Xyloglucan undergoes interpolymeric transglycosylation during binding to the plant cell wall *in vivo*: evidence from $^{13}\text{C}/^3\text{H}$ dual labelling and isopycnic centrifugation in caesium trifluoroacetate

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Xyloglucan from the walls of *Rosa* cells that had been cultured on [^{12}C]- or [^{13}C]-glucose formed bands in caesium trifluoroacetate with mean buoyant densities of 1.575 or 1.616 g/ml respectively. Incubation of a mixture of [$^{13}\text{C},^3\text{H}$]xyloglucan and [$^{12}\text{C},^1\text{H}$]xyloglucan in the presence of xyloglucan endotransglycosylase (XET) activity caused the mean buoyant density of the radioactive material to decrease, indicating that interpolymeric transglycosylation could be detected *in vitro*. We used two $^{13}\text{C}/^3\text{H}$ -dual-labelling protocols to look for interpolymeric transglycosylation *in vivo*. In protocol A, [^{13}C]glucose-grown *Rosa* cells were transferred into [^{12}C]glucose medium 6 h after a ≈ 2 h pulse of L-[^3H]arabinose (which radiolabels the xylose residues of xyloglucan). The mean buoyant density of the wall-bound [^3H]xyloglucan decreased during the following 7 days in culture. This indicates that, during or after the wall-binding of newly synthesized [$^{12}\text{C},^1\text{H}$]xyloglucan, it became covalently attached to previously wall-bound [$^{13}\text{C},^3\text{H}$]xyloglucan. In protocol

B, [^{12}C]glycerol- or [^{12}C]glucose-grown *Rosa* cells were transferred into [^{13}C]glucose medium, 20 or 60 min before a ≈ 2 h pulse of [^3H]arabinose. The buoyant density of the earliest wall-bound [^3H]xyloglucan showed that it had a $^{12}\text{C}/^{13}\text{C}$ ratio of $\sim 1:1$. This indicates that, during (or, implausibly, before) wall-binding, the newly synthesized [$^{13}\text{C},^3\text{H}$]xyloglucan became covalently attached to previously synthesized [^{12}C]xyloglucan. During the following 7 days in culture, the mean buoyant density of the [^3H]xyloglucan increased, showing that later-synthesized [$^{13}\text{C},^1\text{H}$]xyloglucan can be covalently attached to previously wall-bound [$^{12}\text{C},^3\text{H}$]xyloglucan. The only known mechanism by which segments of xyloglucans could become covalently attached to each other in the cell wall is by interpolymeric transglycosylation catalysed by XET. We conclude that XET-catalysed interpolymeric transglycosylation accompanies, and probably causes, the integration of newly secreted xyloglucan into the cell-wall architecture.

INTRODUCTION

The enzymic restructuring of cell-wall polysaccharides *in vivo* is widely held to be important in the control of plant growth [1–3], abscission [4] and fruit ripening [5–7]. Wall enzymes that could restructure polysaccharides include hydrolases and transglycosylases [3]. However, the mere presence of these enzymes in a cell wall does not prove that they catalyse reactions there [3]. To test whether the enzymes do act in the walls of living cells, it is necessary to monitor the metabolism of their polysaccharide substrates *in vivo*. Hydrolase action would decrease the M_r of wall polysaccharides, and such decreases have indeed been seen during normal growth and in response to exogenous auxins or low pH [1,2,8]. However, there is currently no evidence that wall polysaccharides undergo transglycosylation *in vivo*.

Xyloglucan is a major structural polysaccharide of primary walls. It hydrogen-bonds to, and probably tethers, neighbouring microfibrils [9–12]. It was therefore of interest to investigate whether xyloglucan chains undergo transglycosylation in the walls of living plant cells. Xyloglucan endotransglycosylases (XETs) are widespread in land plants [13–19], and numerous correlations between XET activity and wall loosening have been noted [5,7,20–24]. XET cuts a xyloglucan chain (donor substrate)

and transfers the newly formed potentially reducing terminus on to the non-reducing terminus of another chain (acceptor substrate) [15–17]. *In vitro*, the acceptor can be a high- M_r xyloglucan or a short xyloglucan oligomer as small as a pentasaccharide [25]. Transglycosylation between xyloglucan chains in the cell wall could play a role in wall construction [8,26,27] and/or wall loosening [8,14,16,21,22,25,28]. Progress in testing these ideas has been hampered by our inability to say whether XET catalyses interpolymeric transglycosylation *in vivo*. XETs are known to catalyse polymer-to-oligomer transglycosylation *in vitro* [15–17,25], interpolymeric transglycosylation *in vitro* [17,29,30] and polymer-to-oligomer transglycosylation *in vivo* (although not necessarily involving wall-bound polymer) [13,14]. However, there is no direct evidence for interpolymeric transglycosylation *in vivo*.

Suspension-cultured plant cells are a convenient model system in which to study xyloglucan metabolism. They rapidly take up L-[^3H]arabinose and incorporate a substantial proportion of the radioactivity into [xylosyl- ^3H]xyloglucan [8,27]. This incorporation proceeds via the scavenger pathway:

Abbreviations used: CsTFA[®] (Pharmacia), caesium trifluoroacetate; GPC, gel-permeation chromatography; PyAW, pyridine/acetic acid/water (1:1:23, by vol., pH 4.7); $\Delta\rho$, difference in buoyant density; note that, in the names of isotopically labelled compounds, ^1H and ^{12}C are omitted except for emphasis; (c)XET, (cauliflower) xyloglucan endotransglycosylase; XLLGol, the structure of this oligosaccharide is given in the text.

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the last step being catalysed by UDP-xylose 4-epimerase [31]. Thus radioactivity from exogenous [^3H]arabinose is incorporated into the L-arabinose and D-xylose residues of polysaccharides, whereas the hexose and uronic acid residues are not labelled [31,32]. Even without the use of a 'cold chase', a single dose of [^3H]arabinose is utilized rapidly enough to give labelling kinetics analogous to a pulse-chase experiment, in which the pulse (1–2 h) is < 10% of a cell cycle [8,27]. Most of the newly synthesized [^3H]xyloglucan binds to the walls very soon after secretion [27]. *Rosa* cells synthesize xyloglucan as relatively short chains ($M_r \leq 10000$) (J. E. Thompson and S. C. Fry, unpublished work), but upon integration into the wall the chains increase in M_r to ≈ 200000 [8], suggesting that xyloglucans covalently attach to one another during integration. Rapid increases observed in the M_r of xyloglucan in stem segments in response to treatments that decrease wall extensibility [2,33] also suggest covalent attachment. During the 2 days after wall-binding, *Rosa* xyloglucans are trimmed by, on average, 40 kDa and most of the degradation products are sloughed into the culture medium [8]. We suggested that the covalent attachment and/or trimming could be catalysed by XET [8].

It is not straightforward to test for interpolymeric transglycosylation *in vivo* because the substrates may be identical with the products of the reaction. Labelling experiments critically depend on the use of two independent labels: label (1) can be a radiolabel, but label (2) must provide the basis of a physical separatory method. By use of label (2) it would be possible to set up two separable populations of xyloglucan in the same cell wall; subsequent transfer of label (1) from one population to the other would provide evidence for interpolymeric transglycosylation *in vivo*. $^{14}\text{C}/^3\text{H}$ dual labelling is not suitable because there is no useful way of showing whether these two isotopes are part of the same xyloglucan chain.

In the work described here, label (1) was ^3H of relatively low isotopic enrichment. Label (2) was ^{13}C of high isotopic enrichment, creating a population of high buoyant density [^{13}C]xyloglucan chains that were distinguishable from [^{12}C]xyloglucan by isopycnic centrifugation [34]. By ^3H -labelling one of these populations, we have sought to detect interpolymeric transglycosylation between the high- and low-density populations by monitoring changes in the density of the radiolabelled segments of xyloglucan. We have applied this strategy to test whether newly secreted xyloglucan becomes covalently attached to previously wall-bound xyloglucan.

MATERIALS AND METHODS

Cell cultures

Cell-suspension cultures of *Rosa* sp. ('Paul's Scarlet' rose) were grown under constant dim light on an orbital shaker at 25 °C. Cells were subcultured every 2 weeks by 10-fold dilution into fresh medium [35] containing 2% D-glucose or 2% glycerol as the sole carbon source. In the density-labelling experiments, the major carbon source was replaced by 0.5 or 1.0% [^{12}C]- or [^{13}C]-glucose.

Isotopically labelled chemicals

D-[U- ^{13}C]Glucose (98.9% isotopic enrichment) was from MSD Isotopes, Montreal, Canada, and was used without further purification. L-[1- ^3H]Arabinose (92 MBq/ μmol ; i.e. 9% isotopic enrichment at H-1) was custom-synthesized by Amersham International and purified in Edinburgh by preparative paper chromatography. D-[U- ^{14}C]Glucose (300 MBq/ μmol) and [1-

^{14}C]acetic anhydride (0.703 MBq/ μmol) were from Amersham International. [^3H]XLLGol, whose structure is:

(for an explanation of the abbreviated nomenclature, see [36]), was prepared as described [20].

Xyloglucan and Driselase

Tamarind-seed xyloglucan ($M_r \approx 10^6$) was kindly donated by Professor J. S. G. Reid, Department of Molecular and Biological Sciences, University of Stirling, Stirling, U.K. Driselase (Sigma Chemical Co., Poole, Dorset, U.K.) was partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and desalting [37]. Isoprimeverose [$\alpha\text{-D-Xylp-(1}\rightarrow\text{6)-D-Glc}$] was prepared as described in [37].

[^{14}C]Acetylated xyloglucan

Tamarind-seed xyloglucan [1% (w/v); 12 ml] was adjusted to pH 7.0 with pyridine. Acetic anhydride/collidine [1:2 (v/v); 1.2 ml] was added slowly and left stirring for 2 h at 25 °C to N-acetylate any contaminating protein. NaOH (3.38 g) was then added and the solution stirred for 30 min at 25 °C to hydrolyse O-acetyl esters, acidified with 5 ml of acetic acid and dialysed at 4 °C against water; the xyloglucan was dried under vacuum. [^{14}C]Acetic anhydride [20 μl of a 1.3% (w/w) solution in toluene] was added to 5 mg of the pre-treated, dried xyloglucan in a capped tube and left for 24 h at 25 °C. Ice-cold PyAW [pyridine/acetic acid/water (1:1:23, by vol., pH 4.7); 10 ml] was added; the solution was dialysed and the [^{14}C]acetylated xyloglucan solution (1.85 kBq/ml) dried in 200 μl aliquots under vacuum.

Cauliflower XET (cXET)

Finely grated cauliflower florets (200 g) were homogenized (45 s in a blender) in 300 ml of ice-cold 0.35 M sodium succinate buffer, pH 5.5. The homogenate was incubated (0 °C, 2 h) with occasional mixing, then strained through muslin (four layers) and centrifuged (2500 g, 30 min, 4 °C). The supernatant was poured through Miracloth (four layers), dialysed against succinate buffer, labelled 'cXET' and stored frozen at -70 °C.

XET activity was assayed as follows. Substrate solution [20 μl of 0.3% w/v xyloglucan (≈ 60 pmol) containing 1.6 kBq of [^3H]XLLGol (≈ 80 pmol)] was mixed with cXET (10 μl of a 1:10 dilution in succinate buffer) and incubated at 25 °C. At various time points, replicate assays were stopped with formic acid (100 μl , 30% w/v) and paper-binding ^3H -labelled products were assayed [16].

Xyloglucan endohydrolase activity was assayed as follows. Xyloglucan solution (4 ml, 1% w/v in water) was incubated at 25 °C with 0.7 ml of undiluted cXET (or 0.7 ml of succinate buffer as a control). At various time points the viscosity of the solution was assayed at 25 °C by measurement of the efflux time from a fine-bore 0.2 ml glass pipette.

Action of cXET on [^{13}C]xyloglucan *in vitro*

To a mixture of [^{13}C , ^3H]hemicellulose (10 kBq; ≈ 0.25 mg of glucose equivalents by the anthrone assay; from the 12 h time point of protocol A) and [^{12}C , ^1H]hemicellulose (≈ 0.5 mg of

(a) **Protocol A** (b) **Protocol B₁** **Figure 1** Time tables for manipulation of the cultures in protocols A and B₁

Time 0 in protocol A was the second change of carbon source; in protocol B₁ it was the addition of $[^3\text{H}]\text{Arabinose}$. In protocol B₂, the initial medium contained $[^{12}\text{C}]\text{glucose}$ rather than glycerol, and the carbon source was changed at -1 h. ▲, time points interpreted in Figure 7; ★, timing of samples taken for analysis on CsTFA[™] gradients.

glucose equivalents) in 200 μl of water was added succinate buffer (55 μl), followed by cXET extract (45 μl ; either active or denatured at 100 $^{\circ}\text{C}$ for 1 h). After 1 h at 25 $^{\circ}\text{C}$ the reaction was stopped with 1 ml of 30 % (w/v) formic acid. A portion (160 μl) of each assay mixture was subjected to gel-permeation chromatography (GPC) and the remainder to isopycnic centrifugation (with $[^{14}\text{C}]\text{acetylated xyloglucan}$ as internal marker); to monitor the $[^3\text{H}]\text{xyloglucan}$, we digested the GPC and CsTFA[™] fractions with Driselase and assayed the products for $[^3\text{H}]\text{isoprimeverose}$ (see below).

Use of $[^{14}\text{C}]\text{glucose}$ to develop an *in vivo* density-labelling strategy

D-[U- $^{14}\text{C}]\text{Glucose}$ (260 kBq/2 ml of water; autoclaved) was added to 40 ml of fresh medium containing 0.5 % (w/v) glucose and 5 g fresh weight of glucose-grown *Rosa* cells (4 days after subculturing). At time points up to 6 h, aliquots (2 ml) of the suspension were added to trichloroacetic acid [2 ml, 40 % (w/v)], filtered on glass fibre (Whatman GF/A) and washed with 20 % trichloroacetic acid. The residue (proteins, wall-bound polysaccharides etc.) was dried and assayed for ^{14}C .

Density labelling

In Protocol A (Figure 1a), $[^{12}\text{C}]\text{glucose}$ -grown *Rosa* cultures (14 days after subculturing) were filtered through sterile muslin, washed with carbon-free medium (3 $\times$ 200 ml) and gently

squeezed to remove excess medium. Cells (2 g fresh weight) were transferred into 50 ml of medium containing 1.0 % w/v $[^{13}\text{C}]\text{glucose}$ (or $[^{12}\text{C}]\text{glucose}$ in the control) and incubated for 7 days (three or four cell cycles), bringing the cells to a state of rapid growth [8] to deplete ^{12}C pools of UDP-sugars and to saturate the wall with ^{13}C -labelled polysaccharides. $[^3\text{H}]\text{Arabinose}$ (12 MBq/2 ml; autoclaved) was then added to radiolabel newly synthesized xyloglucan. After 6 h the cultures were filtered and washed with carbon-free medium (4 $\times$ 100 ml); cells (10 g fresh weight) were transferred into 50 ml of medium containing 1.0 % D- $[^{12}\text{C}]\text{glucose}$ and 5 mM L- $[^{12}\text{C}]\text{arabinose}$ [38] to compete with further incorporation of ^{13}C and ^3H . Aliquots (4 ml) of the suspensions were dispensed into Sterilin polycarbonate pots (50 ml capacity). Pots were harvested at time points 1 h to 7 days after the $^{13}\text{C} \rightarrow ^{12}\text{C}$ shift. Wall hemicellulose was analysed by isopycnic centrifugation with $[^{14}\text{C}]\text{acetylated xyloglucan}$ as internal marker.

In Protocol B₁ (Figure 1b), glycerol-grown cultures (4 days after subculturing, and therefore rapidly growing [8]) were filtered through sterile muslin, washed with carbon-free medium (3 $\times$ 200 ml) and gently squeezed. Cells (5 g fresh weight) were transferred into 40 ml of medium containing 0.5 % (w/v) $[^{13}\text{C}]\text{glucose}$ (or $[^{12}\text{C}]\text{glucose}$ in the control) and left under normal growth conditions for 20 min to deplete ^{12}C pools. $[^3\text{H}]\text{Arabinose}$ (24 MBq/2 ml; autoclaved) was then added. Aliquots (4 ml) of the suspensions were dispensed into Sterilin pots and harvested 1 h to 7 days after the addition of $[^3\text{H}]\text{arabinose}$. Wall hemicellulose was analysed by isopycnic centrifugation.

In Protocol B₂, the experiment was repeated as in protocol B₁ except that glucose-grown (not glycerol-grown) cells were used, 1 h (not 20 min) was allowed for depletion of ¹³C pools and 12 MBq of [³H]arabinose was added.

Fractionation of cell polysaccharides

Cultures were quickly filtered on a Polyprep column (Bio-Rad) and washed with 6 × 5 ml of water; the 'culture filtrate' was stored frozen. The cells were frozen at -70 °C, resuspended in sonication buffer (20 mM Hepes/2 % Triton X-100/40 mM NaF, pH 7.0; 50 mg fresh weight/ml) and sonicated for 2 min in an MSE Soniprep-150 instrument (3 mm titanium probe; 24 kHz; 18 µm amplitude). The homogenate was immediately filtered on a Polyprep column and washed with 6 × 3 ml of sonication buffer; the filtrate ('protoplast contents') was stored frozen. The wall-rich residue was resuspended in 5 ml of 6 M NaOH containing 1 % w/v NaBH₄ [39] and shaken gently at 37 °C for 24 h. The suspension was then filtered on a Polyprep column and washed with 3 × 3 ml of NaOH/NaBH₄ solution; the filtrate was acidified by slow addition of acetic acid, dialysed against water at 4 °C and stored at -20 °C ('hemicellulose extract').

Buoyant density of xyloglucan

Dried [³H]hemicellulose (≈ 16 kBq) and [¹⁴C]acetylated xyloglucan (0.67 kBq) were dissolved in PyAW, mixed, and adjusted to 4.672 ml with more PyAW. The following were then added: 8 ml of CsTFA[®] solution (2.03 g/ml; Pharmacia Biotech), 347 µl of pyridine, 347 µl of acetic acid and 100 µl of 0.5 % Blue Dextran in PyAW. The mixture (density 1.60 g/ml) was centrifuged for 100 h at 18 °C in a polyallomer tube (Quickseal; 16 mm × 76 mm; Beckman) in a Beckman 70.1 Ti fixed-angle rotor at 45000 rev./min (≈ 100000 g). This formed a stable gradient (≈ 1.4–1.8 g/ml), which was fractionated by downward displacement with white mineral oil (13 ml/h). The density of each fraction was determined from the weight of 200 µl. A portion (50 µl) of each fraction was assayed for total ³H and ¹⁴C after addition of 950 µl of water and 10 ml of Triton scintillant.

A 100 µl portion of each fraction was desalted on Bio-Gel P-2 (2 ml bed volume), equilibrated, and eluted with PyAW. The polymeric material was dried under vacuum and digested with Driselase (see below) to generate [³H]isoprimeverose.

Profiles of radioactive xyloglucan were plotted with a computer graph-drawing package (SigmaPlot). A built-in curve-fitting procedure was used to give a Gaussian curve, whose peak was taken as an estimate of the mean buoyant density of the xyloglucan.

Gel-permeation chromatography

[³H]Hemicellulose in 2 ml of PyAW containing markers (0.4 mg each of 5 MDa dextran and sucrose) was analysed on an 80-ml-bed-volume column (1.5 cm diameter) of Sepharose CL-6B, equilibrated and eluted with PyAW at 7 ml/h. Fractions (1.5 ml) were collected; 200 µl was assayed with anthrone [40] to locate the dextran (*k_{av}* 0) and sucrose (*k_{av}* 1); 200 µl was assayed for ³H; 1 ml was digested with Driselase and the resulting [³H]isoprimeverose assayed.

Digestion with Driselase

Feeding of plant cell cultures with [³H]arabinose results in labelling of the cell walls in their L-arabinose and D-xylose residues, whereas hexoses, uronic acids and amino acids remain non-radioactive [31,32,35]. In cultured *Festuca* cells, the supply

of exogenous [³H]arabinose resulted in labelling of wall-bound xylose residues to a specific activity approximately half that of the wall-bound arabinose residues [32]. The two major hemicelluloses of the primary walls of cultured dicotyledonous-plant cells are xyloglucans and arabinoglucuronoxylans, which are typically present at a ratio of between 2:1 and 5:1 [37]. To monitor specifically the [³H]xyloglucan, we digested the total dried ³H-hemicellulose with Driselase [40 µl; 0.5 % (w/v) in 25 % PyAW] at 37 °C for 48 h; this digests [*xylosyl*-³H]xyloglucan to [³H]isoprimeverose. Under these conditions, arabinoglucuronoxylans yield mainly [³H]arabinose, [³H]xylose and [³H]xylobiose [= β-D-Xylp-(1 → 4)-D-Xyl]. Products were mixed with non-radioactive isoprimeverose as an internal marker and chromatographed on Whatman 3MM paper in ethyl acetate/pyridine/water (9:3:2, by vol.) for 20 h. The dried paper was dipped through dilute stain [0.106 % (w/v) phthalic acid and 0.033 % (v/v) aniline in acetone/diethyl ether/water (49:49:2, by vol.)], re-dried and heated at 105 °C for 5 min. The lightly stained spots of isoprimeverose were cut out and assayed for ³H. The above chromatography solvent was selected [8] because of its ability to resolve isoprimeverose from the four other quantitatively significant [³H]pentose-labelled products present: arabinose, xylose, xylobiose and unidentified material of *R_f* 0 that, on acid hydrolysis, yields [³H]arabinose but no [³H]xylose [8,37]. Driselase contains many hydrolytic enzyme activities, including α- and β-D-galactopyranosidase and highly active α-L-arabinofuranosidase [37] that would quickly convert any α-L-Araf-(1 → 2)-D-Xyl or α-L-Araf-(1 → 3)-D-Xyl to monosaccharides. The only radioactive compound present in the selected disaccharide spot was therefore [³H]isoprimeverose: this has been verified by co-migration of the ³H with authentic non-radioactive isoprimeverose on paper chromatography in five diverse solvent systems [41] and (after reduction with borohydride) by paper electrophoresis in molybdate buffer [41]; in addition, acid hydrolysis gave [³H]xylose as the sole radioactive product, even after reduction with borohydride [41].

Assay of radioactivity

Aqueous samples were mixed with 10 vol. of Triton scintillant [0.33 % (w/v) 2,5-diphenyloxazole, 0.033 % (w/v) 1,4-di-2-(5-phenyloxazolyl)benzene in toluene/Triton X-100 (2:1, v/v); counting efficiency: ≈ 50 % for ³H; ≈ 95 % for ¹⁴C]. Samples on dried paper were soaked with 2 ml of non-Triton scintillant [0.5 % (w/v) 2,5-diphenyloxazole/0.05 % (w/v) 1,4-di-2-(5-phenyloxazolyl)benzene in toluene; counting efficiency for ³H-sugars ≈ 7 %; for [³H]xyloglucan ≈ 42 %]. Staining the paper chromatogram, under the conditions described, did not significantly affect the detection of ³H (results not shown).

RESULTS

Buoyant densities of polysaccharides

Total [³H]hemicellulose from *Rosa* cell walls banded within the density range of CsTFA[®]. [³H]Hemicellulose from [¹³C]glucose-fed cultures (Figure 2a) had a higher buoyant density than that from [¹³C]glucose-fed cultures (Figure 2b). Driselase digestion of each CsTFA[®] fraction demonstrated that the [³H]xyloglucan profile (Figure 3) closely mirrored the total [³H]hemicellulose profile and showed a good fit to a Gaussian curve; the average peak density of [¹³C,³H]xyloglucan was 1.575 g/ml. Blue Dextran, added as a visible marker, formed a very tight blue band of buoyant density 1.655 g/ml.

Figure 2 Representative profiles of density-labelled and normal hemicelluloses after isopycnic centrifugation in CsTFATM

(a) Total *Rosa* [¹³C,³H]hemicellulose (from protocol A, harvested 1 day after the ¹³C → ¹²C shift) (●), ¹⁴C-acetylated tamarind xyloglucan added as internal marker (○) and the density gradient (□). (b) As (a), but with total *Rosa* [¹²C,³H]hemicellulose (●).

In vitro test of the ability of dual-labelling experiments to detect interpolymeric transglycosylation

Characterization of the cXET extract

The cauliflower extract had high XET activity in a radiochemical assay of polymer-to-oligomer transglycosylation: 1 µl of cXET catalysed the incorporation of 2.7 µM [³H]XLLGol (oligomer) into 0.2 % xyloglucan at 16 pmol · h⁻¹ (Figure 4a). Endohydrolase activity, assayed viscometrically [42], was low (results not shown) and did not preclude the estimation of mean buoyant density in CsTFATM.

Action of XET on dual-labelled substrates *in vitro*

To validate the dual-labelling method for detection of interpolymeric transglycosylation, we incubated a mixture of [¹³C,³H]xyloglucan and [¹²C,³H]xyloglucan for 1 h with active or boiled cXET. Before incubation, the two xyloglucan samples had similar *M_r* profiles on Sepharose CL-6B; on incubation with

Figure 3 Representative profiles of density-labelled and normal xyloglucans after isopycnic centrifugation in CsTFATM

The fractions of [³H]hemicellulose shown in Figure 2 were digested with Driselase and the [³H]isoprimeverose produced was assayed. Hence the profiles of [¹³C,³H]xyloglucan (●) and [¹²C,³H]xyloglucan (○) were obtained. Gaussian curves are fitted (—, ---), and the peak of each curve is indicated (↓). A Gaussian curve is also fitted for the [¹⁴C]acetylated xyloglucan internal marker used with the ¹³C-labelled sample (.....). The density gradient is shown (□).

active cXET, the xyloglucans did not change their *M_r* (results not shown), which shows that negligible endohydrolysis occurred. Also, no ³H-labelled oligosaccharides or monosaccharides were produced, showing that α-D-xylosidase was absent or unable to act on the high-*M_r* xyloglucan used. However, the mean buoyant density of the [³H]xyloglucan decreased from 1.611 to 1.601 g/ml (Figure 4b), indicating that segments of [¹²C]- and [¹³C]-xyloglucans became covalently attached to each other.

The most plausible explanation for such covalent attachment is endotransglycosylation, catalysed by XET. No xyloglucan exotransglycosylase is known: plant exoglycosylhydrolases that could potentially act on xyloglucans (α-L-fucosidase, β-D-galactosidase and α-D-xylosidase) are not known to catalyse transglycosylation at the low substrate concentrations used in the present work, and they are more active on xyloglucan oligosaccharides than on high-*M_r* xyloglucans.

Thus, although impure XET was used in this experiment, the data suggest that interpolymeric endotransglycosylation can be detected *in vitro* by a ¹³C/³H-labelling strategy.

Use of [¹⁴C]glucose to develop a density-labelling strategy

In the presence of 0.5 % [¹²C]glucose, *Rosa* cells incorporated ¹⁴C from [¹⁴C]glucose into polymers for at least 6 h at a steady rate of ≈ 0.16 % h⁻¹ after a lag of ≤ 20 min (Figure 5). If there had been appreciable turnover of preformed ¹²C-labelled polymers, such as starch, the [¹²C]hexoses formed would have diluted the ¹⁴C-labelled intermediates. Since the extent of this dilution would have diminished as a pool of [¹⁴C]starch built up, the ¹⁴C-incorporation rate would have accelerated. No such acceleration was observed during the first 6 h, indicating that turnover was negligible. We conclude that, within 20 min, the major endogenous pools of polymer precursors had become essentially saturated with ¹⁴C. Thus we predict that a culture shifted from [¹²C]- to [¹³C]-glucose would, after a lag of ≤ 20 min, be synthesizing ¹³C-labelled polymers with an isotopic enrichment similar to that of the [¹³C]glucose supplied.

Figure 4 Validation of the $^{13}\text{C}/^3\text{H}$ dual-labelling method *in vitro*

(a) A cauliflower extract was assayed radiochemically for XET activity. The donor substrate was tamarind xyloglucan and the acceptor substrate was [^3H]XLLGol; each point is the mean for four replicate assays $\pm$ S.E.M. (b) CsTFATM profiles of the reaction products obtained after incubation of a mixture of [$^{13}\text{C},^3\text{H}$]xyloglucan and [$^{12}\text{C},^1\text{H}$]xyloglucan with cauliflower extract: ●, native; ○, boiled. The curves without data points indicate the profiles of untreated [$^{12}\text{C},^1\text{H}$]xyloglucan (—) and internal marker ^{14}C -acetylated xyloglucan (.....).

Figure 5 Incorporation of ^{14}C from [^{14}C]glucose into the 20%-trichloroacetic-acid-insoluble fraction of glucose-grown *Rosa* cells

Figure 6 Effect of transferring [^{13}C]glucose-grown *Rosa* cells to [^{12}C]glucose 6 h after a pulse of [^3H]arabinose (protocol A)

(a) Incorporation of radioactivity from [^3H]arabinose into the isoprimeverose residues (xyloglucan) of the wall hemicellulose (■), culture filtrate (●) and protoplast contents (▲). Similar data (not shown) were obtained for the ^{12}C -only control cultures. (b) Changes in mean buoyant density (ρ) of the wall-bound [^3H]xyloglucan. The experimental cultures (●) received the [^{13}C]glucose treatment; the controls (○) received [^{12}C]glucose instead. The bars show the approximate periods during which ^{13}C (▨), ^{12}C (▩) and ^3H (▧) were present in the medium.

Dual-labelling experiments to detect interpolymeric transglycosylation *in vivo*

Protocol A (Figure 1a)

With either [^{12}C] or [^{13}C]glucose as the carbon source, *Rosa* cells grew well and rapidly took up [^3H]arabinose (> 95 % within 1 h; results not shown). The ^3H was incorporated into xyloglucan, i.e. polymeric material that yielded [^3H]isoprimeverose upon digestion with Driselase (Figure 6a). The ^3H in the medium at the times shown in Figure 6(a) did not include detectable free [^3H]arabinose (paper-chromatographic results not shown).

The initial difference in density ($\Delta\rho$) between the wall-bound

Figure 7 Intended labelling patterns in the ^{13}C -fed cultures in protocols A, B₁ and B₂

The diagrams represent transects through *Rosa* cell walls taken at about +0.3 h on the time scales shown in Figure 1 (note: 0.3 h is the approximate transit time for the endomembrane traffic system in these cells). Wall-bound xyloglucans are shown by horizontally aligned chains; newly secreted xyloglucans (not yet integrated into the wall) are shown as oblique chains near to the plasma membrane (pm). The xyloglucans expected to be deposited during each phase of protocol A (a) are: (i) $\circ\circ\circ$, [^{12}C]xyloglucan (> 7 days old); (ii) $\bullet\bullet\bullet$, [^{13}C]xyloglucan (bulk of cell wall); (iii) $\circ\circ\circ$, [$^{13}\text{C},^3\text{H}$]xyloglucan; (iv) $\bullet\bullet\bullet$, [^{13}C]xyloglucan (deposited after depletion of [^3H]Ara); (v) $\circ\circ\circ$, [^{12}C]xyloglucan (newly secreted); the xyloglucans expected to be deposited during each phase of protocols B₁ and B₂ (b) are: (i) $\circ\circ\circ$, [^{12}C]xyloglucan (bulk of cell wall); (ii) $\bullet\bullet\bullet$, [$^{13}\text{C},^3\text{H}$]xyloglucan; (iii) $\circ\circ\circ$, [^{13}C]xyloglucan (newly secreted).

[^3H]xyloglucan of [^{12}C]glucose-fed cells and that of [^{13}C]glucose-fed cells was 0.041 g/ml (Figure 6b). The [$^{12}\text{C},^3\text{H}$]xyloglucan in the control cultures exhibited little change in density (slight increase; not statistically significant) as it 'aged' in the cell wall (Figure 6b, $\circ$). This shows that xyloglucan normally undergoes little or no change in density that could complicate the ^{13}C -feeding experiments. In the ^{13}C -fed culture the [^3H]xyloglucan decreased in density (Figure 6b, $\bullet$; significant negative gradient, $P < 0.05$). Protocol A was designed to create the labelling pattern shown in Figure 7(a). The observation that the [^3H]xyloglucan (initially ^{13}C -saturated; shown as white stars within black circles in Figure 7a) decreased in density as it 'aged' in the wall shows that segments of [^{12}C]xyloglucan (joined thin open circles in Figure 7a) became covalently attached to it. Since the unlabelled [$^{12}\text{C},^3\text{H}$]xyloglucan was synthesized at least 4 h after the [^3H]xyloglucan, the results indicate that xyloglucan can undergo transglycosylation with previously wall-bound xyloglucan. However, this experiment does not establish whether the transglycosylation occurs simultaneously with wall-binding of the unlabelled material or some hours later.

Protocol B₁ (Figure 1b)

Protocol B₁ was designed to set up a labelling pattern (Figure 7b) by which we could determine whether the integration of [$^{13}\text{C},^3\text{H}$]xyloglucan into the wall was accompanied by its covalent attachment to existing wall-bound [$^{12}\text{C},^3\text{H}$]xyloglucans. Both [^{12}C]- and [^{13}C]-glucose-fed cells rapidly incorporated ^3H from [^3H]arabinose into the xyloglucan of the wall hemicellulose, culture filtrate and protoplast contents (Figure 8a). There was little intraprotoplasmic [^3H]xyloglucan at any time point, suggesting that newly synthesized xyloglucan was rapidly secreted into the apoplast, where the majority promptly became incorporated into the wall matrix. About 35% of the newly secreted [^3H]xyloglucan quickly passed through the cell wall and accumulated in the culture filtrate, as observed previously [8,27,43]. Accumulation of [^3H]xyloglucan in the culture filtrate continued over the time course at a lower rate, owing to sloughing of wall-bound xyloglucan into the medium [8]. Intraprotoplasmic

and soluble extracellular xyloglucan are of low M_r ([8]; J. E. Thompson and S. C. Fry, unpublished work) and therefore could not be investigated by isopycnic centrifugation.

As expected from protocol A, the [$^{12}\text{C},^3\text{H}$]xyloglucan in the control culture underwent little change in density. The difference in buoyant density between the earliest wall-bound [^3H]xyloglucan in the ^{13}C -fed cells and that in the ^{12}C -fed controls ($\Delta\rho$) was 0.020 g/ml (Figure 8b). This is about half the $\Delta\rho$ value observed in protocol A and indicates that, as soon as any segments of [$^{13}\text{C},^3\text{H}$]xyloglucan were bound to the wall (and were therefore included in the wall hemicellulose fraction), they had already been covalently attached to previously wall-bound [$^{12}\text{C},^3\text{H}$]xyloglucan. This suggests that interpolymeric transglycosylation occurred concurrently with wall-binding, yielding wall-bound [$^{12}\text{C},^{13}\text{C},^3\text{H}$]xyloglucan, but no wall-bound [$^{13}\text{C},^3\text{H}$]xyloglucan. However, an alternative explanation of the low initial $\Delta\rho$ might be that the cells inadequately depleted their endogenous [^{12}C]sugar-nucleotide pools in the 20 min allowed prior to addition of the [^3H]arabinose. Plant cells can accumulate large amounts of glycerol without loss of turgor, and some of the [^{12}C]glycerol could have been carried over when the carbon source was intended to be changed (−0.33 h in Figure 1b).

The experiment was a pulse-labelling in which the synthesis of [^3H]xyloglucan was essentially restricted to a period 1–2 h after the feeding of [^3H]arabinose [8,27]. Despite this, the density of the [^3H]xyloglucan in the ^{13}C -fed culture increased (significant positive gradient; $P > 0.05$) during the 7 days after the [^{12}C]glycerol → [^{13}C]glucose shift (Figure 8b). This indicates that covalent attachment of newly secreted [$^{13}\text{C},^3\text{H}$]xyloglucan to $^{12}\text{C},^{13}\text{C},^3\text{H}$ -labelled wall-bound xyloglucan exceeded any transglycosylation between the $^{12}\text{C},^{13}\text{C},^3\text{H}$ -labelled wall-bound xyloglucan and older wall-bound [$^{12}\text{C},^3\text{H}$]xyloglucan. The latter process would have tended to decrease the density of the ^3H -labelled material.

Protocol B₂

To test whether the low initial $\Delta\rho$ in protocol B₁ was due to excessive carry-over of [^{12}C]glycerol into the ^{13}C -fed cells, we performed an experiment similar to that in Figure 1(b), but with

Figure 8 Effect of transferring [¹²C]glycerol-grown *Rosa* cells to [¹³C]glucose 20 min before a pulse of [³H]arabinose (protocol B₁.)

(a) Incorporation of ³H into xyloglucan of the wall hemicellulose (■), culture filtrate (●) and protoplast contents (▲). Similar data (not shown) were obtained for the ¹²C-only control cultures. (b) Changes in mean buoyant density of the wall-bound [³H]xyloglucan: ●, cultures with [¹³C]glucose; ○, controls with ¹²C only. The bars show the approximate periods during which ¹²C (▨), ¹³C (▧) and ³H (▩) were present in the medium.

cells pre-grown on glucose instead of glycerol and allowed more time to deplete endogenous ¹²C pools. The [³H]arabinose was again rapidly incorporated into xyloglucan (Figure 9a).

The difference in density between the [³H]xyloglucans deposited by ¹³C- and ¹²C-fed cells (initial $\Delta\rho = 0.019$ g/ml; Figure 9b) was similar to that in protocol B₁. This argues against the idea that a large reserve of [¹²C]glycerol had prevented the efficient incorporation of ¹³C into xyloglucan during the pulse-labelling with [³H]arabinose in protocol B₁. The data therefore confirm that newly secreted segments of xyloglucan become integrated into the wall at the same time as becoming covalently attached to previously wall-bound xyloglucan chains.

[¹²C,³H]Xyloglucan in the control culture again showed little change in density over 7 days (Figure 9b). [³H]Xyloglucan in the ¹³C-fed cells appeared to undergo a small increase in density, as

Figure 9 Effect of transferring [¹²C]glucose-grown *Rosa* cells to [¹³C]glucose 1 h before a pulse of [³H]arabinose (protocol B₂.)

Other details are as in the legend to Figure 8.

in protocol B₁, but the positive gradient was not significant ($P = 0.17$). The lack of a decrease in density supports the conclusion that the covalent attachment of newly secreted xyloglucan to existing wall-bound xyloglucan exceeded any transglycosylation between pairs of previously wall-bound xyloglucan molecules.

DISCUSSION

Methodology

CsCl has long been used to band DNA [34], which has a relatively low density owing to its deoxysugar. RNA and polysaccharides in general have too high a density to band in CsCl [44]. CsTFA[™] was introduced for analysis of RNA because (a) CsTFA[™] can form solutions of higher density than CsCl, and (b) polymers become more highly hydrated and therefore exhibit a lower buoyant density in CsTFA[™] than in CsCl. To our knowledge this is the first report of the use of isopycnic centrifugation in CsTFA[™] to study a plant-cell-wall polysaccharide, although it has been used for mammalian mucins [45]. [¹²C]Xyloglucan from *Rosa* was successfully banded in

CsTFA[®] to give an approximation to a Gaussian curve with a peak buoyant density of ≈ 1.575 g/ml. Xyloglucan from ^{13}C -fed *Rosa* cells exhibited an appreciably higher buoyant density.

In protocol A, the initial buoyant density of the wall-bound [^{13}C , ^3H]xyloglucan differed from that of the [^{12}C , ^3H]xyloglucan by 0.041 g/ml. A $\Delta\rho$ value of 0.041 g/ml represents an increase in buoyant density of 2.6%. Dry [U- ^{13}C]xyloglucan at 98.9% isotopic enrichment would be 3.7% denser than dry [^{12}C]xyloglucan [assuming approximate composition (Glc₈-Xyl₆-Gal-Fuc)_n]. If the [^{13}C , ^3H]xyloglucan initially deposited in protocol A had been 98.9% enriched in ^{13}C , the 2.6:3.7 ratio would indicate that, in buffered CsTFA[®] solution, 1 g of xyloglucan is effectively hydrated with 0.43 g of water.

^3H -labelling under the conditions used would not appreciably alter the buoyant density of the xyloglucan because of the low isotopic enrichment and low concentration of the [^3H]arabinose supplied.

By means of an *in vitro* model experiment with cauliflower XET, we showed that interpolymeric transglycosylation can be demonstrated by the transfer of ^3H -labelled segments between equal- M_r xyloglucans differing in buoyant density (Figure 4b). Previous demonstrations of interpolymeric transglycosylation [29,30] have relied on changes in M_r . It was therefore feasible to use $^{13}\text{C}/^3\text{H}$ dual labelling to examine for the first time the occurrence of interpolymeric transglycosylation between xyloglucans *in vivo*.

In the three *in vivo* density-labelling protocols, the rapid utilization of [^3H]arabinose enabled a ^3H pulse-chase type of experiment in which changes in the density of segments of wall-bound [^3H]xyloglucan could be monitored as they 'aged' in a cell wall containing a majority of non-radioactive xyloglucan of a different density. In the non- ^{13}C -fed controls of each protocol, the [^3H]xyloglucan underwent little change in density; the trend, if any, was a slight rise in density during the 7-day period of observation. A rise in density could be due to the loss of some residues of fucose (a deoxysugar). The behaviour of [^{12}C , ^3H]xyloglucan provided an essential reference against which to compare the behaviour of density-labelled [^3H]xyloglucan.

The experimental approach reported here cannot be used to detect transglycosylation between xyloglucan molecules of the same age, because such molecules would all have the same initial density. However, the method does allow investigation of transglycosylation between a particular set of xyloglucan molecules and those synthesized either earlier or later. By varying the experimental design, we have obtained evidence for transglycosylation of xyloglucans with both younger and older molecules.

Interpolymeric transglycosylation *in vivo*

In protocols B₁ and B₂, the initial buoyant density of the wall-bound [^3H]xyloglucan in the ^{13}C -fed cells differed from that in the ^{12}C -fed cells by a much smaller margin ($\Delta\rho \approx 0.020$ g/ml) than in protocol A ($\Delta\rho = 0.041$ g/ml). Therefore, in the ^{13}C -fed cells, the radioactive xyloglucan immediately after its binding to the cell wall was [^{12}C , ^{13}C , ^3H]xyloglucan, whereas in protocol A it was a closer approximation to [^{13}C , ^3H]xyloglucan. In protocol A, the new [^{13}C , ^3H]xyloglucan was secreted into a ^{13}C -labelled wall, whereas in protocol B it was secreted into a ^{12}C -labelled wall. Thus the data indicate that newly secreted xyloglucans become covalently attached to existing xyloglucans during integration into the cell wall. The only known process that could achieve such covalent attachment in the apoplast is interpolymeric transglycosylation, catalysed by XET.

The gradual decrease in density of the [^3H]xyloglucan in the

^{13}C -treated culture of protocol A (Figure 6b) strongly supports the conclusion that newly secreted xyloglucan (in this case [^{12}C , ^3H]xyloglucan) was integrated into the cell-wall matrix by transglycosylation between it and previously wall-bound material (in this case [^{13}C , ^3H]xyloglucan).

In protocols B₁ and B₂, the [^{12}C , ^{13}C , ^3H]xyloglucan was designed to be sandwiched between a thick layer of older [^{12}C , ^3H]xyloglucan and a gradually increasing layer of younger [^{13}C , ^3H]xyloglucan (Figure 7b). In this situation, the [^3H]xyloglucan could increase, decrease or not change in density, depending on the relative rates of two kinds of interpolymeric transglycosylation: (i) transglycosylation of the [^3H]xyloglucan with newly secreted xyloglucan would tend to increase the density of the radiolabelled material, whereas (ii) transglycosylation with older xyloglucan molecules would tend to decrease it. Thus the observed increase in density of the radioactive xyloglucan in the ^{13}C -treated cells (Figures 8b and 9b) indicates that the rate of interpolymeric transglycosylation between wall-bound xyloglucan and newly secreted xyloglucan exceeded any interpolymeric transglycosylation occurring between pairs of previously wall-bound xyloglucan molecules.

The present experiments did not provide evidence for or against the occurrence of interpolymeric transglycosylation between pairs of previously wall-bound xyloglucan chains in cultured *Rosa* cells. However, we demonstrated for the first time the interpolymeric transglycosylation of xyloglucan in the walls of living plant cells. The $^{13}\text{C}/^3\text{H}$ -dual-labelling technique developed here shows that newly secreted xyloglucan chains undergo interpolymeric transglycosylation with existing wall-bound chains during and, we propose, causing the rapid binding of the newly secreted xyloglucans into the wall matrix.

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REFERENCES

- Labavitch, J. and Ray, P. M. (1974) *Plant Physiol.* **54**, 499–502
- Nishitani, K. and Masuda, Y. (1982) *Plant Sci. Lett.* **28**, 87–94
- Fry, S. C. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 497–520
- Roberts, J. and Sexton, R. (1982) *Annu. Rev. Plant Physiol.* **33**, 133–162
- Redgwell, R. J. and Fry, S. C. (1993) *Plant Physiol.* **103**, 1399–1406
- Sakurai, N. and Nevins, D. J. (1993) *Physiol. Plant.* **89**, 681–686
- Cutillas-Iturralde, A., Zarra, I., Fry, S. C. and Lorences, E. P. (1994) *Physiol. Plant.* **91**, 169–176
- Thompson, J. E. and Fry, S. C. (1997) *J. Exp. Bot.* **48**, 297–305
- Fry, S. C. (1989) *Physiol. Plant.* **75**, 532–536
- Hayashi, T. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139–168
- McCann, M. C., Wells, B. and Roberts, K. (1990) *J. Cell Sci.* **96**, 323–334
- Vincken, J.-P., de Keizer, A., Beldman, G. and Voragen, A. G. J. (1995) *Plant Physiol.* **108**, 1579–1585
- Baydoun, E. A.-H. and Fry, S. C. (1989) *J. Plant Physiol.* **134**, 453–459
- Smith, R. C. and Fry, S. C. (1991) *Biochem. J.* **279**, 529–535
- Farkaš, V., Sulová, Z., Stratilová, E., Hanna, R. and MacLachlan, G. (1992) *Arch. Biochem. Biophys.* **298**, 365–370
- Fry, S. C., Smith, R. C., Renwick, K. F., Martin, D. J., Hodge, S. K. and Matthews, K. J. (1992) *Biochem. J.* **282**, 821–828
- Nishitani, K. and Tominaga, R. (1992) *J. Biol. Chem.* **267**, 21058–21064
- Saab, I. N. and Sachs, M. M. (1995) *Plant Physiol.* **108**, 439–440
- Xu, W., Campbell, P., Vargheese, A. K. and Braam, J. (1996) *Plant J.* **9**, 879–889
- Hetherington, P. R. and Fry, S. C. (1993) *Plant Physiol.* **103**, 987–992
- Potter, I. and Fry, S. C. (1993) *Plant Physiol.* **103**, 235–241
- Potter, I. and Fry, S. C. (1994) *J. Exp. Bot.* **45**, 287–294
- Pritchard, J., Hetherington, P. R., Fry, S. C. and Tomos, A. D. (1993) *J. Exp. Bot.* **44**, 1281–1289
- Xu, W., Purugganan, M. M., Antosiewicz, D. M., Polisensky, D. H., Fry, S. C. and Braam, J. (1995) *Plant Cell* **7**, 1555–1567
- Lorences, E. P. and Fry, S. C. (1993) *Physiol. Plant.* **88**, 105–112
- Edelmann, H. G. and Fry, S. C. (1992) *Plant Physiol.* **100**, 993–997
- Edelmann, H. G. and Fry, S. C. (1992) *J. Exp. Bot.* **43**, 463–470

-
- 28 Wu, Y., Spollen, W. G., Sharp, R. E., Hetherington, P. R. and Fry, S. C. (1994) *Plant Physiol.* **106**, 607–615
- 29 McDougall, G. J. and Fry, S. C. (1990) *Plant Physiol.* **93**, 1042–1048
- 30 Nishitani, K. and Tominaga, R. (1991) *Physiol. Plant.* **82**, 490–497
- 31 Fry, S. C. and Northcote, D. H. (1983) *Plant Physiol.* **73**, 1055–1061
- 32 Wende, G. and Fry, S. C. (1997) *Phytochemistry* **44**, 1019–1030
- 33 Talbot, L. D. and Ray, P. M. (1992) *Plant Physiol.* **98**, 369–379
- 34 Meselson, M. and Stahl, F. W. (1958) *Proc. Natl Acad. Sci. U.S.A.* **44**, 671–677
- 35 Fry, S. C. and Street, H. E. (1980) *Plant Physiol.* **65**, 472–477
- 36 Fry, S. C., York, W. S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E. P., MacLachlan, G. A. and McNeil, M. (1993) *Physiol. Plant.* **89**, 1–3
- 37 Fry, S. C. (1988) *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*, Longman, Harlow
- 38 Fry, S. C. (1987) *Planta* **171**, 205–211
- 39 Edelman, H. G. and Fry, S. C. (1992) *Carbohydr. Res.* **228**, 423–431
- 40 Dische, Z. (1962) *Methods Carbohydr. Chem.* **1**, 475–514
- 41 Fry, S. C. (1986) *Planta* **169**, 443–453
- 42 Manning, K. (1981) *J. Biochem. Biophys. Methods* **5**, 189–202
- 43 Becker, G. E., Hui, P. A. and Albersheim, P. (1964) *Plant Physiol.* **39**, 913–920
- 44 Birnie, G. D. and Rickwood, D. (1978) *Centrifugal Separations in Molecular and Cell Biology*, Butterworths, London
- 45 Goso, Y. and Hotta, K. (1994) *Anal. Biochem.* **223**, 274–279
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