly discriminates between these two white round potato varieties on a score plot (Fig. 2B), reflecting the distinctive metabolites and corresponding biological pathways involved in forming their periderms [36]. Moreover, a contribution plot from principal component analysis (Fig. 2C) offers molecular insight into the metabolites responsible for these differences by identifying the chemical shifts, and thus the structural moieties, that are up-regulated or down-regulated in the various periderm tissues. This plot demonstrates up-regulation of CH_nO (oxymethylene) groups and down-regulation of C=C (multiply bonded or aromatic) groups in the Yukon Gold variety, but molecular identification of these metabolites requires more extensive NMR and/or mass spectrometry measurements [36].

4. NMR approaches to plant protective tissues: challenges and limitations

This review presents two examples of how NMR has contributed to our understanding of plant protective tissues at the molecular level, suggesting the prevalence of cross-linkable CHO moieties in cuticle-deficient tomato mutants. Although we illustrate herein the usefulness of this methodology for investigations of intact tomato cutins and potato periderms, it is important to recognize the challenges and limitations of such approaches.

¹³C CP and DP MAS are one-dimensional solid-state NMR experiments that provide limited information regarding molecular structure due to overlap of the broad spectral lines typical of heterogeneous plant biopolymers. Although DP experiments often require long acquisition times, they provide invaluable quantitative estimates for each of the functional

groups and thus offer vital support for the relatively faster CP measurements. At least three strategies have been adopted to overcome the sensitivity and spectral resolution limitations in studies of plant protective tissues. First, ^{13}C -enriched precursors have been used to examine wound-healing potato periderms, highlighting the spectral contributions of those functional groups that incorporate the stable isotope and allowing for the monitoring of polymer development at the molecular level [24, 25]. However, this approach is technically challenging because it requires a model system for the protective barrier, such as wound-healing potato periderm, that can successfully incorporate exogenous metabolic precursors to form the target biopolymers. Secondly, two-dimensional NMR analyses of cutins, suberins, and related materials have been conducted on gel-like swelled samples in dimethylsulfoxide to separate overlapping peaks and make more confident identifications of structural moieties by correlating signals from directly or remotely bound ^{13}C and ^1H nuclei [26–29]. Nonetheless, such studies may be limited by the differing swelling capabilities of the various components, resulting in underrepresentation of moieties in densely cross-linked regions that are insufficiently exposed to the solvent. Thirdly, ionic liquids have been used to dissolve the suberized cell wall [37, 38] to allow characterization of the chemical constituents via solution-state NMR. This latter approach to structural studies is currently limited by incomplete dissolution and by inadvertent modifications of the native materials: disruption of hydrogen bonds involved in essential intra- or intermolecular associations within the protective plant materials or putative degradation at elevated temperatures as observed for lignin [39].

A related approach that couples chromatography to mass spectrometry has provided the metabolite profile of tomato epidermis and potato wound healing periderm [40, 41]. NMR-based metabolomic studies avoid destruction of the sample [42], provide absolute amounts [43], and are not selective with respect to the class of compound, but they require larger samples and complementary mass spectroscopic analysis to identify compounds in complex mixtures [43].

5. Future perspectives

NMR approaches have been developed during the past two decades to study cutinized and suberized tissues of natural plant protective materials such as tomato or lime fruit cuticles [26, 29, 31, 33, 44, 45], potato wound-healing periderms [15, 16, 19, 23–25, 27], and cork oak periderms [17, 21, 22]. The molecular composition information determined for these lipid biopolyesters has been augmented in some instances using FT-IR spectroscopic measurements as a function of developmental stage [46, 47] and tissue location [33, 48, 49]. Moreover, bulk NMR and FT-IR measurements on these protective biomaterials [3, 4, 50] have been complemented by a non-invasive surface-specific view from AFM [51–53], yielding topographic and micromechanical information under both ambient and environmental stress conditions that could be correlated with site-specific bulk flexibility derived from NMR relaxation characteristics [31, 51].

Moreover, functional genomics has recently made many genetically modified cuticles and periderms available with altered protective properties [54–59] and see ref [60] for an *Arabidopsis* inventory. Besides, an ecological and evolutionary functional genomics approach has employed related wild tomato species as new models to evaluate the evolution, morphology and function of the cuticle [61]. Whereas alterations in the composition of the barrier have typically been evaluated using chemical degradation techniques, and such studies have proven very useful for characterizing the genotype and consequent polyester biosynthesis, our understanding of the cell-wall components that contribute to the barrier physiology is far from complete. Physiological analyses of the mutants evidence the

complexity of such tissues, which exhibit no simple correlations of polyester and wax accumulation with protective function.

For instance, ectopic deposition of suberin-like monomers in *Arabidopsis* cuticle led to ultrastructural disorganization accompanied by a lowered resistance to desiccation but preserved fungal resistance to *Alternaria brassicicola* [62]. A set of genetically modified tomato fruits that showed no significant variations in the lipid composition of degraded cuticular materials nevertheless exhibited different degrees of resistance to water desiccation and *Botrytis cinerea* infection [32]. In genetically modified potato, facile water loss was associated with lamellar disorganization attributed to the suberin polyester in tuber periderm, but even higher water permeability was observed in periderms with a ferulate ester deficiency but a typical lamellar structure [54, 55]. These examples show that in addition to the amount and composition of these plant polymers, intermolecular interactions and supramolecular assembly with other cell wall components are likely to play central roles in establishing their barrier functions.

Because NMR analysis may be conducted for intact tissues, each of these previously characterized genotypes with impaired or specialized barrier properties is a promising candidate for studies that relate protective function to biopolyester and cell-wall architecture. As an example, ssNMR analyses (Section 2 and [32]) of the cd2 tomato mutant with higher susceptibility to *B. cinerea* infection than wild type M82 showed a greater proportion of cross-linked cutin structural elements. In a similar fashion, NMR analyses could prove useful for the modified suberin present in roots and/or seed coats of *Arabidopsis* mutants (for an inventory, see [63]), adding to our understanding of suberin macromolecular structure. Finally, NMR-based metabolomics has the potential to track divergent plant biopolymer development resulting from engineered genetic modifications. Thus, the perspectives illustrated in this mini-review demonstrate that NMR spectroscopy and functional genomics together offer exciting synergistic potential for future breakthroughs in our understanding of plant protective tissues.

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Highlights

Solid-state ^{13}C NMR reveals cross-linking capability in intact tomato cutins.

NMR-based metabolomics distinguishes potato varieties from tuber periderm extracts.

NMR offers comprehensive molecular information for engineered protective tissues.

Fig. 1. Solid-state ^{13}C NMR analysis of isolated dewaxed cuticles from red ripe wild-type (M82) and cutin-deficient (cd2) mutant tomato fruits, showing enhanced cross-linking capacity for a mutant that is susceptible to the fungus *Botrytis cinerea* (the causal agent of gray mold) infection [32]. 150 MHz direct-polarization magic-angle spinning (DPMAS) measurements were carried out with an Agilent (Varian) DirectDrive spectrometer on 1–4 mg samples with 10 kHz spinning and a 100-sec recycle delay between each of 2000 successive acquisitions to make quantitative estimates for the major carbon-containing functional groups: long-chain aliphatics (0–45 ppm), oxygenated aliphatics (45–110 ppm), multiply bonded and aromatics (110–160 ppm), and carboxyls (170–175 ppm). The SPINAL method [64] was used to achieve high-power (~170–180 kHz) heteronuclear ^1H decoupling; chemical shifts were referenced externally to the methylene group of adamantane at 38.4 ppm [65]. The integrated signal intensity ratio ((CHO)/(CH₂)_n) was measured to assess the cross-linking capability of the intact cutin biopolyester.

Fig. 2.

Solution-state NMR and multivariate analysis of native tuber periderm polar extracts from two potato varieties. (A) ^1H NMR spectra of polar metabolites of Yukon Gold (black, 7 superimposed replicates) and Atlantic (red, 8 superimposed replicates) varieties, verifying reproducibility. The dried polar extracts were reconstituted in 100 mM pH 7.4 phosphate buffer in 99.96% D_2O with 20 μM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid). 800 MHz data were acquired on 0.5–2.0 mg samples with a Bruker Avance I spectrometer using water suppression and spin echo techniques to optimize spectral quality and a 1-sec delay between each of 128 successive acquisitions. Chemical shifts were referenced to internal DSS [66]. (B) Score plot from principal component analysis of polar metabolites in Yukon Gold (black) and Atlantic (red) periderms, showing metabolite discrimination between two superficially similar potato varieties. (C) Contribution plot of Yukon Gold versus Atlantic periderms, demonstrating up-regulation of oxymethylene (3.3–3.8 ppm) and methyl groups (0.8–1.0 ppm) and down-regulation of aromatic or multiply bonded (6.3 – 6.8 ppm) and aliphatic (~2.0 ppm) groups in the Yukon Gold variety.