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Solid-state ^{13}C NMR study of the mobility of polysaccharides in the cell walls of two apple cultivars of different firmness

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ABSTRACT

Solid-state ^{13}C nuclear magnetic resonance (NMR) was used to compare differences in mobility of the cell wall polysaccharides of 'Scifresh' and 'Royal Gala' apples after 20 weeks of storage. The texture of 'Scifresh' apples was markedly firmer than that of 'Royal Gala' at the end of storage. In a novel approach Two Pulse Phase Modulation (TPPM) decoupling was combined with cross polarisation (CP) and single pulse excitation (SPE) experiments. The resulting high resolution solid-state SPE spectra, unprecedented for apple cell walls, allowed a detailed insight into the physical and chemical properties of very mobile polysaccharides such as the arabinan and galactan side chains of the pectic polysaccharide rhamnogalacturonan I (RG-I). NMR showed that the cellulose rigidity was the same in the two cultivars, while arabinans were more mobile than galactans in both. Unexpectedly, arabinans in 'Scifresh' cell walls were more mobile than those in 'Royal Gala' which was unforeseen considering the greater firmness of the 'Scifresh' cultivar.

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1. Introduction

The physical properties of the plant cell walls (CWs) have a major influence on the texture of fruit, for example they define whether a ripe fruit is crisp like an apple or soft like a kiwifruit. Primary CWs contain three major classes of polysaccharides: (i) cellulose, which constitutes the rigid framework, (ii) hemicelluloses, which in eudicotyledons consist mostly of xyloglucans, and (iii) pectic polysaccharides, which are mainly composed of homogalacturonan (HG), and rhamnogalacturonan-I and rhamnogalacturonan-II (RG-I and RG-II), with RG-I having side chains of arabinan (branched and linear), galactan and arabinogalactan.[1,2] Primary CWs also contain structural proteins and glycoproteins. The influence of a particular CW polysaccharide on the mechanical properties of the intact CW will depend on the polysaccharide's own rigidity and the manner in which it interacts with other wall polysaccharides.

The softening of fruit during ripening is associated with changes in the CW with major changes being reported in pectic polysaccharide composition, solubility and structure.[3,4] The type and extent of changes, which may include loss of galactan and/or arabinan side chain constituents of RG-I as well as some depolymerisation of the polygalacturonan backbone, varies widely among species.[3] Changes may also occur in xyloglucans[3] but with the exception of raspberries[5] not in cellulose.

Determining these changes is usually achieved by isolating the walls from the plant tissue followed by sequential extraction with chelating and alkaline solutions. Such extraction procedures inevitably disrupt the interactions of the component CW polysaccharides with each other and consequently one is no longer studying the CW as it occurs in the plant. In contrast, solid-state ^{13}C nuclear magnetic resonance (NMR) allows examination of intact walls, that have been kept hydrated at all times and hence in their near native state.[6,7]

Various solid-state ^{13}C NMR techniques have been applied to investigate the components of plant primary CWs. Rigid cellulose can readily be observed using conventional CP/MAS (cross polarisation magic angle spinning) techniques.[6] The Iα and Iβ crystalline forms can be detected and their ratio determined.[8–11] In addition the cross-section dimensions of cellulose microfibrils can be calculated.[10] In the case of apple cell walls, the cellulose microfibrils were found to be ~3 nm in cross-section and the ratio of Iα to Iβ was ~1:1.[10] CP/MAS NMR can also be used for investigating the mobility of the semi-rigid non-cellulosic polysaccharides in CWs in species that soften on ripening, including apple.[10] Indeed, it

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has been successful in investigating the physical nature of the CWs of a wide variety of soft and firm fruit and vegetables, including onion,[7,12,13] tomato[14] strawberry,[15] cabbage and pineapple,[7] potato,[16,17] celery,[11,18] kiwifruit[19] and winter squash.[20]

Cross-polarisation is not suitable for investigating the very mobile polysaccharide components of the CW which may include parts of the arabinan and galactan side chains of RG-I. However, these components can be detected using single pulse excitation (SPE) techniques.[6,12,14,16,21] The SPE approach relies on very short repetition intervals (recycle delays) between pulses in order to saturate signals from rigid polysaccharide components with long relaxation times (T_{1C}), which consequently are not observable. However, if the repetition time is very short (100 ms–1 s) the signals for mobile polysaccharides are enhanced. Namely, very short relaxation times are due to the greater mobility of these polysaccharides. SPE experiments require an appropriate proton decoupling scheme during the acquisition in order to further enhance the signals from these very mobile polysaccharides. The conventional approach is to use a continuous wave decoupling. Alternatively, a multiple-pulse decoupling sequence WALTZ-16 designed for solution NMR has proven useful[16] at moderate spinning rates (~10–15 kHz).[22] However, since slow spinning rates are used in CWs investigations (4 kHz) and considering that CWs consist of polysaccharides with a range of mobilities, a TPPM (Two Pulse Phase Modulation) decoupling pulse sequence[23] was applied in both CP and SPE experiments. This should enable one to obtain enhanced signals from both mobile and semi-rigid polysaccharides in the apple CWs.

We have recently reported a comparison of the tissue and cell wall structures of two apple cultivars with different softening behaviours during fruit development and ripening.[24] 'Royal Gala' readily softens during storage whereas 'Scifresh' (commercially marketed as Jazz™) retains its firm and crisp texture. In order to extend this investigation on apple softening, we aimed to find out if there were any differences in the mobility of the constituent cell wall polysaccharides which should cast light on the differences in flesh firmness. To achieve this, we determined the monosaccharide composition of the cell walls in these two apple cultivars using chemical procedures and then examined them using cross-polarisation and single-pulse excitation solid-state ^{13}C NMR techniques. The focus here was on ripe fruit at the end of the storage phase, when the apple cultivars differed most in texture and the NMR technique would be most applicable.

2. Experimental

Mature apples (*Malus domestica* Borkh cvs. 'Scifresh' and 'Royal Gala') were harvested from the Plant & Food Research orchard in Havelock North, New Zealand, at 120 days after full bloom for 'Royal Gala' and at 140 days after full bloom for 'Scifresh' as this cultivar had a longer developmental period. For each cultivar, 20 fruit were harvested from three trees. The fruit were ripened in storage for 20 weeks at 0.5 °C under ambient atmospheric conditions. Fruit firmness was assessed by a puncture test[25] using a texture analyser (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY).

2.1. Cell wall isolation and composition

Cell walls of the apple cortex tissue were isolated in HEPES-KOH buffer (20 mM, pH 6.7) containing 10 mM dithiothreitol (DTT) using a fresh tissue to buffer ratio of 1:2.[26] Ground, frozen apple cortex tissue (20 g) was homogenised in the buffer using a Polytron (IKA®, Germany), centrifuged at 8600*g* for 20 min and filtered onto Miracloth™ (EMD, Millipore, USA). The pellet was washed twice with water (75 mL), centrifuged and re-filtered as above. The residue recovered was weighed and left stirring overnight in 90% aqueous dimethylsulphoxide (DMSO) (Ajax Finechem, Australia) at room temperature. The mixture was centrifuged at 1000*g* for 10 min, filtered onto Miracloth™ and washed extensively with water for 2 h. An aliquot of this preparation was partially dried to reduce the water content to approximately 50% by weight (while keeping it hydrated at all stages), and was used for NMR studies. The actual water contents of the CW preparations are reported in Table 1. The remaining cell wall isolate was freeze dried and used for the chemical analyses.

Cell walls were hydrolysed in 2 M trifluoroacetic acid (TFA) for 1 h at 120 °C to determine the non-cellulosic monosaccharide composition and by a two-stage sulfuric acid hydrolysis to determine the total monosaccharide composition.[27] The monosaccharides were converted to alditol acetates and quantified by GC-FID. Uronic acid (UA) content was determined colorimetrically.[28,29] The degree of methyl esterification (DE) was determined by the release of methanol.[30]

2.2. ^{13}C NMR spectroscopy

The solid-state NMR experiments were performed using a Bruker Avance 300 spectrometer operating at 300.13 MHz proton frequency, employing a multinuclear double-tuned Bruker probe with 7 mm zirconia rotors, retained with Kel-F end-caps. The ^{13}C CP/MAS and SPE/MAS spectra were recorded using a 4.2 μs 90° proton pulse and a spin rate of 4.0 kHz. A contact time of 1 ms was used for the ^{13}C CP/MAS experiments. ^{1}H decoupling was obtained via the continuous wave and TPPM (Two Pulse Phase-Modulation) decoupling sequences.[23] Recycle delays of 1 s and 200 ms were employed for the CP/MAS and SPE/MAS experiments, respectively. The typical number of scans was between 40,000 and 90,000 for the CP/MAS spectra and up to 600,000 for the SPE/MAS spectra. All spectra were referenced (externally) to *tetra*-methylsilane (TMS) and were processed using TopSpin™ NMR Software (Bruker).

3. Results and discussion

3.1. Fruit softening

Flesh firmness, fresh weight and dry matter concentration of the apples (Table 1) are characteristics of ripening. Although the two apple cultivars 'Royal Gala' and 'Scifresh' showed comparable fruit weights after 20 weeks of ripening, the firmness of 'Royal Gala' was significantly lower than that of 'Scifresh'. From harvest to stored fruit, both cultivars showed cell separation and the presence of intercellular air spaces, which in the case of stored apples was slightly larger in 'Royal Gala' compared to 'Scifresh' (See Supporting information Fig. S1). The firmness of ripe 'Royal Gala' fruit after storage had declined by one third compared with 'Scifresh' at harvest, whereas the firmness of 'Scifresh' fruit effectively remained unchanged during ripening. This decline in

Table 1
Fruit firmness, fruit weight, dry matter concentration, cell wall (CW) yield and water content in cultivars 'Royal Gala' and 'Scifresh' fruit ripened at 0.5 °C for 20 weeks

	Royal Gala	Scifresh
Fruit firmness (*N*) ± 8.3	51.4	83.2
% Decrease in firmness from mature to ripe fruit	37.2	7.5
Fruit weight (g) ± 25	213.7	170.3
Dry matter concentration (%) ± 4.2	14.9	15.3
CW yield* (mg g^{-1} fresh weight)	7.8	6.6
Water content of CW preparation (%)	48.2	54.3

Values represent mean ± standard error (SE) of 20 measurements.
* No standard error values as only one CW preparation per cultivar was conducted.

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