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POLYMER MOBILITY IN CELL WALLS OF TRANSGENIC TOMATOES WITH REDUCED POLYGALACTURONASE ACTIVITY

K. M. FENWICK, M. C. JARVIS,* D. C. APPERLEY,† G. B. SEYMOUR‡ and C. R. BIRD§

Agricultural, Food & Environmental Chemistry, Chemistry Department, Glasgow University, Glasgow G12 8QQ, Scotland, UK; †EPSRC Solid-state NMR Unit, University of Durham Industrial Research Laboratories, South Road, Durham, England, UK; ‡Horticultural Research International, Worthing Road, Littlehampton BN17 6LP, U.K. §Zeneca Plant Science, Jealott's Hill Research Station, Bracknell, Berks RG12 6EY, U.K.

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato ripening; antisense RNA; cell walls; polygalacturonase; NMR relaxation.

Abstract—Cell walls were prepared from unripe and red-ripe tomato fruit, cv. Ailsa Craig, and from ripe transgenic fruit carrying antisense genes downregulating the ripening-related polygalacturonase activity. The cell walls were examined by ^{13}C NMR using a cross-polarization/magic-angle spinning experiment with variable contact time to estimate the proton magnetic relaxation parameter $T_{1\rho}$, which is sensitive to molecular motions on the kHz timescale and hence to the rigidity of the polymer network of the cell walls; and the cross-polarization time constant T_{CH} which is sensitive to similar, and more local, motional effects. The proton $T_{1\rho}$ values for the ^{13}C resonances from each cell wall preparation were separated into two groups. One of these showed slow relaxation, implying low mobility, and included the ^{13}C resonances characteristic of cellulose. The other, corresponding to resonances characteristic of pectins only, showed two-component relaxation behaviour with one component relaxing much faster than the previous group, and hence corresponding to the pectic polymers in the middle lamella and perhaps also between the microfibrils. The $T_{1\rho}$ values for the first group shortened on ripening as polymer mobility increased within the cell wall. This was still evident, but less so, in the PG-antisense material, showing that the antisense genes reduced the softening of the cell wall, but did not do so as much as might be expected from the almost complete loss of polygalacturonase activity. The T_{CH} was separated into very fast and slower components corresponding to the times required for local and larger-scale magnetization transfer.

INTRODUCTION

The mechanism by which fruit softens on ripening has been widely studied and reviewed [1] but is still far from clear. Changes of some kind in the structure of the cell walls are almost certainly involved. Loss of cohesion in the cell wall and middle lamella has been observed by electron microscopy for a number of species (e.g. [2]), but remains to be explained in molecular terms. Until recently it was widely held that the essential factor was the enzymic depolymerization of pectin in some species, or in others its metabolic replacement by more highly esterified forms [1, 3]. The classic example was the tomato, where polygalacturonase (PG) activity was shown to increase from zero, in parallel with the onset of softening [4], by *de novo* synthesis [5]. Fruit of the *rin* mutant synthesized little PG and did not soften on ripening [6]. Purified PG from ripe fruit degraded cell walls from unripe fruit in a manner similar to ripening [2, 7]. All this evidence was consistent with a crucial role for PG in cell wall softening. However transgenic tomato fruit, expressing antisense PG RNA and containing down to 1% of the control PG activity, failed to show any detectable difference in softness from the controls [8]. Restoration of the PG gene to the *rin* mutant did not bring about softening [9]. These experiments led to the role of PG in ripening being questioned [9] and to intense activity in search of other enzymes that might be involved, both in tomatoes and in other fruits [10].

More detailed examination of the tomato system showed that the PG-antisense fruit did show some changes in pectin composition from the ripe controls. A water-soluble fraction of the PG-antisense pectins remained higher in M_r during ripening [11], and was tentatively considered [12] to be the cause of a commercially useful increase in the viscosity of the liquid phase of tomato juice [13]. A detailed mechanical study revealed very small increases in firmness, and reduced cell separation [14]. In other experiments firmness was increased, and susceptibility to fungal infection de-

*Author to whom correspondence should be addressed.

creased, during storage of PG-antisense fruit to beyond the normal stage of ripeness [13]. However, the reasons are uncertain at the cellular level [12, 13], and it is far from clear to what extent PG is responsible for the softening of tomatoes during the normal process of ripening.

Firmness, as measured by penetrometer or flat-plate deformation, is a highly complex parameter which is sometimes more dependent on turgor than on the mechanical characteristics of the cell walls [15, 16]. Indeed it is mathematically difficult to extract the mechanical properties of cell walls from measurements on the turgid tissues whose strength they undoubtedly provide, particularly when the cells are not elongated and when they can undergo simultaneous deformation in both shape and volume under stress. One solution to this problem is to use solid-state NMR relaxation methods to measure the micromechanical properties of the isolated cell walls via the mobility of the polymer chains that they contain [17, 18]. Some of these NMR methods can also distinguish the mobilities of individual polymers within the cell wall [18]. Whereas conventional mechanical studies measure the relationship between macroscopic deformation and applied stress, NMR relaxation measures the relationship between internal, molecular mobility and the forces exerted by the impact of other molecules, including water, in thermal motion. A number of different NMR approaches are available, which respond to molecular motions in different frequency bands, and focus on different spatial scales within the material [18].

In this paper we describe a study on transgenic tomato cell walls with polygalacturonase downregulated, using measurements of the rotating-frame spin-lattice relaxation time $T_{1\rho}$ of protons to assess the mobility of large polysaccharide segments in the kHz frequency range. These kHz motions are important in controlling the toughness of synthetic polymers, dissipating impact energy at temperatures close to the glass transition point of the material [19]. Cell walls are so heterogeneous that such mobilities are likely to be spatially variable, but the type of experiments used here allow the observed chain mobility to be averaged over a scale of the order of 10 nm by spin diffusion [18–21]. Since the proton $T_{1\rho}$ is measured through the effects of ^{1}H relaxation on the ^{13}C spectra it can be cross-checked between signals from different ^{13}C nuclei known to be present in the same part of the same molecule, given adequate resolution and detailed resonance assignments as obtained here.

RESULTS AND DISCUSSION

Models for relaxation data

The use of hydrated cell walls gave improved resolution in the cross-polarization/magic-angle spinning (CP-MAS) ^{13}C spectra (Fig. 1), as has been shown in other polysaccharide systems [21–23]. It may be assumed that hydration reduces interchain stresses and frees the polysaccharides to take up a limited range of low-energy conformations, particularly with respect to C–OH bond rotation, thus narrowing the continuous distribution of chemical shifts observed for each carbon nucleus. Signal assignments are shown in Table 1. The resolution of the four carboxyl signals was possible only from the unripe sample: these signals were too small to quantify in the spectra from the other samples. We have observed elsewhere [24] that the signals from pectic carboxyls disappear at high levels of hydration. The relaxation data were obtained by varying the

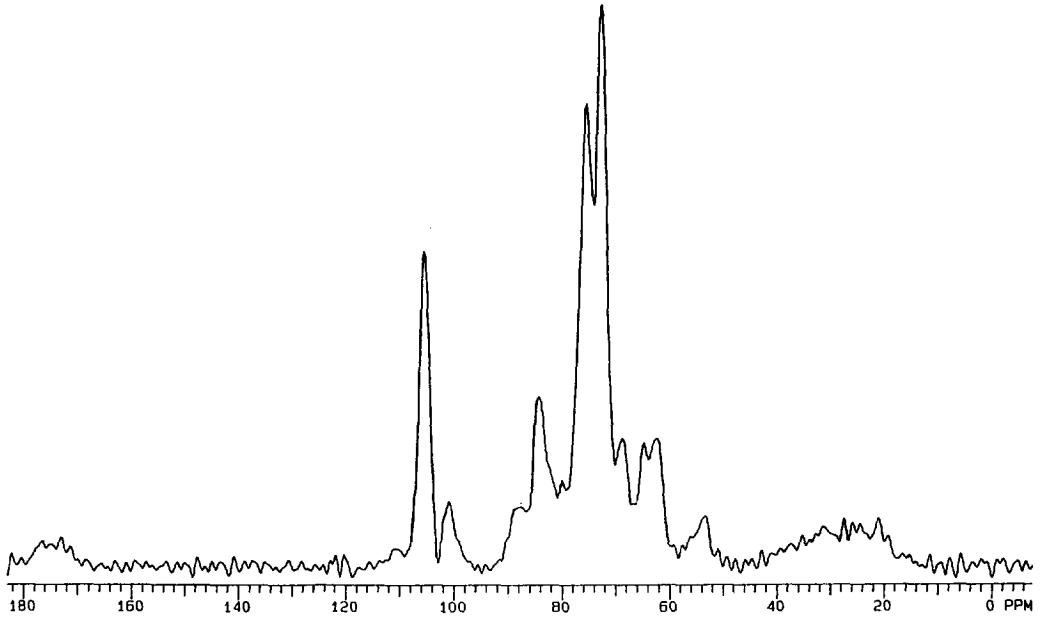

Fig. 1. CP-MAS spectrum of cell walls from ripe tomato. Contact time 0.4 msec.

Table 1. Signal assignments

Chemical shift, ppm	Assignment
177	C-6 of galacturonan with monovalent cation
175	C-6 of galacturonan in calcium form
173	Carboxyl of acetyl
171	C-6 of galacturonan in H- or Me form
105	C-1 of cellulose, some hemicelluloses and *B*(1-4) galactan
101	C-1, predominantly of galacturonan
88	C-4 of crystalline cellulose
84	C-4 of crystallite-surface cellulose
80	C-4, predominantly of galacturonan in random-coil and 3_1 helical forms
75	general carbohydrate C-2, C-3, C-5
72	general carbohydrate C-2, C-3, C-5
69	C-2/C-3 of galacturonan
65	C-6 of crystalline cellulose
62	C-6 of crystallite-surface cellulose and *B*(1-4) galactan
53	pectic methoxyl
21	CH_3 of acetyl

contact time—the time allowed for transfer of magnetization between ^1H and ^{13}C nuclei—in an otherwise standard CP-MAS experiment. Since experiments of this type produce a multiple exponential curve of signal intensity against contact time, which must be analysed to extract the relaxation parameters, they are demanding in the precision of the raw data. The high signal/noise ratio in these experiments permitted accurate curve-fitting with a variety of relaxation models. During the cross-polarization contact time, magnetization is transferred from ^1H to ^{13}C nuclei. This brings them towards magnetic equilibrium at a rate defined by the time constant T_{CH}. The proton magnetization, and the ^{13}C magnetization tracking it, then dissipate much more slowly with time constant $T_{1\rho}$. With materials of this type T_{CH} is typically well under 1 msec (Table 2) and ^1H $T_{1\rho}$ is a few msec [18, 21, 25]. The rate of proton

Table 2. Polarization transfer kinetics for unripe tomato cell walls

ppm	T_{CH}, ms	S_X/S_0
177	0.51	0.10
175	0.52	0.00
173	0.34	0.02
171	0.54	0.02
105	0.18	0.46
101	0.07	0.32
88	0.24	0.47
84	0.18	0.44
80	0.06	0.18
75	0.16	0.44
72	0.18	0.47
69	0.11	0.48
65	0.16	0.49
62	0.15	0.51
54	0.18	0.14

spin diffusion is usually considered sufficient to average the ^1H $T_{1\rho}$ values associated with all ^{13}C nuclei, whatever their chemical environment, within a spatial domain a few nm across [18, 20, 21]. The ^1H $T_{1\rho}$ values observed for plant cell walls and similar materials have been assumed to decrease with increasing molecular mobility (e.g. [17]). This behaviour is common in synthetic polymers below their glass transition temperature [19]. However in the case of some polymers with glass transitions close to ambient temperature, variable-temperature studies have shown that the ^1H $T_{1\rho}$ goes through a minimum: at temperatures above this, the lengthening $T_{1\rho}$ is attributed to increasing amounts of motion associated with the glass transition itself. We cannot, therefore, automatically assume that a shortening ^1H $T_{1\rho}$ indicates increasing molecular motion, and for the tomato cell walls this assumption will be examined in more detail below.

In most earlier models and in the commercially available software, the rising part of the curve at contact times less than 1 msec, where the signal intensity S is controlled by T_{CH}, is modelled as a simple exponential. However a two-phase model for polarization transfer [26; K. M. Fenwick, D. C. Apperley and M. C. Jarvis, unpublished] gave a much closer fit to the experimental data (Fig. 2). The fast phase appeared to be essentially complete by the shortest contact time of 50 μsec and was modelled simply as an intercept on the signal-intensity axis. Thus a single exponential function could still be used to describe the rising part of the curve (Fig. 2), even though two kinetic components were present. Although it is predominantly the rising part of the curve that is affected by T_{CH} there was still a significant effect on the calculated $T_{1\rho}$ values. A more

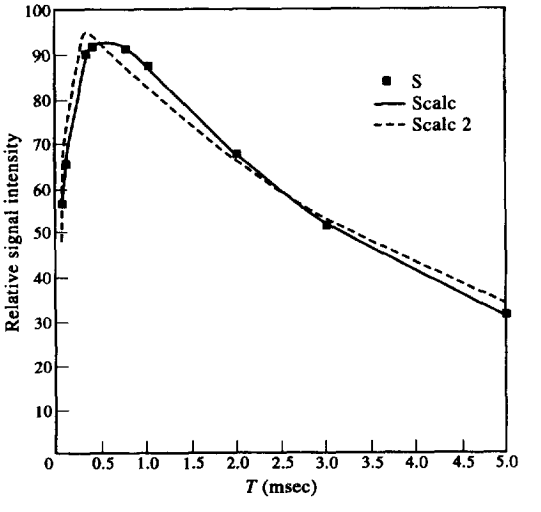

Fig. 2. Observed and calculated signal intensities for a typical peak (105 ppm) in the spectrum of ripe tomato cell walls, as a function of contact time T. The calculated signal intensities are based (solid line) on the single-component relaxation equation described in the Experimental section, or (dotted line) a similar equation with $S_X = 0$, i.e. ignoring the separation of cross-polarization into fast and slow phases.

sophisticated approach will be described elsewhere (K. M. Fenwick, D. C. Apperley and M. C. Jarvis, unpublished) but was not applicable here because it requires more data at contact time <0.1 msec.

The fast initial stage ($t_{1/2}$ = 20–30 msec) has been attributed to direct polarization transfer from protons covalently bonded to methylene or methine carbons [26] and is retarded by increasing motion of these protons. The entire rising part of the curve showed slow cross-polarization for carboxyl carbons which have no covalently bonded protons [26]. In our experiments S_X/S_0, representing the magnitude of the fast component as a fraction of the total, was approximately zero for carboxyl carbons and was smaller for methyl carbons, which are free to rotate, than for methylene or methine carbons (Table 2). Thus freely rotating methyl groups are an example of a system where increasing motion can increase the time required for polarization transfer.

Differences in relaxation behaviour within spectra

Figure 3 shows the $T_{1\rho}$ values calculated on the basis of a model with single exponential functions for both T_{CH} and $T_{1\rho}$ and a fitted intercept S_X. Leaving aside the differences between samples, within each spectrum the resonances may be divided into three groups corresponding broadly to cellulose CHOH carbons, pectic (galacturonan) CHOH carbons and methyl carbons. Some resonances from hemicelluloses and from any pectic side-chains rigid enough to cross-polarize are superimposed on resonances of the pectic group, others on resonances of the cellulosic group. In the two cell wall preparations from ripe fruit the $T_{1\rho}$ values for the pectic group were consistently shorter than for the cellulose group, while those of the methyl resonances were variable and sometimes longer than any of the others. The $T_{1\rho}$s for the 171 and 177 ppm resonances, corresponding respectively to pectic $COOCH_3$ and COO^- Na^+ carbons, were in reasonable agreement with those of the other pectic resonances (Fig. 4). The $T_{1\rho}$ of the calcium-complexed pectic carboxyls (175 ppm) was exceptionally long and quite different from anything else in the cell wall. Since carboxyl carbons have no directly bonded protons it is not clear how they cross-polarize at all, but apparently all except the calcium-bonded pectic carboxyls do so quite efficiently. However, a likely source is hydrogen-bonded hydroxyl groups in close proximity, which are absent in the 'holes' in the egg-box structure for calcium pectate and in the threefold helical aggregates that accompany it in concentrated pectic gels [24], leaving only bound water protons as likely polarization sources.

The acetyl methyl resonance at 23 ppm had a much longer $T_{1\rho}$ than the acetyl carboxyl resonance at 173 ppm, and the pectic methoxyl resonance at 54 ppm had a much longer $T_{1\rho}$ than the resonance at 171 ppm derived primarily from methylated pectic carboxyls. These observations are surprising because the acetyl methyl carbons must be in the same domain as the acetyl carboxyls, and the pectic methoxyl carbons must be in the same domain as the carboxyls to which they are bonded. We have shown (unpublished) that such behaviour is common for mobile segments of hydrated cell walls. It results from the presence of a small proportion of the galacturonan and galactan chains which are so mobile that cross-polarization within them is greatly hindered and their T_{CH} is a few msec, an order of magnitude larger than the rest of the cell wall (Table 2) and comparable with the $T_{1\rho}$s of the more rigid polymers. The increasing contribution of this more mobile material, at contact times above 1 msec,

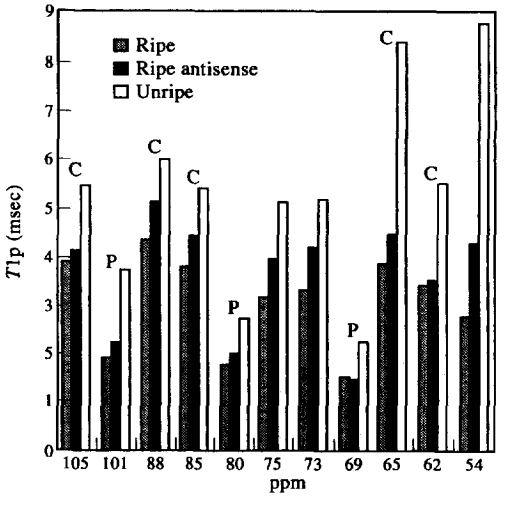

Fig. 3. Single-component $T_{1\rho}$ values for each peak in the spectrum of cell walls from ripe, antisense and unripe fruit. C and P denote peaks associated with cellulose and pectin, respectively.

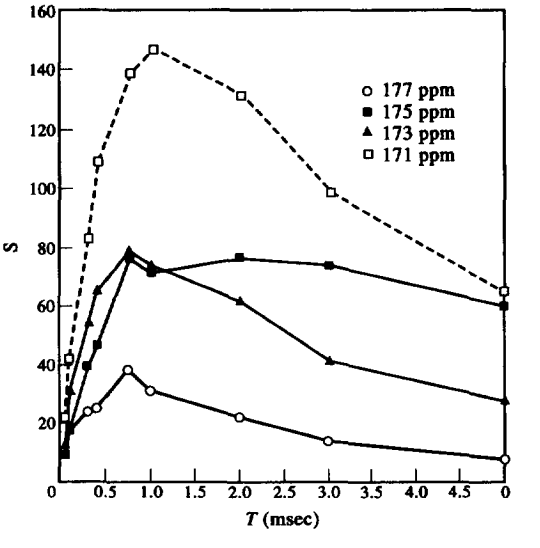

Fig. 4. Cross-polarization kinetics for carboxyl signals in spectra from cell walls of unripe tomatoes. Fitted single-component $T_{1\rho}$ values for the 171 (H/Me pectin) 173 (acetyl), 175 (Ca pectin) and 177 ppm (K pectin) resonances were, respectively, 4.4, 3.9, 11.5 and 2.8 msec.

lengthens the *apparent* $T_{1\rho}$ derived from the composite signal. At the relatively low moisture content of the tomato cell walls used here, this phenomenon is shown clearly only by the rapidly rotating methoxyl and acetyl CH_3 groups (54 and 21 ppm).

For each of the cell wall samples there was close agreement between the $T_{1\rho}$ values for C-1, C-4 and C-6 of cellulose. The $T_{1\rho}$ values for C-1, C-4 and C-2/C-3 of pectins were similar to each other but shorter than those for cellulose. This is evidence that these $T_{1\rho}$ values were averaged by spin diffusion within the proton pool surrounding each polysaccharide, and that pectins and cellulose were embedded in spatially separated domains differing in molecular mobility. The spatial separation was confirmed by detailed examination of the fit between the observed peak intensities and those calculated on the basis of a single $T_{1\rho}$ exponential. For all the resonances in the cellulose group the fit was close: the size of the differences between observed and calculated intensities depended on the signal: noise ratio and hence on the absolute intensity of the peak in question, but was generally small and showed no systematic pattern. For the resonances in the pectic group, however, the observed intensities typically dipped below the calculated ones in the middle of the falling part of the curve, at contact times of about 2–3 msec (Fig. 5). This behaviour is characteristic of a curve derived from two or more additive exponentials, implying that the pectic chains were divided between two or more domains differing in molecular mobility. The number of domains may not correspond exactly with the number of exponential components [27, 28], but a minimal hypothesis is that one domain contained both pectin and cellulose, with a common, long $T_{1\rho}$; while a second domain contained only pectin and had a shorter $T_{1\rho}$. The relaxation curves for the pectic peaks at 69, 80 and 101 ppm were therefore re-analysed on the assumption that one part of the pectin shared the same $T_{1\rho}$ value as cellulose (estimated from the 84, 88 and 105 ppm peaks). This portion was subtracted from the total at each time point and a separate exponential function with shorter $T_{1\rho}$ fitted to the result. Figure 5

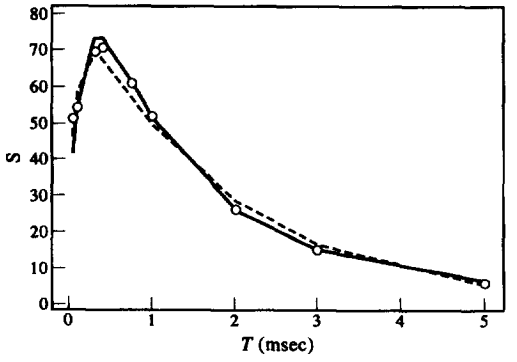

Fig. 5. Relaxation kinetics of the pectic resonance at 69 ppm for cell walls from ripe tomatoes. The dotted line is based on the single-component model and the solid line on the two-component model for $T_{1\rho}$.

shows that when the signal:noise ratio was adequate, the fit was extremely close, supporting the assumptions made. The shorter $T_{1\rho}$ component was in the range 1–2 msec for the pectic resonances at 100, 80, 69 and 54 ppm. On the basis of any reasonable model of the primary cell wall, pectins are less rigid than cellulose, so for these NMR resonances motion shortens the $T_{1\rho}$ as conventionally assumed.

The close agreement between the $T_{1\rho}$ values for crystalline (88, 65 ppm) and crystal-surface (84, 62 ppm) cellulose suggests that these were present in a single domain. While the identification of hemicellulose resonances is problematic, it is likely that they show at least some overlap with the 105, 84 and 62 ppm signals, and if that is correct the hemicelluloses concerned are likely to have been present in the same domain as the cellulose. The presence of about a quarter of the pectin also, in this domain, is consistent with the data. The very approximate dimensions of these domains may be calculated from the rate of spin diffusion amongst the protons, using the relationship $R = (Dt)^{0.5}$, where R is the domain radius, D is the proton spin diffusion coefficient and t is the time allowed for spin diffusion to occur, i.e. approximately the contact time. Since D is about 10^{-15} msec^{-1} the domain size is of the order of a few nm [18, 20, 21], although this estimate may well be affected by other factors such as the geometry of the system and the local concentration of relatively rigid protons [21, 28]. The thickness of the tomato cell walls is about 300 nm [2, 29], so the spatial separation of the cell wall and middle lamella is large enough for these components to show as separate domains according to the calculations above. The data also fit their composition, with the middle lamella containing pectins but no cellulose. However the proportion of the pectins showing fast proton relaxation, about 75%, was too large to be accounted for by the middle lamella fraction alone. CDTA, which is considered to extract middle lamella pectins, removed about half of the uronic acid from both ripe and unripe tomato cell walls [30]. Thus it seems likely, as has been suggested for apple cell walls [31], that some of pectins interstitial between microfibrils of the cell wall also fall within the fast-relaxating group. The diameter of the microfibrils in typical primary cell walls is about 10 nm and they are separated by a similar or slightly greater distance [32], so within the uncertainties of the theoretical calculations, the hypothesis that some of the interfibrillar cell wall pectins are free enough to show rapid proton relaxation is consistent with the data.

Differences between samples

The models used allowed the resonance intensities to be corrected for differences in cross-polarization behaviour and quantitative data on cell wall composition to be extracted. Some highly mobile carbohydrate chains may be missing from the spectra if they were insufficiently rigid to cross-polarize at all [22], but at the moisture content used the amounts are not likely to

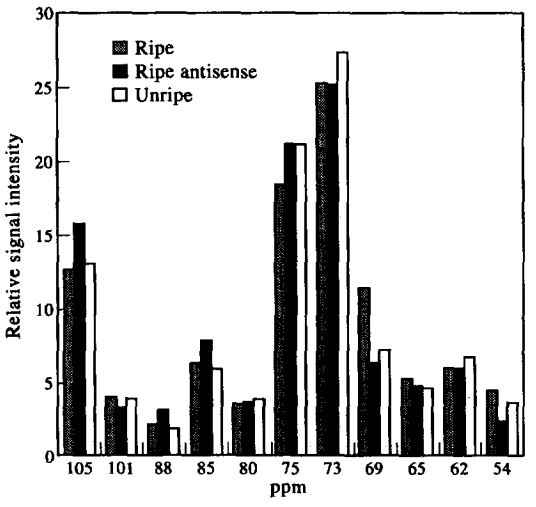

Fig. 6. Relative sizes (S_0) of peaks in the CP-MAS spectra of cell walls from unripe, ripe and ripe PG-antisense tomatoes after correction for cross-polarization efficiency.

be large. Figure 6 shows the spectra in diagrammatic form corrected in this way. It shows that changes in composition due to ripening were relatively small. There may have been some increase in the fast-relaxing pectic component on ripening, and if so it did not occur in the PG-antisense cell walls, but the complex relaxation behaviour of the 69 ppm peak makes the signal intensity less accurate. The C-1 and C-4 pectic peaks at 101 and 80 ppm are not suitable for quantitation by peak height because their width reflects a broad and possibly changing range of glycosidic conformations (M. C. Jarvis and D. C. Apperley, unpublished). A small decrease in the intensity of the 62 ppm signal on ripening may reflect loss of β(1,4)-linked galactan chains.

A clear and consistent effect of ripening is visible in the slowly-relaxing group of $T_{1\rho}$ values, including those for cellulose which are diagnostic of the cell wall itself rather than the middle lamella. Their decreasing $T_{1\rho}$ on ripening may be assumed to result from increased molecular mobility in the kHz frequency range, and hence from a loss of cohesion in the cell wall during the ripening process. The $T_{1\rho}$ values for the PG-antisense cell walls were intermediate. The mean (single-component) $T_{1\rho}$ values for the pectic carbons likewise decreased on ripening, but to rather similar extents in the PG-antisense and control tomatoes. In principle the fast-relaxing component of the pectins, corresponding to middle-lamella and interstitial polymers, could be characterized from the two-component relaxation model. However the problems of fitting a dual exponential function, even to experimental data of this precision, meant that the accuracy was low and it was not possible to decide whether a smaller proportion of the pectins were included in the fast-relaxing domain in the unripe cell walls, or whether its $T_{1\rho}$ was longer. Either hypothesis, however, would imply that the pectic network of the middle lamella and the region between the microfibrils became looser on ripening, and that the PG-antisense gene had little effect on this process.

Our evidence shows that the lack of PG in the antisense tomatoes reduced, but did not prevent, the loss of overall cell wall rigidity during natural ripening. A reasonable hypothesis would be that this effect was due to reduced depolymerisation of pectins. However it would then be expected that in the ripe antisense material, the pectic $T_{1\rho}$s would be closer than the cellulose $T_{1\rho}$s to those of the unripe cell walls, and the reverse was the case. That apparent contradiction can be resolved if only a small fraction of the pectic polymers was susceptible to degradation by the PG that was downregulated, and this fraction had a significant effect on the rigidity of the network of microfibrils. It might, for example, have been a pectic fraction spatially associated with the microfibrils.

EXPERIMENTAL

The introduction of polygalacturonase-antisense genes into tomato, cv. Ailsa Craig, has been described [8, 11]. The transformed line AC105 was compared in this experiment with unripe and red-ripe fruit of cv. Ailsa Craig. Cell walls were prepared as described [30], except that the initial homogenization in acetone was replaced by disintegration of frozen tissue followed by stirring in acetone. Some of the wall-bound Ca^{2+} ions may have been lost during the phenol extraction step of the wall preparation [33]. The cell walls were cryo-milled in a dental amalgamator with the capsule immersed in liquid N_2. The cryo-milling step allowed the mass of cell wall that could be packed into the NMR rotor to be doubled. H_2O was added immediately before packing the rotor to increase the moisture content to *ca* 30%. The unripe cell walls lost some of this H_2O during the experiment, but it was retained by the other two cell wall preparations.

The NMR experiments were carried out on the SERC Varian 3000 MHz spectrometer at Durham. The rotating-frame spin-lattice relaxation time $T_{1\rho}$ and the polarisation transfer time constant T_{CH} were measured by varying the contact time from 50 μsec to 5 msec in an otherwise standard cross-polarization/magic-angle spinning (CP-MAS) experiment with MAS rate approximately 3 kHz and relaxation delay 1 sec.

The data were analysed by least-squares fitting based on the equation:

$$S = S_0 \exp(-t/T_{1\rho})(1 - (1 - S_X/S_0)\exp(-t/T_{CH})),$$

where S is the signal intensity and contact time t, S_0 is the calculated maximum signal intensity, and S_X is the intercept at $t = 0$.

The two-component relaxation analysis of the pectic signals was based on the equation:

$$S = S_{01}\exp(-t/T_{1\rho 1}) + S_{02}\exp(-t/T_{1\rho 2}) \times (1 - (1 - S_X/S_0)\exp(-t/T_{CH})),$$

where the additional suffixes 1 and 2 refer to the fast and slow relaxation components, respectively.

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